

# Evaluation of the Effect of Cobicistat on the *In Vitro* Renal Transport and Cytotoxicity Potential of Tenofovir

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A once-daily single-tablet antiretroviral regimen containing tenofovir (TFV) disoproxil fumarate, emtricitabine (FTC), elvitegravir (EVG), and cobicistat (COBI) is an approved combination for the treatment of patients infected with HIV. COBI and TFV have been reported to interact with distinct transporters in renal proximal tubules; while TFV is renally eliminated by a combination of glomerular filtration and tubular secretion via anion transporters OAT1, OAT3, and MRP4, COBI inhibits renal cation transporters, particularly MATE1, resulting in a measurable decrease in the tubular secretion of creatinine. To investigate the potential for a renal drug-drug interaction between TFV and COBI *in vitro*, the uptake of TFV in the presence and absence of COBI was determined in fresh human renal cortex tissue and in cells expressing the relevant renal transporters. At concentrations exceeding clinical protein-unbound plasma levels, COBI did not significantly inhibit the transport of TFV by the anion transporters OAT1, OAT3, and MRP4 (50% inhibitory concentrations [IC<sub>50</sub>s] of >15, 6.6, and 8.5  $\mu$ M, respectively). Conversely, TFV had little or no effect on the cation transporters OCT2 and MATE1 (IC<sub>50</sub> > 100  $\mu$ M). Consistent with studies using individual transporters, no increase in the accumulation of TFV in freshly isolated human renal cortex tissue or renal proximal tubule cells (RPTECs) was observed in the presence of COBI. Finally, COBI alone or in combination with FTC and EVG did not affect the sensitivity to TFV of cultured primary RPTECs or cells coexpressing OAT1 and MRP4. These results illustrate that COBI and TFV interact primarily with distinct renal transporters and indicate a low potential for pharmacokinetic renal drug-drug interaction.

STRIBILD, an antiretroviral single-tablet regimen, consists of two nucleoside/nucleotide HIV reverse transcriptase inhibitors, tenofovir (TFV) disoproxil fumarate (TDF) and emtricitabine, combined with the integrase strand transfer inhibitor elvitegravir and the pharmacokinetic enhancer cobicistat (COBI), which effectively blocks the CYP450-mediated metabolic clearance of elvitegravir (1–4). Once-daily treatment with this regimen showed noninferior and durable efficacy and safety in treatment-naïve HIV-infected patients when compared head-to-head with either efavirenz (5, 6)- or atazanavir (7–9)-containing once-daily treatment regimens. A small, early, and nonprogressive increase in the levels of serum creatinine due to a COBI-mediated blockade of creatinine active tubular secretion without an effect on glomerular filtration or overall renal function has been observed during clinical trials (10).

The orally administered prodrug TDF is unstable in plasma and is rapidly hydrolyzed, releasing the parent nucleotide TFV. TFV is renally eliminated by a combination of glomerular filtration and active tubular secretion via renal transporters (11–15). TFV is taken up into proximal tubules by renal organic anion transporter 1 (OAT1) and OAT3, and its luminal efflux is mediated by ABC efflux pump multidrug resistance-related protein 4 (MRP4) (Fig. 1A) (13, 16, 30). OAT1 appears to represent the major renal uptake pathway for TFV, while OAT3 plays a secondary role (11, 17). While TFV interacts with renal organic anion transporters, recent studies have shown that COBI can inhibit cationic transporters, including the efflux pump multidrug and toxin extrusion protein 1 (MATE1) (Fig. 1B) (10, 18). The inhibition of MATE1 is the primary mechanism by which COBI affects the renal tubular secretion of creatinine, causing the serum creatinine increases observed in patients without affecting actual glomerular filtration (10, 18).

While the above findings indicate that COBI is likely to interact

with a different set of renal transporters than TFV, the potential for their renal drug-drug interactions remains to be characterized. Therefore, the present studies seek to assess the potential of COBI and the boosting agent ritonavir (RTV) to affect the active tubular secretion and/or the cytotoxicity profile of TFV in various *in vitro* renal cell and tissue models. We observed that COBI under pharmacologically relevant conditions exhibited minimal interference with the transport pathways currently known to be involved in the renal tubular secretion of TFV. In addition, COBI did not affect the cytotoxicity of TFV in renal cell models. It should be noted, however, that the observations of this study do not affect the requirement for monitoring renal functions in patients treated with TDF-FTC-EVG-COBI in accordance with the STRIBILD prescribing information.

## MATERIALS AND METHODS

**Compounds.** TFV and COBI were synthesized by Gilead Sciences. RTV was purchased from Toronto Research Chemicals (North York, ON, Canada). Probenecid was purchased from Sigma (St. Louis, MO). A stock solution of TFV was prepared in water and adjusted to pH 7.0. COBI, RTV, and probenecid were dissolved in dimethyl sulfoxide (DMSO) and diluted directly into cell culture media according to individual experimental designs. [Adenine-2,8-<sup>3</sup>H]TFV (15 Ci/mmol) and [adenine-8-

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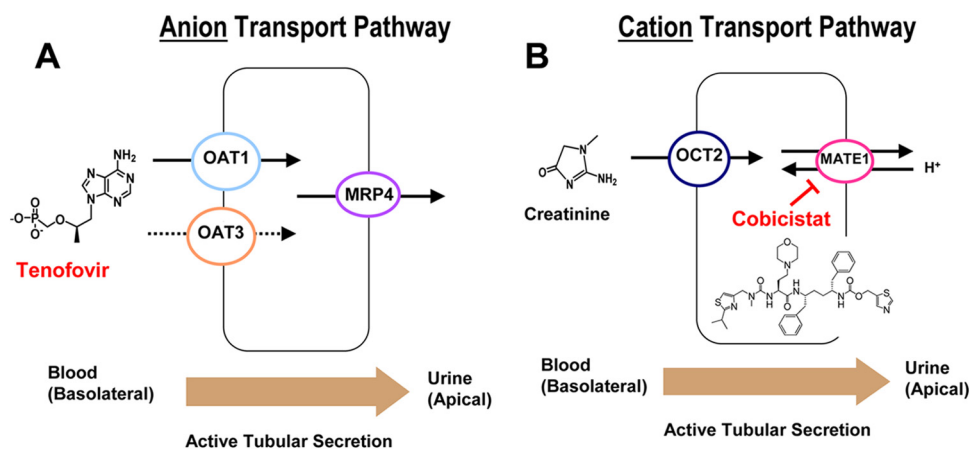
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**FIG 1** Renal tubular transporters interacting with TFV and COBI. (A) The active tubular secretion of TFV is mediated by the organic anion pathway (OATs and MRP4). (B) COBI interacts with the organic cation pathway involved in the active secretion of creatinine (MATE1) (18).

