Effects of Human Immunodeficiency Virus Protease Inhibitors on the Intestinal Absorption of Tenofovir Disoproxil Fumarate In Vitro[▽]

Leah Tong,¹ Truc K. Phan,¹ Kelly L. Robinson,¹ Darius Babusis,¹ Robert Strab,²
Siddhartha Bhoopathy,² Ismael J. Hidalgo,² Gerald R. Rhodes,¹
and Adrian S. Ray^{1*}

Gilead Sciences, Inc., Foster City, California 94404,¹ and Absorption Systems, L. P., Exton, Pennsylvania 19341²

Received 22 May 2007/Returned for modification 5 July 2007/Accepted 19 July 2007

Human immunodeficiency virus protease inhibitors (PIs) modestly affect the plasma pharmacokinetics of tenofovir (TFV; –15% to +37% change in exposure) following coadministration with the oral prodrug TFV disoproxil fumarate (TDF) by a previously undefined mechanism. TDF permeation was found to be reduced by the combined action of ester cleavage and efflux transport in vitro. Saturable TDF efflux observed in Caco-2 cells suggests that at pharmacologically relevant intestinal concentrations, transport has only a limited effect on TDF absorption, thus minimizing the magnitude of potential intestinal drug interactions. Most tested PIs increased apical-to-basolateral TDF permeation and decreased secretory transport in MDCKII cells overexpressing P-glycoprotein (Pgp; MDCKII-MDR1 cells) and Caco-2 cells. PIs were found to cause a multifactorial effect on the barriers to TDF absorption. All PIs showed similar levels of inhibition of esterase-dependent degradation of TDF in an intestinal subcellular fraction, except for amprenavir, which was found to be a weaker inhibitor. All PIs caused a dose-dependent increase in the accumulation of a model Pgp substrate in MDCKII-MDR1 cells. Pgp inhibition constants ranged from 10.3 μ M (lopinavir) to >100 μ M (amprenavir, indinavir, and darunavir). Analogous to hepatic cytochrome P450-mediated drug interactions, we propose that the relative differences in perturbations in TFV plasma levels when TDF is coadministered with PIs are based in part on the net effect of inhibition and induction of intestinal Pgp by PIs. Combined with prior studies, these findings indicate that intestinal absorption is the mechanism for changes in TFV plasma levels when TDF is coadministered with PIs.

Tenofovir disoproxil fumarate (TDF; Viread, Gilead Sciences, Inc.), a prodrug of the nucleotide reverse transcriptase inhibitor tenofovir (TFV), is used to effectively deliver TFV across the gut wall. Following absorption, TDF rapidly degrades to TFV and TDF is not observed in the systemic circulation. When administered by itself, TFV has poor oral bioavailability (7). TDF is a common once-a-day backbone for use with human immunodeficiency virus (HIV) protease inhibitors (PIs). Clinical trials have shown TDF with emtricitabine in combination with either lopinavir (LPV) or atazanavir (ATV), each boosted with ritonavir (RTV, or “r” when referred to as a boosting agent), to be efficacious and well tolerated (22, 30).

Polypharmacy in HIV patients creates the potential for drug interactions (8, 35). No interaction between PIs and TDF would be anticipated due to the lack of cytochrome P450 involvement in the elimination pathway of TDF or TFV (25). However, modest changes in TFV plasma pharmacokinetic parameters have been reported for TDF coadministered with PIs. As summarized in Table 1, PIs can be categorized into three different groups based on their effects on TFV plasma pharmacokinetics. The first group encompasses PIs that cause modest increases in TFV plasma exposure (area under the concentration-time curve from 0 h to time t [AUC_{0-t}]) when coadministered with TDF and includes saquinavir (SQV)/r,

darunavir (DRV)/r, LPV/r, the experimental PI brexanavir (BRV)/r, and ATV/r. The second group of PIs do not change the TFV AUC_{0-t} , although slight increases in the TFV maximal or minimal plasma concentration (C_{max} and C_{min} , respectively) have been reported, and include indinavir (IDV) and nelfinavir (NFV). The third group includes PIs that cause slight decreases in TFV levels. Tipranavir (TPV)/r causes a dose-dependent decrease in the TFV C_{max} . Fosamprenavir (FPV; the phosphate prodrug of amprenavir [APV]) dosed alone causes a decrease in the TFV AUC_{0-t} , C_{max} , and C_{min} , while FPV dosed as FPV/r causes a lesser decrease in TFV plasma C_{max} , with no change in the AUC_{0-t} or C_{min} .

The goal of the research described here was to determine if PIs can alter the intestinal absorption of TDF, causing the slight perturbations in the level of TFV observed in the systemic circulation. These studies were undertaken in light of the following observations suggesting that PIs may affect TDF absorption: (i) TDF absorption can be increased by a high-fat meal (3, 25); (ii) TDF intestinal absorptive flux was previously reported to be limited in vitro by esterase activity and efflux transport, based on increased Caco-2 cell permeability observed in the presence of fruit extracts and the transport inhibitor cyclosporine A (CsA) (43); (iii) esterase activity has also been noted to affect the absorption of TFV prodrugs in vivo, based on the correlation between increased esterase stability and higher oral bioavailability in the dog for different ester prodrugs of TFV (42); and (iv) PIs are known to inhibit the intestinal efflux transporter P-glycoprotein (Pgp) (27). Detailed in vitro studies were designed to identify if Pgp is in-

* Corresponding author. Mailing address: Department of Drug Metabolism, Gilead Sciences, Inc., 333 Lakeside Dr., Foster City, CA 94404. Phone: (650) 522-5536. Fax: (650) 522-1892. E-mail: adrian.ray@gilead.com.

[▽] Published ahead of print on 30 July 2007.

