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

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Abstract

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

Design—Prospective prevention study in hu-BLT mice.

Methods—Using an oil-in-water emulsion solvent evaporation technique, TAF+EVG drugs were entrapped together into NPs containing poly(lactic-co-glycolic acid) (PLGA). *In vitro* prophylaxis studies (IC₉₀) compared NPs to drugs in solution. Humanized-BLT (n=5/group) mice were given 200 mg/kg SubQ, and vaginally challenged with HIV-1 (5×10⁵ TCID₅₀) 4 and 14 days (d) post-NP administration (PI). Control mice (n=5) were challenged at 4 d. Weekly plasma viral load (pVL) 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 (ISH). In parallel, CD34⁺ humanized mice (3/ time point) compared tenofovir (TFV) and EVG drug levels in vaginal tissues from NPs and solution. TFV and EVG were analyzed from tissue using LC-MS/MS.

Results—TAF+EVG NPs were < 200 nm in size. *In-vitro* prophylaxis indicates TAF+EVG NPs IC₉₀ was 0.002 µg/mL and TAF+EVG solution was 0.78 µg/mL. TAF+EVG NPs demonstrated detectable drugs for 14 days and 72 h for solution, respectively. All Hu-BLT control mice became infected within 14 d after HIV-1 challenge. In contrast, hu-BLT mice that received NPs and challenged at 4 d PI, 100% were uninfected, and 60% challenged at 14 d PI were uninfected (p = 0.007; Mantel-Cox test). ISH confirmed these results.

Conclusions—This proof-of-concept study demonstrated sustained protection for TAF+EVG NPs in a hu-BLT mouse model of HIV vaginal transmission.

Keywords

tenofovir alafenamide; elvitegravir; PLGA nanoparticles; HIV-1 prevention; humanized mouse model

Introduction

Presently, approximately 36.9 million people worldwide are living with human immunodeficiency virus-1 (HIV-1) (1). Orally administered antiretroviral drugs (ARVs) for pre-exposure prophylaxis (PrEP) as a preventative strategy have demonstrated efficacy in diverse groups of high-risk individuals (2–6). Indeed, oral ARV therapy has shown significant protection from HIV-1 among people who are at high risk such as men having unprotected sex with men (MSM), injection drug users, and serodiscordant couples (7). However, several clinical trials with oral tenofovir (TFV) and tenofovir disoproxil fumarate/emtricitabine (TDF/FTC) have been terminated early due to lack of efficacy (8,9) predominantly due to non-adherence 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” ARVs, 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 preventative 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 ARV tissue concentration and prevention efficacy. In the CAPRISA 004 trial of 1% TFV vaginal gel, efficacy was correlated with cervico-vaginal fluid levels >1000 ng/mL (12). However, there are significant differences in Nucleoside Reverse Transcriptase Inhibitor (NRTI) drug accumulation pattern in different organs. Kashuba, 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 ARV accumulation. The results showed TFV gel achieved 130-times higher levels of TFV active metabolite (TFV di-phosphate) in vaginal tissue compared to oral TFV therapy (14). Yet, preventative 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 > 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 route of administration and ARV 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 tenofovir 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 FDA-approved polymer to encapsulate ARVs into nanoparticles (NPs) to develop a novel sustained drug-delivery module for HIV-1 prevention. Through this report we confirm successful encapsulation of two combination ARVs (TAF + EVG) into nanoparticles (TAF +EVG NPs) for PrEP delivery. We pursued combination antiretroviral (cARV) for encapsulation into the NP because clinical trials have documented a non-significant advantage to > 1 drug in prevention trials compared to TFV alone (2,3,5,15). Present results demonstrate SubQ administration of TAF+EVG NP shows efficacy in the humanized-BLT (hu-BLT) mouse model against HIV-1 vaginal transmission.

