

Tenofovir alafenamide and elvitegravir loaded nanoparticles for long-acting prevention of HIV-1 vaginal transmission

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Objective: This report presents tenofovir (TFV) alafenamide (TAF) and elvitegravir (EVG) fabricated into nanoparticles for subcutaneous delivery as prevention strategy.

Design: Prospective prevention study in humanized bone marrow–liver–thymus (hu-BLT) mice.

Methods: Using an oil-in-water emulsion solvent evaporation technique, TAF + EVG drugs were entrapped together into nanoparticles containing poly(lactic-co-glycolic acid). In-vitro prophylaxis studies (90% inhibition concentration) compared nanoparticles with drugs in solution. Hu-BLT ($n = 5/\text{group}$) mice were given 200 mg/kg subcutaneous, and vaginally challenged with HIV-1 [5×10^5 tissue culture infectious dose for 50% of cells cultures (TCID₅₀)] 4 and 14 days post-nanoparticle administration (post-nanoparticle injection). Control mice ($n = 5$) were challenged at 4 days. Weekly plasma viral load was performed using RT-PCR. Hu-BLT mice were sacrificed and lymph nodes were harvested for HIV-1 viral RNA detection by in-situ hybridization. In parallel, CD34⁺ humanized mice (3/time point) compared TFV and EVG drug levels in vaginal tissues from nanoparticles and solution. TFV and EVG were analyzed from tissue using liquid chromatograph-tandem mass spectrometry (LC-MS/MS).

Results: TAF + EVG nanoparticles were less than 200 nm in size. In-vitro prophylaxis indicates TAF + EVG nanoparticles 90% inhibition concentration was 0.002 $\mu\text{g/ml}$ and TAF + EVG solution was 0.78 $\mu\text{g/ml}$. TAF + EVG nanoparticles demonstrated detectable drugs for 14 days and 72 h for solution, respectively. All hu-BLT control mice became infected within 14 days after HIV-1 challenge. In contrast, hu-BLT mice that received nanoparticles and challenged at 4 days post-nanoparticle injection, 100% were uninfected, and 60% challenged at 14 days post-nanoparticle injection were uninfected ($P = 0.007$; Mantel–Cox test). In-situ hybridization confirmed these results.

Conclusion: This proof-of-concept study demonstrated sustained protection for TAF + EVG nanoparticles in a hu-BLT mouse model of HIV vaginal transmission.

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Introduction

Currently, approximately 36.9 million people worldwide are living with HIV-1 [1]. Orally administered

antiretroviral drugs for preexposure prophylaxis (PrEP) as a preventive strategy have demonstrated efficacy in diverse groups of high-risk individuals [2–6]. Indeed, oral antiretroviral therapy has shown significant protection

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from HIV-1 among people who are at high risk such as MSM, injection drug users, and serodiscordant couples [7]. However, several clinical trials with oral tenofovir (TFV) and TFV disoproxil fumarate/emtricitabine (TDF/FTC) have been terminated early due to lack of efficacy [8,9] predominantly due to nonadherence issues. Trial participants have needed to ingest TFV or TDF/FTC daily for HIV prevention. The use of 'on demand' TDF/FTC (Truvada) has also demonstrated significant efficacy for MSM [10]. In the iPERGAY study of 'on demand' antiretrovirals, TDF/FTC were taken before and after sexual activity. However, there are significant side effects with daily Truvada that can negatively impact adherence. An international survey among high-risk individuals has documented positive responses for a long-acting preventive option [11]. Thus, a long-acting injectable delivery system could be the 'on demand' requirement for PrEP against HIV-1 infection. This would promote prevention and provide significant adherence.