TABLE 1. Reported effects of coadministration of PIs with TDF (300 mg once daily) on the plasma pharmacokinetics of TFV in clinical drug interaction studies

PI(s)	Dosing regimen (mg) ^b	% Change ^a			Reference
		AUC	C _{max}	C _{min}	
ATV/r	300/100 QD	↑ 37	↑ 34	↑ 29	1
BRV/r	300/100 BID	↑ 32	↑ 24	NR ^c	12
LPV/r	400/100 BID	↑ 32	↑ 15	↑ 51	23, 26
DRV/r	300/100 BID	↑ 22	↑ 24	↑ 37	2
SQV/r	1,000/100 BID	↑ 14	↑ 15	↑ 23	5
IDV	800 TID	↔	↑ 14	NR ^c	24
NFV	1,250 BID	↔	↔	↑ 9	4
TPV/r	500/100 BID	↔	↓ 23	↔	40
	750/250 BID	↔	↓ 38	↔	
FPV/r	700/100 BID	↔	↓ 18	↔	29
FPV	1,400 BID	↓ 15	↓ 25	↓ 12	

^a ↔, ↑, and ↓ denote a statistically significant lack of change, an increase, and a decrease in TFV plasma pharmacokinetics, respectively.

^b QD, BID, and TID denote once-, twice-, and thrice-daily dosing, respectively.

^c NR, not reported.

volved in TDF efflux and to determine the magnitude of the effect it has in limiting the absorption of TDF in the gastrointestinal tract. The effects of PIs on the permeation of TDF in vitro and the respective abilities of PIs to alter factors affecting TDF intestinal absorption, including esterase cleavage and efflux transport, were then determined. Results from these studies in combination with literature reports of similar drug interactions and the induction potential of PIs were used to develop a model explaining the relative effects of PIs on TFV circulating levels.

(This work was presented in part as poster 49 at the 7th International Workshop on Clinical Pharmacology of HIV Therapy, Lisbon, Portugal, 20 to 22 April 2006 [38].)

MATERIALS AND METHODS

Reagents. TDF, its monoester intermediate metabolite (monoPoc TFV), and TFV were obtained from Gilead Sciences, Inc. (Foster City, CA). SQV was purchased from Moravak Biochemicals, Inc. (Brea, CA). NFV (mesylate salt), IDV (sulfate salt), RTV, APV, and LPV were purchased from Toronto Research Chemicals, Inc. (North York, Ontario, Canada). ATV (sulfate salt) was isolated from capsules. BRV and DRV were chemically synthesized. The transport inhibitor CsA, the Pgp substrate calcein-AM, and the esterase inhibitor di-isopropyl fluorophosphate were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture supplies were obtained from Invitrogen (Carlsbad, CA), and HyClone fetal bovine serum was purchased from Fisher Scientific (Pittsburgh, PA). All other reagents were the highest quality available from Sigma-Aldrich.

Caco-2 and MDCKII cell transepithelial transport assays. Parental Madin-Darby canine kidney (MDCKII) cells and MDCKII cells stably transfected with the human *MDR1* gene (encoding Pgp; MDCKII-MDR1 cells) were kindly provided by Piet Borst of The Netherlands Cancer Institute. MDCKII-MDR1 cells have been characterized in a prior report (10) and were maintained as previously described (37). MDCKII cells were seeded in 24-well plates at an initial density of 2.1×10^4 cells/well and grown to confluence over 5 days prior to assay initiation. The permeability assay buffer for the donor and receiver chambers was Hanks balanced salt solution containing 10 mM HEPES and 15 mM glucose. The donor and receiver buffers were at pH 6.5 and 7.4, respectively, and the receiver well contained 1% bovine serum albumin. In some studies, the assay buffers also contained PIs (20 μ M) or the transport inhibitor CsA (10 μ M). All chambers were preincubated for 60 min with transport buffer containing the appropriate inhibitor to saturate any transporter binding sites. Following preincubation, fresh assay buffer containing inhibitor and TDF was added, and the assay was started. At each time point, 1 and 2 h, an aliquot was taken from the receiver chamber and replaced with fresh assay buffer. Samples were taken from

the donor chamber at 0 and 2 h. Cells were dosed on the apical (A) or basolateral (B) side to determine A-to-B and B-to-A permeability, respectively, and were incubated at 37°C with 5% CO₂ in a humidified incubator. Each determination was performed in duplicate, and the permeation of control compounds (atenolol, propranolol, and vinblastine) was determined to meet acceptance criteria for each assay plate.

Bidirectional permeability studies were done using confluent monolayers of the human colon carcinoma cell line Caco-2 (16) seeded in 12-well plates essentially as previously reported (37). The effects of PIs on the permeation of TDF were studied following a 30-min preincubation of cell monolayers with 20 μ M PIs in transport buffer to allow for saturation of transporter binding sites. Following preincubation, fresh assay buffer containing inhibitor and TDF was added, and the assay was started. Each determination was performed in duplicate, and the permeation of control compounds (atenolol, propranolol, and digoxin) was determined to meet acceptance criteria for each batch of assay plates. Levels of TDF and its metabolites in Caco-2 and MDCKII cell permeability studies were analyzed by liquid chromatography coupled to triple-quadrupole mass spectrometry (LC/MS/MS) as described below.

Pgp inhibition assay. The inhibition of Pgp-mediated transport was studied using the Pgp substrate calcein-AM and MDCKII-MDR1 cells. Studies were done with a 96-well fluorescence assay essentially as described previously (37). Twofold serial dilutions of PIs, with a starting concentration of 50 μ M (seven individual concentrations were tested), were added simultaneously with calcein-AM to parental and MDCKII-MDR1 cells. All studies were done in duplicate.

Inhibition of TDF degradation in a human intestinal subcellular fraction. Human intestinal S9 (a subcellular fraction) was purchased from XenoTech LLC (Lenexa, KS). Reactions were done with a 1-mg/ml protein concentration. TDF was incubated at 2 μ M in the presence or absence of PIs or the known esterase inhibitor di-isopropyl fluorophosphate. All PI inhibition studies were done at 50 μ M, except for those with RTV, where the dose dependence of inhibition was assessed at concentrations of 10, 50, and 100 μ M. The potent esterase inhibitor di-isopropyl fluorophosphate was incubated at 2 μ M. Reactions were done in duplicate at 37°C. After 30 and 60 min, TDF degradation was stopped by the addition of 67% acetonitrile in water. The protein precipitate was removed by centrifugation, and the supernatant was analyzed by LC/MS/MS as described below.

