

# Long-acting combination anti-HIV drug suspension enhances and sustains higher drug levels in lymph node cells than in blood cells and plasma

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**Objective:** The aim of the present study was to determine whether a combination of anti-HIV drugs – tenofovir (TFV), lopinavir (LPV) and ritonavir (RTV) – in a lipid-stabilized nanosuspension (called TLC-ART101) could enhance and sustain intracellular drug levels and exposures in lymph node and blood cells above those in plasma.

**Design:** Four macaques were given a single dose of TLC-ART101 subcutaneously. Drug concentrations in plasma and mononuclear cells of the blood (PBMCs) and lymph nodes (LNMCs) were analysed using a validated combination LC-MS/MS assay.

**Results:** For the two active drugs (TFV, LPV), plasma and PBMC intracellular drug levels persisted for over 2 weeks; PBMC drug exposures were three- to four-fold higher than those in plasma. Apparent terminal half-lives ( $t_{1/2}$ ) of TFV and LPV were 65.3 and 476.9 h in plasma, and 169.1 and 151.2 h in PBMCs. At 24 and 192 h, TFV and LPV drug levels in LNMCs were up to 79-fold higher than those in PBMCs. Analysis of PBMC intracellular TFV and its active metabolite TFV-diphosphate (TFV-DP) indicated that intracellular exposures of total TFV and TFV-DP were markedly higher and persisted longer than in humans and macaques dosed with oral TFV prodrugs, tenofovir disoproxil fumarate (TDF) or tenofovir alafenamide (TAF).

**Conclusions:** A simple, scalable three-drug combination, lipid-stabilized nanosuspension exhibited persistent drug levels in cells of lymph nodes and the blood (HIV host cells) and in plasma. With appropriate dose adjustment, TLC-ART101 may be a useful HIV treatment with a potential to impact residual virus in lymph nodes.

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*AIDS* 2017, **31**:765–770

**Keywords:** drug combination nanoparticles, enhanced and persistent drug exposure, HIV treatment, intracellular HIV drug exposures, long-acting, lopinavir, lymphatic drug insufficiency, lymphatic targeting, targeted drug delivery, tenofovir

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Received: 23 November 2016; revised: 13 December 2016; accepted: 28 December 2016.

DOI:10.1097/QAD.0000000000001405

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## Introduction

In the U.S.A., of all HIV-infected individuals – those taking and not taking oral combination antiretroviral therapy (cART) – only 30% have viral suppression [1]. We first proposed [2] and others confirmed in prospective studies [3,4] that orally administered drugs exhibit drug insufficiency in lymph nodes [3–5]. We recently reported a stable, scalable triple-drug anti-HIV formulation sustained drug levels in nonhuman primate (NHP) plasma and peripheral blood mononuclear cells (PBMCs) for over 1 week, and enhanced drug levels in lymph node mononuclear cells (LNMCs) [6]. This strategy holds promise as a long-acting injectable that could improve patient adherence – by retaining patients who have challenges with oral therapy in care – and enhance viral suppression in blood and lymphoid tissues.

Although several long-acting injectables, including cabotegravir and rilpivirine, are in development, these are currently formulated as separate agents [7]. Having drug combinations in a fixed dosage formulation could improve patient acceptability and potentially better address concerns about viral resistance. However, incorporating multiple drugs into a single long-acting injectable formulation poses considerable challenges with respect to pharmaceuticals (formulation, scalability), pharmacokinetics (absorption, disposition, safety) and pharmacodynamics (efficacy against disease).

Previously, we described a lymphatic-targeted HIV drug combination (TFV, LPV, RTV) [6,8]. In the present study, we further validate and characterize the long-acting intracellular and plasma exposure over 2 weeks using a similar but optimized injectable combination formulation called TLC-ART101.

## Materials and methods

### Reagents

The reagents used were DSPC, DSPE-mPEG2000 phospholipids (GMP grade) (Corden Pharma, Liestal, Switzerland) and tenofovir (TFV; PMPA), lopinavir (LPV), ritonavir (RTV; Waterstone, Carmel, Indiana, USA).