Materials and Methods

Materials

Poly(lactide co-glycolide) [PLGA 75:25 lactide:glycolide ratio; Mw 4,000–15,000], poly (vinyl alcohol) (PVA) (M.W. 13,000–23,000), dichloromethane (DCM), acetonitrile (ACN), potassium dihydrogen phosphate (KH_2PO_4) and Phosphate Buffered Saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pluronic F127 (PF-127) and dimethyl sulfoxide (DMSO) were purchased from D-BASF (Edinburgh, UK) and Fisher BioReagents (Fair Lawn, NJ, USA), respectively. TAF and EVG (~100% purity) were generously provided by Gilead Sciences Inc. (Foster City, CA, USA). LC-MS grade methanol, acetonitrile, formic acid, and trifluoroacetic acid were purchased (Fisher). Cell line TZM-bl (PTA-5659) was purchased from ATCC® (University Boulevard Manassas, VA 20110 USA.) Dulbecco's High Glucose Modified Eagles Medium (DMEM), fetal bovine serum (FBS), L-Glutamine, trypsin and penicillin-streptomycin (Penstep) 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 NPs (TAF+EVG NPs) were prepared by interfacial polymer deposition technique as described in our previous publication (17,18) with some modifications as described in supplementary Figure 1. Briefly, 100 mg of poly lactic-co-glycolic acid (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 minutes using a probe sonicator having 10 sec bursts (90% amplitude and 0.9 cycle). The organic phase was evaporated overnight (O/N). Finally, the surfactants, free TAF+EVG and PVA were washed from NPs using dialysis cassette (MWCO 30kDa; Thermo Scientific; Rockford, IL, USA) in MilliQ (MQ water; 18.2 MΩ). Washed TAF+EVG NPs were freeze-dried in the Millrock LD85 lyophilizer (Kingston, NY, USA).

For physical characterization, an appropriate amount of freeze dried TAF+EVG NP was dispersed in MQ water at room temperature (RT) 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, NY, USA). The topography of the TAF+EVG NPs were evaluated by Scanning Electron Microscopy (SEM) imaging using Hitachi S-4700 Field-emission SME (New York, NY, USA) following method described in our previously published articles (16,20,21). Experiments were performed in triplicate on five different batches of TAF+EVG NPs.

The percentage encapsulation efficiency (%EE) and drug loading (% DL), were evaluated by high performance liquid chromatography (HPLC) analysis (Shimadzu, Kyoto, Japan). Briefly, TAF+EVG NPs (1 mg) were dissolved in 40% DMSO, and were spun (at 14000×g for 5 mins at 4°C) and filtered through Amicon®Ultra Centrifugal filters (MWCO 30KDa; 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 TAF and EVG was determined by HPLC analysis as explained (19) previously with modifications (method: Isocratic; Mobile phase: 25mM KH₂PO₄ 45%: ACN 55%; TAF and EVG: absorbance maximum at 260 and 313 nm respectively, retention time 4 min and 20 min respectively). The TAF and EVG loading concentrations in 1 mg of TAF+EVG NPs, was estimated based on the respective standard curve. The %EE (Equation 1) and % DL (Equation 2) were estimated by the following formulas:

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

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

In-vitro prophylaxis study

Short-term (1 day pre-treatment) *in vitro* prophylaxis TAF+EVG NPs 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 (10⁵ cells/mL) were seeded in 96-well plate. After O/N incubation, cells were treated with different TAF+EVG concentrations (0.001–10 µg/mL) as TAF+EVG NP 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 (100ng) 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, WI, USA) assay. The Synergy HT Multi-Mode Microplate Reader (BioTeck, Winooski, USA) was used to read the luminescence. Luminescence was determined as relative luminescence units (RLU). The % protection from HIV-1 infection was calculated by using the equation below:

$$\%HIV-1\ inhibition\ (\% protection\ from\ HIV-1\ infection) = \frac{(L_{untreated} - L_{treated})}{L_{untreated}} \times 100$$

Equation 3

where $L_{untreated}$ 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 NP and TAF+EVG solution treated plus HIV-1 infected TZM-bl cells (treated/infected cells). These experiments results are shown as mean $\pm$ SE of three independent experiments.

Pharmacokinetic assessment in humanized mice

CD34+ humanized NOD.Cg-Prkdc^{scid}IL2rg^{tm1Wjl}/Szj (NOD/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 seven days. At the start of the experiments, mice were injected with 200 mg/kg (each drug) of TAF and EVG NP in 1 mL of 5% dextrose administered subcutaneously. At 1, 2, 4, 7, 10, 14 days after injection of TAF+EVG NP, 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.) 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, 14) from mice treated with 200 mg/kg each of TAF+EVG in NP formulation or solution. Vaginal tissue homogenate sample (100 μ L) was mixed with 25 μ L of internal standard spiking solution followed by 100 μ L of 1% trifluoroacetic acid. Samples were vortexed and placed into SPE cartridges. The eluent was evaporated to dryness under a stream of nitrogen, reconstituted with 100 μ L of 50% acetonitrile in water and 5 μ L was injected into the LC-MS/MS instrument. Chromatographic separation was carried out based on our previous method (19). Using a Restek Pinnacle DB Biph (2.1mm $\times$ 50mm, 5 μ m) 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 based on our previously published method. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. Electrospray ionization (ESI) source was operated in positive mode. Average inter-day and intra-day variability of the assay was < 10% according to the FDA analysis guidelines (22).