Quantitation of TDF and its metabolites by LC/MS/MS. Levels of TDF and its metabolites were quantified using LC/MS/MS methods by comparing the relative peak areas of samples to standard curves for known concentrations prepared with appropriate sample matrices. Levels of TDF and monoPoc TFV were determined in MDCKII cell permeability assays and intestinal S9 stability assays by reversed-phase chromatography on a BEH C₁₈ column (1.7 μ m by 50 mm by 2.1 mm), with a gradient from 1% to 80% acetonitrile over 3.5 min and a flow rate of 0.9 ml/min, on an Acquity ultra-performance LC system coupled to a Micromass Quattro Premier XE MS/MS system running in positive ionization and multiple reaction monitoring modes (the column, LC system, and mass spectrometer were all purchased from Waters, Milford, MA). Due to insufficient sensitivity for TFV with the Micromass instrument, the levels of TFV, monoPoc TFV, and TDF were determined in select intestinal S9 stability and MDCKII cell permeability studies by using an API 4000 MS/MS system (Applied Biosystems/MDS Sciex, Foster City, CA) running in positive ionization mode and monitoring the parent/daughter mass transitions of 520/270, 404/270, and 288/176, respectively. An LC flow rate of 1 ml/min, with 0.2% formic acid as the buffer and a linear gradient from 0 to 62% acetonitrile over 3 min, was used. A Synergy Hydro RP 80 column (4 μ m by 50 mm by 4.6 mm; Phenomenex, Torrance, CA) was used to separate the analytes. Similarly, levels of TDF were quantitated following the addition of acetonitrile containing 0.4% formic acid in Caco-2 cell assays by a reversed-phase high-performance LC method coupled to positive-mode MS/MS at Absorption Systems (Exton, PA).

Data analysis. The A-to-B and B-to-A apparent permeability coefficients (P_{app}) for Caco-2 and MDCKII cells were calculated using the equation $P_{app} = (dC/dt) \times V_r / (A \times C_0)$, where dC/dt is the change in concentration over time, as measured at 60 and 120 min, in μ M/s; V_r is the volume of the receiver well, in cm³; A is the area of the cell monolayer, in cm²; and C_0 is the initial concentration in the donor well, in μ M. The percent recovery was calculated by comparing the total amount of material in the donor and receiver chambers at the end of the experiment relative to the amount added to the donor chamber at the beginning of the experiment. The efflux ratio (ER) was defined as the B-to-A P_{app} divided by the A-to-B P_{app} . The binding constant for efflux saturation was calculated by comparing the ER measured at the lowest concentration of TDF tested (1 μ M) to that observed at higher concentrations, using the following equation: efflux saturation = $[(ER_1 - 1) - (ER_i - 1)/(ER_1 - 1)] \times 100\%$, where ER_1 was

FIG. 1. Saturation of efflux by pharmacologically relevant intestinal concentrations of TDF in Caco-2 cell experiments. (A) Increases in the apparent A-to-B P_{app} of TDF were observed at incubation concentrations approaching those anticipated in the gastrointestinal tract. TDF was applied to the apical side of Caco-2 cell monolayers at concentrations ranging from 1 to 1,000 μ M, and its A-to-B P_{app} was determined. (B) Pharmacologically relevant intestinal TDF concentrations were found to partially saturate efflux. The percent saturation of efflux was calculated by comparing the ER for 1 μ M TDF of 25 to that obtained for a higher concentration of TDF. At pharmacologically relevant intestinal TDF concentrations, anticipated to be ≥ 1 mM, minimal changes in the A-to-B P_{app} of TDF due to inhibition of efflux transport are possible (as indicated by the shaded area). Values represent the averages for two independent experiments done in duplicate. Results were consistent between the two independent studies and with TDF permeation observed at other test concentrations.

measured at 1 μ M and ER_T was determined at the respective test concentration. The amount of PI required to inhibit Pgp-dependent calcein-AM transport was determined by fitting the percent increase in intracellular fluorescence in the presence of different PI concentrations to a hyperbolic curve. The rates of TDF degradation in intestinal S9 studies were determined by fitting the data to a single-exponential-decay curve. The percent inhibition of TDF degradation was then obtained by comparing the observed rate of TDF degradation with the addition of dimethyl sulfoxide (control) to that obtained in the presence of inhibitor. Where appropriate, the statistical significance of differences observed was assessed using two-tailed Student's *t* tests.

RESULTS

Dose-dependent effect of efflux transport on TDF permeation. TDF (formula weight, 635) is given as a single 300-mg tablet once a day, and assuming rapid dissolution into approximately 250 ml of intestinal content, the pharmacologically relevant intestinal concentration should be around 2 mM. The dose-dependent bidirectional permeation of TDF was deter-

FIG. 2. Effects of PIs (20 μ M) or the transport inhibitor CsA (10 μ M) on the bidirectional permeation of 10 μ M TDF through confluent monolayers of MDCKII-MDR1 cells. PIs and CsA decreased the Pgp-mediated ER of TDF. Results for PIs are organized left to right in order of increasing Pgp inhibition, and the ER for each treatment condition is indicated above the respective bars. Values represent the means $\pm$ standard deviations for at least three independent experiments done in duplicate. The statistical significance of changes in the ER of TDF compared to that in MDCKII-MDR1 cells in the absence of coinubation was assessed using Student's unpaired two-tailed *t* tests, assuming equal variance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

mined at concentrations approaching levels anticipated in the gastrointestinal tract, using the Caco-2 cell intestinal permeability model. The A-to-B P_{app} of TDF increased linearly >10 -fold between concentrations of 1 and 1,000 μ M (Fig. 1A). Over this TDF concentration range, the ER for TDF was found to be saturable, yielding an apparent binding constant for efflux saturation of 144 μ M (concentration required to cause 50% saturation) (Fig. 1B), reflecting a decrease in the ER, from 25 at 1 μ M TDF to 2.6 at 1,000 μ M TDF.

