A Concept Evaluation Study of a New Combination Bictegravir plus Tenofovir Alafenamide Nanoformulation with Prolonged Sustained-Drug-Release Potency for HIV-1 Preexposure Prophylaxis

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ABSTRACT The antiretroviral treatment (ART) approach is the best-prescribed approach to date for preexposure prophylaxis (PrEP) for high-risk individuals. However, the daily combination antiretroviral (cARV) regimen has become cumbersome for healthy individuals, leading to nonadherence. Recent surveys showed high acceptance of parenteral sustained-release ART enhancing PrEP adherence. Our approach is to design a parenteral nanoparticle (NP)-based cARV sustained-release (cARV-SR) system as long-acting HIV PrEP. Here, we report a new combination of two potent ARVs (tenofovir alafenamide fumarate [TAF] and bictegravir [BIC]) loaded as a nanoformulation intended as a cARV-SR for PrEP. The BIC+TAF NPs were fabricated by using a standardized in-house methodology. *In vitro* intracellular kinetics, cytotoxicity, and HIV-1 protection studies demonstrated that BIC+TAF encapsulation prolonged drug retention, reduced drug-associated cytotoxicity, and enhanced HIV protection. In human peripheral blood mononuclear cells, nanoformulated BIC+TAF demonstrated significant ($P < 0.05$) improvement in the drug's selectivity index by 472 times compared to the BIC+TAF in solution. *In vivo* pharmacokinetic study of BIC, TAF, and respective drug metabolites in female BALB/c mice after single subcutaneous doses of BIC+TAF NPs demonstrated plasma drug concentrations of BIC and tenofovir above the intracellular 50% inhibitory concentration during the entire 30-day study period and prolonged persistence of both active drugs in the HIV target organs, including the vagina, colon, spleen, and lymph nodes. This report demonstrates that the encapsulation of BIC+TAF in a nanoformulation improved its therapeutic selectivity and the *in vivo* pharmacokinetics of free drugs. Based on these preliminary studies, we hypothesize that cARV-SR has potential as an innovative once-monthly delivery treatment for PrEP.

KEYWORDS tenofovir alafenamide, bictegravir, HIV, long acting, PrEP, pharmacokinetics, nanoformulation, antiretroviral

The acceptance of long-acting antiretroviral therapy (LA ART) has paved the way for new and innovative sustained-release drug delivery systems for human immunodeficiency virus (HIV) preexposure prophylaxis (PrEP). The LA ART potential has prompted the National Institute of Allergy and Infectious Diseases (NIAID) to launch an initiative to advance LA ART options to prevent and treat HIV infections. In fact, NIAID has already initiated a clinical trial (i.e., Long-Acting Therapy to Improve Treatment Success in Daily Life [LATITUDE]) (1) to evaluate LA ART HIV potency in patients who face challenges to remain adherent to conventional oral ART regimen. The patient acceptance of LA ART is striking (2–4) and may become standard therapy for antiretrovirals (ARVs) in the future (5). Cabotegravir, a long-acting injectable drug, has shown efficacy in HIV

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PrEP application. An ongoing HPTN 083 randomized, double-blind, phase 3 study (6) interim analysis (18 May 2020) indicated cabotegravir (administered once every 8 weeks) demonstrated improved efficacy compared to daily oral Truvada for PrEP (7).

Two-ARV ART regimens for PrEP are well accepted by at-risk individuals due to its ability to prevent HIV with acceptable tolerability. However, prescription uptake of PrEP regimens has not grown significantly. Healthy individuals may not wish to take an oral therapy for HIV prevention, and an LA nanoformulation may be well received. A recently approved integrase strand transfer inhibitor (INSTI), bictegravir (BIC; GS-9883) (8, 9) has gained popularity as an alternative to dolutegravir (DTG) due to its improved safety profile (10, 11). The 96-week results from a randomized, double-blind, phase 3 trial indicated that BIC is safe and well tolerated in chronic HIV infections (12). Tenofovir alafenamide (TAF), a tenofovir (TFV) prodrug, is a comparatively new nucleotide reverse transcriptase inhibitor (NRTI) compared to tenofovir disoproxil (TDF). TAF has gained popularity because of its lower plasma TFV levels, higher intracellular TFV-diphosphate (TFV-dp) levels, leading to a superior safety profile due to its fewer renal and bone adverse events compared to TDF (13–15). The use of TAF/emtricitabine/BIC, two NRTIs, and an INSTI combination (Biktarvy) is already available as a single tablet regimen for HIV treatment (16).

In this report, we evaluated the LA properties of a new combination ARV (BIC+TAF) as a potential next-generation regimen for PrEP. Our aim was to fabricate a long-acting, sustained-release (LA/SR) delivery system loaded with BIC+TAF for PrEP. Several patient surveys have determined enthusiasm and acceptability to parenteral LA ARVs (3, 4, 17). Furthermore, LA/SR delivery systems could reduce HIV transmission rate using a self-administered, subcutaneous (s.c.) route and could potentially improve ART adherence especially for PrEP (5, 18).

Therefore, we fabricated a unique combination of BIC, the second-generation integrase inhibitor, and TAF, a standard NRTI, as a two-drug-loaded nanoformulation with the aim to reduce adverse effects without compromising PrEP efficiency. For the LA nanoformulation, poly(lactide-coglycolide) (PLGA), a U.S. Food and Drug Administration (FDA)-approved biocompatible and biodegradable polymer, was utilized with already-established potency as the LA ARV delivery system (19–21). Our hypothesis is that s.c. application of BIC+TAF nanoformulation improves the drug retention kinetics in plasma as well as in the HIV target organs and thus could potentially become an alternative strategy to improve patient adherence to ART.