Strong evidence has been accumulating that there is a correlation between the antiretroviral tissue concentration and prevention efficacy. In the CAPRISA 004 trial of 1% TFV vaginal gel, efficacy was correlated with cervico-vaginal fluid levels more than 1000 ng/ml [12]. However, there are significant differences in nucleoside reverse transcriptase inhibitor (NRTI) drug accumulation pattern in different organs. Thompson *et al.* reported that FTC concentration is high in vaginal tissue, whereas rectal tissue shows TFV dominance after oral administration of Truvada [13]. Microbicide Trials Network (MTN-001) clinical trial compared vaginal tissue TFV levels after oral and vaginal preparations in HIV-1 negative women. Also drug administration route has shown differences in antiretroviral accumulation. The results showed TFV gel achieved 130-times higher levels of TFV active metabolite (TFV diphosphate) in vaginal tissue compared with oral TFV therapy [14]. Yet, preventive results with 1% TFV gel showed only 39% protection. Moreover, the Partners-in-PrEP clinical trial randomized oral TDF or TDF/FTC drugs among HIV-1 uninfected members of serodiscordant couples showed that plasma TFV levels at least 40 ng/ml were associated with an estimated 88% protective efficacy when TFV alone was ingested and 91% protection when consumed TDF/FTC combination [15]. TFV plasma threshold in this clinical trial was similar to the plasma level when patients take the drug daily without any missed doses. Therefore, a major factor is the route of administration and antiretroviral tissue accumulation. Thus our hypothesis is subcutaneous (SubQ) dosing indicates promise over other factors responsible for the less than optimal results. Therefore, current study was designed to investigate if tissue drug levels are among the critical factors that play a role in HIV-1 protection using a long-acting nanomedicines combining TFV alafenamide (TAF) and elvitegravir (EVG).

Our laboratory has been developing nanotechnology-based long-acting delivery systems for HIV treatment and

prevention [16–21]. The nano-formulation involved use of a Food and Drug Administration (FDA)-approved polymer to encapsulate antiretrovirals into nanoparticles to develop a novel sustained drug-delivery module for HIV-1 prevention. Through this report, we confirm successful encapsulation of two combination antiretrovirals (TAF + EVG) into nanoparticles (TAF + EVG nanoparticles) for PrEP delivery. We pursued combination antiretroviral (cARV) for encapsulation into the nanoparticle because clinical trials have documented a nonsignificant advantage to more than one drug in prevention trials compared with TFV alone [2,3,5,15]. Present results demonstrate SubQ administration of TAF + EVG nanoparticle shows efficacy in the humanized-bone marrow -liver -thymus (hu-BLT) mouse model against HIV-1 vaginal transmission.

Materials and methods

Materials

Poly(lactide-*co*-glycolide) (PLGA 75 : 25 lactide : glycolide ratio; M_w 4000–15 000), poly(vinyl alcohol) (PVA) (M_w 13 000–23 000), dichloromethane (DCM), acetonitrile (ACN), potassium dihydrogen phosphate (KH_2PO_4), and PBS were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Pluronic F127 (PF-127) and dimethyl sulfoxide (DMSO) were purchased from D-BASF (Edinburgh, UK) and Fisher BioReagents (Fair Lawn, New Jersey, USA), respectively. TAF and EVG (~100% purity) were generously provided by Gilead Sciences Inc. (Foster City, California, USA). LC-MS grade methanol, ACN, formic acid, and trifluoroacetic acid were purchased (Fisher). Cell line TZM-bl (PTA-5659) was purchased from ATCC (University Boulevard Manassas, Manassas, Virginia, USA). Dulbecco's High Glucose Modified Eagles Medium, fetal bovine serum, L-glutamine, trypsin, and penicillin-streptomycin solution were purchased from Hyclone (Logan, Utah, USA). All reagents were used as received without further purification.

Nanoparticle preparation and characterization

TAF + EVG loaded PLGA nanoparticles (TAF + EVG nanoparticles) were prepared by interfacial polymer deposition technique as described in our previous publication [17,18] with some modifications as described in Supplementary Fig. 1, <http://links.lww.com/QAD/B21>. Briefly, 100 mg of PLGA was dissolved in the 2.5 ml DCM (organic phase) containing an equivalent amount of PF-127 as stabilizer. TAF (50 mg) and EVG (50 mg) were added to the above organic phase, under constant magnetic stirring. The above organic phase with TAF + EVG + PLGA + PF-127 was then added to 15 ml of 1% PVA solution (aqueous phase). The above oil-in-water (o/w) emulsion was sonicated for 5 min using a probe sonicator having 10 s bursts (90% amplitude and 0.9 cycle). The organic phase was evaporated

overnight. Finally, the surfactants, free TAF + EVG and PVA were washed from nanoparticles using dialysis cassette (MWCO 30 kDa; Thermo Scientific; Rockford, Illinois, USA) in MilliQ water (18.2 MΩ). Washed TAF + EVG nanoparticles were freeze-dried in the Millrock LD85 lyophilizer (Kingston, New York, USA).