Pgp-mediated transport of TDF in MDR1-transfected MDCKII cells. Earlier studies have indirectly inferred that TDF absorption is affected by Pgp-mediated efflux based on the effect of the efflux transport inhibitor CsA (38, 43). To verify that Pgp contributes to TDF efflux, permeability studies were done with parental and MDR1-transfected MDCKII cells. TDF had a 3.2-fold lower forward permeation level and a 16-fold higher ER in MDCKII-MDR1 cells than in parental cells (data not shown). Quantitation of the TDF metabolites monoPoc TFV and TFV during TDF permeability studies with MDCKII-MDR1 cells showed minimal conversion (combined recovery in the donor and receiver wells was $<5\%$ of the original TDF concentration) and no marked contribution to total permeation (data not shown).

Effects of PIs on bidirectional permeation of TDF through monolayers of MDCKII-MDR1 and Caco-2 cells. Lower than pharmacologically relevant concentrations of TDF were used in cell-based permeability studies in order to increase efflux, facilitating the accurate assessment of the relative effects of PIs on TDF permeation. Having established the Pgp-dependent efflux of TDF, the effects of PIs on the efflux of TDF in MDCKII-MDR1 cells were assessed. PIs decreased the efflux of TDF in Pgp-overexpressing cells (Fig. 2). The PIs BRV, NFV, LPV, and RTV were the most potent inhibitors of TDF efflux, while DRV, IDV, and APV showed smaller effects. We could not assess the effects of TPV due to insufficient material,

FIG. 3. Effects of select PIs on bidirectional permeation of 50 μ M TDF through monolayers of Caco-2 cells. The effects of 20 μ M APV, IDV, SQV, ATV, LPV, and RTV were compared to those of a potent transport inhibitor, CsA (10 μ M), and the esterase inhibitor di-isopropyl fluorophosphate (DFP; 2 μ M). All PIs except APV and IDV caused significant increases in the TDF A-to-B P_{app} . All PIs except IDV caused a significant inhibition of TDF efflux. Results for PIs are organized left to right in order of increasing efflux inhibition, and the ER for each treatment condition is indicated above the respective bars. Values represent the means $\pm$ standard deviations for three independent experiments done in duplicate. The statistical significance of changes in the A-to-B P_{app} values and ERs compared to the values for Caco-2 cells in the absence of coincubation was determined by using Student's unpaired two-tailed t tests, assuming equal variance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). While the change in TDF A-to-B P_{app} caused by CsA did not reach statistical significance based on unpaired analyses of all data, it did cause a reproducible increase in side-by-side assays, as illustrated by Student's paired two-tailed t test (#, $P = 0.03$).

but it has been reported to be a weak Pgp inhibitor (Aptivus package insert; Boehringer Ingelheim).

In order to study the effects of select PIs on the permeation of TDF in a more physiologically relevant model of gastrointestinal absorption, bidirectional permeability studies were done with Caco-2 cells (16). The effects of LPV, ATV, RTV, SQV, IDV, and APV on the permeation of TDF were compared to those of the potent efflux inhibitor CsA and the esterase inhibitor di-isopropyl fluorophosphate (Fig. 3). CsA and di-isopropyl fluorophosphate increased the forward permeation of TDF by 1.4- and 2.5-fold, respectively. The results for CsA and di-isopropyl fluorophosphate observed here were not unlike those found previously by others for CsA and fruit extracts (43). RTV, LPV, ATV, and SQV caused greater increases in the TDF A-to-B P_{app} than did CsA. Comparison of permeation in both the A-to-B and B-to-A directions showed that CsA caused a near complete blockage of TDF efflux transport, while di-isopropyl fluorophosphate similarly increased the P_{app} of TDF in the A-to-B and B-to-A directions. Consistent with the results from MDCKII-MDR1 cells, APV and IDV were among the weakest and LPV and RTV were among the strongest inhibitors of TDF efflux in Caco-2 cells.

Inhibition of intestinal ester hydrolysis of TDF by PIs. The potential for inhibition of TDF hydrolysis by PIs was assessed in a human intestinal subcellular fraction (S9). The major product of TDF degradation in S9 was the intermediate monoester metabolite of TFV (monoPoc TFV), with lower levels of TFV (data not shown). RTV caused a dose-dependent de-

FIG. 4. Inhibition of intestinal esterase-dependent degradation of TDF by PIs. (A) RTV showed a dose-dependent inhibition of TDF degradation in a human intestinal S9 fraction. The rate of TDF degradation was 0.031, 0.021, 0.016, and 0.012 min^{-1} in the presence of 0 (dimethyl sulfoxide control), 10, 50, and 100 μ M RTV, respectively. (B) Percent inhibition of TDF degradation in intestinal S9 fraction by the esterase inhibitor di-isopropyl fluorophosphate (DFP; 2 μ M) or by PIs incubated near their solubility limits (50 μ M). Values represent the means $\pm$ standard deviations for three to six independent experiments done in duplicate. The statistical significance of changes in the rate of TDF degradation in the presence of inhibitors relative to that with no inhibitor was assessed by Student's unpaired two-tailed t tests, assuming equal variance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

crease in TDF degradation (Fig. 4A). Di-isopropyl fluorophosphate and PIs were found to inhibit TDF degradation (Fig. 4B) and, concomitantly, to decrease the appearance of its metabolites. PIs were tested at a concentration near their solubility limits to model pharmacologically relevant concentrations potentially formed following dosing in the gastrointestinal tract (50 μ M). While most PIs showed a similar inhibition of TDF degradation, IDV and DRV reproducibly showed less marked inhibition ($\leq 40\%$ inhibition). The results for APV did not reach statistical significance.