RESULTS

Physicochemical characteristics of BIC+TAF NPs. The oil-in-water (o-w) emulsion interfacial polymer deposition nanoformulation method yielded well-defined spherical nanoparticles (see Fig. S1 in the supplemental material). The size distribution by dynamic light scattering (DLS) analysis demonstrated the obtained BIC+TAF NPs were 228.8 ± 14.9 nm with uniform size distribution (polydispersity index [PDI] = 0.124 ± 0.035) and weak negative surface potential (-20.2 ± 1.4 mV). The shape and surface morphology (see Fig. S1) evaluation by scanning electron microscopy showed that the NPs were uniform, spherical, and smooth surfaced, similar to blank NPs (19, 20, 22). The percent encapsulation efficiency (%EE) values for both BIC and TAF in the BIC+TAF NPs, as evaluated by high-performance liquid chromatography (HPLC), were >50%. The percent drug loading (%DL) values showed that the BIC+TAF NPs were loaded with BIC ($6.5 \pm 1.2\%$) and TAF ($6.1 \pm 0.99\%$) at 1:1 ratios.

In vitro intracellular uptake/release and conversion kinetics of BIC+TAF NPs. The intracellular uptake/release, as well as the conversions of TAF to TFV (active drug form) to TFV-diphosphate (TFV-dp, active drug metabolite) and BIC from the nanoformulation compared to BIC+TAF solution, was evaluated in TZM-bl cells (23). Liquid chromatography-mass spectrometry (LC-MS)-based analysis was used to evaluate the unbound intracellular free drugs and metabolites. The uptake/release study over 24 h (Fig. 1) demonstrated that BIC+TAF NP endosomal uptake and release (20) induced biphasic drug-release kinetics, i.e., initial rapid release, followed by the sustained

FIG 1 *In vitro* intracellular uptake, release, and conversion. (A) Schematic diagram illustrates the intracellular PK of the (pro)drugs. In the endosome, due to low pH (3.6 to 5.5), the encapsulated TAF and BIC are released from the NP and transported into the cytosol. In the cytosol, BIC does not need conversion, whereas TAF (prodrug) gets conversion to TFV (active-drug) to TFV-diphosphate (TFV-dp; active-drug metabolite, that inhibits reverse transcriptase [RT] activity). (B to E) Comparative intracellular kinetics of BIC (B), TAF (C), TFV (D), and TFV-dp (E) upon BIC+TAF NP and BIC+TAF solution treatment. Each data point represents the means $\pm$ the SEM of three independent experiments.

release of both drugs (Fig. 1B and C). As expected, BIC, a hydrophobic drug, demonstrated enhanced retention (Fig. 1B) compared to TAF (Fig. 1C). The nanoformulation substantially prolonged intracellular elimination half-life ($t_{1/2}$) (see Table S1) and demonstrated a higher maximum concentration (C_{max}) and area under the plasma concentration-time curve (AUC_{all}) of BIC by 10-, 530-, and 1,377-fold compared to the drug in solution. Similarly, nanoformulated TAF demonstrated increased $t_{1/2}$, C_{max} , and AUC_{all} by 29-, 241-, and 323-fold, respectively, compared to TAF in solution.

The intracellular TFV and TFV-dp demonstrated a gradual intracellular increase over time, eventually reaching dynamic equilibrium between $TAF \rightleftharpoons TFV \rightleftharpoons TFV-dp$ (Fig. 1D and E). Before treatment wash-off, both BIC+TAF NP and BIC+TAF solution had similar drug-release kinetics of TFV or TFV-dp, indicating nanoencapsulation does not add substantially in the conversion rate of $TAF \rightleftharpoons TFV \rightleftharpoons TFV-dp$. However, in the absence of treatment pressure (drug treatment washoff 24 h posttreatment), the nanoformulated TAF demonstrated a spike in conversion and retention rate of TFV and TFV-dp compared to TFV derived from the solution (Fig. 1C and D). In either condition, the nanoformulation demonstrated higher accumulation and retention for BIC and TFV compared to drugs in solution. Overall, these results illustrate that BIC+TAF nanoencapsulation improves the drug/active metabolite intracellular concentration which is needed for effective inhibition of reverse transcriptase and integrase activity.

TABLE 1 Comparative study illustrating cytotoxicity (CC_{50}), HIV-1 protection (IC_{50}), and SI of BIC+TAF NPs versus BIC+TAF solution^a

Cell type	Treatment	Mean concn ($\mu\text{g/ml}$, each drug) $\pm$ the SEM		SI
		CC_{50}	IC_{50}	
TZM-bl cells	BIC+TAF NPs	52.3 ± 2.6	0.002 ± 0.003	27,526
	BIC+TAF solution	11.8 ± 1.9	0.04 ± 0.04	295
PBMCs	BIC+TAF NPs	35.7 ± 2.7	0.008 ± 0.002	4,246
	BIC+TAF solution	9.5 ± 2.3	1.1 ± 3.8	9

^aData are presented as the means of three independent experiments ($n = 3$) determined using three different batches of BIC+TAF NPs versus BIC+TAF solution (where, during each experiment, the treatments were considered in duplicate) on TZM-bl cells and on three healthy donors (where, during each donor study, the treatments were considered in triplicate) for PBMCs. SI, selectivity index; SEM, standard error of the mean.