For physical characterization, an appropriate amount of freeze dried TAF + EVG nanoparticle was dispersed in MilliQ water at room temperature and size, polydispersity index (PDI) as well as zeta potential (surface charge) analysis were performed using a ZetaPlus Zeta Potential Analyzer instrument (Brookhaven Instruments Corporation, Holtsville, New York, USA). The topography of the TAF + EVG nanoparticles were evaluated by scanning electron microscopy (SEM) imaging using Hitachi S-4700 Field-emission SEM (New York, New York, USA) following method described in our previously published articles [16,20,21]. Experiments were performed in triplicate on five different batches of TAF + EVG nanoparticles.

The percentage encapsulation efficiency and percentage drug loading, were evaluated by high-performance liquid chromatography (HPLC) analysis (Shimadzu, Kyoto, Japan). Briefly, TAF + EVG nanoparticles (1 mg) were dissolved in 40% DMSO and were spun (at $14\,000 \times g$ for 5 min at 4 °C) and filtered through AmiconUltra Centrifugal filters (MWCO 30 kDa; Merck KGaA, Darmstadt, Germany). Similar procedure was followed with TFV + EVG solution (concentration range from 500 to 0.48 µg/ml of each drug in the mix) to generate the standard curve ($r^2 = 0.99$). The respective concentration of TFV and EVG was determined by HPLC analysis as explained [19] previously with modifications (method: Isocratic; Mobile phase: 25 mmol/l KH₂PO₄ 45%: ACN 55%; TFV and EVG: absorbance maximum at 260 and 313 nm, respectively, retention time 4 and 20 min, respectively). The TAF and EVG loading concentrations in 1 mg of TAF + EVG nanoparticles were estimated on the basis of the respective standard curve. The percentage encapsulation efficiency [Eq. (1)] and percentage drug loading [Eq. (2)] were estimated by the following formulas:

$$\%EE = \frac{\text{Amount of drug entrapped in NPs}}{\text{Amount of drug added to the emulsion}} \times 100 \quad (1)$$

$$\%DL = \frac{(\text{Amount of drug in the NPs})}{(\text{Amount of polymer} + \text{drug})} \times 100 \quad (2)$$

In-vitro prophylaxis study

Short-term (1-day pretreatment) in-vitro prophylaxis TAF + EVG nanoparticles vs TAF + EVG solution against HIV-1_{NL4-3} was performed using TZM-bl cell line as HIV-1 infection indicator cells [16,20]. Briefly, TZM-bl cells (10^5 cells/ml) were seeded in 96-well plate. After overnight incubation, cells were treated with different TAF + EVG concentrations (0.001–10 µg/ml)

as TAF + EVG nanoparticle and TAF + EVG solution. After 24-h treatment, the cells were washed thrice with warm PBS followed by inoculation with HIV-1_{NL4-3} virus (100 ng) for 4 h. The cells were then washed and incubated in fresh medium. Again, untreated/uninfected cells and untreated/infected cells were used as negative and positive controls, respectively. After 96-h incubation as a measure of HIV-1 infection, duplicate wells were used for determination of the luminescence using the BrightGLO (Promega, Madison, Wisconsin, USA) assay. The Synergy HT Multi-Mode Microplate Reader (BioTeck, Winooski, Vermont, USA) was used to read the luminescence. Luminescence was determined as relative luminescence units. The % protection from HIV-1 infection was calculated by using the equation below:

$$\begin{aligned} \%HIV-1 \text{ inhibition} (\% \text{ protection from HIV-1 infection}) \\ = \frac{(L_{\text{untreated}} - L_{\text{treated}})}{L_{\text{untreated}}} \times 100 \end{aligned} \quad (3)$$

where $L_{\text{untreated}}$ is the luminescence of untreated but HIV-1 infected TZM-bl cells (untreated/infected cells), and L_{treated} is the luminescence of respective TAF + EVG nanoparticle and TAF + EVG solution treated and HIV-1 infected TZM-bl cells (treated/infected cells). These experiments results are shown as mean ± SE of three independent experiments.