$^3\text{H}$ ]TDF (2.6 Ci/mmol) were purchased from Moravек Biochemicals (Brea, CA). *p*-[Glycyl-2- $^3\text{H}$ ]aminohippuric acid (PAH; 2.53 Ci/mmol) was purchased from PerkinElmer (Boston, MA).

**Cells and tissues.** Human epithelial kidney 293T (HEK293T) cells were kindly provided by Warner Greene (Gladstone Institute of Virology and Immunology, San Francisco, CA). Cryopreserved and freshly isolated human renal proximal tubule cells (RPTECs) were provided by Lonza (Walkersville, MD; catalog no. C2553) and Celsis *In Vitro* Technologies (Halethorpe, MD), respectively. Renal cortex tissue slices were prepared by Vitron (Tucson, AZ) from freshly harvested human kidneys obtained from the Association of Human Tissue Users (Tucson, AZ) with donor consent. The donor was a healthy 36-year-old male treated with no medications who died of intracranial hemorrhaging. The kidneys were explanted from the donor under a protocol used for organ transplantation and delivered on ice in Viaspan cold preservation solution. Following decapsulation, renal cortical slices (200  $\mu\text{m}$  thick) were prepared using a Brendel/Vitron tissue slicer under oxygenated, ice-cold V-7 preservation solution (Vitron). Each organ slice was placed on a titanium roller insert, blotted, and placed into glass vials containing incubation media (Waymouth's MB 752/1 tissue culture medium containing 10% fetal calf serum, 0.35 g/liter L-glutamine, 10 ml/liter Fungi-Bact, and 84 mg/liter gentamicin sulfate). This reproducible precision cut technique produces uniformly sized tissue slices, allowing for a comparison of tissue drug levels without a need for determining the relative accumulation per milligram of tissue protein.

**Transient expression of OAT1, OAT3, and MRP4 transporters.** Full-length cDNA clones in pCMV-based expression vectors containing coding sequences of OAT1 variant 1 (OAT1v1; SC303532), OAT1v2 (SC123302), OAT3 (SC122673), and MRP4 (SC121947) were obtained from Origene (Rockville, MD). HEK293T cells were seeded in 6-well plates 24 h prior to transfection. Subconfluent cells were transiently transfected with the above cDNA constructs using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) according to the manufacturer's recommendations. Cells were incubated at 37°C in a humidified chamber with 5%  $\text{CO}_2$  for 24 h, then trypsinized and reseeded into 12-well plates coated with poly-L-lysine (BD BioCoat; BD Sciences, San Jose, CA) at  $0.3 \times 10^6$  cells per well in phenol red-free minimal essential medium (MEM) (Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS). Plates were incubated another 24 h prior to use in transporter assays.

**OAT1 and OAT3 uptake assays.** HEK293T cells expressing OATs were incubated with 1.2  $\mu\text{M}$  [ $^3\text{H}$ ]TFV or 1.5  $\mu\text{M}$  [ $^3\text{H}$ ]PAH and either DMSO, a specified concentration of the tested compounds, or 50  $\mu\text{M}$  probenecid (control inhibitor of OAT-dependent uptake) for 3 to 6 min (OAT1) or 10 min (OAT3) at 37°C in Waymouth buffer (135 mM NaCl, 5 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgCl}_2$ , 0.8 mM  $\text{MgSO}_4$ , 28 mM

glucose, and 13 mM HEPES, pH 7.2). At the end of the incubation, the cells were washed 3 times with ice-cold phosphate-buffered saline (PBS; 2 ml/well) and lysed directly on the plate by adding 0.4% Triton X-100 (1 ml/well) for 15 min. The intracellular level of TFV was quantified by scintillation counter after a mix with 5.0 ml Ready Safe scintillation fluid (Beckman Instruments, Fullerton, CA). The percentage of OAT-mediated uptake of [ $^3\text{H}$ ]TFV or [ $^3\text{H}$ ]PAH in the presence of each concentration of COBI or RTV was calculated according to the formula % TFV transport =  $100 \times (\text{Rad}_{\text{INH}} - \text{Rad}_{\text{PBC}}) / (\text{Rad}_{\text{CTRL}} - \text{Rad}_{\text{PBC}})$ , where  $\text{Rad}_{\text{INH}}$ ,  $\text{Rad}_{\text{PBC}}$ , and  $\text{Rad}_{\text{CTRL}}$  represent the  $^3\text{H}$  radioactivity signal in the presence of tested compound, 50  $\mu\text{M}$  probenecid (0% TFV transport), or DMSO (100% TFV transport), respectively. Each inhibitor concentration was tested in at least 4 independent replicates, and the resulting % TFV transport was summarized as the mean  $\pm$  standard deviation (SD). Concentrations of tested compounds reducing the OAT-mediated uptake by 50% ( $\text{IC}_{50}$ s) were calculated from individual dose response experiments using nonlinear regression analysis in XLfit software (IDBS, Guildford, United Kingdom).