In vitro cytotoxicity evaluation. The cytotoxicity of the nanoformulated BIC+TAF compared to BIC+TAF drugs in solution were evaluated on the TZM-bl cell line and human peripheral blood mononuclear cells (PBMCs) (Table 1). The untreated cells (negative control) were considered 100% viable, and all the data presented were evaluated using equation 3 (see below). The cytotoxicity evaluation in TZM-bl cells demonstrated that the nanoformulation reduced cytotoxicity (half-maximal cytotoxic concentration [CC_{50}]) of BIC+TAF by 5-fold ($52.3 \pm 2.6 \mu\text{g/ml}$) compared to free drugs in solution ($11.8 \pm 1.9 \mu\text{g/ml}$). In PBMCs (primary cells), nanoformulated TAF+BIC demonstrated a 3.7-fold-higher CC_{50} (nanoformulation $35.67 \pm 2.69 \mu\text{g/ml}$) compared to the BIC+TAF solution (nanoformulation $9.53 \pm 2.32 \mu\text{g/ml}$). The results suggest drug nanoencapsulation reduces the cytotoxicity of free BIC and TAF.

In vitro HIV protection study. The HIV protection potency of the BIC+TAF nanoformulation compared to the free BIC+TAF solution was based on a short-term HIV-1 protection study in TZM-bl cells and human PBMCs (Table 1). The HIV infectivity study of TZM-bl cells demonstrated nanoencapsulation the reduced the half-maximal inhibitory concentration (IC_{50}) significantly ($P < 0.05$) 21-fold ($0.0019 \pm 0.0033 \mu\text{g/ml}$) compared to free drugs in solution ($0.04 \pm 0.039 \mu\text{g/ml}$), whereas the nanoformulation lowered the IC_{50} significantly for PBMCs ($P < 0.05$) 131-fold ($0.0084 \pm 0.0016 \mu\text{g/ml}$) compared to BIC+TAF drugs in solution ($1.12 \pm 3.8 \mu\text{g/ml}$). Based on previous studies (19, 20, 22) and these *in vitro* results, nanoencapsulated BIC+TAF could significantly improve the protective efficacy of free drug for PrEP.

To sum up, nanoencapsulation of BIC+TAF compared to same drugs in solution demonstrated a significant improvement in *in vitro* protection and a reduction in cytotoxicity. Thus, nanoformulation (BIC+TAF NPs) significantly improved the selectivity index (SI) of drugs compared to soluble BIC+TAF (Table 1) by ~ 472 -fold in PBMCs (primary target cells) and 93-fold in TZM-bl cells (cell line).

In vivo drug distribution and pharmacokinetics study. The *in vivo* biodistribution and pharmacokinetics (PK) of BIC and TAF, as well as its metabolites (i.e., TFV and TFV-dp), were evaluated in BALB/c mice (6 weeks old, female) over 30 days after a single BIC+TAF NP s.c. administration (at 200 mg/kg/drug) by LC-MS analysis (Table 2 and Fig. 2). The s.c. administration of the nanoformulation demonstrated peak concentrations (C_{max}) of 10.5 ± 0.2 and $11.4 \pm 4.1 \mu\text{g/ml}$ of BIC and TFV in plasma, respectively, and the TFV-dp concentration averaged $12.2 \pm 1.1 \text{ pmol}/10^6$ in PBMCs (Table 2). The vaginal C_{max} of TFV ($49.4 \pm 17.2 \mu\text{g/g}$) was ~ 4 -fold higher compared to colonic tissue ($13.3 \pm 1.5 \mu\text{g/g}$), whereas BIC showed an ~ 2 -fold higher vaginal C_{max} ($11.4 \pm 1.5 \mu\text{g/g}$) compared to colonic tissue ($6.5 \pm 1.2 \mu\text{g/g}$). This is consistent with previously published studies, which show hydrophilic drugs tend to accumulate faster in vaginal compared to colonic tissue (20). TFV (a hydrophilic active drug form [24, 25] derived from TAF prodrug) had a faster assimilation rate T_{max} of 1 h than BIC (hydrophobic drug, T_{max} of 6 h). The AUC_{all} demonstrated 2-fold lower TFV concentrations in tissues (vagina [$453.6 \pm 84.3 \text{ h } \mu\text{g/g}$] compared to colon [$1,038.1 \pm 82.3 \text{ h } \mu\text{g/g}$]), whereas BIC had the

TABLE 2 Biodistribution of BIC and TFV in plasma and tissue after s.c. BIC+TAF NP treatment of BALB/c mice ($n = 4$)^a

PK parameter	Plasma		Vagina		Colon		PBMCs
	BIC	TFV	BIC	TFV	BIC	TFV	TFV-dp
Mean $C_{max} \pm SEM$	10.5 $\pm$ 0.2 ^A	11.4 $\pm$ 4.1 ^A	11.4 $\pm$ 1.5 ^B	49.4 $\pm$ 17.2 ^B	6.5 $\pm$ 1.2 ^B	13.3 $\pm$ 1.5 ^B	12.2 $\pm$ 1.1 ^C
T_{max} (h)	1	1	6	1	1	6	1
Mean $AUC_{all} \pm SEM$	2,276.6 $\pm$ 228.8 ^E	70.5 $\pm$ 12.7 ^E	1,053.6 $\pm$ 120.4 ^F	453.6 $\pm$ 84.3 ^F	572.1 $\pm$ 56.3 ^F	1,038.1 $\pm$ 82.3 ^F	294.6 $\pm$ 19.8 ^G
$t_{1/2}$ (h)	112.8	105.6	105.7	41.3	103.1	57.2	49.1

