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# Fexofenadine Transport in Caco-2 Cells: Inhibition with Verapamil and Ritonavir

Michael D. Perloff, PhD, Lisa L. von Moltke, MD, and David J. Greenblatt, MD

*This study investigated fexofenadine (FXD) transport and the inhibition of FXD transport in Caco-2 cell monolayer transwells, using rhodamine 123 (RH123) transport as a positive control. FXD transport from the basolateral (B) to apical (A) compartment was fivefold higher than A to B transport. FXD transport was linear with respect to time (up to 270 min) and concentration (up to 300  $\mu\text{M}$ ). Similar results were seen with the positive control RH123. Ritonavir (100  $\mu\text{M}$ ) and verapamil (100  $\mu\text{M}$ ) reduced transport of FXD and RH123 by more than 80%, whereas transport was not inhibited by 100  $\mu\text{M}$  indomethacin or 2  $\text{mM}$  probenecid. This suggests pre-*

*dominantly P-glycoprotein (P-gp)-mediated transport as opposed to transport by multidrug resistance protein. In concentration-response experiments, FXD transport was inhibited by verapamil and ritonavir with  $\text{IC}_{50}$  values of 6.5  $\mu\text{M}$  and 5.4  $\mu\text{M}$ , respectively. Results from this in vitro study demonstrate differential transport of FXD across Caco-2 cell monolayers and inhibition of FXD transport by established P-gp inhibitors. The findings support the use of FXD as an index or probe compound to reflect P-gp activity in vivo.*

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The nonsedating antihistamine fexofenadine (FXD) has been employed as an index or probe compound for studies of P-glycoprotein (P-gp)-mediated transport.<sup>1-3</sup> Although FXD, like digoxin, is a substrate for cellular uptake via organic anion transport protein (OATP) transport,<sup>4,5</sup> it is nonetheless of value as a probe of P-gp-mediated transport in drug-drug interaction, drug bioavailability, and disposition studies. FXD is nonmetabolized, in that more than 95% of a dose is recovered intact in urine and feces.<sup>1</sup> In *md1a(-/-)* mice, FXD plasma and brain levels are five- and ninefold higher, respectively, compared to wild-type mouse controls after oral and IV administration.<sup>4</sup> Use of FXD as a probe in human studies represents a safe option with low incidence of toxicity, as opposed to digoxin, which could cause cardiac complications at elevated levels.<sup>6</sup>

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While various methods exist to study intestinal absorption, Caco-2 cell monolayer transwells are a widely accepted model of human intestinal absorption.<sup>7-13</sup> The expression of multiple transporters in Caco-2 cells is similar to expression profiles in human jejunum, including P-gp and MRP1.<sup>13</sup> Specific transporters also have been studied by employing cDNA-transfected cell lines with elevated levels of transporter (e.g., P-gp, MRPs, etc.).<sup>14-17</sup> Nonetheless, a number of studies have demonstrated that Caco-2 cells closely resemble human and rat small intestinal mucosa and exhibit similar transport kinetics for different model substrates.<sup>7,11,12</sup> This study was designed to validate FXD as a probe of P-gp-mediated transport in Caco-2 cell monolayers.

## MATERIALS AND METHODS

### Chemicals

FXD was extracted into methanol solution from commercially available capsules (Aventis, Bridgewater, NJ), with purity verified by high-performance liquid chromatography (HPLC). Ritonavir (RIT) was kindly provided by Abbott Laboratories (North Chicago). Rhodamine 123 (RH123), verapamil (VER),

indomethacin (INDO), and probenecid (PRO) were obtained from Sigma Chemical Co. (St. Louis, IL).

### Cell Lines

The human colon adenocarcinoma cell line Caco-2 was kindly provided by Douglas Jefferson, PhD (Tufts University School of Medicine and Tufts–New England Medical Center, Boston) and used at passages 30 to 40.<sup>18-21</sup> Cells were grown in DMEM (Dulbecco's Modified Eagle's Medium, GibcoBRL, Rockville, MD) supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids (GibcoBRL), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Validation of P-gp expression and function in this system has been described previously.<sup>18-21</sup>

### Transport Experiments

Caco-2 cells were seeded at  $2 \times 10^4/\text{cm}^2$  in polycarbonate membrane transwell plates (2.5 cm diameter, 3  $\mu\text{m}$  pore size) (Corning Costar Corp., Cambridge, MA) and grown in a humidified chamber (37°C, 5% CO<sub>2</sub>) with media changes every 3 to 5 days. Experiments were conducted on days 18 to 21 postseeding. Drug solutions were prepared in Opti-MEM serum-free media (GibcoBRL) containing 0.5% DMSO.