**MRP4 efflux assays.** HEK293T cells expressing MRP4 were incubated with 1  $\mu\text{M}$  [ $^3\text{H}$ ]TDF or 0.02  $\mu\text{M}$  dehydroepiandrosterone sulfate (DHEAS) for 90 min under ATP-depleting conditions consisting of glucose-free Dulbecco's modified Eagle medium (DMEM) supplemented with 10 mM  $\text{NaN}_3$  and 10 mM 2-deoxy-D-glucose to load the cells with TFV (13). Following incubation, the cells were washed twice with ice-cold PBS to remove extracellular [ $^3\text{H}$ ]TDF and supplemented with prewarmed complete DMEM containing DMSO, a specified concentration of tested compounds, or 50  $\mu\text{M}$  MK-571 (control inhibitor of MRP4-dependent efflux). In some assays, 10% FBS or 100% pooled human serum (Sigma, St. Louis, MO) was added to the incubation media. Cells were incubated at 37°C for 90 min. At the end of the incubation, the cells were washed 3 times with ice-cold PBS (2 ml/well) and lysed directly on the plate by adding 0.4% Triton X-100 (1 ml/well) for 15 min. The intracellular level of  $^3\text{H}$  radioactivity was quantified by scintillation counter after a mix with 5.0 ml Ready Safe scintillation fluid (Beckman Instruments, Fullerton, CA), and the percentage of MRP4-mediated efflux of TFV in the presence of each inhibitor concentration was calculated as described above for the OAT-mediated transport.

**OCT2 and MATE1 transport assays.** MATE1 and OCT2 studies were performed in stably transfected Chinese hamster ovary cell lines at SOLVO Biotechnology (Budaörs, Hungary). Twenty-four hours after the cells were plated into 96-well plates, the cultures were washed with Krebs-Henseleit buffer and incubated for 5 min (OCT2) or 20 min (MATE1) in the same buffer containing 3.6  $\mu\text{M}$  [ $^{14}\text{C}$ ]tetraethylammonium ([ $^{14}\text{C}$ ]TEA) substrate and TFV at the range of tested concentrations. Following the incubation, the medium was aspirated and cells were washed

twice prior to solubilization with 0.1 M NaOH. The amount of cell-associated radioactivity was determined by liquid scintillation counter. Results were compared to those for parent control CHO cells (without human OCT2 or MATE1) treated in the same manner. Verapamil (100  $\mu$ M) and quinidine (100  $\mu$ M) were tested in parallel as the positive-control inhibitors for OCT2 or MATE1, respectively. Fractional transport activity with the [ $^{14}$ C]TEA substrate was calculated from the equation activity (%) =  $(\text{CHO-TRANS}_{\text{test}} - \text{CHO}_{\text{test}})/(\text{CHO-TRANS}_{\text{vehicle}} - \text{CHO}_{\text{vehicle}}) \times 100\%$ , where CHO-TRANS refers to transfected cells, CHO refers to the parental cell line, and subscripts test and vehicle refer to the test compound and vehicle control, respectively.  $\text{IC}_{50}$ s were calculated from the fractional transport activity in the presence of the test compounds, with the vehicle control representing 100% transport activity.  $\text{IC}_{50}$ s were determined by nonlinear regression using GraphPad Prism 5.0.

**TFV accumulation in fresh RPTECs.** Fresh RPTEC cells were suspended at  $2 \times 10^6$  cells per ml in InVitroGRO renal proximal tubule cell culture media (Celsis In Vitro Technologies, Halethorpe, MD) and prewarmed to 37°C. TFV (1.2  $\mu$ M) was added into RPTEC suspensions and incubated in the presence and absence of COBI (1.5  $\mu$ M and 15  $\mu$ M), RTV (1.5  $\mu$ M and 15  $\mu$ M), or probenecid (50  $\mu$ M) for 60 min at 37°C. The entire reaction mixtures were then overlaid onto preprepared microcentrifuge tubes containing 100  $\mu$ l 2 N NaOH (bottom layer) and 100  $\mu$ l filtration oil (top layer; 74.5:25.5 silicon oil-mineral oil mix). Samples were centrifuged immediately at  $13,600 \times g$  for 30 s to pellet the cells. The samples were then allowed to sit at room temperature for 2 h to complete cell lysis and frozen for at least 1 h at  $-80^\circ\text{C}$ . Cell lysates were collected, neutralized with 2 N HCl solution, and extracted with acetonitrile. Following the analysis of TFV content by liquid chromatography/tandem mass spectrometry (LC/MS/MS), the percentage of TFV uptake in the presence of inhibitors relative to that for untreated controls (100%) was calculated. Mean values  $\pm$  standard deviations of TFV levels were calculated from four independent measurements, and the percentage of TFV accumulation in the presence of COBI or ritonavir relative to a control in the absence of compound was determined.

**TFV accumulation in fresh renal cortical tissue.** Incubation of freshly prepared renal slices with tested drugs was conducted at Vitron, Inc. (Tucson, AZ). Each individual tissue slice was incubated in 1.7 ml incubation media containing 1.2  $\mu$ M [ $^3\text{H}$ ]TFV in the presence and absence of COBI (1.5  $\mu$ M and 15  $\mu$ M), RTV (1.5  $\mu$ M and 15  $\mu$ M), or probenecid (200  $\mu$ M). Samples were gently agitated for 60 min at 37°C, washed 3 times with ice-cold phosphate-buffered saline, frozen at  $-80^\circ\text{C}$ , and shipped to Gilead Sciences. When the samples were received, each tissue slice was lysed overnight in 0.5 ml 0.5 N NaOH at 37°C, followed by neutralization with 0.125 ml 2.0 N HCl. The amount of [ $^3\text{H}$ ]TFV accumulated in each tissue slice was quantified by scintillation counter after a mix with 5.0 ml Ready Safe scintillation fluid (Beckman Instruments, Fullerton, CA). Three individual renal tissue slices were analyzed for each tested condition and incubation time. Mean values  $\pm$  standard deviations of TFV levels were calculated from the three independent samples, and the percentage of TFV accumulation in the presence of COBI or ritonavir relative to that for untreated controls (100%) was determined.