Pharmacokinetic assessment in humanized mice

CD34⁺ humanized NOD.Cg-Prkdc^{scid}IL2rg^{tm1Wjl}/Szj (non-obese diabetic (NOD)/severe combined immunodeficient (SCID)/IL2rgnull; NSG) mice (hu-CD34-NSG) were purchased from Jackson Laboratory. Mice ($n = 3$ /time point) were allowed to acclimate to the animal facility for 7 days. At the start of the experiments, mice were injected with 200 mg/kg (each drug) of TAF and EVG nanoparticle in 1 ml of 5% dextrose administered subcutaneously. At 1, 2, 4, 7, 10, and 14 days after injection of TAF + EVG nanoparticle, mice were sacrificed by carbon dioxide inhalation and cervical dislocation. Blood and organs were harvested. The organs included vagina [19]. Pharmacokinetic software (Phoenix WinNonLin; Certara Inc., St Louis, Missouri, USA) was used to determine pharmacokinetic parameters from the tissue concentration-time data. Area-under-the-vaginal-concentration-time data was determined using the trapezoid rule from the software. These tissue concentration-time data were used to determine the amount of drug at specific times at the site of infection.

LC-MS analysis of vaginal drug levels

To evaluate the in-vivo vaginal tissue pharmacokinetics of TFV and EVG, vaginal tissue samples were collected at different time points (day 1, 2, 4, 7, 10, and 14) from mice treated with 200 mg/kg each of TAF + EVG in nanoparticle formulation or solution. Vaginal tissue homogenate sample (100 µl) was mixed with 25 µl of

internal standard spiking solution followed by 100 μ l of 1% trifluoroacetic acid. Samples were vortexed and placed into SPE (Thermo-Fisher Scientific St. Louis, Missouri, USA) cartridges. The eluent was evaporated to dryness under a stream of nitrogen, reconstituted with 100 μ l of 50% ACN in water and 5 μ l was injected into the LC-MS/MS instrument. Chromatographic separation was carried out on the basis of our previous method [19]. Using a Restek (Bellefonte, Pennsylvania, USA) Pinnacle DB Biph (2.1 \times 50 mm², 5 μ m) column with isocratic mobile phase consisting of 0.1% formic acid in water and 0.1% formic acid in ACN (48:52 v/v) at a flow rate of 0.250 ml/min based on our previously published method. The mass spectrometer was operated in multiple reaction monitoring mode. Electrospray ionization source was operated in positive mode. Average inter-day and intraday variability of the assay was less than 10% according to the FDA analysis guidelines [22].

Generation of humanized-BLT mice

Hu-BLT mice were generated by following the previously published protocols [23,24]. Briefly, 6-week-old to 8-week-old NOD.Cg-Prkdc^{scid}IL2rg^{tm1Wjl}/Szj (NOD/SCID/IL2rgnull, NSG) mice (The Jackson Laboratory) were purchased and maintained in pathogen-free conditions at University of Nebraska-Lincoln Life Sciences Annex. Human fetal livers and thymus tissues were procured from Advanced Bioscience Resources (Alameda, California, USA). On the day of surgery, mice received 12 cGy/g of mouse body weight with an RS200 radio-graph irradiator (Rad Source Technologies, Suwanee, Georgia, USA). The irradiated mice were transplanted with two pieces of human fetal liver and one piece of thymic tissue fragments under the left kidney capsules, followed by injection of $1.5\text{--}2.3 \times 10^5$ fetal liver-derived CD34⁺ human stem cells intravenously. These mice were allowed to grow for another 12–16 weeks to regenerate human immune system. Those hu-BLT mice that showed the human leukocytes to total leukocytes ratio greater than 50% in the peripheral blood were considered for challenge with HIV-1 infection.

Ethics statement

All hu-NSG mouse pharmacokinetic experiments were performed at Creighton University and approved by the Institutional Animal Care and Use Committee (Protocol 0989). All mouse prophylaxis experiments adhered to the NIH Guide for the Care and Use of Laboratory Animals (Institutional Animal Care and Use Committee (Protocol no. 1322), University of Nebraska-Lincoln; UNL) [25]. The UNL IACUC Committee approved the Protocol 1322.

HIV-1 vaginal challenge in humanized-bone marrow-liver-thymus mouse

The hu-BLT ($n=15$) mice with more than 50% engraftment of human CD4⁺ cells were considered for these experiments. Of those, 10 mice treatment (Rx)

received 1 ml of TAF + EVG-nanoparticles (equivalent to 200 mg/kg each of TAF and EVG) in 5% dextrose subcutaneously. On day 4 and 14 days ($n=5$ each time point) post-nanoparticle injection treatment (Rx) mice received 5×10^5 TCID₅₀ in 20 μ l from two HIV transmission/founder viruses (WITO.c/2474 and SUMA.c/2821) from acutely infected patients intravaginally. In parallel, control mice ($n=5$) received blank nanoparticles and were infected with the same transmission/founder viruses. Rx-treated mice were divided into two Rx groups ($n=5$ each). Post-SubQ injection (post-nanoparticle injection) of TAF + EVG nanoparticles, first and second set of Rx group was vaginally challenged on day 4 and 14, respectively. To evaluate when mice became infected after HIV-1 challenge, weekly plasma viral load (pVL) was estimated and Kaplan-Meier curve fitting was used to evaluate the HIV-1 infectivity (Fig. 3).

