Kinetic Identification of Membrane Transporters That Assist P-glycoprotein-Mediated Transport of Digoxin and Loperamide through a Confluent Monolayer of MDCKII-hMDR1 Cells

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ABSTRACT:

A robust screen for compound interaction with P-glycoprotein (P-gp) has some obvious requirements, such as a cell line expressing P-gp and a probe substrate that is transported solely by P-gp and passive permeability. It is actually difficult to prove that a particular probe substrate interacts only with P-gp in the chosen cell line. Using a confluent monolayer of MDCKII-hMDR1 cells, we have determined the elementary rate constants for the P-gp efflux of amprenavir, digoxin, loperamide, and quinidine. For amprenavir and quinidine, transport was fitted with just P-gp and passive permeability. For digoxin and loperamide, fitting required a basolateral transporter ($p < 0.01$), which was inhibited by the P-gp inhibitor N-4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isooquinolinyl)ethyl]-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918). This means that when digoxin is used as a probe substrate and a compound is shown to inhibit digoxin flux, it could be that the inhibition occurs at the basolateral transporter rather than at P-gp. Digoxin basolateral>apical efflux also required an apical importer ($p < 0.05$). We propose that amprenavir and quinidine are robust probe substrates for assessing P-gp interactions using the MDCKII-hMDR1 confluent cell monolayer. Usage of another cell line, e.g., LLC-hMDR1 or Caco-2, would require the same kinetic validation to ensure that the probe substrate interacts only with P-gp. Attempts to identify the additional digoxin and loperamide transporters using a range of substrates/inhibitors of known epithelial transporters (organic cation transporters, organic anion transporters, organic ion-transporting polypeptide, uric acid transporter, or multitarget resistance-associated protein) failed to inhibit the digoxin or loperamide transport through their basolateral transporter.

The importance of membrane transporters in the metabolism and disposition of drugs is well recognized (Mizuno et al., 2003; Collett et al., 2005; Spears et al., 2005; Shitara et al., 2006; Robertson and Rankin, 2006; Sekine et al., 2006). Although it seems clear that membrane transporters mediate the transcellular transport of compounds across epithelial and endothelial barriers, it has been challenging to identify which uptake and efflux transporters are involved with a particular compound in vivo (Lau et al., 2006). Cell lines overexpressing individual transporters have proven to be quite useful in this respect, in identifying both substrates and as inhibitors of the transporter in question.

The human multidrug resistance transporter P-glycoprotein (P-gp) (Juliano and Ling, 1976) is the product of the hMDR1 (ABCB1) gene and is widely expressed in human epithelial tissue as a protection against xenobiotics (Dean et al., 2001). Polarized confluent cell monolayers overexpressing P-gp have been used extensively as a model system to study P-gp transport mechanisms and to assess the risk of P-gp-mediated drug-drug interactions (Tang et al., 2002; Rautio et al., 2006; Bartholomé et al., 2007; Korjamo et al., 2007). Using the polarized Madin-Darby canine kidney II cell line that overexpresses human MDR1 (MDCKII-hMDR1) confluent cell monolayer, which overexpresses human P-gp in the apical plasma membrane and a detailed mass action analysis of P-gp efflux kinetics, we have been able to answer several fundamental questions about P-gp function (Bentz et al., 2005; Tran et al., 2005; Acharya et al., 2006). With P-gp as the only transporter in the kinetic model, we have found that amprenavir and quinidine were well fitted, whereas loperamide showed more efflux than could be explained by just P-gp (Tran