### TLC-ART101 TFV–LPV–RTV combination

TLC-ART101 composed of DSPC, DSPE-mPEG2000, TFV, LPV and RTV was prepared as described previously [2,8]. TLC-ART101 contained 17.1 mmol/l TFV, 18.3 mmol/l LPV and 4.5 mmol/l RTV; fraction in particle-associated form was 11.2%, 92.2% and 91.1%, respectively. Mean diameter was  $69.0 \pm 8.3$  nm.

### Pharmacokinetic study

Four male macaques (*Macaca nemestrina*, 10.5, 9.8, 6.5, 8.3 kg) were enrolled under an approved Institutional Animal Care and Use Committee protocol. No adverse

events occurred. Macaques were dosed 10.6, 25.0, 7.0 mg/kg TFV, LPV, RTV, subcutaneously once in the back mid-scapular region. Blood was collected at 0, 0.25, 0.5, 1, 3, 5, 8, 24, 48, 120, 168, 192, 336 h; an inguinal lymph node was excised at 24 and 192 h. Plasma, PBMCs and LNMCs were isolated as described [8]; LPV, RTV, TFV concentrations were analysed with a validated LC-MS/MS assay [8]. A noncompartmental model was used to estimate pharmacokinetic parameters with Phoenix v6.4 (Certara, Mountain View, California, USA). Intracellular TFV and its active metabolite TFV-diphosphate (TFV-DP) were analysed in the context of reported values after TFV, tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide (TAF) dosing.

### Statistical analysis

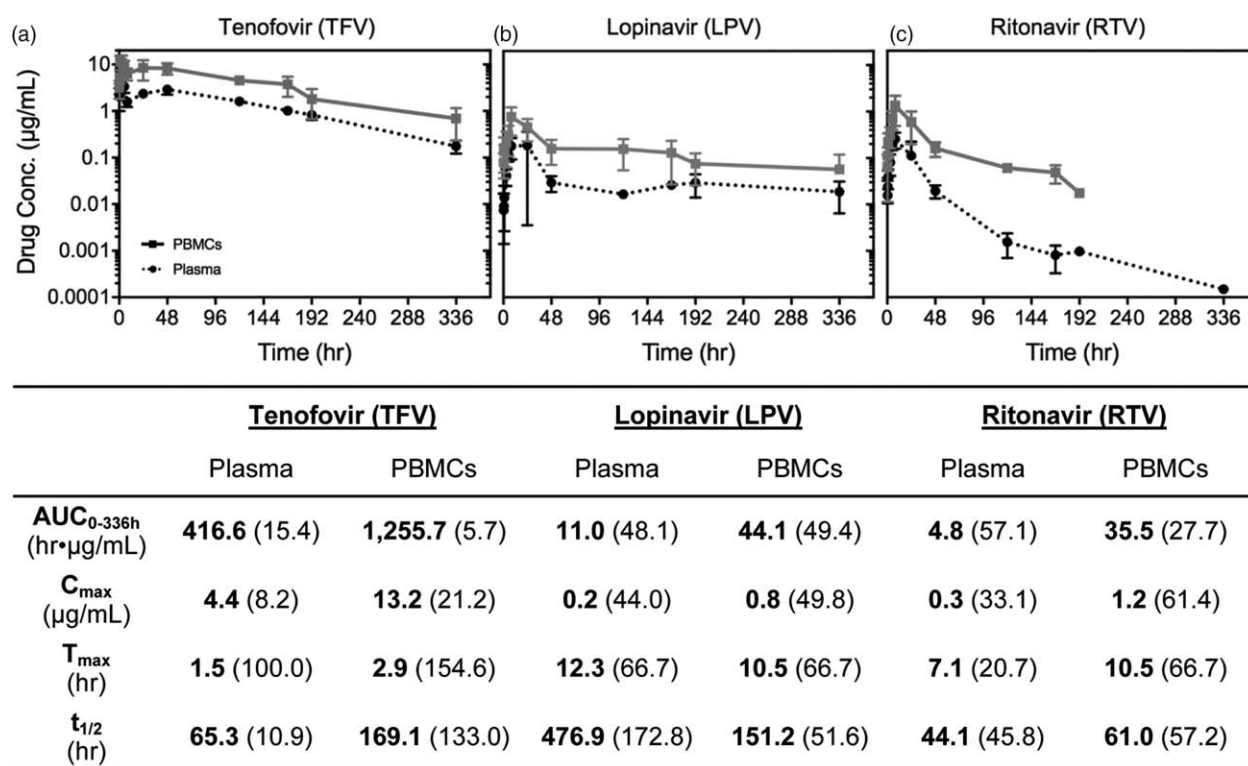
All statistical tests were two-tailed *t*-tests with  $\alpha$  value equal to 0.05.