**In vitro cytotoxicity assay in cultured RPTECs.** Cryopreserved RPTEC cells were thawed upon receipt and seeded at a density of  $3.5 \times 10^3$  cells per  $\text{cm}^2$  in prewarmed REBM culture medium (Lonza, Walkersville, MD) supplemented with a growth factor cocktail (Lonza catalog no. CC 4127). Cells were utilized for cytotoxicity assays within 6 passages of thawing. Cells were seeded at a density of  $7.0 \times 10^3$  cells/well in 96-well plates (Costar; Corning, Corning, NY). Twenty-four hours after seeding, medium containing TFV serially diluted 3-fold from a starting concentration of 4,000  $\mu$ M was added either alone or in combination with fixed concentrations of COBI, elvitegravir, or emtricitabine. The final amount of DMSO was maintained at 1% across the plate. The concentrations used in combination with TFV corresponded to their respective peak plasma levels in treated HIV-infected patients. Cells were incubated at 37°C in a humidified chamber with 5%  $\text{CO}_2$ . After 5 days, culture medium was

removed and the amount of lactate dehydrogenase (LDH) released into the medium was quantified using the Cytotox-One homogeneous membrane integrity assay (Promega, Madison, WI). In addition, the cell viability was determined using CellTiter Glo (Promega, Madison, WI). Fluorescence and luminescence signals were quantified on an Envision plate reader (Perkin-Elmer, Waltham, MA).

The  $\text{CC}_{50}$  value was defined as the concentration inducing a 50% decrease in cell viability or an LDH release corresponding to a 50% of maximum LDH release. Data were analyzed using XLFit software (IDBS, Guildford, United Kingdom).  $\text{CC}_{50}$  values were calculated by nonlinear regression analysis using the sigmoidal dose-response (variable-slope) equation (four parameter logistic equation)  $Y = (T - B) * \text{CC}_{50}^h / (\text{CC}_{50}^h + x^h) + B$ , where T is the top, B is the bottom, and h is the hill slope. The bottom and top values were fixed at 0 and 100, respectively.  $\text{CC}_{50}$  values were calculated as averages of four independent experiments.

**In vitro cytotoxicity assay in HEK293T cells.** Subconfluent human embryonic kidney 293T cells were transfected with transporter expression plasmids using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) according to the manufacturer's recommendations, incubated at 37°C in a humidified chamber with 5%  $\text{CO}_2$  for 24 h, trypsinized, and reseeded at a density of  $7.0 \times 10^3$  cells/well in poly-D-lysine-coated 96-well plates (VWR, Pittsburgh, PA). After further incubation for 24 h, medium containing TFV serially diluted 3-fold from a starting concentration of 2,000  $\mu$ M was added, either alone or in combination with fixed concentrations of COBI, elvitegravir, or emtricitabine. The final amount of DMSO was maintained at 1% across the plate. The concentrations used in combination with TFV corresponded to their respective peak plasma levels in treated HIV-infected patients. Cells were incubated at 37°C in a humidified chamber with 5%  $\text{CO}_2$ . After 4 days, cell viability was determined by the addition of 100  $\mu$ l CellTiter Glo viability reagent (Promega, Madison, WI) to each well. Luminescence signals were quantified on an EnVision luminometer (Perkin-Elmer, Waltham, MA). The  $\text{CC}_{50}$  values were defined as described above for RPTEC cytotoxicity assays and were calculated as averages of three independent experiments.

## RESULTS

**OAT-mediated uptake of TFV.** To characterize the effect of COBI, *in vitro* models based on the transient expression of two genetic variants of OAT1 and a consensus variant of OAT3 in HEK293T cells were established and validated. OAT1 variant 1 (OAT1v1) differs from OAT1v2 by a 13-amino-acid deletion encompassing residues 523 to 535. Transient transfection of each of the two OAT1 variants induced similar levels of TFV transport, while the transfection of OAT3 induced substantially lower levels of TFV transport than were induced by the two variants of OAT1 (see Fig. S1 in the supplemental material). The transport of TFV mediated by all three transporters was sensitive to the anion transport inhibitor probenecid. In addition, probenecid-sensitive uptake of PAH was established, indicating proper function of all three transiently expressed OATs.

COBI at concentrations ranging from 0.5 to 15  $\mu$ M was tested for an effect on the active transport of TFV by OAT1 and OAT3, and its effect was compared to the effect of RTV at the same concentrations under identical conditions. To more broadly assess the effects of both pharmacokinetic boosting agents on the activity of the tested OATs, PAH was also included as a prototypic organic anion substrate. COBI showed no inhibition of TFV active transport by the two OAT1 variants up to the highest concentration tested (15  $\mu$ M) (Fig. 2). Identical results were obtained with PAH as a substrate (data not shown). In comparison, RTV showed an effect on the activity of both variants of OAT1 at 15  $\mu$ M, exhibiting 30 to 40% inhibition of the transport of both TFV (Fig. 2) and PAH (data not shown). The effect of RTV was dose dependent,

