

In Vitro Virology Profile of Tenofovir Alafenamide, a Novel Oral Prodrug of Tenofovir with Improved Antiviral Activity Compared to That of Tenofovir Disoproxil Fumarate

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Tenofovir alafenamide (TAF) is an investigational oral prodrug of the HIV-1 nucleotide reverse transcriptase inhibitor tenofovir (TFV). Tenofovir disoproxil fumarate (TDF) is another TFV prodrug, widely used for the treatment of HIV-1 infection. TAF is converted mostly intracellularly to TFV and, in comparison to TDF, achieves higher tenofovir diphosphate (TFV-DP) levels in peripheral blood mononuclear cells. As a result, TAF has demonstrated potent anti-HIV-1 activity at lower doses than TDF in monotherapy studies. Here, the *in vitro* virology profile of TAF was evaluated and compared to that of TDF. TAF displayed potent antiviral activity against all HIV-1 groups/subtypes, as well as HIV-2. TAF exhibited minimal changes in the drug concentration needed to inhibit 50% of viral spread (EC₅₀) upon removal of the prodrug, similar to TDF, demonstrating intracellular antiviral persistence. While TAF and TDF exhibited comparable potencies in the absence of serum pretreatment, TAF maintained activity in the presence of human serum, whereas TDF activity was significantly reduced. This result demonstrates TAF's improved plasma stability over TDF, which is driven by the different metabolic pathways of the two prodrugs and is key to TAF's improved *in vivo* antiviral activity. The activity of TAF is specific for HIV, as TAF lacked activity against a large panel of human viruses, with the exception of herpes simplex virus 2, where weak TAF antiviral activity was observed, as previously observed with TFV. Finally, *in vitro* combination studies with antiretroviral drugs from different classes showed additive to synergistic interactions with TAF, consistent with ongoing clinical studies with TAF in fixed-dose combinations with multiple other antiretroviral drugs for the treatment of HIV.

Tenofovir [(R)-9-(2-phosphonomethoxypropyl)adenine] (TFV) is a human immunodeficiency virus (HIV) nucleotide reverse transcriptase (RT) inhibitor (NtRTI), also frequently referred to as member of the nucleoside RT inhibitor (NRTI) class (1). TFV contains a phosphonate group that is equivalent to the first phosphate group present in natural monophosphate nucleotides (2). In comparison to the natural phosphoric group, the TFV phosphonate moiety has the C—O bond switched, which is not recognized by host enzymes and thus makes it less prone to phosphatase activities. Consequently, TFV activation requires only two phosphorylation steps (3). Tenofovir is intracellularly phosphorylated to the active metabolite tenofovir diphosphate (TFV-DP), which is incorporated into viral DNA by HIV RT and acts as a DNA chain terminator (4). However, the negative charges harbored by TFV from its phosphonate moiety reduce its cellular permeability, limiting its absorption and oral bioavailability. Tenofovir disoproxil fumarate (TDF) (Fig. 1) is a prodrug of TFV widely used for the treatment of HIV-1 infection and is the preferred NtRTI for use in combination with other antiretroviral agents for the treatment of HIV-1 infection (5–7). TDF was developed to improve TFV permeability and to allow systemic delivery of TFV via oral administration. However, TDF is quickly metabolized to TFV by gut and serum esterases *in vivo*, and high drug exposures are necessary to allow proper loading of target cells.

Tenofovir alafenamide (TAF) (formerly GS-7340) (Fig. 1) is an investigational prodrug of TFV. In contrast to TDF, TAF has been shown to be significantly more stable in blood and plasma (Fig. 2) and is quickly converted to TFV within lymphocytes, improving intracellular accumulation of TFV-DP in HIV target cells (8). The cellular enzyme mostly responsible for the intracellular conversion of TAF has previously been identified as the hydrolase cathep-

sin A (CatA) or carboxyesterase 1 (CES1), depending on the cell type (9, 10). In preclinical studies in dogs, TAF (administered orally) has been shown to distribute preferentially into peripheral lymphocytes and lymphatic tissues (8). In clinical studies, TAF has demonstrated favorable pharmacological properties, with higher intracellular levels of TFV-DP and lower circulating levels of TFV than of TDF. The higher TFV-DP levels in peripheral blood mononuclear cells (PBMCs) have resulted in improved antiviral activity at lower doses than TDF in monotherapy studies (11, 12). The efficacy of low doses of TAF has been confirmed in phase 2 clinical trials in combination with elvitegravir (EVG, or E), cobicistat (COBI, or C), and emtricitabine (FTC, or F). Moreover, patients receiving E/C/F/TAF experienced decreased changes in kidney function (estimated glomerular filtration rate [eGFR] and tubular proteinuria) and bone mineral density compared to the control arm of E/C/F/TDF (13). No resistance was detected in the E/C/F/TAF arm compared to 3.4% of patients in the E/C/F/TDF

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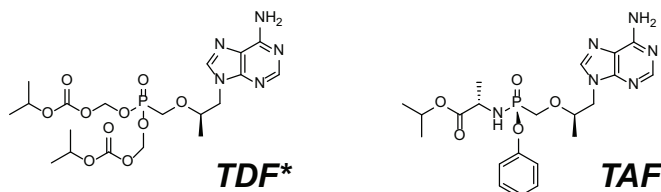


FIG 1 Chemical structures of TAF and TDF. *, the fumarate salt is not represented for TDF.

arm, supporting the potential improved resistance profile of TAF over TDF (13).