**Transport kinetics.** Opti-MEM containing FXD (30, 100, and 300  $\mu\text{M}$ ) was added to the apical (A, 1.5 ml) or basolateral (B, 2.5 ml) chamber. Cells were incubated at 37°C, 5% CO<sub>2</sub> for 90 to 270 minutes, and 50  $\mu\text{l}$  samples were taken from the chamber initially not containing FXD. Sample protein was precipitated with the addition of 150  $\mu\text{l}$  acetonitrile; phenacetin was added as an internal standard, and samples were subject to HPLC analysis. Alternatively, Opti-MEM containing RH123 as a positive control P-gp substrate (1-100  $\mu\text{M}$ ) was added to the A or B chamber. Cells were incubated at 37°C, in 5% CO<sub>2</sub> for 30 to 240 minutes, and 50  $\mu\text{l}$  samples were taken from the chamber initially not containing RH123. Samples were diluted with 500  $\mu\text{l}$  methanol and subjected to fluorometric analysis.

**Transport inhibition.** Opti-MEM containing RH123 (5  $\mu\text{M}$ ) or FXD (100  $\mu\text{M}$ ) was added to the A or B chamber, with the inhibitor (PRO 2 mM, INDO 100  $\mu\text{M}$ , VER 1-100  $\mu\text{M}$ , or RIT 1-100  $\mu\text{M}$ ) present in both chambers. Cells were incubated for 180 minutes, and drug concentrations in the chamber initially not containing RH123 or FXD were determined by fluorometric or HPLC analysis. IC<sub>50</sub> values for FXD transport inhibition (B to A transport, correcting for A to B passive diffusion

baseline) were determined using nonlinear regression (SigmaPlot 4.0, SPSS Inc., Chicago) based on variations of the Hill equation, as previously described.<sup>19,22,23</sup>

**Drug stability.** In tissue culture media alone (with no Caco-2 cells), nearly 100% ( $\pm 3\%$ ) of all drugs used were recoverable after 270 minutes of incubation, indicating no drug degradation. In actual Caco-2 transwell experiments, > 85% of all drugs were accounted for at the end of transport experiments when quantifying both the A and B chambers. The remaining (~15%) of unquantified drug was assumed to be intracellular within the Caco-2 monolayer or otherwise unrecovered from the system.

### Fluorometric and HPLC Detection

RH123 was quantified with fluorometric analysis, with detection wavelengths of 500 nm (excitation) and 550 nm (emission), using a Perkin Elmer LS50B luminescence spectrometer.<sup>8,9,18-21</sup> The FXD HPLC mobile phase consisted of 400 ml of acetonitrile and 600 ml of 10 mmol/L phosphate buffer, with pH adjusted to 4.0 with phosphoric acid and a flow rate of 1.4 ml/min. The analytical column (25 cm  $\times$  4.6 mm) was a 5 $\mu\text{m}$  ODS-ultrasphere reverse-phase C-18 Symmetry (Beckman Co., Fullerton, CA). Column effluent was monitored by ultraviolet absorbance at 214 nm. The average coefficient of variance (CV) for triplicate samples was 6.7% at 100  $\mu\text{M}$  FXD. The detection limit was 2 ng FXD (per 50  $\mu\text{l}$  injection). Picomoles of RH123 or FXD in culture samples were determined based on calibration curves constructed from a series of standards containing varying known amounts of RH123 (0.57 to 57 ng) alone or FXD (2.7 to 267 ng) together with an internal standard.

## RESULTS

### Transport Kinetics

FXD transport from apical (A) to basolateral (B) chambers (attributed to passive diffusion) was less than 20% of uninhibited B to A transport (attributed to active transport). There was a greater than five-fold differential transport of FXD between chambers (Figure 1A, B). FXD transport was linear with respect to time (up to 270 min) and concentration (up to 300  $\mu\text{M}$ ). The within-day CV for FXD (100  $\mu\text{M}$ ) transport was 18.5% (B to A) and 21.2% (A to B). FXD transport could not be tested at concentrations higher than 300  $\mu\text{M}$  due to lack of solubility in tissue culture media. Accordingly, sub-