^aTFV, tenofovir; BIC, bicitrigravir; TFV-dp, tenofovir diphosphate; C_{max} , maximum concentration; $t_{1/2}$, half-life; T_{max} , time of maximum concentration. The superscript letters A, B, and C indicate the C_{max} values in plasma ($\mu\text{g/ml}$), tissue ($\mu\text{g/g}$), and PBMCs (pmol/ 10^6 cells), respectively. AUC_{all} , area under the concentration-time curve. The superscript letters E, F, and G indicate AUC_{all} values in plasma ($\text{h } \mu\text{g/ml}$), tissue ($\text{h } \mu\text{g/g}$), and PBMCs ($\text{h pmol}/10^6$ cells), respectively. Mean AUC_{all} and $C_{max} \pm SEM$ values were obtained from four independently treated mice ($n = 4$).

opposite trend of ~ 2 -fold-higher vaginal ($1,053.6 \pm 120.4 \text{ h } \mu\text{g/g}$) compared to the colonic ($572.1 \pm 56.3 \text{ h } \mu\text{g/g}$) tissue. The plasma elimination half-life ($t_{1/2}$) averaged 105.6 and 112.8 h for TFV and BIC, respectively, whereas, in PBMCs, the vagina, and the colon, the TFV $t_{1/2}$ averaged 49.1, 41.3, and 51.2 h, respectively. BIC demonstrated a longer $t_{1/2}$, averaging 105.7 and 103.1 h in the vagina and the colon, respectively. In addition, both TAF and BIC drug showed biphasic drug-elimination kinetics (Fig. 2; see also Fig. S2). The extended plasma and tissue half-lives for both drugs demonstrate the LA nature of the nanoformulation. Figure 2 demonstrates that throughout the 30-day experiment, the TFV and BIC concentrations were maintained above their respective IC_{50} s in plasma. In the spleen and lymph node, both BIC and TFV concentrations were above the respective IC_{50} value (see Fig. S2). The nanoformulation injection site maintained high BIC+TFV levels similar to a drug-depo and observed high drug concentrations until the end of the study at the injection site (see Fig. S2).

DISCUSSION

Current sustained-release ARV strategies hold promises for the prevention of HIV, mainly to improve patient adherence (2–4). Improving adherence would reduce the incidence of new HIV infections for at-risk individuals. In these experiments, a new ARV combination was explored for PrEP. Our strategy was to explore LA nanoformulation for newer ARVs. Various approaches to achieve LA ARV, such as polymeric nanoformulation, wet milling, and the use of solid lipid NPs, have been evaluated to be potential candidates that could prolong ARV plasma half-life (19, 20, 22, 26–31). We adapted the polymeric nanoformulation method (19, 20, 22, 26, 27) to introduce sustained-release properties to prolong BIC+TAF retention for HIV protection.

The nanoencapsulation of BIC+TAF in PLGA polymer-based nanoformulation introduces various unique properties to the soluble BIC+TAF drugs. These properties include reducing drug elimination and prolonging systemic retention (19, 20, 22, 27, 32), as well as improved drug solubility and stability (32–34). The BIC+TAF nanoformu-

FIG 2 Graphical presentation of *in vivo* BIC (left) and TFV (right) level in plasma and primary HIV infection and entry site (i.e., vagina and colon) over 30 days after s.c. administration of BIC+TAF NP (200 mg/kg for each drug). Data are presented as means $\pm$ the SEM ($n = 4$). The dotted straight line represents the respective drug's IC_{50} values.

lation was uniformly sized at <300 nm with a weak surface negative charge (see Fig. S1), ensuring that the nanoparticle will not cause fine capillary clogging and reduced nonspecific binding (35–37). The NP's small size will also ensure invisibility to the immune system (38, 39). In addition, upon intracellular endosomal degradation, the PLGA polymer degrades to lactic acid and glycolic acid, which could be either utilized in the Krebs cycle or could be eliminated as other cellular by-products through the kidney or liver, resulting in a minimal residual effect (40, 41).

The difference in the BIC and TAF retention patterns was observed because of the difference in the release mechanism from the polymeric scaffold (pScaffold). The drug molecules get entrapped in the pScaffold by adsorption and remain attached due to physical interaction (hydrophobic interaction, as well as weak interaction forces such as H-bonding and Van der Waals forces); however, no covalent bonding between the drug and polymeric component was expected. The hydrophobic interactions are stronger than weak interaction forces (42–44). Hence, both drugs have different adherence affinities to the pScaffold. TAF, a hydrophilic drug (0.236 mg/ml in water), gets adsorbed due to weak interaction forces only; thus, it is retained in plasma for 14 days. In contrast, BIC, a highly hydrophobic drug (0.0537 mg/ml in water), gets adsorbed due to hydrophobic interaction, as well as weak interaction forces, and thus is retained longer (day 20). Therefore, due to differences in physicochemical characteristics, both drugs show different PK behaviors even when they are in the same pScaffold. Both TAF (45) and BIC (22) lipophilicity (46, 47) and PLGA nanoformulation drug release properties (20, 48) could be the reason behind the prolonged bimodal-elimination kinetics (Fig. 2 and Fig. S2). The biphasic-elimination kinetics of BIC are more evident due to its higher lipophilic characteristics compared to TAF (26, 27). After release from the pScaffold, the drug molecule acts like the free drug and follows the same fate as the free drug.