Plasma viral load

Plasma viral RNA was extracted from plasma using QIAamp Viral RNA Mini kit (Qiagen, Valencia, California, USA). Viral RNA (vRNA) was extracted from the pellet with Proteinase K (2.5 μ g/ μ l; Life Technology, St Louis, Missouri, USA) and the High Pure Viral RNA kit (Diagnostics Corp., Indianapolis, Indiana, USA). Eluted vRNA (100 μ l) was eluted in 50 μ l, from which 20 μ l were reverse transcribed using MultiScribe Reverse Transcriptase (Life Technology) in a 50- μ l gene-specific reaction. Of which 14 μ l of cDNA were added to *TaqMan* gene expression master mix (Life Technology), along with primers: forward 5'-GCCTCAATAAA-GCTTGCTTGA-3', reverse 5'-GGGCGCCACTG-CTAGAGA-3' and a probe 5'-FAM/CCAGAGT-CACACAACAGACGGGCACA/BHQ_1/-3' targeting the gag region of HIV-1, and subjected to 45 cycles of qPCR analyses. Fluorescence signals were detected with an Applied Biosystems 7500 Sequence Detector (Life Technology). Data were captured and analyzed with Sequence Detector Software (Life Technology). Viral copy numbers were calculated by plotting C_t values obtained from samples against a standard curve generated with in vitro-transcribed RNA representing known viral copy numbers [26]. The limit of detection of the assay was 800 copies/ml plasma.

HIV-1 viral RNA detection in tissues using in-situ hybridization

In-situ hybridization (ISH) was conducted according to previously published method [26,27]. In brief, animal tissues of cervical, axillary, and mesenteric lymph nodes were collected after euthanasia and fixed in 4% paraformaldehyde. An amount of 6- μ m tissue sections of the cervical, lymph node tissues were cut and adhered to a SuperFrost Plus slide (Fisher Scientific, Hampton, New Hampshire, USA) fixed and air-dried. The sections were then rehydrated, permeabilized, and acetylated before hybridization to ³⁵S-labeled HIV antisense

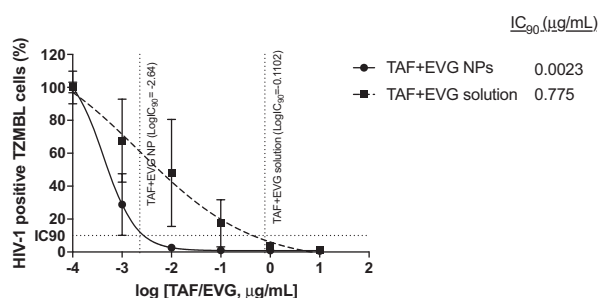


Fig. 1. Short-term prophylaxis study. TZM-bl indicator cells were used to determine 90% inhibition concentration of tenofovir alafenamide + elvitegravir nanoparticle compared. To tenofovir alafenamide + elvitegravir solution. The data presented mean \pm SE of five independent experiments (each performed in duplicate).

riboprobes that covered more than 90% of HIV-1 genome [26] and sense riboprobes as negative control. After washing and digestion with RNase, sections were coated with nuclear track emulsion, exposed for 7 days. Sections were then developed and counterstained with H&E stain (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Statistical analysis

All experiments represent values as mean \pm SEM of the obtained data. The 90% inhibition concentration was analyzed on the basis of log (agonist) vs response by using GraphPad Prism 5 software (La Jolla, California, USA). However, to evaluate HIV-1 viral infectivity function, Kaplan–Meier curve fitting (a nonparametric statistic) was performed. A *P* value of 0.05 or less was considered statistically significant.