ABBREVIATIONS: P-gp, the P-glycoprotein product of the hMDR1 gene; MDCKII-hMDR1, Madin-Darby canine kidney II cell line that overexpresses human multidrug resistance 1; bcrp/BRCP, breast cancer resistance protein; GF120918, N-4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isooquinolinyl)ethyl]-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; DMEM, Dulbecco’s modified Eagle’s medium; A>B (or B>A), transport across the confluent cell monolayer when the donor chamber is apical (or basolateral) and the receiver chamber is basolateral (or apical); TM, transport medium; BT, basolateral transporter; AT, apical transporter; RMS, root-mean-square; OAT, organic anion transporters; OATP, organic ion-transporting polypeptide; OCT, organic cation transporters; MRP, multidrug resistance-associated protein.
et al., 2005; Acharya et al., 2006). Previous work had shown other transporters in model cell lines. Secretory transport of rhodamine 123 across Caco-2 cell monolayers involves a basolateral uptake transporter in addition to P-gp (Troutman and Thakker, 2003). MDCKII wild-type cells and MDCKII cells overexpressing bcrp1 contain an apical influx transporter for the BCRP substrate mitoxantrone (Pan and Elnequist, 2007).

These observations led 1) to the speculation that there was another transporter involved for loperamide in the MDCKII-hMDR1 cells and 2) to the question of the extent to which other transporters in these cells might affect inhibition of a probe substrate in screening for drug–drug interactions with P-gp. A “robust” probe substrate for P-gp inhibition screening should be transported across the cell monolayer solely by P-gp and the passive permeability, which is measured in the presence of a potent P-gp inhibitor, such as GF120918. In this case, the P-gp-mediated efflux can be roughly estimated by the difference between the two curves, i.e., the total efflux (−GF120918) − the passive efflux (+GF120918). We say “roughly” because backflow, from the receiver side to the donor side, must be ignored (Tran et al., 2005).

If a probe substrate also uses another transporter, then there are two possibilities. The first case is easy. If this new transporter is not inhibited by GF120918, then the +GF120918 efflux would contain both the contributions from the other transporter and from passive permeability. In this case, the difference between the two curves would still be due to P-gp alone. One just needs to be clear that “passive permeability,” +GF120918, measurements can include transporters. The second case is the problem. If the novel transporter is inhibited by GF120918, then the passive permeability, +GF120918, will underestimate the amount of probe substrate actually reaching P-gp in the −GF120918 experiment. Then the difference between the curves yields an overestimate of the P-gp-mediated efflux. Now, if a novel compound binds to and inhibits the other transporter, −GF120918, then less probe substrate will reach P-gp, thus reducing its efflux kinetics. Such a reduction would probably be misinterpreted as the novel compound being a P-gp inhibitor.

Here we have used our kinetic analysis to detect the presence of novel transporters of loperamide and digoxin in the basolateral membrane of this cell line, as well as a digoxin transporter in the apical membrane. Digoxin has been used as a probe substrate with MDCKII-hMDR1 confluent cell monolayers to screen novel compounds for P-gp inhibition (Rautio et al., 2006). A rigorous statistical analysis confirms the need for these additional transporters and thus supports our kinetic findings. For amprenavir and quinidine, only P-gp and passive efflux (−GF120918) are required to fit the data, suggesting that they are robust probe substrates for this cell line.

Materials and Methods

Materials. Amprenavir and GF120918 were from GlaxoSmithKline Pharmaceuticals (Research Triangle Park, NC), and loperamide and quinidine were from Sigma-Aldrich (St. Louis, MO). [3H]Loperamide (10 Ci/mmol) and [3H]amprenavir (21 Ci/mmol) were custom synthesized by GE Healthcare (Little Chalfont, Buckinghamshire, UK). [3H]Quinidine (20 Ci/mmol) was from MP Biomedicals, Inc. (Solon, OH). Dulbecco’s modified Eagle’s medium (DMEM) was from MediaTech, VWR International, Inc. (West Chester, PA). DMEM with 25 mM HEPES buffer, high glucose (4.5 g/l), L-glutamine, fetal bovine serum, pyridoxine hydrochloride, without sodium pyruvate, and with phenol red was from Invitrogen (Carlsbad, CA). The same medium without phenol red, denoted transport media, was used for transport experiments. Transwell 12-well plates with polycarbonate inserts (0.4-μm pore size and 12-mm diameter) were obtained from Corning Life Sciences (Acton, MA). All compounds tested in Table 2 were from Sigma Chemical.