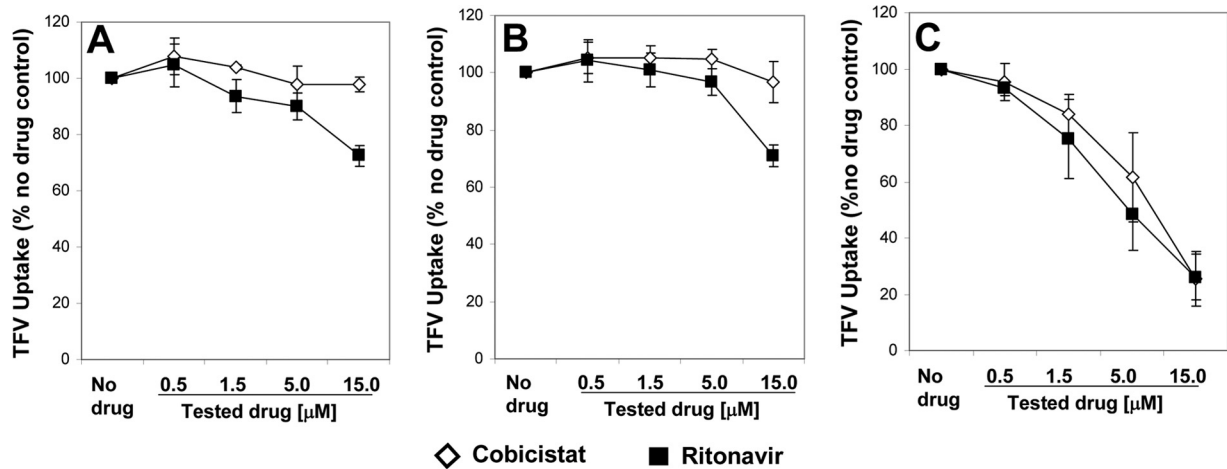


FIG 2 Effect of COBI and RTV on the transport of tenofovir by OAT1v1 (A), OAT1v2 (B), and OAT3 (C). Cells expressing OATs were incubated with 1.2  $\mu\text{M}$  [ $^3\text{H}$ ]TFV and either DMSO or serially diluted tested compounds for 3 to 6 min (OAT1) or 10 min (OAT3) at 37°C. The intracellular level of [ $^3\text{H}$ ]TFV was quantified, and the percentage of OAT1- or OAT3-specific uptake relative to that for the untreated control was calculated after subtracting background determined in the presence of 50  $\mu\text{M}$  probenecid. Results represent means  $\pm$  SD from at least three independent experiments.

with <10% inhibition of OAT1 detected at the concentration of 1.5  $\mu\text{M}$ .

In comparison to that of OAT1, the activity of OAT3 was sensitive to inhibition by both COBI and RTV in a concentration-dependent manner, with similar effects observed on both tested substrates. OAT3-mediated transport of TFV was inhibited by COBI and RTV with  $\text{IC}_{50}$ s of 6.6 and 4.8  $\mu\text{M}$ , respectively. The effect of the two boosting agents on OAT3 was slightly weaker when PAH was used as a substrate. Table 1 summarizes the  $\text{IC}_{50}$ s of COBI and RTV for the transport of TFV and PAH by the three tested OATs.

**MRP4-mediated efflux of TFV.** The development and validation of a transient expression model system for MRP4 in HEK293T cells have been previously described (19). In this system, MRP4-expressing cells are first preloaded with TFV using its prodrug TDF under ATP-depleting conditions to minimize MRP4 efflux activity. The active efflux of TFV from the cells is

then monitored after removing TDF and unblocking ATP synthesis. At the end of preloading with TDF, the intracellular concentration of TFV reached approximately 20  $\mu\text{M}$ , a level substantially below the saturation of MRP4 efflux (16). In this system, both COBI and RTV inhibited the efflux of TFV by MRP4 in a dose-dependent manner (Fig. 3A), with  $\text{IC}_{50}$ s of 8.5 and 12.3  $\mu\text{M}$ , respectively (Table 1). A somewhat weaker effect of COBI and RTV on the activity of MRP4 was observed with the prototype substrate dehydroepiandrosterone sulfate (DHEAS;  $\text{IC}_{50}$  = 14.5 and >20  $\mu\text{M}$ , respectively). Additional transport experiments were performed using MRP4-containing membrane vesicles and DHEAS as the substrate. Results similar to those in the cell-based assay were obtained, with  $\text{IC}_{50}$ s of 20.7 and >20  $\mu\text{M}$  for COBI and RTV, respectively, indicating a correlation between the two frequently used *in vitro* efflux models.

Because the inhibition of MRP4-mediated efflux of TFV could potentially increase intracellular levels of TFV and may affect its renal toxicity profile, further evaluation under pharmacologically relevant conditions was needed to fully understand this interaction. As the free fractions of both COBI and RTV in human serum reach only 2 to 5% of the total concentration (data not shown) due to extensive binding by serum proteins, including albumin and  $\alpha$ -acid glycoprotein, the serum proteins will decrease the availability of both drugs for interacting with MRP4. Cell-based assays in the presence of human serum are frequently used to test the interactions of drugs with intracellular targets in a more physiological environment (20, 21). To directly test the effect of protein binding, the inhibition of MRP4 by COBI and RTV was evaluated in the presence of 100% human serum. Under these conditions, neither of the two boosting agents inhibited the efflux of TFV by MRP4 up to the highest concentration tested (20  $\mu\text{M}$ ) (Fig. 3B).

**Accumulation of TFV in fresh RPTECs and renal cortical tissue.** To confirm that the active transport of TFV is functional in the primary human renal models selected for *ex vivo* studies, freshly isolated RPTECs and precisely cut renal cortex slices were incubated with [ $^3\text{H}$ ]TFV in the absence and presence of the OAT inhibitor probenecid. TFV accumulated in both RPTECs and renal tissue slices in a time-dependent manner, with the accumula-

TABLE 1 Effects of COBI and RTV on the activity of renal organic anion transporters

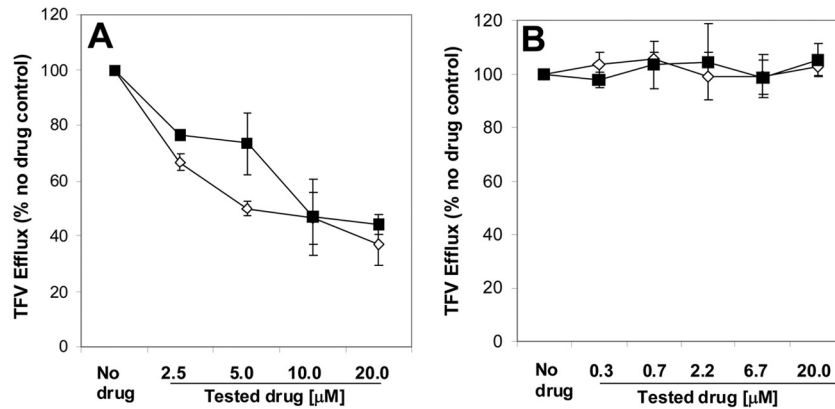
Transporter	Function	$\text{IC}_{50}$ [ $\mu\text{M}$ ] <sup>a</sup> for transport of:			
		TFV		Prototype substrate <sup>b</sup>	
		COBI	RTV	COBI	RTV
OAT1v1	Uptake	>15	>15	>15	>15
OAT1v2	Uptake	>15	>15	>15	>15
OAT3	Uptake	6.6 $\pm$ 1.8	4.8 $\pm$ 1.2	14.6 $\pm$ 6.8	8.7 $\pm$ 3.4
MRP4	Efflux	8.5 $\pm$ 2.6	12.3 $\pm$ 1.3	14.5 $\pm$ 2.8	>20
MRP4 + 100% HS <sup>c</sup>	Efflux	>20	>20	ND <sup>d</sup>	ND

<sup>a</sup>  $\text{IC}_{50}$ s are presented as means  $\pm$  SD determined in at least 3 independent measurements.