Results

Characterization of nanoparticles

The interfacial polymer deposition by an oil-in-water emulsion solvent evaporation method resulted in well defined TAF + EVG nanoparticles. The dynamic light scattering analysis reveals TAF + EVG nanoparticles

averaged 190.2 ± 2.3 nm in size, with PDI of 0.14 ± 0.01 , and surface charge averaged -19.2 ± 1.7 ($n=5$; Supplementary Table 1, <http://links.lww.com/QAD/B21>). The percentage encapsulation efficiency for TAF and EVG into the polymeric nanoparticles averaged 54.1 ± 3.6 and $44.6 \pm 2.4\%$, respectively ($n=5$). Morphological analysis by SEM image (Supplementary Fig. 1, <http://links.lww.com/QAD/B21>) demonstrated that the nanoparticles obtained were well defined spherical particles with very uniform size distribution. Low PDI value (<0.2) is in agreement with the SEM image finding.

In-vitro prophylaxis studies

To evaluate the antiretroviral properties of TAF + EVG entrapped in nanoparticles, we performed a short-term prophylactic study on TZM-bl indicator cells (Fig. 1). The results of the in-vitro HIV-1 prophylaxis study ($n=5$) indicate the 90% inhibition concentration of the TAF + EVG nanoparticles was low at $0.0036 \mu\text{g/mL}$, and TAF + EVG solution was $0.107 \mu\text{g/mL}$. Therefore, in in-vitro condition TAF + EVG nanoparticles is ~ 30 times more efficacious compared with TAF + EVG solution *in vitro*. These results suggest the high intracellular drug levels from the nanoparticle formulation could potentially be responsible for preventing HIV-1 infection in TZM-bl cells at a lower drug concentration (nanogram) compared with TAF + EVG solution. These results demonstrate the nano-formulation improves the intracellular delivery, retention, and controlled release of TAF + EVG for sustained protection from HIV-1 challenge.

Pharmacokinetic experiments in hu-CD34-NSG mice

Drug concentrations in vaginal tissues were compared for nanoparticle formulation and soluble drugs (Fig. 2). Data revealed that TAF + EVG when entrapped in the nanoparticle leads to detectable antiretroviral concentrations through the entire 14-day study period, whereas soluble drugs administered in solution had detectable drug concentrations for only 72 h. Area-under-the-vagina-concentration-time profile ($\text{AUC}_{0-\text{last conc}}$) from the nanoparticle formulation averaged $168\,967.2 \text{ ng/mL}$ for TFV and $33\,993.6 \text{ h ng/mL}$ for EVG, respectively. This

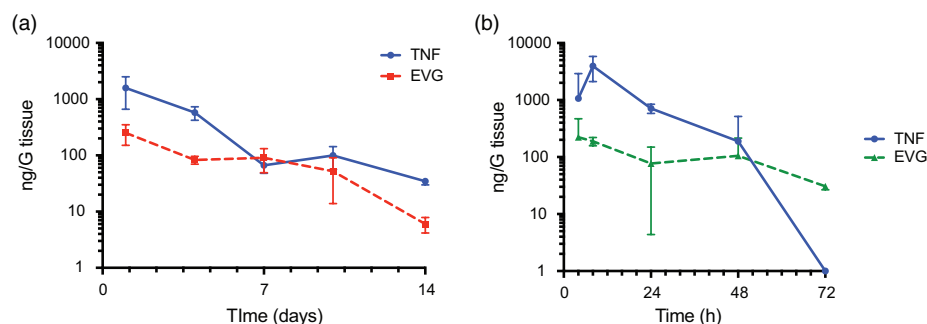


Fig. 2. Antiretroviral drugs (TNF + elvitegravir) concentrations over time (three mice/time point) from nanoparticles (a) or solution (b) given as 200 mg/kg per drug subcutaneous. Vaginal tissue samples were analyzed by LC–MS/MS.

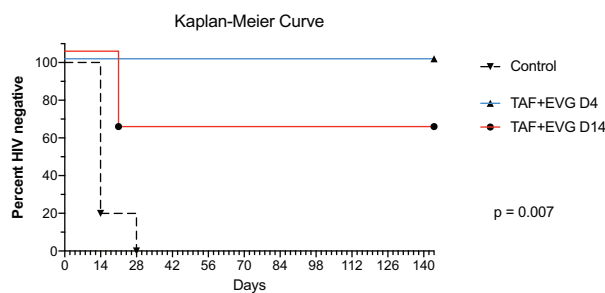


Fig. 3. Kaplan–Meier curve for humanized-BLT mice challenged with two strains of HIV-1 4 and 14 days after tenofovir alafenamide + elvitegravir nanoparticles given subcutaneous as 200 mg/kg dose. At 2-week postinfection (post-nanoparticle injection) plasma viral load was performed weekly to determine infectivity rate until week 6. Data represent percentage infection in five mice at each time point.

is compared with averaged 62 593.9 and 8802.8 h ng/ml for TFV and EVG, respectively when the same dose of TAF + EVG was administered in solution. Noncompartmental analysis reveals TFV and EVG from nanoparticle formulation results in respectively 2.6 and 3.8 times higher drug retention compared with drug administered in solution. In addition, EVG AUC was times higher in nanoparticle formulation than drug in solution. This demonstrates the sustained release properties and ease to tissue penetration of the nanoparticle formulation as compared with drugs in solution.