Cell Line and Culture Conditions. The MDCKII-hMDR1 was purchased from the Netherlands Cancer Institute (Amsterdam, The Netherlands). MDCKII-hMDR1 cells were grown in 175-cm² culture flasks using DMEM with 10% fetal bovine serum, 1% L-glutamine, and 50 U/ml penicillin and 50 mg/ml streptomycin at 37°C in 5% CO₂ atmosphere. Cells were split twice a week to 70 to 80% confluence in a ratio of 1:40, after at least two washes in phosphate-buffered saline and trypsinization with 0.25% trypsin-EDTA. All transport assays were done with cells from passages 30 to 55. Cells were kept at 37°C in 5% CO₂.

Single Substrate Efflux Assay. Cells were seeded in 12-well Costar transwell plates with polycarbonate membrane inserts at a density of 175,000 cells per insert and grown for 4 days in culture medium. Cells were given fresh media 1 day after seeding. On the day of the experiment, culture medium was replaced, and cells were preincubated for 30 min with either transport media alone or transport media with 2 μM GF120918, an inhibitor of P-gp. Efflux of amprenavir, digoxin, loperamide, and quinidine across the confluent monolayer of cells was measured in both directions, i.e., apical to basolateral (A>B) and basolateral to apical (B>A) in the presence and absence of GF120918. [3H]Amprenavir, [3H]digoxin, [3H]loperamide, or [3H]quinidine (0.5 μCi/ml) was added to each respective substrate concentration to allow quantitation of efflux from donor to receiver chambers. Lucifer yellow (100 μM) was added to the donor chamber to monitor integrity of the confluent cell monolayer.

Samples (25 μl) were taken over a period of 4 or 6 h, as shown, from both donor and receiver chambers into 96-well Lumaplates and dried overnight, after which the radioactivity was counted by using a TopCount model 9912 counter (PerkinElmer Life and Analytical Sciences, Boston, MA). The initial concentration measurement was taken at 6 min after the addition of substrate in the first well (Tran et al., 2005), and subsequent measurements were taken at multiple time points up to 6 h for all experiments (Tran et al., 2005). After each aliquot was taken, the transwell plates were placed in a shaker at a speed of 30 rpm at 37°C in 5% CO₂. The fluorescence of lucifer yellow (excitation maximum, 438 nm; emission maximum, 530 nm) was measured at time 0 from aliquots taken directly from the vials and compared with that of samples aliquoted at the end of the experiment from both the basolateral and apical chambers; the fluorescence was analyzed using a SpectraMax microplate reader. Passive permeability of lucifer yellow was always <10 nm/s over the entire experiment.

Cell Stability and Substrate Metabolism. The cells do not lose any capacity for drug transport over 6-h incubations (Tran et al., 2005; Acharya et al., 2006), implying that cellular ATP levels remain adequate for full P-gp function throughout the experiment. We showed previously that the stability of the cell monolayer and plasma membrane with respect to passive and active transport was not affected by the prolonged exposure times to amprenavir for at least 6 h (data not shown) (Tran et al., 2005). It was also shown that metabolism or decomposition was insignificant for amprenavir, quinidine, and loperamide on this time scale using radio-high-performance liquid chromatography (data not shown) (Tran et al., 2005).

Inhibition Studies. Cells were seeded and fed as described above. On the day of the experiment culture medium was removed by aspiration. The cells were then preincubated with the inhibitor/substrate drugs in both chambers for 30 min. During the preincubation, half of the wells received inhibitor solution without GF120918 (to study active transport) and the other half received inhibitor solution with GF120918 (to study passive transport). After 30 min, the preincubation solutions were removed by aspiration and the labeled substrate was added to the donor chamber, and both chambers were refilled with fresh inhibitor drug solution, again one half without GF120918 and the other half with it.