<sup>b</sup> PAH (1.5  $\mu\text{M}$ ) was used as a prototype substrate for OAT1s and OAT3; dehydroepiandrosterone sulfate (DHEAS; 0.02  $\mu\text{M}$ ) was used as a prototype substrate for MRP4.

<sup>c</sup> MRP4 was tested in the presence of human serum (HS) to thoroughly assess the potential for *in vivo* drug-drug interaction under pharmacologically relevant conditions.

<sup>d</sup> ND, not determined.



**FIG 3** Effect of COBI and RTV on the MRP4-mediated efflux of TFV in the absence (A) or presence (B) of human serum. The results represent means  $\pm$  SD from at least 3 independent measurements. MK-571 (50  $\mu$ M) was used as a positive-control inhibitor of MRP4 to establish the complete inhibition of TFV transport (0% efflux).

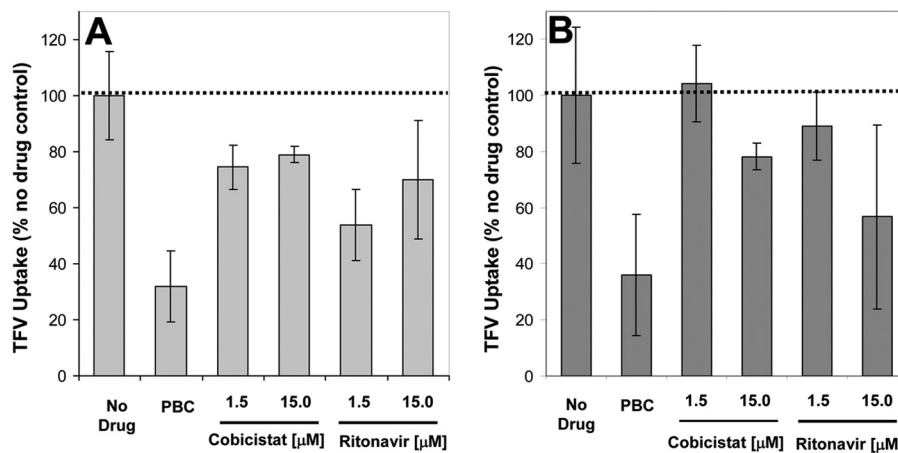
tion substantially reduced in the presence of probenecid (32.0% and 36.3% uptake relative to untreated control,  $P = 0.013$  and  $0.012$ , respectively), confirming the presence of functional renal transport of TFV in both primary cells and fresh tissue models (Fig. 4).

The *ex vivo* accumulation of [ $^3$ H]TFV in RPTECs and fresh cortex slices was tested in the presence of COBI and RTV at 1.5 and 15  $\mu$ M concentrations, representing clinically relevant and supra-therapeutic levels of the two compounds, respectively. Following a 1-hour incubation, COBI at 1.5 and 15  $\mu$ M reduced the accumulation of TFV in RPTECs by approximately 25% and 21%, respectively. In the renal cortex tissue, 1.5  $\mu$ M COBI showed no effect on the accumulation of TFV, while 15  $\mu$ M COBI reduced the accumulation of TFV by approximately 22%, an effect similar to that observed in RPTECs. In comparison, RTV showed more-substantial effects, reducing the levels of TFV accumulation in RPTECs and renal cortex tissue by 30% and 43%, respectively, when tested at the concentration of 15  $\mu$ M (Fig. 4).

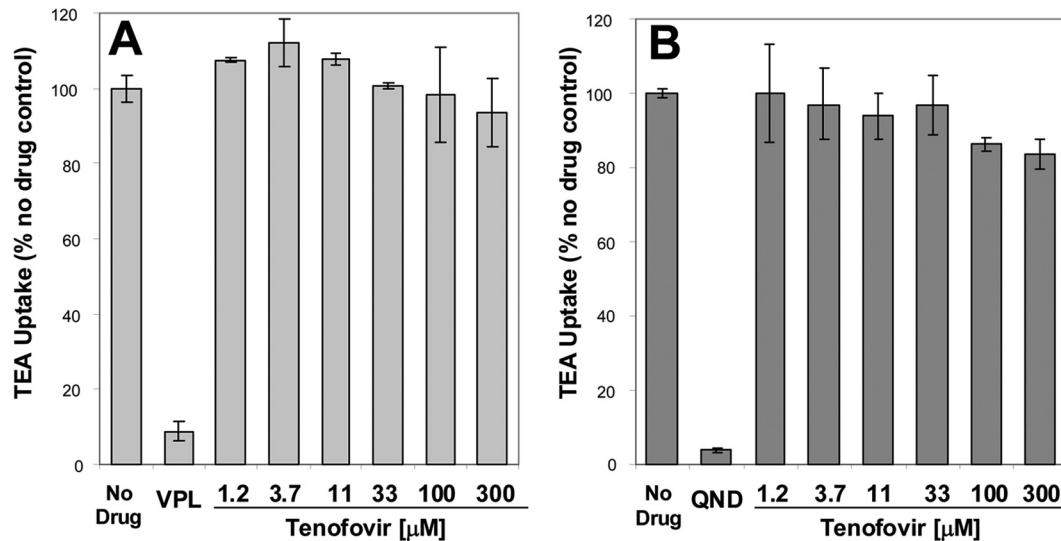
**Effect of TFV on OCT2- and MATE1-mediated transport.** TFV was tested for its potential effect on the transport activity of OCT2 and MATE1, two renal cationic transporters that are in-

involved in the active tubular secretion of creatinine and that have been shown to interact with COBI (18). In CHO cells stably expressing OCT2, TFV showed no effect on the OCT2-mediated transport of [ $^3$ H]TEA up to the highest tested concentration of 300  $\mu$ M (Fig. 5). In CHO cells stably expressing MATE1, TFV showed only a weak effect on the MATE1-mediated transport of [ $^3$ H]TEA, with no inhibition up to the 33  $\mu$ M concentration and a weak, approximately 20% inhibition, at the 100 to 300  $\mu$ M concentrations. While the assay conditions do not allow for full determination of potential effects of TFV phosphorylated metabolites on MATE1, the likelihood that these highly negatively charged forms of TFV would interfere with MATE1 functions is very low based on the strong preference of the transporter for cationic molecules (22).