This study evaluated the *in vitro* virology profile of TAF, including its antiviral activity against laboratory and clinical isolates, as well as its selectivity, *in vitro* potency, stability, persistence, and suitability to be coformulated with other antiretroviral drugs (ARVs).

MATERIALS AND METHODS

TAF, TFV, and TDF were evaluated in parallel for their antiviral activities in several assays using multiple cell types. In assays not specifically evaluating the role of the prodrug moiety, TFV was evaluated rather than TDF due to its better stability *in vitro*.

Antiviral drugs. TAF (GS-7340), TDF (GS-4331), TFV (GS-1278), EVG (GS-9137), COBI (GS-9350), and emtricitabine (FTC) (GS-9036) were synthesized by Gilead Sciences. Nevirapine (NVP), raltegravir (RAL), dolutegravir (DTG), atazanavir (ATV), and efavirenz (EFV) were isolated from therapeutic formulations. Darunavir (DRV) was purchased from Toronto Research Chemicals (North York, ON, Canada). Ribavirin (RBV) and didanosine (ddI) were purchased from Sigma (St. Louis, MO). Stavudine (d4T) was supplied by Bristol-Myers Squibb (Princeton, NJ). The antiviral control compounds used in the human virus panel were provided by Southern Research Institute (SRI) (Frederick, MD, USA).

Cells and viruses. MT-2 cells (provided by Stanford University, Palo Alto, CA) and MT-4 cells (AIDS Research and Reference Reagent Program) were maintained in RPMI 1640 medium supplemented with antibiotics and 10% fetal bovine serum (FBS). The cells were passaged twice a week and kept at a density of $<0.6 \times 10^6$ cells/ml. Human PBMCs were isolated from donor buffy coats (Stanford Blood Bank, Palo Alto, CA), using centrifugation in Ficoll Paque Plus (Amersham Biosciences, Piscataway, NJ), and activated for 5 days in RPMI 1640 medium with 20% FBS, antibiotics, interleukin-2 (IL-2) (20 units/ml), and phytohemagglutinin (PHA) (1 μ g/ml). Human CD4⁺ T lymphocytes were purified from activated PBMCs. Macrophages were removed by cell adhesion. CD4⁺ T cells were purified from the nonadherent fraction (peripheral blood lymphocytes [PBLs]) by negative magnetic bead sorting with an AutoMACS using a CD4⁺ T cell Isolation Kit II (Miltenyi Biotec). Non-CD4⁺ T cells were indirectly labeled by using a cocktail of biotin-conjugated antibodies, in combination with anti-biotin microbeads. Purified CD4⁺ T cells from several donors (up to 4) were pooled to reduce donor variability, improve assay reproducibility, and increase total cell numbers. The HIV-1 strains HIV-1_{IIB} and BaL (Advanced Biotechnologies, Columbia, MD) were used for infection of MT-2 or MT-4 cells and of primary CD4 cells, respectively. SRI provided the panel of human viruses and cells.

HIV antiviral assay. MT-2 cells (2.4 million) were incubated with virus for 3 h at 37°C in 1-ml screw-cap tubes. Virus was normalized to yield a signal-to-noise ratio (uninfected cells to infected cells) between 4 and 7, which was equivalent to a multiplicity of infection (MOI) of ~ 0.003 for wild-type HIV-1_{IIB}. Fivefold drug dilutions were transferred in triplicate to the inside wells of 96-well plates (50 μ l per well). The signal-to-noise ratio for each plate was calculated from the no-drug control (maximum cell killing) and the 300 μ M TFV control (minimum cell killing). After incubation, infected MT-2 cells were seeded at 8,500 per

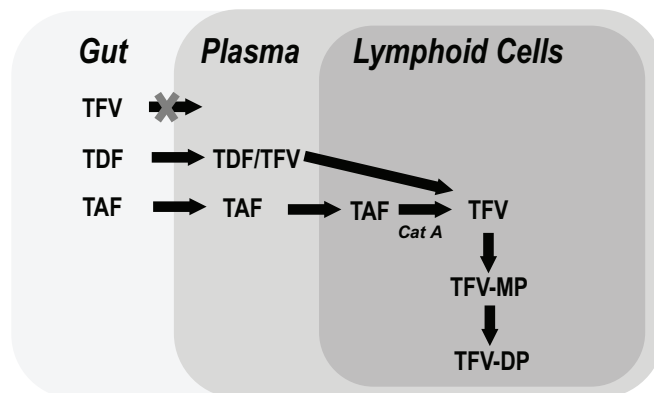


FIG 2 TFV conversion pathways for TAF and TDF. The schematic shows the different pathways by which TAF and TDF are converted to TFV. TAF remains stable in plasma and is mostly converted intracellularly to TFV by cathepsin A (CatA). In contrast, TDF is mostly converted to TFV by gut and plasma hydrolases. For both TAF and TDF, TFV is then converted by cellular kinases to TFV monophosphate (TFV-MP), followed by TFV-DP, the active form of the drug.