The intracellular release kinetics study (Fig. 1B and C) indicates BIC+TAF nanoformulation resulted in higher TAF and BIC intracellular concentrations compared to the BIC+TAF solution. This could be due to intracellular kinetics dependency on the drug retention time. Intracellular drug retention depends on two factors: metabolism and excretion (49). Cells maintain cellular homeostasis by inducing drug metabolism occurs mainly by drug transformation, whereas efflux transporters (such as ATP-binding cassette [ABC] transports) are involved in excreting molecules out of the cell (50). The nanoformulated drugs demonstrated biphasic drug release (20, 48) and could be attributed to the higher and prolonged retention of both drugs, compared to soluble drugs. In both forms—as NPs and in solution—TAF showed a lower intracellular concentration compared to BIC (Fig. 1B and C) due to TAF conversion to active TFV and TFV-dp. Intracellular TAF is metabolized to TFV and TFV-dp (as schematically depicted in Fig. 1A), reaching a thermodynamic equilibrium: $\text{TAF} \rightleftharpoons \text{TFV} \rightleftharpoons \text{TFV-dp}$. Once thermodynamic equilibrium is reached and the equilibrium is established across the membrane, according to Nernst theory the intracellular TFV and TFV-dp concentrations are maintained at same intracellular concentration. This was observed in both BIC+TAF solution and BIC+TAF NP-treated cells (Fig. 1D and E). In the absence of factors that influence the equilibrium (absence of cellular target or poor efflux), the intracellular TFV and TFV-dp concentrations act as a rate-limiting factor (51). However, during HIV infection it is expected that the high TAF concentration in NPs compared to solution (Fig. 1C) would be able to contribute in prolonging maintenance of the equilibrium. Further, BIC is a high membrane permeable drug, maintaining high concentrations upon equilibrium even in the absence of HIV infection (Fig. 1B). Hence, the observed high intracellular availability of BIC and TFV (compared to TAF) for the nanoformulated drugs could be a factor that promotes *in vitro* HIV protection efficacy (IC_{50} , Table 1). The observed gradual reduction of intracellular drug concentration over time could be due to drug transformation or active ABC efflux transporters that remove excess TAF and BIC free drug molecules from the cell (50).

The *in vitro* cytotoxicity evaluation demonstrated a substantial lowering of toxicity

compared to soluble drugs (Table 1). In addition, the gradual shrinkage of the s.c. depo area at the site of s.c. injection without any sign of necrosis demonstrates that polymers from the nanoformulation gradually degrade without showing any inflammatory response (see Fig. S3). This observation is of interest since a reported TAF prodrug administered in an s.c. implant resulted in local inflammation and tissue necrosis in both rabbits and macaques (52). Based on these observations, we believe that PLGA nanoencapsulation reduces direct contact of drug molecules within cells, which indirectly would further reduce cytotoxicity associated with the free drugs (15, 53, 54).

The *in vitro* short-term protection experiments in both TZM-bl cells and PBMCs demonstrated that the BIC+TAF nanoformulation provides considerable protection against HIV infection (Table 1). In PBMCs, nanoencapsulation of BIC+TAF substantially lowered the IC_{50} by ~131-fold. Prolonged intracellular concentrations of BIC and TAF from nanoformulation could be the reason for enhanced HIV-1 protection at low nanogram concentrations (Fig. 1). Indeed, the selectivity index (SI) values are substantially improved by 472-fold compared to drugs in solution. These *in vitro* results demonstrate that BIC+TAF nanoencapsulation reduces free drug cytotoxicity and significantly lowers the IC_{50} against HIV-1 improving the therapeutic index (based on the SI value) of the combination for PrEP application. Finally, ARV concentrations greater than the IC_{50} are essential to avoid the possibility of drug resistance (52).

The BIC+TAF nanoformulation demonstrated a prolonged elimination half-life ($t_{1/2}$) (Table 2) and maintenance of the plasma TFV and BIC concentration above the measured IC_{50} during the entire study period (Fig. 2). At the HIV infection sites, a detectable TFV level was observed until 14 (vagina) and 21 (colon) days due to assay limitation (lower limit of quantification, 0.5 ng/g). The result signifies that the TFV concentration was maintained significantly above the protective level at least until days 14 and 21 in the vagina and colon, respectively. The BIC concentrations at all sites were maintained above IC_{50} for the entire study period. This observation is essential because, after reaching PK steady state, the free drug concentration in plasma is in equilibrium with the drug in tissue (55). Therefore, the IC_{50} value of novel therapeutics is a major pharmacologic factor that could elucidate the pharmacologic efficacy of a therapeutic treatment (56). However, protection studies (Fig. 2 and Table 1), as predicted in nonhuman primates, lasting 28 days (57) or longer require further investigation to confirm the HIV-1 PrEP efficacy of BIC+TAF NP.