Inhibition of Pgp by PIs. The relative inhibition of Pgp by PIs was studied by monitoring the effects of coincubation on the Pgp-dependent accumulation of the fluorescent compound calcein (metabolite of the Pgp substrate calcein-AM) in MDCKII-MDR1 cells. All PIs showed a dose-dependent inhibition of Pgp. The effects of PIs ranged from the most potent, LPV, to the least potent, DRV, with observed inhi-

TABLE 2. Inhibition of Pgp-dependent efflux of calcein-AM following incubation of MDCKII-MDR1 cells with the Pgp inhibitor CsA or with PIs

Compound	IC ₅₀ (μM) ^a
CsA.....	9.34 ± 4.33
LPV.....	10.3 ± 4.4
NFV.....	19.9 ± 21.1
BRV.....	24.1 ± 9.2
RTV.....	39.6 ± 19.4
ATV.....	67.8 ± 27.7
SQV.....	100 ± 68
APV.....	>100 ^b
IDV.....	>100
DRV.....	>100

^a Values represent means ± standard deviations for 3 to 10 independent assays, each including seven inhibitor concentrations, done in duplicate.

^b HIV PIs were tested at concentrations of up to 50 μM in the absence of serum proteins. All PIs showed significant dose-dependent inhibition of Pgp during the assay, although many did not attain ≥25% inhibition at 50 μM, and their IC₅₀ values are reported as >100 μM.

bition in 50 μM incubations of approximately 100% and 15%, respectively. The calculated 50% inhibitory concentrations (IC₅₀s) for the PIs and CsA are summarized in Table 2. The rank order of PIs was similar to that observed based on their effects on the bidirectional permeation of TDF through MDCKII-MDR1 cells. When tested at concentrations ranging between 0.78 and 50 μM, the most potent PIs, LPV, NFV, and BRV, inhibited the efflux of calcein-AM by 50% at approximately 20 μM. APV, IDV, and DRV were the weakest inhibitors (IC₅₀ > 100 μM).

DISCUSSION

The observation of efflux in Caco-2 cells illustrates that TDF is a substrate for one or more intestinal efflux transport pumps. The differential transport of TDF observed in parental and Pgp-overexpressing MDCKII cells suggests that this highly expressed intestinal efflux pump is at least partly responsible for this phenomenon. There is a synergistic interaction between intestinal Pgp transport and cytochrome P450 3A4 degradation in limiting the oral bioavailability of substrates for both proteins (34, 46). The interaction is facilitated by Pgp increasing the intracellular residence time of compounds in intestinal cells and allowing for further degradation by cytochrome P450 3A4. Our results support the hypothesis that a similar interplay between intestinal Pgp and esterase activity may play a role in limiting the intestinal absorption of TDF. However, the measurement of an apparent binding constant for efflux transport of 144 μM, combined with the high solubility of TDF, suggests that efflux is largely saturated during TDF absorption (Fig. 1B). Of primary importance in the current discussion of drug interactions, this suggests the potential for only minimal changes in TDF absorption and resulting TFV circulating levels upon coadministration with agents affecting either esterase cleavage or Pgp transport.

PIs were established to be inhibitors of the molecular barriers to TDF absorption, based on results in bidirectional permeability, intestinal extract stability, and fluorescent Pgp inhibition assays. Their multifactorial effect on efflux transport and esterase-dependent permeation of TDF through Caco-2

cell monolayers was likely the reason that SQV, ATV, LPV, and RTV caused similar or greater effects on the TDF A-to-B P_{app} than did the near complete blockage of efflux by CsA. Evidence for the role of Pgp in the efflux component of this interaction can be drawn from the effects of PIs on Pgp-dependent efflux in MDCKII-MDR1 cells. However, these results do not eliminate the potential for a contribution by other intestinal efflux pumps known to be expressed in Caco-2 cells (16). RTV, ATV, and LPV were observed in both MDCKII-MDR1 and Caco-2 cells to increase the A-to-B P_{app} of TDF and to decrease TDF efflux, likely explaining the presence of these PIs in combinations causing increases in TFV exposure in vivo. Alternatively, the lower inhibition caused by APV and IDV in transepithelial permeability, esterase activity, and Pgp transport assays may explain why FPV and IDV do not cause increased TFV levels in patients.

There is, however, a partial disconnect between the increase in TDF permeation observed for most PIs in vitro and the spectrum of effects that PIs and their combinations have on plasma TFV levels clinically. This inconsistency is most apparent in the decreases in TFV levels observed when TDF is coadministered with TPV/r, FPV/r, and most significantly, FPV. A clue to the mechanism for the decreased TFV levels observed with these PIs is evident in the drug interaction between the strong intestinal Pgp inducer rifampin (14, 15) and TDF. When TDF was coadministered with rifampin, a slight decrease in TFV plasma levels (16% C_{max} and 12% AUC_{0-4}) was observed (9). The similarity in the effects of rifampin and these PIs suggests a common mechanism for the decreased TFV levels. Consistent with this hypothesis, TPV is known as a strong inducer of metabolizing enzymes (Aptivus product insert; Boehringer Ingelheim). While they are less marked inducers than TPV or rifampin, both NFV and APV induce intestinal Pgp after repeat dosing in the rat (18). Studies with a colonic cell line have shown the rank order of Pgp induction of tested PIs to be RTV > NFV ≥ APV > SQV > IDV (33), and NFV also induces Pgp expression in CD4⁺ and CD8⁺ lymphocytes in vitro (11). Indeed, the intestinal Pgp induction potential of NFV, APV, rifampin, and TPV may not be coincidental with their minimal (NFV) or slightly detrimental (FPV, rifampin, and TPV) effects on TFV circulating levels.

The ability of PIs to both inhibit and induce Pgp illustrates that their net effect on intestinal Pgp activity will be a balance between their inhibition and induction potentials. Digoxin levels have been shown to be sensitive to intestinal Pgp activity (14, 17), and the strong Pgp inducers and weak Pgp inhibitors TPV/r and rifampin have been shown to reduce digoxin's AUC_{0-4} (14, 45). Alternatively, the digoxin AUC_{0-4} is increased by the potent Pgp inhibitors RTV and LPV/r (21, 31). Similarly, net inhibition of intestinal Pgp has been observed for LPV/r, based on its effects on the oral bioavailability of the Pgp substrate fexofenadine in healthy volunteers (44). While it was observed to be an inducer of Pgp, the net effect of ATV on Pgp activity in vitro has been shown to be inhibition (32). A model for the effects of PIs on TDF absorption, including intestinal esterase inhibition and the net effect of intestinal Pgp inhibition and induction, is presented in Fig. 5.