Digoxin Cell Concentration Exclusion Assay. Cells were seeded and fed as described above. On the day of the experiment, culture medium was removed by aspiration. The cells were then preincubated for 30 min with transport medium (TM), TM plus quinidine (20 μM), TM plus GF120918 (2 μM), or TM plus quinidine (20 μM) plus GF120918 (2 μM) in both chambers. The same protocol was repeated with an additional 1 μM benzbromarone. All of these preincubation conditions were repeated in triplicate. Preincubation solutions were then removed by aspiration. Donor and receiver solutions contained the radiolabeled substrate digoxin (5 μM), in addition to the respective preincubation condition. Basolateral and apical chambers were filled with donor and receiver solutions, respectively. Transport from B>A was measured.
over 4 h. Because the same concentration of digoxin was used on both sides, a flat curve showing steady state was obtained. At the 4th h, all solutions were removed. Both chambers were washed three times with cold transport media containing 2 μM GF120918. The polycarbonate membrane inserts with the cells were carefully cut out, and radioactivity associated to the cells on the membrane was counted by liquid scintillation.

**Numerical Integrations.** We used the stiffest integrator in MATLAB, ode23s, with absolute and relative tolerances set to 10⁻⁸. Other MATLAB integrators, although faster, were not accurate enough at the later times of simulations. In data fitting, all concentration curves are simultaneously fitted, so that despite the fact that the A:B curve, i.e., substrate concentration in the apical chamber, is the donor, is the most visually striking, all curves contribute to minimizing the difference between data and simulated curves. MATLAB finisimnaris minimizes the coefficient of variation between the data and the simulated curves. Further details can be found in Tran et al. (2005) and Bentz et al. (2005).

When the fitting included the novel basolateral and apical transporters, both Vmax and Km were fitted independently using eq. 2 in the mass action kinetic model for transport (Tran et al., 2005; Acharya et al., 2006). For the best fits, the Vmax and Km values did not converge, but their ratio was constant for all concentrations of loperamide (Vmax/Km = 100 s⁻¹) and digoxin (Vmax/Km = 30 s⁻¹) (Table 1). For loperamide, this value was the average of the best fits for a concentration range of 0.012 to 10 μM, i.e., 3 orders of magnitude, suggesting that a single transporter is responsible for the increased flux.

**Statistical Analysis.** For the digoxin and loperamide data, we sought statistical validation for the proposed basolateral (BT) and apical (AT) transporters. To compare the predictions by the kinetic model and actual measured amounts of digoxin in apical and basolateral compartments over time, we sought to evaluate four hypotheses: that predictions from adequate models would 1) be highly correlated with empirical measurements, 2) have small root-mean-square (RMS) prediction errors, 3) be linearly related to empirical measurements, and 4) have a slope of 1 for the regression of empirical data on the predictions. Regressions were performed using singular value decomposition (Press et al., 1992). Refinement of predictions by allowing curvilinear regressions was measured by the contribution of second-order terms to minimize prediction errors (Rao, 1998; Quinn and Keough, 2002). Regression slopes were compared with unity using t tests comparing the estimated slopes to the hypothetical values (Sokal and Rohlf, 1995; Rao, 1998).

Because the amounts of digoxin remaining in the basolateral compartment or transported to the apical compartment were measured, sequential measurements were potentially autocorrelated and hence, not independent, violating an assumption of regression analyses. To circumvent this violation, we performed autocorrelation analyses and showed that with lag times of 2 to 3 h, autocorrelation (r²) fell to <0.01 with p > 0.70. Hence, values were independent after this time lag. All analyses reported here used sequential time points every 3 h. Loperamide trials were shorter than the stitched digoxin trials (6 versus 30 h), but transport experiments were run for several initial concentrations of loperamide. Assuming that parameters remained constant at all loperamide concentrations allowed us to construct an analysis analogous to a multiple linear regression analysis, treating each transporter (P-gp-based transport, basolateral transport by a BT, and apical transport by an AT) as a new predictor using 1 df. The need to add further transporters to a minimal model could be tested analogously to the need to add new predictors in a multiple linear regression model, by testing the improvement of the prediction via the decrease in the residual error of the prediction versus measurement regression (Rao, 1998; Quinn and Keough, 2002). Because adding a new transporter yielded an entirely new simulation and an entirely new prediction, this approach technically does violate one of the assumptions of the linear analysis, i.e., independence of the transporters. Therefore, the probability estimates must be regarded as approximate.