## Results

To determine the time-course of TFV, LPV and RTV, macaques were dosed with TLC-ART101 subcutaneously. Figure 1 shows the concentrations of TFV, LPV and RTV in plasma and PBMCs [9]. The two active drugs (TFV, LPV) in TLC-ART101 exhibited sustained plasma and intracellular (PBMC) levels for 336 h (2 weeks), with higher intracellular TFV and LPV levels than in plasma (Fig. 1). PBMC intracellular exposures to TFV and LPV over 2 weeks ( $AUC_{0-336h}$ ) were 3.01- to 4.02-fold higher than in plasma ( $P < 0.0001$  and  $P = 0.0522$ , respectively) (Fig. 1, table insert). Peak drug concentrations ( $C_{max}$ ) of TFV and LPV were also higher in PBMCs than in plasma ( $P = 0.0076$  and  $P = 0.0539$ , respectively). In plasma and PBMCs, TFV concentrations peaked initially at nearly 2 h postdose prior to rebounding to a secondary peak level at nearly 24 to 48 h. LPV and RTV levels in plasma and PBMCs peaked between 8 and 14 h (Fig. 1). Plasma apparent terminal half-lives ( $t_{1/2}$ ) were nearly 2.7 days for TFV and nearly 20 days for LPV; PBMC  $t_{1/2}$  were nearly 7 days for TFV and nearly 6.3 days for LPV (Fig. 1). Unlike TFV and LPV, RTV levels fell below the detection limit in plasma by 2 weeks and PBMCs after 8 days (Fig. 1).

As TLC-ART101 is designed to accumulate drug combinations in lymph nodes, we evaluated LNMC intracellular drug levels. Intracellular TFV and LPV levels in LNMCs persisted at days 1 and 8; TFV and LPV levels in LNMCs were 0.9- to 79.2-fold higher than in PBMCs and 3.1- to 197.8-fold higher than in plasma (Table 1) [9]. Even the booster RTV exhibited enhanced intracellular drug levels in LNMCs versus those in PBMCs.

Data in Table 2 indicate that compared with subcutaneous TFV, oral TDF and oral TAF, a single subcutaneous TLC-ART101 dose provided higher PBMC intracellular



**Fig. 1. Time-course of plasma and PBMC intracellular concentrations of TFV, LPV and RTV after a single SC dose of the TLC-ART101 drug combination nanosuspension, as well as descriptive PK parameters.** (a–c) show consistently higher TFV, LPV and RTV concentrations in peripheral blood mononuclear cells (PBMCs) at each time point than those in plasma. The squares (■) represent PBMC intracellular drug concentrations, and circles (●) represent plasma drug concentrations. Each time point represents arithmetic mean  $\pm$  SD of plasma and PBMC intracellular drug concentrations from  $N=4$  male pigtail macaques. PBMC cell volume assumed to be 0.2829 pl/cell [9]. Limit of detection (LOD) = plasma/PBMCs, TFV: 100/110; LPV: 5/2; RTV: 25/2.5 pg/ml. Limit of quantification (LOQ) = plasma/PBMCs, TFV: 250/250; LPV: 10/5; RTV: 50/5 pg/ml. RTV (intended as a booster for LPV PK) in plasma was <LOQ in  $N=2$  at 192 and 336 h, and was <LOQ in PBMCs of  $N=3$  at 192 and  $N=4$  at 336 h. The summary table lists PK parameter estimates based on noncompartmental analysis (NCA) using Phoenix. Each PK parameter is presented as the geometric mean (% coefficient of variation) of the individual PK parameters from  $N=4$  macaques. The PBMC TFV  $AUC_{0-366h}$  of 1255.7 h·µg/ml was converted to the molar equivalent value (equal to 4372.06 h·µmol/l) for data comparisons presented in Table 2.  $AUC_{0-336h}$ , area under the concentration–time curve from 0 to 336 h;  $C_{max}$ , maximum drug concentration reached;  $T_{max}$ , time at which  $C_{max}$  was reached;  $t_{1/2}$ , apparent terminal elimination half-life.