Overall, the *in vivo* biodistribution data in mice were robust, which provides clear evidence of the long-acting nature of the nanoformulation. The drug concentrations from the PK experiments were maintained above the IC_{50} , avoiding the long-lasting "tail" (52). The nanoformulation avoids sustained subtherapeutic drug concentrations, which is a reason behind the development of drug-resistant viral strains (58, 59). Further study with multiple doses is needed to verify this claim.

Based on these preliminary nonclinical pharmacokinetic results, the use of BIC+TAF NPs s.c. injections might drastically reduce the dosing frequency of a current BIC-based daily regimen in humans to a monthly regimen. In addition, the nanoformulation s.c. administration site could act as a potential depo for PrEP patients. All of these studies collectively support the use of nanoformulations as a new ARV combination. Therefore, BIC+TAF NPs could provide an alternative to HIV PrEP for improved patient adherence.

MATERIALS AND METHODS

BIC+TAF-loaded nanoformulation and characterization. To formulate a BIC+TAF-loaded nanoformulation, a previously reported standardized nanoformulation method was used (20, 22). Briefly, to 20 ml of 1% poly(vinyl alcohol) (Millipore Sigma, Darmstadt, Germany), we added 5 ml of dichloromethane (Sigma-Aldrich; MO) containing PLGA (75:25 ratio; M_w 4,000 to 15,000; Sigma-Aldrich; MO), pluronic F127 (stabilizer; d-BASF, Edinburg, UK), TAF (Gilead Sciences, Inc., CA), and BIC (Biochempartner Co., Ltd., China) at a 1:1:1:1 ratio (wt/wt/wt/wt) dropwise under high-speed continuous-stirring conditions at room temperature. The oil-in-water (o-w) emulsion described above was then probe sonicated on ice for 5 min using a UP100H ultrasonic processor (100 W, 30 kHz; Hielscher, Inc., NJ), under a 90% amplitude setting with a pulse of 0.9 cycle/bursts. This sonicated o-w emulsion was left in the hood overnight under constant stirring condition to evaporate the organic phase. After organic phase removal, the aqueous phase was removed by lyophilization using Millrock LD85 lyophilizer (NY). The dry BIC+TAF

NPs were stored at 4°C until used. The complete procedure was carried out in a biosafety cabinet to maintain sterility.

For the physical property evaluation, 5 mg of BIC+TAF NP was dispersed in 10 ml of ultrapure autoclaved water at room temperature and sonicated briefly (1 min) in an Ultrasonic Bath (Branson Ultrasonics Corp., CT). The size and polydispersity index (PDI) of the freshly dispersed BIC+TAF NPs was determined by dynamic light scattering (DLS) analysis using ZetaPlus Zeta Potential Analyzer (Brookhaven Instruments Corporation, NY). The PDI value reflects the size homogeneity and size distribution pattern of the obtained BIC+TAF NP batch. A PDI value of <0.2 defined the homogeneous NP population with narrow Gaussian distribution. The surface charge was determined by zeta potential analysis using a ZetaPlus Zeta potential analyzer. The morphology and shape of the obtained BIC+TAF NPs and blank PLGA NPs were evaluated by scanning electron microscopy according to standardized methodology on an S-4700 field-emission scanning electron microscope (Hitachi, New York, NY) (60).

The %EE with respect to the starting drug amount added and %DL to excipients of BIC+TAF NPs was evaluated by HPLC according to a standardized methodology (20, 22). The BIC+TAF NPs (1 mg) were dissociated in 50 μ l of dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO); to this was added 150 μ l of mobile phase 45% 25 mM potassium dihydrogen phosphate (Sigma-Aldrich) plus 55% acetonitrile (Sigma-Aldrich), and the sample was briefly sonicated. Following the same procedure, the BIC+TAF standards (each drug concentration ranged from 0.5 to 0.0078 mg/ml) were prepared. Both the BIC+TAF standards and the dissociated BIC+TAF NP solution were spin filtered through EMD Amico Ultra centrifugal filter units (Millipore Sigma, Burlington, MA). The chromatography separation was performed on a Phenomenex C-18 column (150 $\times$ 4.6 mm; particle size, 5 μ m) using the isocratic mobile phase at a 0.5-ml/min flow rate, a temperature of 25°C, and detection at 260 nm. An HPLC instrument (Shimadzu Scientific Instruments, MD) equipped with SIL-20AC auto-sampler, LC-20AB pumps, and SPD-20A UV/Visible detector was used for this study. The quantification of each drug was determined by evaluating the area under the curve (AUC) at its respective retention time (4 min for TAF and 6.5 min for BIC). The amount of TAF and BIC loaded in the BIC+TAF NPs was analyzed based on the standard curve (linear correlation, $r^2 \geq 0.99$) obtained from TAF and BIC standard concentrations analyzed from respective standard AUCs. The interday and intraday variability of the instrument was determined to be <10%.

The %EE (equation 1) and %DL (equation 2) of each drug in each BIC+TAF NPs batch were calculated according to the following formulas. The data are presented as means $\pm$ the standard errors of the mean (SEM) of three BIC+TAF NPs batches ($n = 3$).