well in 100 μ l of medium. After incubation for 5 days at 37°C, 100 μ l of CellTiterGlo reagent (Promega, Madison, WI, USA) was added to each well of the assay plates, and luminescence was measured using an Envision plate reader (PerkinElmer, Shelton, CT, USA).

Serum treatment. Potency experiments were performed in MT-2 cells as described above in the presence of ARV drugs pretreated or mock treated for 30 min in type AB human serum (HS) (catalog number 14-491E; Lonza, Walkersville, MD). The HS was pooled from multiple donors. The final concentration of HS was adjusted to 5% for all samples prior to infection.

Cytotoxicity assays. Cells (MT-2 and MT-4 cells) were mixed in 96-well plates with 5-fold serial dilutions of compounds at a density of 20,000 cells/well in a final volume of 200 μ l. After a 5-day incubation at 37°C, the cells were mixed with CellTiterGlo as described above. Cell viability was expressed as a percentage of the signal from untreated samples (0% cytotoxicity) after subtraction of the signal from samples treated with 1 μ M podophyllotoxin (Sigma) (100% cytotoxicity). The drug concentration that reduced cell viability by 50% (CC_{50}) was determined by nonlinear regression analysis (Prism 4; GraphPad, San Diego, CA).

HIV-1 and HIV-2 primary isolate assays. Infections with HIV-1 primary isolates were conducted at SRI using freshly isolated human PBMCs seronegative for HIV and hepatitis B virus (HBV) (14). Wild-type HIV primary isolates representing HIV-1 groups M (subtypes A to G), N, and O, as well as HIV-2 isolates, were tested for susceptibility to TAF and AZT. Assay readout was performed using an RT activity assay as described below.

CD4 single-cycle infection assay. Primary CD4⁺ T lymphocytes were infected with a high-titer stock of HIV-1 BaL at a high multiplicity of infection (MOI = 1) to achieve a strong synchronous infection and virus detection during a single viral cycle (15, 16). After 2 h of virus incubation, the cells were washed twice with medium containing 50% fetal calf serum (FCS) and seeded into 96-well plates at 100,000 cells/well. Serial drug dilutions were added, and 36 h later, the supernatants were collected and virus production was measured using a HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) (Beckman Coulter, Miami, FL). Regression analysis was used to determine the drug concentrations needed to inhibit 50% of viral spread (EC_{50} s).

Antiviral persistence assay in CD4 primary cells. The antiviral activities of TAF and TDF were determined in an HIV-1 CD4 single-cycle infection assay (see above) using either constant drug exposure (EC_{50}) or pulsed drug exposure (50% persistence concentration [PC_{50}] assay), as previously described (R. M. Ledford, J. E. Vela, A. S. Ray, C. Callebaut,

TABLE 1 Antiviral activity and selectivity of TAF versus TFV

Cell type ^a	TAF (μM)			TFV (μM)		
	EC ₅₀	CC ₅₀	SI	EC ₅₀	CC ₅₀	SI
MT-2 ^b	0.005 ± 0.002	42	8,853	1.4 ± 0.5	>1,000	>690
MT-4 ^c	0.005 ± 0.002	4.7	903	4.2 ± 0.8	>1,000	>238
PBMCs ^d	0.007 ± 0.004	9	1,385	3.5 ± 0.7	>1,000 ^e	>286

^a Cells were infected with HIV-1_{IIIB}.

^b EC₅₀ assay, *n* = 3 experiments; CC₅₀ assay, *n* = 2 experiments.

^c EC₅₀ assay, *n* = 2 experiments; CC₅₀ assay, *n* = 1 experiment.

^d EC₅₀ assay, *n* = 2 experiments; CC₅₀ assay, *n* = 1 experiment.

^e Twenty-five percent inhibition was observed at 1,000 μM.

M. D. Miller, and D. J. McColl, presented at the 19th International Conference on Antiviral Research, San Juan, Puerto Rico, 2006). Briefly, in the persistence assay, cells were pulsed with drug for 4 h, with HIV infections limited to the last 2 h; external drug was removed after 4 h by washing with medium containing 50% FCS (see Fig. S1 in the supplemental material). In the constant drug exposure assay, cells were incubated with virus for 2 h, washed twice with medium containing 50% FCS, and incubated in medium plus drugs. As the end of reverse transcription has been established in this experimental system to be ~6 h postinfection by time-of-addition experiments (not shown), the time for the drugs to act in each setup was ~4 h. Virus production was determined by p24 antigen ELISA as described above for the CD4 infection assay. The EC₅₀, the PC₅₀, and the relative antiviral persistence ratio (*P*_{rel}) (PC₅₀/EC₅₀) were determined.