**In vitro cytotoxicity of TFV in RPTECs.**  $CC_{50}$  values for TFV were above the highest concentration tested (4,000  $\mu$ M) in 4 individual experiments using RPTECs from 2 donors (Table 2). Emtricitabine, elvitegravir, and COBI were also tested for cytotoxicity toward RPTECs, with resulting  $CC_{50}$  values of >100, 13.7, and 26.2  $\mu$ M, respectively. These values were consistent with the generally low cytotoxicity of TFV in RPTECs reported



**FIG 4** Effect of COBI and RTV on the accumulation of TFV in freshly isolated RPTECs (A) and human renal cortical slices (B). The results represent means  $\pm$  SD from 4 independent measurements in RPTECs from two separate donors and 3 independent measurements in renal cortical tissue. Probenecid (PBC; 200  $\mu$ M) was used as a control inhibitor of the active uptake of TFV.



**FIG 5** Effect of TFV on the transport of tetraethylammonium (TEA) by renal organic cation transporters OCT2 (A) and MATE1 (B). The results represent means from two independent measurements. The difference in individual measurements was <13.5%. Verapamil (VPL; 100 μM) and quinidine (QND; 100 μM) were used as control inhibitors of OCT2 and MATE1, respectively.

previously (11, 23–25). Coincubation of TFV with either COBI alone or COBI, elvitegravir, and emtricitabine in combination at pharmacologically relevant concentrations showed no measurable cytotoxicity within the range of tested concentrations of TFV (Table 2).

***In vitro* cytotoxicity assay in cells expressing the renal anion transporters.** Functional expression of OAT1 and MRP4 following their transient transfection in HEK293T cells was confirmed by the uptake and efflux of TFV, respectively, as previously described in Materials and Methods. Both transporters showed the expected functional effect upon their expression (see Fig. S2 and S3 in the supplemental material). While TFV was not cytotoxic in control mock-transfected cells up to 2,000 μM, its cytotoxicity in cells expressing OAT1 increased substantially ( $CC_{50} = 78.7 \pm 1.3$  μM), an effect consistent with previously described results (24, 26) (Table 3). TFV was less toxic in cells expressing OAT1 and MRP4 ( $CC_{50} = 299.5 \pm 81.3$  μM) than in cells expressing OAT1

alone due to its reduced intracellular accumulation via MRP4-mediated efflux. Puromycin was also tested as a control for cytotoxicity in mock-transfected and transporter-expressing cells, with resulting  $CC_{50}$  values of 0.2 to 0.3 μM, respectively, irrespective of the transporter expression. Notably, in cells transiently expressing OAT1 or OAT1 and MRP4 together, the combination of TFV with either COBI or COBI, elvitegravir, and emtricitabine at pharmacologically relevant concentrations produced no significant change in cytotoxicity relative to TFV alone (Table 3). These data indicate that COBI, elvitegravir, and emtricitabine at pharmacologically relevant levels do not change the cytotoxic potential of TFV associated with its active accumulation in renal cells.

## DISCUSSION

Approval of the fixed-dose combination of TDF, emtricitabine, elvitegravir, and COBI (TDF-FTC-EVG-COBI; STRIBILD) in 2012 further expanded the first-line, single-tablet regimen options available to HIV-infected patients. In a 48-week head-to-head comparison with the fixed-dose combination of TDF, emtricitabine, and efavirenz (5) or TDF and emtricitabine combined with

**TABLE 2** Effect of COBI, emtricitabine, and elvitegravir on the cytotoxicity of TFV in human RPTECs

Compound	$CC_{50}$ [μM] <sup>a</sup> for:	
	Cell viability	LDH release
TFV	>4,000	>4,000
COBI	26.2 ± 5.3	39.4 ± 0.8
Elvitegravir	13.7 ± 0.1	32.7 ± 0.1
Emtricitabine	>100	>100
TFV + COBI (2 μM) <sup>b</sup>	>4,000	>4,000
TFV + COBI (2 μM) + elvitegravir (4.5 μM) + emtricitabine (8 μM) <sup>b</sup>	>4,000	>4,000

<sup>a</sup> The results represent means ± SD from 4 independent experiments performed in RPTECs from 2 independent donors.  $CC_{50}$  values were determined in parallel from cell viability readout (CellTiter Glo) and from lactate dehydrogenase (LDH) release readout.

<sup>b</sup> The tested concentrations of COBI, elvitegravir, and emtricitabine correspond to peak plasma levels ( $C_{max}$ ) in HIV-infected patients treated with a clinical dose of each compound (31, 32).

**TABLE 3** Effect of COBI, emtricitabine, and elvitegravir on the cytotoxicity of TFV in HEK293T cells transiently expressing renal anion transporters

Compound	$CC_{50}$ [μM] <sup>a</sup> for:		
	Control	OAT1	OAT1 + MRP4
Puromycin	0.35 ± 0.02	0.21 ± 0.10	0.22 ± 0.06
TFV	>2,000	78.7 ± 1.3	299.5 ± 81.3
TFV + COBI (2 μM) <sup>b</sup>	>2,000	68.3 ± 0.4	230.9 ± 82.6
TFV + COBI (2 μM) + elvitegravir (4.5 μM) + emtricitabine (8 μM) <sup>b</sup>	>2,000	68.0 ± 4.1	228.8 ± 51.2

<sup>a</sup> The results represent means ± SD from 3 independent experiments performed in HEK293T cells.  $CC_{50}$  values were determined from cell viability (CellTiter Glo) readout.