It has been proposed that the clinically observed changes in TFV circulating levels are due to PI inhibition of the multidrug resistance-related protein 2 (MRP2)-dependent apical renal

FIG. 5. Factors affecting the intestinal absorption of TDF and the potential for PIs to modulate its oral absorption. The absorption of TDF and the subsequent appearance of TFV in the blood are modulated by the synergistic action of intestinal efflux pumps, including Pgp, by increasing intracellular residence times, and by esterase (Es) cleavage. Depending on their local and temporal concentrations in intestinal cells, PIs can affect TDF absorption by altering Pgp activity through the net effect of inhibition and induction and by inhibiting esterase cleavage.

transport of TFV in the kidney during the process of active tubular secretion. Furthermore, blockage of MRP2-mediated renal elimination of TFV has been proposed to cause the accumulation of TFV in renal proximal tubule cells, resulting in an alteration in the renal safety profile of TDF (20, 28, 39, 47). However, two independent reports have shown that the renal transport of TFV is likely mediated by MRP4, not MRP2 (19, 36, 37). Further studies have shown that PIs have minimal potential to inhibit the active renal tubular secretion of TFV mediated by influx from the blood via the human organic anion transporters 1 and 3 and by efflux from proximal tubule cells into the urine by MRP4 (6). Renal accumulation of TFV due to PIs is also not consistent with other observations, including (i) the low incidence of TDF-related renal adverse events in the context of combination therapy, with or without PIs (the TDF renal safety profile was recently reviewed by Sax et al. [41] and by Gitman et al. [13]); and (ii) the lack of overlapping recognition of TFV and PIs by renal efflux transporters (6), unlike the common interaction of PIs and TDF with the intestinal efflux transporter Pgp illustrated here.

We have shown that all PIs have the potential to modulate the intestinal absorption of TDF. The differential changes in circulating TFV levels observed clinically with different PIs are likely related to the relative abilities of PIs to (i) inhibit TDF hydrolysis in intestinal tissue, (ii) inhibit Pgp-mediated efflux of TDF, and (iii) induce Pgp expression. Our work also suggests that the magnitude of perturbations in TDF absorption caused by concomitant agents should be limited by partial saturation of intestinal transport.

ACKNOWLEDGMENTS

We thank Piet Borst (Netherlands Cancer Institute) for providing transfected MDCKII cells; Tomas Cihlar, Christian Callebaut, Kirsten Stray, and Lianhong Xu (Gilead Sciences, Inc.) for graciously providing some of the PIs used; Eugene Eisenberg for advice on conducting intestinal S9 studies; and Matthew Wright, Brian Kearny, and Bernard Murray (Gilead Sciences, Inc.) for thoughtful discussions of the data.