Drug combination studies. The TAF activity in combination with various ARVs was determined in MT-2 cells infected with HIV-1_{IIIB}. Briefly, compound serial dilutions were performed in 100% dimethyl sulfoxide (DMSO) in 384-well polypropylene plates using a Biomek FX workstation (Beckman Coulter, Inc., Brea, CA). The first compound was serially diluted in nine steps of 1:2 dilutions toward the horizontal direction in plate A, with the second compound serially diluted in seven steps of 1:2 dilutions toward the vertical direction in plate B. Equal volumes of serially diluted compounds were combined in plate C, achieving a defined set of drug concentrations and ratios. For each drug, the starting concentration was selected so that the EC₅₀ was the midpoint concentration tested in plate C. All dilutions were performed in triplicate within the same 384-well plate. Controls containing untreated infected cells and infected cells treated with 300 μM tenofovir were included in each assay plate, representing 0% and 100% inhibition, respectively. The HIV infection was carried out as described above (see “HIV Antiviral Assay”) in a 384-well format with 2,000 cells per well. The DMSO concentration in the final assay wells was 0.4%. After a 5-day incubation at 37°C, the virus-induced cytopathic effect was determined using CellTiter Glo reagent (Promega, Madison, WI), with the signal quantified on a Victor V3 reader (PerkinElmer, Wellesley, MA) following a 15-min incubation. The combination effect (synergy, additivity, and antagonism) of each combination was determined with MacSynergy II software (17) using a previously described algorithm (18). Combination volume values were calculated at the 95% confidence level, and combination effects were ranked as previously defined (19): strong synergy (>100 μM²% [the unit represents the combination volumes resulting from each drug concentration, expressed as the percentage of the control]), moderate synergy (>50 and ≤100 μM²%), minor synergy (>25 and ≤50 μM²%), additivity (≤25 and >-25 μM²%), minor antagonism (≤-25 and >-50 μM²%), moderate antagonism (≤-50 and >-100 μM²%), and strong antagonism (≤-100 μM²%). For each combination study, three independent experiments were performed.

Human virus panel. TAF antiviral activity against human viruses was evaluated at SRI. Viruses and cell lines were obtained from the American Type Culture Collection (ATCC) or the BEI Research Resource Repository (BEIR) or from specific sources (see Tables S1 and S2 in the supplemental material). All the methodologies utilized at SRI are summarized in the supplemental material.

RESULTS

Antiviral activity of TAF in primary lymphoid cells and T-cell lines. Previous studies demonstrated the potent *in vitro* anti-HIV-1 activity of TAF under limited conditions (8). In this study, the antiviral activity of TAF was evaluated in two lymphoblastoid T-cell lines (MT-2 and MT-4) infected with HIV-1_{IIIB}, as well as in PBMCs from multiple donors infected with HIV-1 BaL (Table 1). The drug concentrations needed to inhibit 50% of the viral spread (EC₅₀), as well as to induce 50% cell death (CC₅₀), were determined in 5-day assays, using TFV as a comparator. TAF antiviral activities were similar across all cell types, ranging from 5 to 7 nM, while the CC₅₀ varied from 4.7 to 42 μM for MT-4 and MT-2 cells, respectively. The resulting selectivity index (SI) (the ratio of CC₅₀ to EC₅₀) for TAF was greater than 900 for all 3 cell types (903, 1,385, and 8,853, for MT-4 cells, PBMCs, and MT-2 cells, respectively). The *in vitro* anti-HIV-1 potency of TAF was greatly improved over that of TFV, likely due to the higher cellular permeability of TAF. TFV antiviral activity and cytotoxicity were in the micromolar (1.4 to 4.2 μM) and millimolar (>1 mM) ranges, respectively. The SI for TFV was greater than 200 for all 3 cell types (>238, >286, and >690 for MT-4 cells, PBMCs, and MT-2 cells, respectively). These results show that TAF has similar antiviral effects with high selectivity across all 3 cell types tested compared to TFV. These results are similar to those previously published showing the anti-HIV activity of TAF across a diverse donor panel of primary CD4 T cells and monocyte-derived macrophages (20). However, TAF and TFV displayed different ranges of activity, mostly driven by differences in their permeabilities, resulting in a 500-fold improvement in anti-HIV-1 activity with TAF compared to TFV in PBMCs (Table 1).