Generation of hu-BLT mice

Hu-BLT mice were generated by following the previously published protocols (23,24). Briefly, six- to eight-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, CA, USA). On the day of surgery, mice received 12 cGy/g of mouse body weight with an RS200 X-ray irradiator (Rad Source Technologies). 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 (HSCs) intravenously (IV). 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 #1322), University of Nebraska-Lincoln; UNL) (25). The UNL IACUC committee approved the protocol #1322.

HIV-1 vaginal challenge in hu-BLT mouse

The hu-BLT (n=15) mice with > 50% engraftment of human CD4⁺ cells were considered for these experiments. Of those, 10 mice treatment (Rx) received 1 mL of TAF+EVG-NPs (equivalent to 200 mg/kg each of TAF and EVG) in 5% dextrose subcutaneously. On days 4 and 14 days (n=5 each time point) post NP injection (PI) treatment (Rx) mice received 5×10^5 TCID₅₀ in 20 µL from two HIV transmission/founder (T/F) viruses (WITO.c/2474 and SUMA.c/2821) from acutely infected patients intravaginally. In parallel, control (Ctr) mice (n=5) received blank NPs and were infected with the same T/F viruses. Rx treated mice were divided into two Rx groups (n=5 each). Post-SubQ injection (PI) of TAF+EVG NPs, 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 pVL was estimated and Kaplan-Meier curve fitting was used to evaluate the HIV-1 infectivity (Figure 3).

Plasma viral load (pVL)

Plasma viral RNA was extracted from plasma using QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA). Viral RNA (vRNA) was extracted from the pellet with Proteinase K (2.5 µg/µL; Life Technology) and the High Pure Viral RNA kit (Roche). Eluted vRNA (100 µL) was eluted in 50 µL, from which 20 µL were reverse transcribed using MultiScribe Reverse Transcriptase (Life Technology) in a 50-µL gene-specific reaction. Of which 14 µL of cDNA were added to TaqMan gene expression master mix (Life Technology), along with primers: forward 5'-GCCTCAATAAAGCTTGCCTTGA-3', reverse 5'-GGGCGCCACTGCTAGAGA-3' and a probe 5'-FAM/CCAGAGTCACACAACAGACGGGCACA/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. Data were captured and analyzed with

Sequence Detector Software (Life Technology). Viral copy numbers were calculated by plotting Ct 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 per ml plasma.

HIV-1 vRNA detection in tissues using *In situ* hybridization (ISH)

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. Six- μ m tissue sections of the cervical, lymph node tissues were cut and adhered to a SuperFrost Plus slide (Fisher Scientific, Hampton, NH USA) fixed and air-dried. The sections were then rehydrated, permeabilized, and acetylated prior to hybridization to 35 S-labeled HIV anti-sense riboprobes that covered >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 seven days. Sections were then developed and counterstained with H&E stain (Thermo Fisher Scientific, Waltham, MA USA).

Statistical Analysis

All experiments represent values as mean $\pm$ standard error of means (SE) of the obtained data. The 90 % Inhibition Concentration (IC₉₀) was analyzed based on log (agonist) vs. response by using GraphPad Prism 5 software (La Jolla). However, to evaluate HIV-1 viral infectivity function, Kaplan-Meier curve fitting (a nonparametric statistic) was performed. A p-value of 0.05 was considered statistically significant.