REFERENCES

- Agarwala, S., T. Eley, C. Villegas, Y. Wang, E. Hughes, and D. Grasela. 2005. Pharmacokinetic interaction between tenofovir and atazanavir coadministration with ritonavir in healthy subjects, abstr. 16. Abstr. 6th Int. Workshop Clin. Pharmacol. HIV Ther.
- Back, D. J., V. Sekar, E. Lefebvre, M. De Pauw, E. De Paepe, T. Vangeneugden, and R. Hoetlmans. 2006. Use of TMC114 in combination with other drugs: guidance from pharmacokinetic studies, abstr. PL5.1. Abstr. 8th Int. Congr. Drug Ther. HIV Infect.
- Barditch-Crovo, P., S. G. Deeks, A. Collier, S. Safrin, D. F. Coakley, M. Miller, B. P. Kearney, R. L. Coleman, P. D. Lamy, J. O. Kahn, I. McGowan, and P. S. Lietman. 2001. Phase I/II trial of the pharmacokinetics, safety, and antiretroviral activity of tenofovir disoproxil fumarate in human immunodeficiency virus-infected adults. *Antimicrob. Agents Chemother.* **45**:2733–2739.
- Boffito, M., A. Pozniak, B. P. Kearney, C. Higgs, A. Mathias, L. Zhong, and J. Shah. 2005. Lack of pharmacokinetic drug interaction between tenofovir disoproxil fumarate and nelfinavir mesylate. *Antimicrob. Agents Chemother.* **49**:4386–4389.
- Chittick, G. E., J. Zong, M. R. Blum, J. J. Sorbel, J. A. Begley, N. Adda, and B. P. Kearney. 2006. Pharmacokinetics of tenofovir disoproxil fumarate and ritonavir-boosted saquinavir mesylate administered alone or in combination at steady state. *Antimicrob. Agents Chemother.* **50**:1304–1310.
- Cihlar, T., A. S. Ray, G. Laflamme, J. E. Vela, L. Tong, M. D. Fuller, A. Roy, and G. R. Rhodes. 2007. Molecular assessment of the potential for renal drug interactions between tenofovir and HIV protease inhibitors. *Antivir. Ther.* **12**:267–272.
- Cundy, K. C., C. Sueoka, G. R. Lynch, L. Griffin, W. A. Lee, and J. P. Shaw. 1998. Pharmacokinetics and bioavailability of the anti-human immunodeficiency virus nucleotide analog 9-[(R)-2-(phosphonomethoxy)propyl]adenine (PMPA) in dogs. *Antimicrob. Agents Chemother.* **42**:687–690.
- Dasgupta, A., and P. C. Okhuysen. 2001. Pharmacokinetic and other drug interactions in patients with AIDS. *Ther. Drug Monit.* **23**:591–605.
- Droste, J. A., C. P. Verweij-van Wissen, B. P. Kearney, R. Buffels, P. J. Vanhorrssen, Y. A. Hekster, and D. M. Burger. 2005. Pharmacokinetic study of tenofovir disoproxil fumarate combined with rifampin in healthy volunteers. *Antimicrob. Agents Chemother.* **49**:680–684.
- Evers, R., M. Kool, A. J. Smith, L. van Deemter, M. de Haas, and P. Borst. 2000. Inhibitory effect of the reversal agents V-104, GF120918 and Pluronic L61 on MDR1 Pgp-, MRP1- and MRP2-mediated transport. *Br. J. Cancer* **83**:366–374.
- Ford, J., E. R. Meaden, P. G. Hoggard, M. Dalton, P. Newton, I. Williams, S. H. Khoo, and D. J. Back. 2003. Effect of protease inhibitor-containing regimens on lymphocyte multidrug resistance transporter expression. *J. Antimicrob. Chemother.* **52**:354–358.
- Ford, S., S. Murray, M. Anderson, J. Ng-Cashin, M. Johnson, and M. Shelton. 2006. Tenofovir renal clearance decreased following co-administration with brecaonavir/ritonavir, abstr. 38. Abstr. 7th Int. Workshop Clin. Pharmacol. HIV Ther.
- Gitman, M. D., D. Hirschwerk, C. H. Baskin, and P. C. Singhal. 2007. Tenofovir-induced kidney injury. *Expert Opin. Drug Saf.* **6**:155–164.
- Greiner, B., M. Eichelbaum, P. Fritz, H. P. Kreichgauer, O. von Richter, J. Zundler, and H. K. Kroemer. 1999. The role of intestinal P-glycoprotein in the interaction of digoxin and rifampin. *J. Clin. Investig.* **104**:147–153.
- Hamman, M. A., M. A. Bruce, B. D. Haehner-Daniels, and S. D. Hall. 2001. The effect of rifampin administration on the disposition of fexofenadine. *Clin. Pharmacol. Ther.* **69**:114–121.
- Hidalgo, I. J., T. J. Raub, and R. T. Borchardt. 1989. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* **96**:736–749.
- Hoffmeyer, S., O. Burk, O. von Richter, H. P. Arnold, J. Brockmoller, A. John, I. Cascorbi, T. Gerloff, I. Roots, M. Eichelbaum, and U. Brinkmann. 2000. Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. *Proc. Natl. Acad. Sci. USA* **97**:3473–3478.
- Huang, L., S. A. Wring, J. L. Woolley, K. R. Brouwer, C. Serahjit-Singh, and J. W. Polli. 2001. Induction of P-glycoprotein and cytochrome P450 3A by HIV protease inhibitors. *Drug Metab. Dispos.* **29**:754–760.
- Imaoka, T., H. Kusuhara, M. Adachi, J. D. Schuetz, K. Takeuchi, and Y. Sugiyama. 2007. Functional involvement of multidrug resistance-associated protein 4 (MRP4/ABCC4) in the renal elimination of the antiviral drugs adefovir and tenofovir. *Mol. Pharmacol.* **71**:619–627.
- Izzedine, H., V. Launay-Vacher, and G. Deray. 2005. Antiviral drug-induced nephrotoxicity. *Am. J. Kidney Dis.* **45**:804–817.
- Jetter, A., U. Fuhr, R. Aarnoutse, A. Lazar, F. Abdulrazik, N. Schmeiber, E. Schomig, D. Burger, G. Fatkenheuer, and C. Wyen. 2006. The ABC transporter P-glycoprotein is inhibited by lopinavir and low-dose ritonavir both in HIV-infected patients and healthy volunteers, abstr. 37. Abstr. 7th Int. Workshop Clin. Pharmacol. HIV Ther.
- Johnson, M., B. Grinsztajn, C. Rodriguez, J. Coco, E. DeJesus, A. Lazzarin, K. Lichtenstein, V. Wirtz, A. Rightmire, L. Odeshoo, and C. McLaren. 2006.