Antiviral activity of TAF against HIV-1 and HIV-2. The antiviral activity of TAF was evaluated against a panel of HIV-1 and HIV-2 isolates, including HIV-1 group M subtypes A to G, as well as group N and O isolates. Overall, for the 29 primary HIV-1 isolates tested in PBMCs, TAF EC₅₀s ranged from 0.10 to 12.0 nM, with a mean EC₅₀ of 3.5 nM compared to a mean EC₅₀ of 11.8 nM for AZT, which was used as an internal control. For the HIV-2 isolates, the mean EC₅₀s were 1.8 nM for TAF and 6.4 nM for AZT. Overall, there were no significant differences in the mean TAF EC₅₀s for any of the HIV-1 subtypes/groups evaluated, as well as HIV-2, with mean values within ~3 times that of subtype B HIV-1 (Table 2; see Fig. 5).

Persistence of TAF antiviral activity. TDF has been shown to have a long intracellular half-life (as TFV-DP) in clinical studies (21). An assay was developed to assess the persistence of TAF compared to that of TDF as a prototype compound with a pro-

TABLE 2 TAF antiviral activities against HIV primary isolates

HIV type	Isolate group	Isolate subtype	Isolate	EC ₅₀ (nM) ^a		
				TAF	AZT	
HIV-1	M	A	92UG029	8.75	26.4	
			92UG037	0.69	2.12	
			92RW016	0.71	1.53	
		B	93BR021	9.69	48.0	
			JR-CSF	1.05	1.95	
			90US873	3.87	6.03	
			92BR025	4.16	9.00	
		C	98BR004	1.75	7.56	
			93IN101	1.50	5.49	
			92UG001	3.79	11.3	
	D	92UG046	0.99	3.33		
		92UG024	6.27	7.21		
	E	93TH073	1.18	3.54		
		CMU06	1.36	4.09		
		CMU08	5.88	13.1		
	F	93BR019	0.73	4.87		
		92BR024	5.97	17.1		
		93BR029	0.14	1.28		
	G	93BR020	2.22	7.41		
		G3	5.34	14.0		
RU570		12.0	66.4			
N	NA ^b	JV1083	9.85	27.4		
		YBF30	1.98	5.23		
		BCF02	1.30	1.23		
		BCF03	3.01	7.20		
		BCF07	0.10	3.81		
		O	NA	CDC310319	2.63	15.6
				CDC310342	1.96	1.39
				CBL-20	0.91	2.18
		HIV-2	NA	NA		

^a The mean TAF EC₅₀ values for each subtype/group were as follows: A, 3.4 nM; B, 4.9 nM; C, 2.5 nM; D, 3.7 nM; E, 2.8 nM; F, 2.3 nM; G, 9.1 nM; N, not applicable; O, 1.5 nM; HIV-2, 1.8 nM.

^b NA, not applicable.

longed effect. Two experimental conditions were evaluated, in which primary CD4 T cells were subjected to either constant drug incubation or pulsed drug incubation (see Fig. S1 in the supplemental material). Under both conditions (constant drug or pulsed drug incubation), the effective drug exposure was 4 h, as drug incubations started 2 h after initial infection and reverse transcription is usually completed about 6 h postinfection. Antiviral activity

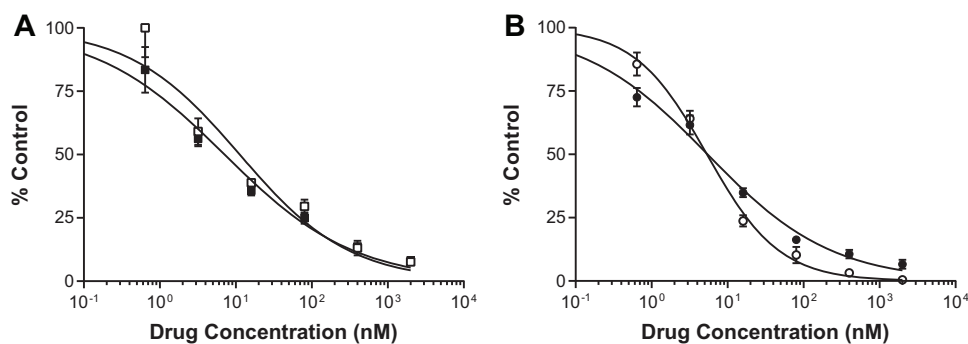


FIG 3 Antiviral activities for TAF (A) and TDF (B) in the persistence assay. The solid symbols indicate constant drug conditions, and the open symbols indicate pulsed drug conditions (see Fig. S1 in the supplemental material for experimental details). Samples were evaluated for antiviral efficacy with triplicate measurements; standard deviations are indicated by the error bars.