exposures to TFV and estimated TFV-DP (even after dose-normalization) [10–13].

## Discussion

Here, we describe a further optimized formulation with a fixed-dose LPV/RTV molar ratio of 4 : 1. This optimized TLC-ART101 preparation provided sustained levels of two active drugs (TFV, LPV) in plasma and PBMCs for over 2 weeks (Fig. 1). In addition, intracellular TFV and LPV concentrations in LNMCs were higher than in PBMCs (Table 1). Thus, this treatment strategy could potentially overcome drug insufficiency in lymph nodes with oral regimens [3–5]. PBMC intracellular concentrations of TFV and LPV were also consistently higher than plasma levels in all NHPs throughout the 2-week study. Collectively, subcutaneous TLC-ART101

**Table 1. Enhanced TFV, LPV and RTV intracellular concentrations in inguinal lymph nodes excised one and eight days after a single subcutaneous dose of TLC-ART101, and comparisons to drug levels at the same time points in PBMCs and plasma.**

SC dose (mg/kg)	Tenofovir 10.58	Lopinavir 25.00	Ritonavir 6.96
LNMCs			
C <sub>24h</sub> (µg/ml)	7.12 (56.5)	9.02 (89.2)	4.50 (28.7)
C <sub>192h</sub> (µg/ml)	5.49 (175.8)	5.16 (127.0)	0.98 (157.6)
LNMCs/PBMCs			
C <sub>24h</sub>	0.93 (79.6)	23.04 (162.7)	8.88 (44.3)
C <sub>192h</sub>	4.41 (198.0)	79.20 (65.9)	531.37 (NA)
LNMCs/Plasma			
C <sub>24h</sub>	3.05 (50.4)	89.41 (119.3)	77.94 (95.9)
C <sub>192h</sub>	6.77 (165.3)	197.78 (93.7)	3068.15 (111.2)

Geometric mean (% coefficient of variation). NA, not available due to three macaques having RTV levels <LOQ in PBMCs. LNMC and PBMC cell volume assumed to be 0.2829 pl/cell [9]. C<sub>24h</sub> and C<sub>192h</sub>, drug concentration at 24 and 192 h; LNMCs, lymph node mononuclear cells; PBMCs, peripheral blood mononuclear cells; SC, subcutaneous.

**Table 2. Comparison of intracellular TFV-DP and total TFV exposure in PBMCs after dosing with TFV, TDF, TAF or TLC-ART101 formulations.**

Drug formulation	Route	Dose (mg/kg)	TFV-DP	TFV (total)	Ref
TFV	SC	4	4.65	8.67	Durand-Gasselín <i>et al.</i> [10]
TDF	PO	30	36.60	53.43	
TAF	PO	1.5	0.05	0.46	Massud <i>et al.</i> [11]
TAF	PO	4.5	1.13	3.39	
TFV in TLC-ART101 <sup>a</sup>	SC	10.6	2666.96	4372.06	Figure 1, table insert

Intracellular drug exposure (AUC in h· $\mu$ mol/l) after a single dose in NHPs is estimated on the basis of intracellular concentration ( $\mu$ mol/l) measured over time in PBMCs as TFV-DP, total TFV or both. AUC, area under the concentration-time curve; NHPs, nonhuman primates; PBMCs, peripheral blood mononuclear cells; PO, *per os* (oral); SC, subcutaneous; TAF, tenofovir alafenamide; TDF, tenofovir disoproxil fumarate; TFV, tenofovir; TFV-DP, tenofovir diphosphate.