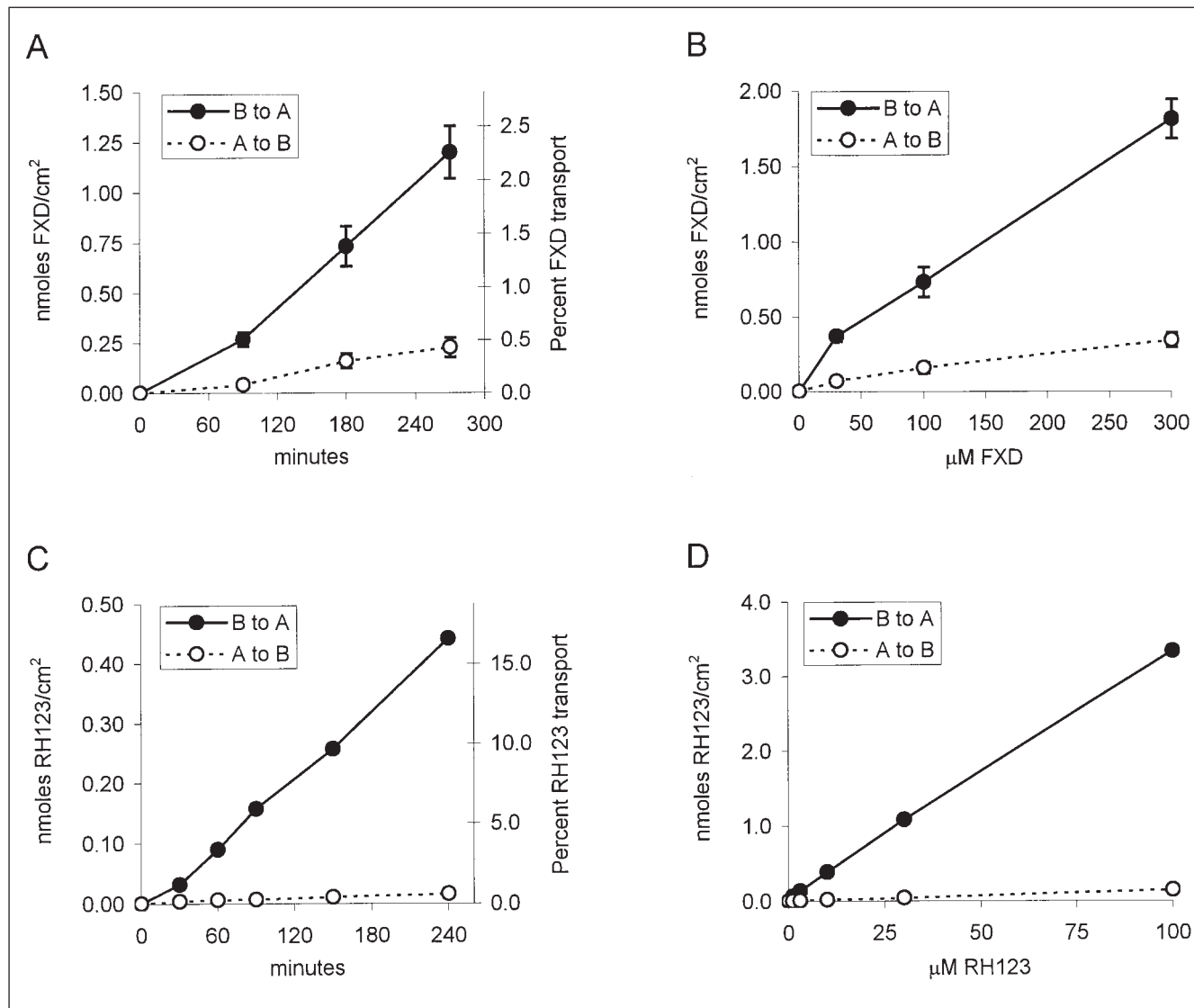


Figure 1. Fexofenadine and rhodamine 123 transport kinetics. Fexofenadine transport across Caco-2 cell monolayers was determined versus time (graph A) and concentration (graph B). Time kinetics were investigated at 100  $\mu\text{M}$  fexofenadine and concentration response at 180 minutes. Each data point represents the mean of triplicate assays ( $\pm$  SD). Rhodamine 123 transport across Caco-2 cell monolayers was determined versus time (graph C) and concentration (graph D). Time kinetics were investigated at 5  $\mu\text{M}$  rhodamine 123 and concentration response at 180 minutes. Each data point represents the mean of duplicate assays, which did not differ more than 10%. Closed circles connected by solid lines represent basal to apical (B to A) passage. Open circles connected by dashed lines represent apical to basal (A to B) passage.

sequent transport inhibition experiments in Caco-2 monolayers were performed with 100  $\mu\text{M}$  FXD and 180-minute incubations.