HIV prevention experiments in humanized-BLT mice

To evaluate the prevention efficacy of TAF + EVG nanoparticle *in vivo*, we performed prevention experiment in hu-BLT mice. Based on pVL (Fig. 3), mice challenged with HIV-1 transmission/founder viruses at 4 days post-nanoparticle injection TAF + EVG nanoparticles were all (100%) protected from HIV-1 infection throughout the study period (6 weeks after challenge). However, 14 days post-nanoparticle injection challenged mice showed 60% protected from HIV-1 infection over the entire study period ($P < 0.004$; Mantel–Cox test).

All of the mice were sacrificed 140 days after the end of the experiment and cervical, axillary, and mesenteric lymph nodes were harvested for ISH to evaluate for presence of HIV-1 viral RNA. As expected, all control animals were vRNA⁺ (Fig. 4). No additional animals were HIV positive by ISH for the 4-day and 14-day challenge.

Discussion

Currently, for PrEP against HIV-1 infection a long-acting injectable delivery system is the ‘on-demand’ requirement. Therefore, the goal of present experiments was to determine the sustained-release efficacy of a novel long-

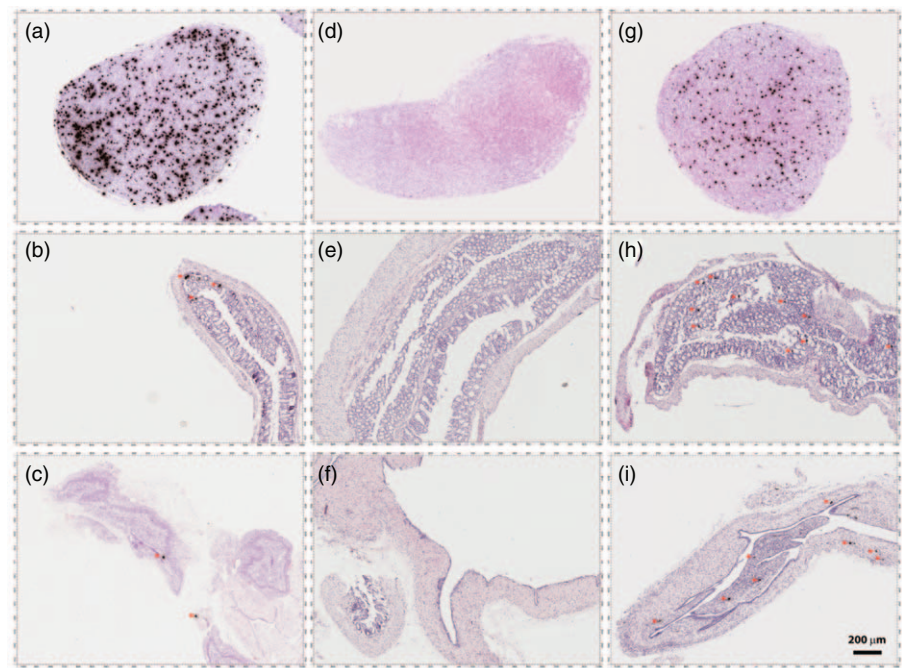


Fig. 4. HIV-1 vRNA detection in control (a–c), day 4 (d–f) and day 14 HIV(+) tissues (g–i). Representative images of HIV-1 vRNA detected in peritoneal lymph nodes (a, d, and g), female reproductive tract (cervix and vagina; b, e, and h) and colon tissues (c, f, and i). The clusters of black silver grains overlay HIV vRNA positive cells after radioautography of 35S-labeled HIV-specific riboprobes. Day 4 tissues serve as representative for Day 14 HIV-protected animals. * Shows HIV (+) cells. Scale bar = 200 μm for all images.

acting nanoformulation to prevent vaginal HIV-1 infection in hu-BLT mouse model. In parallel, we also determined tissue pharmacokinetics at the site of infection in hu-CD34-NSG mice. To the best of our knowledge, this is the first report of combining antiretroviral drugs in a single nano-formulation as a potential long-acting PrEP delivery modality to protect from HIV-1 infection. Furthermore, this is the first report of conducting simultaneous tissue pharmacokinetic study of TAF and EVG in the organs of hu-NSG mouse model.