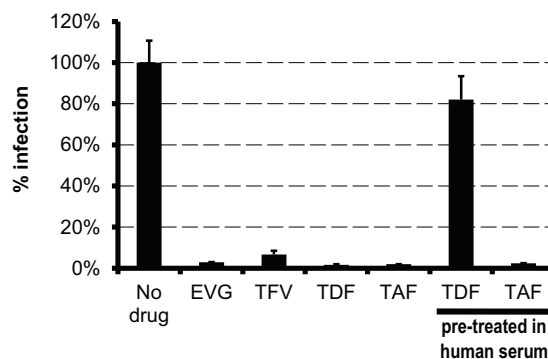


FIG 4 Comparative potencies and stabilities of TAF and TDF. The potency was assessed for each drug tested at 10× the EC₅₀ against HIV-1_{IIIB} in MT-2 cells. TAF and TDF were also pretreated in human serum for 30 min. Human serum was adjusted for all drugs to a final concentration of 5%. The error bars indicate standard deviations.

ity was measured 48 h postinfection. The antiviral activity of TAF was measured at 7.1 nM (EC₅₀) under constant drug incubation (“standard” conditions) (Fig. 3). Antiviral activity was maintained within 2 times the standard conditions under pulsed drug incubation (“persistence” conditions), where it was measured at 11.2 nM (PC₅₀). Similar to TAF, the EC₅₀ and PC₅₀ for TDF were within 2-fold of each other (EC₅₀, 5.5 nM; PC₅₀, 5.4 nM). The calculated P_{REL} (PC₅₀/EC₅₀) values for TAF and TDF were 1.6 and 1, respectively, indicating less than a 2-fold change in antiviral activity over a 24-h period in primary CD4 T cells. In contrast, when a protease inhibitor (PI) (22) was evaluated under the same conditions, the calculated P_{REL} value was >1,000, indicating at least a 200-fold decrease in antiretroviral activity under the same experimental conditions (data not shown).

Antiviral activity and stability of TAF in human serum. *In vitro*, TAF has been shown to be significantly more stable in blood and plasma than TDF (8) (Fig. 2). To determine if the greater stability resulted in higher potency, TAF *in vitro* activity was evaluated in MT-2 cells using a 5-day infectivity assay, with drug pretreated for 30 min in medium (standard conditions) or in 100% HS to mimic plasma exposure. As shown in Fig. 4, the potencies of the two TFV prodrugs (TAF and TDF) were similar in the absence of HS pretreatment. In contrast, TAF maintained its potency after HS pretreatment, reflecting its known

TABLE 3 TAF antiviral activities against human viruses

Virus	TAF		Tenofovir		Positive control
	EC ₅₀ (nM)	CC ₅₀ (nM)	EC ₅₀ (μM)	CC ₅₀ (μM)	
HIV-1 NL4-3	5.8	>1,000	2.3	>100	AZT
HIV-1 BaL	2.0	>1,000	1.0	>100	AZT
SIV mac239	1.21	>1,000	0.73	>1,000	AZT
SIV mac251	0.51	>1,000	0.35	>1,000	AZT
Adenovirus	>1,000	>1,000	>1,000	>1,000	Ribavirin
Dengue virus	>1,000	>1,000	>1,000	>1,000	Ribavirin
Hepatitis C virus	>1,000	>1,000	>1,000	>1,000	rIFN-α ^e
Coxsackie B virus	>1,000	>1,000	>1,000	>1,000	Enviroxime
Rhinovirus	>1,000	>1,000	>1,000	>1,000	Enviroxime
Vaccinia virus	>1,000	>1,000	>1,000	>1,000	Cidofovir
Influenza A virus	>1,000	>1,000	>1,000	>1,000	Zanamivir
Human parainfluenza virus	843	>1,000 ^c	>1,000	>1,000	Enviroxime
Respiratory syncytial virus	>1,000	>1,000	>1,000	>1,000	Ribavirin
VZV ^a	>1,000	>1,000	>1,000	>1,000	Acyclovir
HCMV ^b	>1,000	>1,000	>1,000	>1,000	Ganciclovir
HSV-1	>1,000	>1,000	>1,000 ^d	>1,000	Acyclovir
HSV-2 (KW)	424	>1,000	146	>1,000	Acyclovir
HSV-2 (MS)	697	>1,000	278	>1,000	Acyclovir

^a VZV, varicella-zoster virus.

^b HCMV, human cytomegalovirus.

^c Cell growth inhibition was observed at the highest concentration.

^d Viral inhibition was observed at the highest concentration.

^e rIFN-α, recombinant alpha interferon.

plasma stability, while TDF activity was more than 90% reduced after HS pretreatment.

TAF antiviral activity against human viruses. Antiviral activity assays against a panel of 18 human viruses were performed with TAF and TFV (Table 3). The result for each virus was validated with a control drug that exhibited the expected levels of antiviral activity (data not shown). With the exception of HIV-1 and simian immunodeficiency virus (SIV) isolates, which were potently inhibited by TAF and TFV, no antiviral activity was observed against 12 of the remaining 14 human viruses evaluated (Table 3). TFV weakly inhibited the herpes simplex virus 2 (HSV-2) strain KW (a clinical isolate) with an EC₅₀ of 146 μM, which is consistent with data previously published (23). TAF had an EC₅₀ of 424 nM for that viral isolate. Overall, TAF and TFV activities against HSV-2 KW were 150- to 200-fold weaker than those against HIV. Similarly weak activity was observed for HSV-2 MS (a laboratory isolate). The only other human virus that TAF inhibited was human parainfluenza virus, with an EC₅₀ of 843 nM. While the EC₅₀s against human parainfluenza virus indicate minimal TAF antiviral activity, cell growth inhibition was observed at the 1,000 nM concentration; thus, the observed effect may be due to cell inhibition. Neither TAF nor TFV exhibited cytotoxicity up to the highest concentrations used for these evaluations (1,000 nM for TAF and 1,000 μM for TFV). Overall, the results indicate TAF is a potent inhibitor of immunodeficiency viruses, such as HIV and SIV, and a weak inhibitor of HSV-2.