- 96-week comparison of once-daily atazanavir/ritonavir and twice-daily lopinavir/ritonavir in patients with multiple virologic failures. *AIDS* **20**:711–718.
23. Jullien, V., J. M. Treluyer, E. Rey, P. Jaffray, A. Krivine, L. Moachon, A. Lillo-Le Louet, A. Lescoat, N. Dupin, D. Salmon, G. Pons, and S. Urien. 2005. Population pharmacokinetics of tenofovir in human immunodeficiency virus-infected patients taking highly active antiretroviral therapy. *Antimicrob. Agents Chemother.* **49**:3361–3366.
 24. Kearney, B. P., J. Flaherty, J. Wolf, J. Sayre, S. Gill, and D. Coakley. 2001. Lack of clinically relevant drug-drug interactions between tenofovir DF and efavirenz, indinavir, lamivudine and lopinavir/ritonavir in healthy subjects, abstr. P171. Abstr. 8th Eur. Conf. Clin. Aspects Treat. HIV Infect.
 25. Kearney, B. P., J. F. Flaherty, and J. Shah. 2004. Tenofovir disoproxil fumarate: clinical pharmacology and pharmacokinetics. *Clin. Pharmacokinet.* **43**:595–612.
 26. Kearney, B. P., A. Mathias, A. Mittan, J. Sayre, R. Ebrahimi, and A. K. Cheng. 2006. Pharmacokinetics and safety of tenofovir disoproxil fumarate on coadministration with lopinavir/ritonavir. *J. Acquir. Immune Defic. Syndr.* **43**:278–283.
 27. Lee, C. G., M. M. Gottesman, C. O. Cardarelli, M. Ramachandra, K. T. Jeang, S. V. Ambudkar, I. Pastan, and S. Dey. 1998. HIV-1 protease inhibitors are substrates for the MDR1 multidrug transporter. *Biochemistry* **37**:3594–3601.
 28. Louie, S., J. Lam, M. Neely, and P. Beringer. 2005. Multi-drug resistance protein-2 (MRP2) inhibition by ritonavir increases tenofovir-associated renal epithelial cell cytotoxicity, abstr. WePe3.3C09. Abstr. 3rd Int. AIDS Soc. Conf. HIV Pathog. Treat.
 29. Lubner, A., D. Slowinski, M. Andrews, K. Olson, C. Peloquin, G. Pakes, K. Pappa, M. Shelton, and D. Condoluci. 2006. Steady-state pharmacokinetics (PK) of tenofovir (TDF) and fosamprenavir (FPV) after once daily (QD) TDF with unboosted or ritonavir (R)-boosted twice daily (BID) FPV in healthy volunteers, abstr. P274. Abstr. 8th Int. Congr. Drug Ther. HIV Infect.
 30. Molina, J. M., A. Wilkin, P. Domingo, R. Myers, J. Hairrell, C. Naylor, T. Podsadecki, M. King, and G. Hanna. 2005. Once-daily vs. twice-daily lopinavir/ritonavir in antiretroviral-naïve patients: 96-week results, abstr. WePe12.3C12. Abstr. 3rd Int. AIDS Soc. Conf. HIV Pathog. Treat.
 31. Penzak, S. R., J. M. Shen, R. M. Alfaro, A. T. Remaley, V. Natarajan, and J. Falloon. 2004. Ritonavir decreases the nonrenal clearance of digoxin in healthy volunteers with known MDR1 genotypes. *Ther. Drug Monit.* **26**:322–330.
 32. Perloff, E. S., S. X. Duan, P. R. Skolnik, D. J. Greenblatt, and L. L. von Moltke. 2005. Atazanavir: effects on P-glycoprotein transport and CYP3A metabolism in vitro. *Drug Metab. Dispos.* **33**:764–770.
 33. Perloff, M. D., L. L. von Moltke, J. M. Fahey, J. P. Daily, and D. J. Greenblatt. 2000. Induction of P-glycoprotein expression by HIV protease inhibitors in cell culture. *AIDS* **14**:1287–1289.
 34. Raciassi, S. D., I. J. Hidalgo, J. Segura-Aguilar, and P. Artursson. 1999. Interplay between CYP3A-mediated metabolism and polarized efflux of terfenadine and its metabolites in intestinal epithelial Caco-2 (TC7) cell monolayers. *Pharm. Res.* **16**:625–632.
 35. Ray, A. S. 2005. Intracellular interactions between nucleos(t)ide inhibitors of HIV reverse transcriptase. *AIDS Rev.* **7**:113–125.
 36. Ray, A. S., and T. Cihlar. 2007. Unlikely association of multidrug-resistance protein 2 single-nucleotide polymorphisms with tenofovir-induced renal adverse events. *J. Infect. Dis.* **195**:1389–1390.
 37. Ray, A. S., T. Cihlar, K. L. Robinson, L. Tong, J. E. Vela, M. D. Fuller, L. M. Wieman, E. J. Eisenberg, and G. R. Rhodes. 2006. Mechanism of active renal tubular efflux of tenofovir. *Antimicrob. Agents Chemother.* **50**:3297–3304.
 38. Ray, A. S., L. Tong, K. L. Robinson, B. P. Kearney, and G. R. Rhodes. 2006. Role of intestinal absorption in increased tenofovir exposure when tenofovir disoproxil fumarate is co-administered with atazanavir or lopinavir/ritonavir, abstr. 49. Abstr. 7th Int. Workshop Clin. Pharmacol. HIV Ther.
 39. Rollet, F., E. M. Nazal, L. Chauvelot-Moachon, C. Kelaidi, N. Daniel, M. Saba, S. Abad, and P. Blanche. 2003. Tenofovir-related Fanconi syndrome with nephrogenic diabetes insipidus in a patient with acquired immunodeficiency syndrome: the role of lopinavir-ritonavir-didanosine. *Clin. Infect. Dis.* **37**:e174–e176.
 40. Roszko, P., K. Curry, B. Brazina, A. Cohen, E. Turkie, J. Sabo, T. MacGregor, and S. McCallister. 2003. Standard doses of efavirenz (EFV), zidovudine (ZDV), tenofovir (TDF), and didanosine (ddI) may be given with tipranavir/ritonavir (TPV/r), abstr. 865. Abstr. 2nd Int. AIDS Soc. Conf. HIV Pathog. Treat.
 41. Sax, P. E., J. E. Gallant, and P. E. Klotman. 2007. Renal safety of tenofovir disoproxil fumarate. *AIDS Read.* **17**:90–104.
 42. Shaw, J. P., C. M. Sueoko, R. Oliyai, W. A. Lee, M. N. Arimilli, C. U. Kim, and K. C. Cundy. 1997. Metabolism and pharmacokinetics of novel oral prodrugs of 9-[(R)-2-(phosphonomethoxy)propyl]adenine (PMPA) in dogs. *Pharm. Res.* **14**:1824–1829.
 43. van Gelder, J., S. Deferme, L. Naesens, E. De Clercq, G. van den Mooter, R. Kinget, and P. Augustijns. 2002. Intestinal absorption enhancement of the ester prodrug tenofovir disoproxil fumarate through modulation of the biochemical barrier by defined ester mixtures. *Drug Metab. Dispos.* **30**:924–930.
 44. van Heeswijk, R. P., M. Bourbeau, P. Campbell, I. Seguin, B. M. Chauhan, B. C. Foster, and D. W. Cameron. 2006. Time-dependent interaction between lopinavir/ritonavir and fexofenadine. *J. Clin. Pharmacol.* **46**:758–767.
 45. Vourvahis, M., J. Dumond, K. Patterson, N. Rezk, N. White, S. Jennings, H. Tien, J. Saabo, T. MacGregor, and A. Kashuba. 2007. Effects of tipranavir/ritonavir on the activity of hepatic and intestinal cytochrome P450 3A4/5 and P-glycoprotein: implication for drug interactions, abstr. 563. Abstr. 14th Conf. Retrovir. Opportun. Infect.
 46. Wachter, V. J., C. Y. Wu, and L. Z. Benet. 1995. Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: implications for drug delivery and activity in cancer chemotherapy. *Mol. Carcinog.* **13**:129–134.
 47. Zimmermann, A. E., T. Pizzoferrato, J. Bedford, A. Morris, R. Hoffman, and G. Braden. 2006. Tenofovir-associated acute and chronic kidney disease: a case of multiple drug interactions. *Clin. Infect. Dis.* **42**:283–290.