<sup>a</sup>The steady-state fraction of 61% of total TFV is used to estimate the fraction of TFV-DP [10–13] [e.g. 1255.70 h· $\mu$ g/ml  $\times$  (1000  $\mu$ mol·ml)/(287.21  $\mu$ g·l)] = 4372.06 h· $\mu$ mol/l; 0.61  $\times$  4372.06 h· $\mu$ mol/l total TFV = 2666.96 h· $\mu$ mol/l TFV-DP.

enhanced intracellular LPV and TFV levels in LNMCs for at least 8 days, and extended PBMC and plasma drug levels for over 2 weeks. Thus, TLC-ART101 could be considered for development as a long-acting, fixed-dose injectable combination for HIV treatment for adults and children.

The exact mechanism of TLC-ART101 enhancing LNMC drug levels and sustaining levels in plasma and PBMCs is not clear. It is likely, however, that, from the subcutaneous space, TFV, LPV and RTV associated in TLC-ART101 nanoparticles are too large to penetrate blood capillary endothelial cells. Instead, they are preferentially taken up together by more permeable lymphatic capillaries as a single drug combination unit. Subsequently, each drug particle could be retained in lymph vessels, and due to the stability of the drug-nano complex, they are likely to be transported throughout the lymphatic system as stable units. Without significant degradation, the size of the drug combination particles (60–80 nm) likely prevents extravasation of drug from lymph vessels, thus delaying drug appearance in the blood. This size of TLC-ART101 particles could fill lymph node sinus spaces ( $\sim$ 1–500  $\mu$ m) [14] to capacity before moving to other nodes interconnected by lymphatic vessels. Uptake of TFV, LPV and RTV into cells as a stable particle unit may have contributed to higher intracellular drug concentrations in LNMCs and PBMCs than in the plasma; the time to peak levels in PBMCs for all drugs being nearly 12–24 h supports this hypothesis (Fig. 1, table insert). Should the drug-lipid particle disintegrate, the released soluble drugs would be cleared into nearby blood vessels (wherein flow is  $\sim$ 100–500-fold faster than the lymph system) and eliminated within hours (akin to the  $\sim$ 1–12 h half-life of the respective parent soluble drugs). The hypothesis of preferential lymph vessel uptake and lymph node retention, followed by redistribution of the drug combination in TLC-ART101 to the blood, is consistent with the higher LNMC intracellular concentrations (Table 1) wherein the first exposure to drug particles occurs (versus their PBMC counterparts). Regardless of the exact

mechanisms, the pharmacokinetic data indicate that preferential lymphatic drug-combination particle uptake, distribution, retention and cellular uptake may have contributed to the early peak drug levels followed by sustained drug levels for over 2 weeks.

Given the long-standing clinical experience with LPV and TFV (including TDF and TAF), development of TLC-ART101 could leverage available in-vitro antiviral potency data and target plasma drug levels. Compared with equivalent free drug formulations, a drug-lipid fixed-dose combination exhibited nearly 30-fold lower EC<sub>50</sub> (higher potency); EC<sub>50</sub> values for TFV and LPV in TLC-ART101 against HIV-infected CEM-174 cells were 1.5  $\pm$  0.1 and 3.0  $\pm$  0.8 nmol/l, respectively [8]. Macaque PBMC intracellular concentrations at 0–336 h were nearly 100- to 10 000-fold higher than these EC<sub>50</sub> values after one TLC-ART101 dose. Dose-response and efficacy studies in a NHP HIV treatment model are planned.