Combination studies with TAF and other ARVs. The *in vitro* antiretroviral activity of TAF was tested in various combinations with drugs representing the major ARV classes, including NRTIs (TFV and FTC), nonnucleoside reverse transcriptase inhibitors (NNRTIs) (EFV and NVP), integrase strand transfer inhibitors (INSTIs) (EVG, RAL, and DTG), and PIs (ATV and DRV). Combinations of ddI plus RBV, d4T plus RBV, and TAF with itself were

used as controls for synergy, antagonism, and additivity, respectively. The combination of TAF with TFV resulted in an additive effect, as expected, as both deliver TFV-DP to cells. When combined with any of the NRTIs or NNRTIs, TAF exhibited moderate to high synergistic effects, with synergy scores ranging from 41 to 131 (Table 4). The combination of TAF with INSTIs resulted in the highest level of synergy (271, 205, and 179 for EVG, RAL, and DTG, respectively). Moderate synergy resulted when TAF was combined with PIs, with synergy scores of 96 and 56 for ATV and DRV, respectively (Table 4). The synergy values observed for TAF with all these drugs were comparable to that of TFV, evaluated in parallel (not shown), and to the values previously reported for TFV (24). Importantly, none of the drug combinations containing TAF exhibited antagonistic antiviral effects.

DISCUSSION

The experiments described in this report were conducted to characterize the *in vitro* virology activity of TAF, an investigational prodrug of TFV, which is currently being evaluated in clinical trials in combination with other anti-HIV-1 drugs. Compared to TDF, TAF demonstrated either similar or improved characteristics in all *in vitro* assays. These results are aligned with phase 1/2 clinical trial results showing the superior antiviral activity of TAF compared to TDF, even with the dose of TAF being 1/10 of the TDF dose (11, 12).

The antiviral activity assays performed in multiple cell types with a panel of laboratory-derived and clinical isolates demonstrated that TAF has nanomolar potency against HIV-1 and HIV-2 (Fig. 5). These results are comparable to what has been observed with TDF, which has been shown to be effective globally in HIV-infected individuals (2). In addition, drug combination studies with TAF demonstrated synergistic effects with all drug classes evaluated and no observed antagonism. These results sup-

TABLE 4 TAF antiviral activities in combination with other antiretroviral drugs

Drug combination	Class	Net effect	Synergy score ^a	Antagonism score ^a
TAF + TFV	NRTI	Additive	24	-14
TAF + FTC	NRTI	Strong synergy	131	-9
TAF + EFV	NNRTI	Moderate synergy	100	-7
TAF + NVP	NNRTI	Slight synergy	41	-14
TAF + EVG	INSTI	Strong synergy	271	-9
TAF + RAL	INSTI	Strong synergy	205	-10
TAF + DTG	INSTI	Strong synergy	179	-10
TAF + ATV	PI	Moderate synergy	96	-10
TAF + DRV	PI	Moderate synergy	56	-12
TAF + COBI	PK enhancer	Additive	17	-22
TAF + TAF	Control	Additive	20	-17
ddI + RBV	Control	Strong synergy	302	-20
d4T + RBV	Control	Strong antagonism	20	-340

^a The data shown represent the means of the results of >3 independent experiments performed in triplicate.

port combining TAF with elvitegravir, cobicistat, and emtricitabine (E/C/F/TAF), as currently evaluated in clinical studies. Furthermore, the results support the use of TAF or FTC/TAF as part of an alternative treatment regimen with the potential to be combined with ARVs from other classes, including NNRTIs, PIs, and INSTIs.

The antiviral specificity of TAF is also identical to those of TFV/TDF, with potent activity observed only with HIV and SIV isolates. Another study has recently demonstrated potent TAF activity against HBV (28). Modest antiviral activity was observed with TAF against both HSV-2 isolates. These results were not unexpected, given the results of the CAPRISA-004 study, which investigated 1% TFV vaginal gel for preexposure prophylaxis (PrEP) (25). In addition to observing a 39% reduction in HIV infection, a 51% reduction in HSV-2 infection was also observed in this PrEP study, which has been attributed to the high local concentration of TFV present in the cervicovaginal fluid and relevant tissues following treatment with 1% tenofovir gel (23). However, as these concentrations of TFV are not likely to be achieved through oral delivery, no antiherpetic activity is anticipated from the TAF oral formulation currently in clinical development.