RH123 transport from the B to A chamber was linear with respect to time (up to 4 h) and drug concentration (up to 100  $\mu\text{M}$ ) (Figure 1C, D). RH123 transport from A to B was less than 5% of uninhibited B to A transport.

Although the  $K_m$  of FXD and RH123 transport in this system is not established, transport linearity was demonstrated up to at least 300  $\mu\text{M}$  FXD and 100  $\mu\text{M}$  RH123. This suggests that the drug concentrations (100  $\mu\text{M}$  FXD and 5  $\mu\text{M}$  RH123) used in the present inhibition studies are well below the transport  $K_m$  and that  $\text{IC}_{50}$  values are likely to be close to  $K_i$  values for transport.

**Table I** Drug Transport and Inhibition in Caco-2 Monolayers

Substrate	Transport Rate (B to A) <sup>a</sup>	Transport Rate (A to B) <sup>a</sup>	Verapamil IC <sub>50</sub> ( M)	Ritonavir IC <sub>50</sub> ( M)
Rhodamine 123 (5 μM)	$1.70 \times 10^{-3}$	$7.74 \times 10^{-5}$	2.6 <sup>b</sup>	6.7 <sup>c</sup>
Fexofenadine (100 μM)	$3.83 \times 10^{-3}$	$7.24 \times 10^{-4}$	6.5	5.4

Values were generated with data from three trials. B, basolateral; A, apical; IC<sub>50</sub>, inhibitor concentration that reduces transport to 50% of the uninhibited control.

a. Expressed in nmoles/cm<sup>2</sup>/min.

b. von Moltke et al.<sup>35</sup>

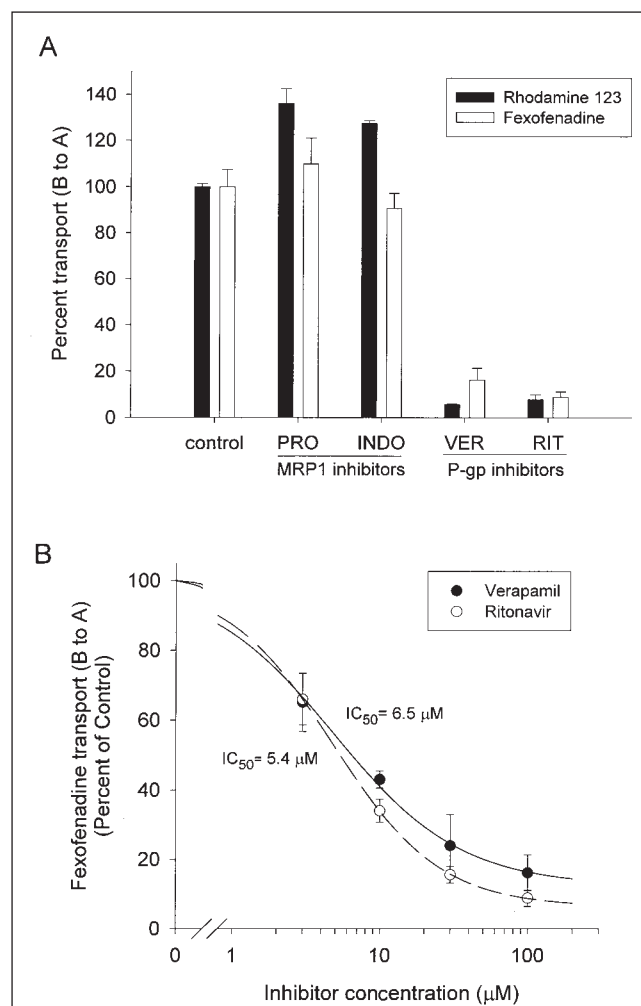
c. Perloff et al.<sup>21</sup>

## Inhibition

FXD and RH123 transport in Caco-2 monolayers was tested in the presence of validated P-gp and multidrug resistance protein 1 (MRP1) inhibitors. Potential inhibition was screened at high concentrations (one order of magnitude higher than reported IC<sub>50</sub>) of drugs established as inhibitors of P-gp (VER and RIT)<sup>6,9,15,24,25</sup> or MRP1 (INDO and PRO).<sup>26-29</sup> While 100 μM INDO and 2 mM PRO demonstrated no inhibition of the transport of FXD and RH123, extensive inhibition was seen with 100 μM VER and 100 μM RIT (Figure 2A). In concentration-response experiments, FXD B to A transport was inhibited by VER and RIT with IC<sub>50</sub> values of 6.5 μM and 5.4 μM, respectively (Figure 2B). FXD and RH123 A to B transport was unaffected by potential inhibitors.