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

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

Intracellular drug-kinetics study. The *in vitro* BIC+TAF uptake and retention of BIC+TAF NPs and BIC+TAF solution was compared using TZM-bl cells. The TZM-bl cells were seeded (10^4 cells/well) in a 96-well plate with complete HiDMEM medium (Thermo Scientific, OK), supplemented with 10% fetal bovine serum (VWR International, PA) and $1 \times$ antibiotic-antimycotic (Thermo Scientific, OK). After overnight adherence, the respective wells were treated with BIC+TAF NPs or BIC+TAF solution, each drug at a 10 μ g/ml concentration in BIC+TAF NPs (determined by HPLC as described above) or in solution. For cellular uptake/release experiments, the treated and control TZM-bl cells at respective time points (i.e., at 1, 6, 18, and 24 h) were washed twice with warm phosphate-buffered saline (PBS; Sigma-Aldrich) and detached by trypsin-EDTA (25%; Thermo Scientific). After washing off the trypsin-EDTA, the detached cells were counted at each time point to determine cell count. The cells were then air dried, lysed with 70% methanol, and stored at -80°C until analysis. For the drug-retention study, after 24 h of treatment, the treated and control cells were washed three times with warm PBS and maintained in a fresh complete HiDMEM medium without treatment. At time points 1, 6, 24, and 72 h after 24 h of treatment washoff, which corresponded to 25, 30, 48, and 96 h, respectively, the cells were rewashed, detached using trypsin-EDTA, counted, air dried, subjected to cell lysis, and stored as described above. The drug, active-drug, and drug-metabolite concentrations in the samples were analyzed using standard LC-MS with minor modifications (11, 20, 22, 61).

For intracellular BIC, TAF, TFV, and TFV-dp concentration evaluations, the respective cell lysates were centrifuged at $17,530 \times g$ for 5 min at 4°C, and the supernatant was collected. An aliquot of 100 μ l of supernatant and 300 μ l of internal standard spiking solution (10 ng/ml [each] of DTG, TAF-d5, and TFV-d6, and 100 ng/ml of TFV-dp-d6 in acetonitrile) was added, and the sample was vortexed. The samples were then dried at 45°C under nitrogen stream and reconstituted with 100 μ l of 50% acetonitrile. The drugs and metabolites were quantified from the same sample using the LC-MS/MS instruments.

To quantify TAF, TFV, and BIC, 0.5 μ l of the processed sample was injected onto an LC-MS/MS apparatus (5500 QTrap; AB Sciex, CA) operated in electrospray ionization (ESI)-positive mode. The chromatographic separation was performed on a Restek Pinnacle DB Biphenyl column (50 $\times$ 2.1 mm, 5 μ m) with a mobile phase composed of 0.5% formic acid-acetonitrile (48:52 [vol/vol]) at a flow rate of 0.25 ml/min. The mass transitions used for TAF, TFV, BIC, and respective internal standards were 477.1 $\rightarrow$ 270, 288 $\rightarrow$ 176.2, 450.1 $\rightarrow$ 289.1, 482.1 $\rightarrow$ 270 (TAF-d5), 293.9 $\rightarrow$ 182.3 (TFV-d6), and 420.1 $\rightarrow$ 277.1 (DTG) respectively. The calibration range was 0.01 to 500 ng. The chromatographic run time for each sample was 3 min with retention times of 0.61, 1.1, and 1.48 min for TFV, BIC, and TAF, respectively.

For the quantification of TFV-dp, 5 μ l of the processed sample was subjected to LC-MS/MS (AB Sciex 5500 QTrap) operated in ESI-positive mode. Chromatographic separation was performed on a Kinetex C₁₈ column (75 $\times$ 4.6 mm, 2.6 μ m) with a mobile phase consisting of 10 mM ammonium acetate (pH 10.5) and acetonitrile (70:30 [vol/vol]) at a flow rate of 0.25 ml/min. The mass transitions used for TFV-dp and respective internal standards were 448.1 $\rightarrow$ 270 and 454.2 $\rightarrow$ 276 (TFV-dp-d6), respectively. The calibration range was 0.01 to 100 ng. The chromatographic run time for each sample was 3.5 min, with retention times of 2.2 min for both TFV-dp and TFV-dp-d6. The acceptance criteria were set as interday and intraday variabilities of <15%, which corresponds to FDA bioanalytical guidelines (62).

In vitro cytotoxicity evaluation. The *in vitro* cytotoxicity of BIC+TAF NP was compared to the BIC+TAF solution in TZM-bl cells and human PBMCs (obtained from healthy donors) using the CellTiter-Glo luminescent assay method, as described previously (26). Briefly, the TZM-bl cells (10⁴ cells/well) and PBMCs (10⁵ cells/well) were treated in triplicate with BIC+TAF NP and BIC+TAF-free solution, respectively, at concentrations of 20, 10, 1, 0.1, and 0.01 μ g/ml/drug for 96 h. Untreated cells (equal-volume PBS treated) and 5% dimethyl sulfoxide (Sigma-Aldrich)-treated cells were considered as negative and positive controls, respectively. The cytotoxicity was evaluated by using a CellTiter-Glo luminescent cell viability assay kit (Promega) according to the manufacturer protocol, and the luminescence intensity (relative luminescence unit [RLU]) was read on a Synergy II multimode reader with Gen5TM software (BioTek, VT). The percentage cytotoxicity (% cytotoxicity) was calculated as the percent normalized viability against the untreated (negative) control group. The experiment was performed three independent times using three independent batches of BIC+TAF NPs and BIC+TAF solutions. The percent cytotoxicity was evaluated by applying equation 3, and the results are presented as means $\pm$ the SEM of three independent experiments:

$$\% \text{Cytotoxicity} = \frac{\text{RLU}_{\text{untreated}} - \text{RLU}_{\text{treated}}}{\text{RLU}_{\text{untreated}}} \times 100 \quad (3)$$