Thus far the Partners-in-PrEP clinical trial reported that plasma TFV concentrations more than 40 ng/ml had shown significantly higher protection [15]. This along with other studies have also conferred that people receiving TDF/FTC for prevention have shown higher protection against HIV-1 compared with those receiving TDF alone [2,3,5,22]. However, the above-reported maintenance of plasma antiretroviral drug concentration is only possible when the volunteers remain adhered to daily drug intake regime. Due to nonadherence, several clinical trials have shown lack of efficacy leading to early study termination [8,9].

We chose TAF (an FDA-approved NRTI drug) as ester prodrug of TFV over TDF, as TAF has been reported to be as effective as TDF in HIV-1 suppression and less toxic to kidneys and bone. Systemically TAF penetrates into tissue better than TDF [28]. As another component of antiretroviral combination regimen, we chose EVG, an anti-HIV integrase strand transfer inhibitor [29]. However, not much literature could be found regarding the concentration–response relationship for EVG. Oral administered EVG (150 mg daily) was found to have peak plasma concentrations at 4 h, and the plasma half-life corresponds to 7.6 h. Massud *et al.* reported in macaques administration of 50 mg/kg EVG orally the plasma drug concentrations were similar to humans [29], and it shows the highest penetration in rectal and vaginal fluids. However, EVG administered orally was detectable in vaginal secretions from macaques for only 24 h. Therefore, keeping this in mind we formulated a potential long-acting combination antiretrovirals (TAF + EVG) nanoparticles for PrEP application. These two drugs in the formulation could show positive suppression of vaginal HIV-1 infectivity.

To date, there is no clear report that specifies the vaginal TFV concentration that is necessary for protection against HIV-1 [30]. So far, the vaginal tissue concentrations after a single oral dose of Truvada showed TDF ester prodrug concentration to be 7 ng/g [31]. Veselinovic *et al.* determined TFV (prodrug form of TDF) levels at steady-state after oral gavage of TDF (61.5 mg/kg) in humanized and nonhumanized mice [31]. They reported a median peak vaginal TFV level to be 729 ng/g after oral administration in Rag [2] knockout mice. The present study demonstrates SubQ administration of TAF + EVG

nanoparticles leads to higher accumulation of TFV in humanized mouse vaginal tissues. Therefore, higher and steady antiretroviral levels within vaginal tissue due to nano-encapsulation could potentially show better protection from HIV-1 infection.

Next we evaluated the TAF + EVG nanoparticle protection efficacy against HIV-1 challenge. The hu-NSG mouse is a good model to use to determine tissue drug levels that closely resemble the hu-BLT mice. Median TFV and elvitegravir vaginal drug levels at day 4 averaged 577.7 and 83 ng/g, respectively, resulted in 100% protection. This compares with median TFV and EVG vaginal drug levels at day 14 were 34.6 ng/g and less than 10 ng/g, respectively, resulted in 60% efficacy in hu-BLT mice (Figs. 2 and 4). Moreover, the in-vitro 90% inhibition concentration concentrations in TZM-bl cells demonstrated a low concentration (3.6 ng/ml) for the combined TAF + EVG nanoparticle formulation (Fig. 1). The in-vitro 90% inhibition concentration estimation could explain the 60% prevention efficacy on day 14 challenged mice. Certainly, a larger animal model would lend to a more accurate assessment of HIV-1 protection when assessing the efficacy of these combination nanoparticles.

This preliminary proof-of-concept study demonstrated TAF + EVG fabricated into a nanoformulation using a FDA-approved polymer resulting in detectable drug concentrations in humanized mice for up to 14 days. HIV-1 efficacy was shown over a prolonged period 100% when challenged at 4 days post-nanoparticle injection and 60% when challenged at 14 days post-nanoparticle injection. Further research using other antiretrovirals encapsulated into nanoformulations would be of interest. Also, an interesting future study could be SubQ delivery of the combination antiretroviral nanoformulations and then rectal challenge with HIV-1.

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Conflicts of interest

There are no conflicts of interest.

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