## DISCUSSION

FXD is becoming increasingly used as a probe compound to monitor P-gp-mediated transport in vivo.<sup>1,2</sup> FXD is cleared relatively unmetabolized, and alterations in FXD clearance are associated with alterations in P-gp expression and activity.<sup>1</sup> FXD is also transported by drug pumps other than P-gp, including OATP and potentially MRPs.<sup>3,14,30</sup> While total transport of FXD across Caco-2 cell monolayers was small (1%-2% of total drug after 4 h), differential transport from the basolateral (B) chamber to the apical (A) was fivefold higher than the A to B transport (Figure 1A, B), suggestive of predominantly P-gp-mediated transport. FXD transport was linear up to at least 300 μM (Figure 1B), consistent with apparent efflux transport of FXD by intestinal enterocytes in vivo, even at the transiently high intraluminal concentrations achieved after oral dosage. Nonetheless, it is possible that OATP or other uptake transporters could influence FXD bioavailability.<sup>30</sup> In addition, the various MRPs may cause FXD cellular export (both apical and basal).<sup>14</sup> While MRP expression and activity have been demonstrated in nontransfected Caco-2 cells,<sup>7,13,31</sup> FXD transport was unaffected by the established MRP1 inhibitors INDO and PRO (Figure 2A).<sup>26-29</sup>



**Figure 2.** Inhibition of fexofenadine and rhodamine 123 transport. (A) Inhibition of transport of fexofenadine and rhodamine 123 across Caco-2 monolayers (mean  $\pm$  SD,  $n = 3$ ) by probenecid (PRO), indomethacin (INDO), verapamil (VER), and ritonavir (RIT) was assessed by comparison to vehicle control (2 mM PRO; all others were 100 M). (B) Concentration inhibition response and IC<sub>50</sub> were determined for fexofenadine transport with verapamil and ritonavir. Data represent drug transport from the basolateral to the apical chamber ( $n = 3$ ) as a percentage of the uninhibited control. Each data point represents the mean ( $\pm$  SD). IC<sub>50</sub> values were determined by nonlinear regression as described previously.<sup>19,22,23</sup> R<sup>2</sup> values (goodness of fit) exceeded 0.99, and relative asymptotic standard errors of the parameter estimates were less than 11%.

Although RH123 has not been used in human studies, it is extensively used as an index or probe of P-gp-mediated transport in rodent and tissue culture models.<sup>10,16,18-21,32-34</sup> RH123 was used as a positive control for P-gp-mediated transport in Caco-2 monolayers. While both MRP1 and breast cancer resistance protein (MXR) may transport RH123, neither contributed significant activity to RH123 transport in Caco-2 monolayers. MRP1 is thought to have relatively low efficiency as a transporter of RH123,<sup>16,33</sup> and accordingly, RH123 Caco-2 monolayer transport was unaffected by MRP1 inhibitors INDO and PRO, while P-gp inhibitors (RIT and VER) virtually eliminated RH123 transport activity (Figure 2A). Since MXR transport is unaffected by the classic P-gp inhibitor verapamil,<sup>17</sup> its role in RH123 and FXD transport in the Caco-2 monolayer transwell models appears to be minimal.

OATP substrate and inhibition specificity overlap substantially with that of P-gp.<sup>3</sup> Therefore, absolute separation of activity was not possible. Interestingly, IC<sub>50</sub> values determined for the inhibition of FXD (B to A) transport in Caco-2 cell monolayers by RIT and VER (6.5 and 5.4  $\mu$ M for VER and RIT, respectively) were very similar to IC<sub>50</sub> values determined with inhibition of OATP.<sup>3</sup> Recently, single nucleotide polymorphisms in *MDR1* (the gene that encodes human P-gp) were associated with reduced FXD area under the plasma level-time curve, apparently attributable to enhanced P-gp activity.<sup>2</sup> This, along with our in vitro data, supports the use of FXD as an index of P-gp-mediated activity in vivo. Nonetheless, additional investigation is needed since coadministration of grapefruit juice was found to diminish bioavailability of oral FXD through inhibition of OATP activity.<sup>30</sup>

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