In vitro HIV protection study. The comparative *in vitro* protection between BIC+TAF NP and BIC+TAF solution was evaluated against HIV-1_{NL4-3} in TZM-bl cells (an HIV-1 infection reporter cell line) and against HIV_{ADA} in human PBMCs (obtained from healthy donors) by using a previously reported method (26, 27). TZM-bl cells (10⁴ cells/well) and PBMCs (10⁵ cells/well) were seeded in 96-well plates in HiDMEM and RPMI complete medium, respectively. The cells were treated with different concentrations of BIC+TAF (20, 10, 1, 0.1, and 0.01 μ g/ml/each drug) either as BIC+TAF NP or as BIC+TAF solution. The untreated/uninfected cells and untreated/infected cells were negative and positive controls, respectively. After 24 h of treatment, the TZM-bl cells were infected with HIV-1_{NL4-3} virus (multiplicity of infection [MOI] = 0.1) for 8 h, whereas PBMCs were infected with HIV-1_{ADA} virus (MOI = 0.1) for 16 h. The infected and control cells were rewashed with warm PBS (three times) and maintained in the respective fresh complete medium for 96 h. The HIV-1 infectivity for TZM-bl cells was evaluated based on the luminescence intensity using a Steady-Glo luciferase assay (Promega) according to the manufacturer's protocol. For PBMCs, the HIV infectivity was evaluated by using a Retro-Tek HIV-1 p24 antigen ELISA kit (ZeptoMetrix Co., NY) according to the manufacturer's method. The HIV-1 infection was evaluated based on the luminescence intensity, i.e., the RLU in TZM-bl cell infection study, and was based on optical density (OD) values for PBMCs, as read on a Synergy HT multi-mode microplate reader (BioTek). The percent HIV-1 infection was calculated by using equation 4:

$$\% \text{HIV protection} = \frac{\text{RLU or OD}_{\text{untreated/infected}} - \text{RLU or OD}_{\text{treated/infected}}}{\text{RLU or OD}_{\text{untreated/infected}}} \times 100 \quad (4)$$

The *in vitro* protection study was performed three independent times with three different batches of BIC+TAF NP and BIC+TAF solution (each performed in duplicate) for TZM-bl cells. In the *in vitro* protection study with PBMCs, the experiments were performed on PBMCs from three different donors (each treatment variable was assessed in triplicate for each donor). Finally, the SI was evaluated by using equation 5:

$$\text{SI} = \frac{\text{CC}_{50}}{\text{IC}_{50}}, \quad (5)$$

where CC₅₀ and IC₅₀ values were evaluated based on *in vitro* cytotoxicity and protection data by nonlinear regression curve-fitting analysis, as described under statistical analyses above.

In vivo biodistribution study in BALB/c mice. BALB/c female mice (6 weeks old, each ~23 g) purchased from Jackson Laboratories (ME) were used to assess the plasma and tissue drug biodistribution. The BIC+TAF NPs (200 mg/kg of each drug) were reconstituted in PBS and administered as an s.c. injection. At 1 h, 6 h, and 1, 4, 7, 14, 21, and 30 days posttreatment, mouse groups ($n = 4$ /time point) were euthanized using CO₂ inhalation and cervical dislocation. From the euthanized mouse, whole blood, along with organs of interest, including the vagina, colon, spleen, lymph nodes, and injection sites, were harvested. Whole blood was collected in a K₂EDTA collection tube (BD Microtainer MAP, NJ) and centrifuged at 394 $\times g$ for 20 min to harvest the plasma; the plasma was then frozen until analyzed. PBMCs were isolated from whole blood using Ficoll-Paque density gradient separation (GE Healthcare Life Sciences, Uppsala, Sweden) according to the manufacturer's protocol and kept frozen until analysis for TFV active

metabolite (TFV-dp). TFV and BIC (plasma and tissue) sample processing was performed according to a previously published method (11, 61). For TFV-dp, the samples were processed and evaluated by LC-MS/MS, as explained in the supplemental material (i.e., by the intracellular drug concentration evaluation method). Phoenix WinNonlin 8.1 (Certara, Inc., NJ) was used to determine the PK parameters from plasma and tissue using noncompartmental modeling of the concentration-time data. All PK mouse experiments adhered to the NIH *Guide for the Care and Use of Laboratory Animals*, and the protocol we used was approved by the Animal Care and Use Committee (protocol 1111 [Creighton University, Omaha, NE]).

Statistical analysis. All study results presented here are expressed as means $\pm$ the SEM of the obtained data from at least three independent experiments. The CC_{50} value was determined using nonlinear regression curve fitting based on log (BIC or TAF concentration) versus normalized luminescence (three-parameter logistics) of cytotoxicity response curves. The IC_{50} value was evaluated by the nonlinear regression inhibitory curve fitting of log versus log (BIC or TAF concentration) versus normalized luminescence (three parameters). All the statistical analyses presented here were determined by using Prism 7 software (GraphPad, CA). Significant differences between treated (NP and free drugs in solution) versus control groups were determined at a P value of <0.05 using an unpaired t test.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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