Tenofovir has been shown to have both a long plasma half-life (17 h) and a long intracellular half-life (>60 h) in PBMCs from patients on TDF-containing regimens (21, 26). In addition, the

pharmacokinetics (PK) of intracellular TFV-DP is likely the primary contributor to establishing and maintaining antiviral suppression, even after missed doses (21, 26). Similar to what has been observed *in vivo*, the *in vitro* persistence assay results from this study demonstrate that TDF maintains its antiviral activity over a full viral cycle. TAF demonstrated comparable maintenance of antiviral activity, suggesting that in the clinical setting, TAF should have an intracellular pharmacokinetic profile similar to that of TDF. A similar study found that TFV had a P_{REL} value of 2.4, while abacavir (ABC) had a P_{REL} value of >243 (R. M. Ledford, J. E. Vela, A. S. Ray, C. Callebaut, M. D. Miller, and D. J. McColl, presented at the 19th International Conference on Antiviral Research, San Juan, Puerto Rico, 2006), highlighting the persistence of TFV and its prodrugs compared to another NRTI. Such high intracellular persistence of TFV prodrugs may be due to the chemical linkage of the phosphonate group, which imparts a stable negative charge to TFV, thus reducing cell membrane permeability relative to those of unphosphorylated parent nucleoside analogs.

While TAF and TDF are both prodrugs of TFV, their conversion to TFV occurs by different pathways. TAF has been shown to be stable in plasma, resulting in the delivery of TAF to its target cells, followed by intracellular conversion to TFV (8), with the first step being driven by CatA or CES1, depending on the cell type (9). In contrast, most TDF is converted to TFV by gut and serum hydrolases extracellularly, resulting in delivery of the less permeable TFV to the target cells (Fig. 2). The human serum stability data in this study reflect the differential sensitivities of the two prodrugs to serum hydrolases, with TAF maintaining its potency after serum pretreatment and TDF losing most of its potency after serum pretreatment.

The improved stability of TAF (8) leads to the improvement of two PK parameters compared to TDF *in vivo*. First, the increased TAF stability results in a decrease in TFV systemic exposure, reducing the potential for bone and renal adverse events, which may be related to overall TFV exposure. This is supported by the week 48 data from study GS-US-292-0102, where patients receiving E/C/F/TAF experienced reduced changes in kidney function (eGFR and tubular proteinuria) and bone mineral density compared to patients receiving E/C/F/TDF (13). Second, the improved stability of TAF results in an increase in intracellular TFV-DP, which allows a lower dose of TAF to be used in clinical settings. In

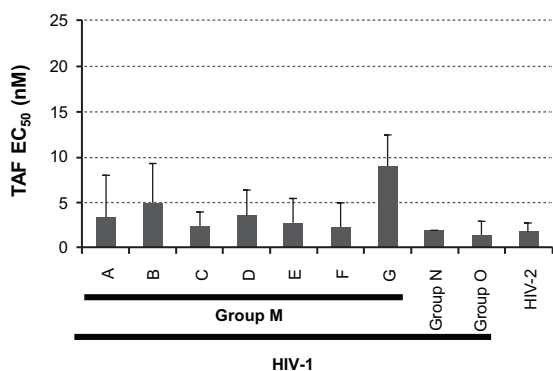


FIG 5 TAF activities against primary HIV isolates. The mean EC₅₀s and standard deviations are shown for HIV-1 group M subtype A to G, group N, and group O isolates, as well as HIV-2 isolates. The mean EC₅₀s and standard deviations were calculated from the data summarized in Table 2.

a phase 1 study, 8 mg TAF produced viral load (VL) declines comparable to those with 300 mg TDF over a 10-day period (11). In the same study, the 25-mg TAF dose resulted in better reduction of the VL, as well as 5 times more TFV-DP in PBMCs, than a 300-mg TDF dose. Finally, the increase in TFV-DP levels may also lead to an improved resistance profile for TAF over TDF. *In vitro*, the resistance profile of TAF is similar to that of TDF/TFV (see our accompanying paper [27]). However, as previously demonstrated, the intracellular TFV-DP levels achieved with TAF were 5 times higher than those with TDF. Therefore, the TAF inhibitory quotient (IQ) should be 5 times higher than that of TDF, suggesting that TAF could have the potential to inhibit previously defined TDF-resistant viruses and may have a higher resistance barrier. In support of this observation, a 48-week phase 2 clinical study comparing E/C/F/TAF versus E/C/F/TDF has shown that no patient receiving E/C/F/TAF developed resistance, whereas 3.4% of patients receiving E/C/F/TDF developed resistance (13). Additionally, very low levels of resistance (<1%) were observed in ongoing phase 3 studies after 48 weeks of treatment (29).

In summary, this study has demonstrated that TAF either equals or improves upon the *in vitro* virologic profile of TDF. Ongoing clinical studies will determine the clinical benefits of TAF.

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