Results

Characterization of NPs

The interfacial polymer deposition by an oil-in-water emulsion solvent evaporation method resulted in well-defined TAF+EVG NPs. The dynamic light scattering analysis reveals TAF+EVG NPs 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). The %EE for TAF and EVG into the polymeric nanoparticles averaged $54.1 \pm 3.6\%$ and $44.6 \pm 2.4\%$, respectively (n=5). Morphological analysis by SEM image (Supplementary figure 1) demonstrated that the NPs 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 NPs, we performed a short-term prophylactic study on TZM-bl indicator cells (Figure 1). The results of the *in-vitro* HIV-1 prophylaxis study (n=5) indicate the IC₉₀ of the TAF+EVG NPs 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 NPs is ~30 times more efficacious compared to TAF+EVG solution *in vitro*. These results suggest the high intracellular drug levels from the NP formulation could potentially be responsible for preventing HIV-1 infection in TZM-bl cells at a lower drug concentration (nanogram) compared to 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 NP formulation and soluble drugs. (Figure 2) Data revealed that TAF+EVG when entrapped in the nanoparticle leads to detectable ARV concentrations through the entire 14 days study period, whereas soluble drugs administered in solution had detectable drug concentrations for only 72 h. Area-under-the-vagina-concentration-time profile ($AUC_{0\text{-last conc}}$) from the NP formulation averaged 168,967.2 h*ng/mL for TFV and 33,993.6 h*ng/mL for EVG, respectively. This is compared to averaged 62,593.9 h*ng/mL and 8,802.8 h*ng/mL for TFV and EVG, respectively when the same dose of TAF+EVG was administered in solution. Non-compartmental analysis reveals TFV and EVG from NP formulation results in respectively 2.6 and 3.8 times higher drug retention compared to drug administered in solution. Additionally, EVG AUC was times higher in NP formulation than drug in solution. This demonstrates the sustained release properties and ease to tissue penetration of the NP formulation as compared to drugs in solution.

HIV prevention experiments in Hu-BLT mice

To evaluate the prevention efficacy of TAF+EVG NP *in vivo*, we performed prevention experiment in hu-BLT mice. Based on pVL (Figure 3), mice challenged with HIV-1 T/F viruses at 4 days PI TAF+EVG NPs were all (100%) protected from HIV-1 infection throughout the study period (6 weeks after challenge). However, 14 days PI 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 *in situ* hybridization (ISH) to evaluate for presence of HIV-1 viral RNA. As expected all control animals were vRNA⁺ (Figure 4). No additional animals were HIV positive by ISH for the 4-day and 14-day challenge.

Discussion

Presently, 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-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 ARV 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 > 40 ng/mL had shown significantly higher protection (15). This along with other studies have

also conerred that people receiving TDF/FTC for prevention have shown higher protection against HIV-1 compared to those receiving TDF alone (2,3,5,22). However the above reported maintenance of plasma ARV drug concentration is only possible when the volunteers remain adhered to daily drug intake regime. Due to non-adherence, several clinical trials have shown lack of efficacy leading to early study termination (8,9).

We chose TAF (a Food and Drug Administration (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 ARV 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 (150mg daily) was found to have peak plasma concentrations at 4 h and the plasma half-life correspond 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 (31) 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 ARVs (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. 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 non-humanized mice [32]. 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 NPs leads to higher accumulation of TFV in humanized mouse vaginal tissues. Therefore, higher and steady ARV levels within vaginal tissue due to nano-encapsulation could potentially show better protection from HIV-1 infection.

Next we evaluated the TAF+EVG NP 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 tenofovir and elvitegravir vaginal drug levels at day 4 averaged 577.7 ng/g and 83 ng/g, respectively resulted in 100% protection. This compares to median TFV and EVG vaginal drug levels at day 14 were 34.6 ng/g and < 10 ng/g, respectively, resulted in 60% efficacy in hu-BLT mice (Figure 2, 4). Moreover, the *in vitro* IC₉₀ concentrations in TZM-bl cells demonstrated a low concentration (3.6 ng/mL) for the combined TAF+EVG NP formulation (Figure 1). The *in-vitro* IC₉₀ 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 PI and 60% when challenged at 14 days PI. Further research using other antiretroviral drugs encapsulated into nanoformulations would be of interest. Also, an interesting future study could be SubQ delivery of the combination ARV nanoformulations and then rectal challenge with HIV-1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Short-term prophylaxis study. TZM-bl indicator cells were used to determine IC₉₀ of TAF +EVG NP compared To TAF+EVG solution. The data presented mean ± SE of five independent experiments (each performed in duplicate)

Figure 2. Antiretroviral drugs (TNF+EVG) concentrations over time (3 mice/time point) from nanoparticles (A) or solution (B) given as 200 mg/kg/drug SubQ. Vaginal tissue samples were analyzed by LC-MS/MS.

Figure 3. Kaplan-Meier curve for Hu-BLT mice challenged with two strains of HIV-1 4 and 14 days after TAF+EVG NPs given SubQ as 200 mg/kg dose. At 2 weeks post-infection (PI) pVL was performed weekly to determine infectivity rate Until week 6. Data represents percentage infection in five mice at each time point.

Figure 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, G), female reproductive tract (cervix and vagina; B, E, H) and colon tissues (C, F, I). The clusters of black silver grains overlay HIV vRNA positive cells after radioautography of ³⁵S-labelled 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.