<sup>b</sup> The tested concentrations of COBI, elvitegravir, and emtricitabine correspond to peak plasma levels ( $C_{max}$ ) in HIV-infected patients treated with a clinical dose of each compound.

RTV-boosted atazanavir (7), a noninferior efficacy and differentiated safety profile for TDF-FTC-EVG-COBI was observed. Recent data from the 96-week analyses of both trials established the longer-term robust and durable efficacy of this regimen (6, 9). Minor elevation in serum creatinine attributed to the direct inhibitory effect of COBI on renal organic cation transporters has been noted in clinical studies with the TDF-FTC-EVG-COBI regimen (5, 7, 18). The effect of COBI on serum creatinine is similar to those observed with other drugs commonly used in this patient population such as RTV (9), dolutegravir (27), and cimetidine (28).

In the present study, we assessed the potential of COBI to directly affect the renal pharmacology of TFV. While previous findings suggested that COBI is likely to interact with a different set of renal transporters than TFV (18), the potential for renal drug-drug interaction between these two components of TDF-FTC-EVG-COBI required further characterization. Therefore, we used several complementary *in vitro* and *ex vivo* models to assess whether COBI and its structurally similar pharmacological enhancer RTV (1) exhibit a potential to affect the active renal transport and the cytotoxicity of TFV.

OAT1 and OAT3 mediate the active basolateral uptake of anionic substrates, including TFV, into proximal tubule cells during the process of tubular secretion (11, 17). COBI did not affect the transport of TFV by two variants of OAT1, even at concentrations 10-fold above its peak plasma level following the 150-mg dose used for clinical pharmacokinetic boosting. COBI affected the OAT3-mediated transport of TFV, with an  $IC_{50}$  of 6.7  $\mu$ M, but had a minor (<20%) inhibitory effect on the transport of TFV by OAT3 at concentrations equal to its peak plasma levels. However, this effect on OAT3 transport of TFV is unlikely to be clinically relevant as OAT3 has been shown to be secondary to OAT1 in active uptake of TFV into proximal tubule cells (11, 17). In addition, reduced uptake of TFV into proximal tubules due to the interference with OAT3 would not adversely affect renal safety of TFV as it would effectively reduce the intracellular accumulation of TFV.

The ABC pump MRP4 is involved in the apical efflux of anionic substrates, including TFV, from proximal tubule cells into the nephron lumen (13). Our results showed that, while the MRP4-mediated efflux of TFV is sensitive to COBI at relatively low concentrations, the effect is eliminated in the presence of serum protein binding that effectively reduces the levels of COBI available for interacting with the transporter, indicating that COBI is unlikely to affect the renal efflux of TFV under pharmacologically relevant conditions. These results are consistent with previous observations characterizing the effect of human serum on the inhibition of MRP4 by RTV (19).

Notably, the low potential of COBI or RTV to interact with OAT3 and MRP4 was confirmed with other anion substrates such as PAH and DHEAS. It should also be noted in this context that recent studies suggested the potential involvement of MRP7 (ABCC10) in the renal efflux of tenofovir in addition to MRP4 (29). The potential for interactions between TFV and COBI at the level of MRP7 will require additional studies.

COBI has been shown in earlier studies to interact primarily with cation transporters OCT2 and MATE1, involved in the pathway of active secretion of creatinine, acting as their effective inhibitor (18). Hence, COBI showed a propensity for interacting with both cation and anion transporters. In contrast, TFV did not show

any effect on OCT2 or MATE1 transporters even at extremely high concentrations, indicating its selective interaction with anion transporters.

We extended our assessment of the renal interaction between TFV and COBI to two physiologically more relevant primary cell/tissue models: human renal proximal tubule epithelial cells and fresh human renal cortical tissue slices. The presence of functional active uptake of TFV was confirmed by the effect of probenecid, a prototype inhibitor of organic anion transporters. COBI at levels mimicking clinical systemic exposure exhibited only a minor effect on the accumulation of TFV in RPTECs. Similarly, TFV accumulation in renal cortical tissue was only weakly affected in the presence of COBI at the highest tested concentration (15  $\mu$ M). Consistent with the observations made in OAT1- and OAT3-transfected cells, COBI inhibited TFV uptake to an equal or lesser degree than RTV. It is important to note that the inhibition of TFV uptake, while not predicted to have any effect at pharmacologically relevant concentrations, would decrease intracellular levels of TFV in the proximal tubule.

The present study also assessed the effect of COBI and the combination of COBI, elvitegravir, and emtricitabine on the cytotoxicity of TFV in two *in vitro* renal cell culture models. TFV by itself exhibited minimal cytotoxicity in RPTECs, even at high concentrations, an observation consistent with prior studies (24, 25). Neither COBI alone nor the combination of COBI, elvitegravir, and emtricitabine affected the cytotoxicity of TFV in human RPTECs. However, it should be noted that the level of expression of transporters in primary RPTECs may fluctuate with the duration of primary culture. Therefore, an additional assay in which OAT1 and MRP4 transporters were coexpressed at high levels in HEK293T embryonic kidney cells was developed. Similar to the observations made in the primary culture model, the cytotoxicity of TFV in cells coexpressing OAT1 and MRP4 was not affected by COBI or the combination of COBI, elvitegravir, and emtricitabine at concentrations matching their clinical systemic peak exposures.

In summary, COBI under pharmacologically relevant conditions exhibited minimal interference with the functions of transport pathways currently known to be involved in the active renal tubular secretion of TFV. These conclusions were supported by results from primary *in vitro* and *ex vivo* renal tissue models and were corroborated by the lack of the effect of COBI on the cytotoxicity of TFV in renal cell cultures, indicating a low potential for renal drug-drug interaction between these two components of the TDF-FTC-EVG-COBI single-tablet regimen.

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