Long-acting injectable drugs, including cabotegravir and rilpivirine, are in development. However, to our knowledge, none are co-formulated. Cabotegravir and rilpivirine (two different dosage forms), injected in separate intramuscular sites, allow drug release predominantly from each injection site. As a result, it takes days to build up plasma drug levels in humans and the  $T_{max}$  of each is variable (median 9–69 days [15], 6–11 days [16], depending on the dose). Among other considerations, the delay in reaching therapeutic levels requires an initial dose supplement for these formulations. In contrast, TFV and LPV levels after TLC-ART101 peaked in plasma at  $\sim$ 1.5 and  $\sim$ 12.3 h and likely reach therapeutic levels (above EC<sub>50</sub>) within hours (Fig. 1). These early peak plasma and cell levels may overcome the need to have a lead-in oral dose for TLC-ART101.

Previously, we used a LPV/RTV molar ratio of 2:1 [6,8] and TFV and LPV levels in NHP plasma and PBMCs persisted for over 1 week. In the present study, TLC-ART101, with a LPV/RTV molar ratio of 4:1 (as in *Kaletra* tablets [17]), extended TFV and LPV levels in

plasma and PBMCs to over 2 weeks. However, inexplicably, at 1 week postdosing, there were no significant differences for LPV plasma and PBMC  $AUC_{0-168h}$ ,  $C_{max}$  and  $T_{max}$  between LPV/RTV 2:1 versus 4:1, yet the LPV/RTV 4:1 formulation exhibited a significantly higher PBMC intracellular TFV  $AUC_{0-168h}$  ( $P < 0.005$ ). In this study, as with our previous study [8], markers of renal and hepatic function remained within normal levels. Cerebral spinal fluid drug levels in macaques 24h after TLC-ART101 dosing were 16- to 1660-fold lower than those in plasma.

In TLC-ART101, we used TFV, as the bioavailability of TFV via the subcutaneous route is not limited by oral absorption. Prodrugs TDF and TAF are intended to overcome limited oral bioavailability of TFV. Interestingly, PBMC intracellular TFV exposures after a single subcutaneous TLC-ART101 dose were much higher than equivalent or recommended doses of subcutaneous TFV, oral TDF and oral TAF in NHPs (Table 2). At steady state, the intracellular TFV-DP ratio was ~61% in both human and macaque PBMCs [10–13]. Even with TFV, which exhibits 10- to 100-fold lower cell uptake *in vitro* [18], our data suggest that TLC-ART101 enhanced intracellular TFV and TFV-DP drug concentrations in NHP PBMCs and LNMCs, and substantially enhanced exposure. Even after weight-based adjustments, TLC-ART101 still provided a several thousand-fold increase in intracellular drug exposure versus TDF or TAF. The increase in TFV exposure enabled by TLC-ART101 may allow the use of a lower TFV dose. This may improve the safety of TFV, an important consideration for adults and children.

In summary, subcutaneous TLC-ART101 (TFV, LPV and RTV in a nanosuspension) enabled persistent drug levels in LNMCs, PBMCs and plasma in NHPs; significant enhancements were noted in overall intracellular drug exposures of hydrophilic TFV over 2 weeks. The long-acting behaviour and higher intracellular levels in lymph nodes and blood over plasma levels may address the adherence issues and drug insufficiency reported in lymph nodes after current oral HIV regimens. These data support TLC-ART101 development as a potential platform for a subcutaneous long-acting combination antiretroviral regimen.

## Acknowledgements

We thank all the TLC-ART members who contributed to study discussions. We especially thank Sophia Song for assistance with the preparation of the TLC-ART101 formulation, as well as the Washington National Primate Research Center staff (Baoping Tian, Michael Gough, Jason Ogle, Wonsok Lee, and Chris English) for their support with the macaque studies.

JCK and RJYH wrote the article, designed studies, collected data and analysed the data. LAM, JK and LK designed studies, collected data and proofread the article. CC, DDS and ACC designed studies and provided substantive input into the article.

This study was supported in part by NIH grants UM1 AI120176, P51OD010425.

## Conflicts of interest

The authors have no conflicts of interest to declare.

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