**Mass Action Kinetic Model.** Fig. 1 is a cartoon model of a confluent cell monolayer, featuring the polarized MDCKII-tMDR1 cells, where the basolateral membrane is attached to the polycarbonate filters and P-gp (upward arrows) expressed on the apical surface. The apical and basolateral chambers are kept separate by the tight junctions. Active transport by P-gp occurs vectorially, with substrate binding to a site on P-gp within the apical membrane inner monolayer and with efflux into the apical chamber (Loo and Clarke, 2005; Lugo and Sharam, 2005).

With the confluent cell monolayer system, we measured the concentration of substrate in the apical chamber, denoted CA, and in the basolateral chamber, denoted CB. However, the concentration of substrate in the cytosol, denoted CC, and in the inner plasma membrane in contact with the P-gp binding site, denoted Cpc, cannot be measured rigorously in real time. These internal concentrations are variables of the mass action model and were fitted by elementary rate constants for well defined kinetic barriers, according to the measured values of CB and CC over time (Tran et al., 2005; Acharya et al., 2006).

The simplest Michaelis-Menten mass action reaction to model P-gp is
Confluent Monolayer of Polarized Cells

The kinetic parameters for P-gp transport published in Acharya et al. (2006) were determined under the assumption that P-gp was the sole transporter. This model worked very well for amperavir and quinidine. However, the “P-gp alone” model gave adequate fits for loperamide only with concentrations > 3 μM (Tran et al., 2005; Acharya et al., 2006). At lower concentrations, more loperamide efflux was observed than could be fitted assuming that the passive permeability coefficients fitted +GF120918 and that the P-gp kinetic parameters fitted at high (≥3 μM) loperamide concentrations.

The loperamide data have been fitted again, now allowing a basolateral transporter, modeled by the Michaelis-Menten equation, eq. 2. Both B>A and A>B transport data were used to find the consensus value of $V_{\text{max}}/K_m$. Here, for economy, we will only show the data and fits for the B>A transport of loperamide across MDCKII-hMDR1 monolayers. The A>B transport data fitted just as well.

Figure 2 shows the concentration of loperamide over time in each chamber and the fits for three of the eight loperamide concentrations we have examined, from 12 nM to 30 μM. The B>B>A and A>B>A curves denote the concentrations of drug in the basolateral and apical chambers when the basolateral chamber was the donor, i.e., transport runs B>A. Data are shown by □ when the receiver apical chamber is sampled and by ○ when the donor basolateral chamber is sampled. All data points represent triplicate measurements at each time point with the corresponding S.D. With the best fits, the $V_{\text{max}}$ and $K_m$ values did not converge, but their ratio was constant for all concentrations of loperamide <μM, where P-gp saturated and contributes little to total transport. $V_{\text{max}}/K_m$ is 100 ± 20 s⁻¹ (Table 1) was the average of the best fits for loperamide concentrations from 0.012 to 10 μM, i.e., 3 orders of magnitude, suggesting that a single transporter is responsible for the increased basolateral flux. The fits are shown by a dotted line for P-gp alone (Acharya et al., 2006) and by a solid line when the basolateral facilitated transporter, denoted BT, is added using eq. 2. In addition, all curves contain the predicted passive efflux, +GF120918.

Figure 2A shows that with 0.03 μM loperamide there is a substantial underestimate of the data for P-gp alone. With the added basolateral transporter the data fitted very well. Figure 2B shows that with 1 μM loperamide, the two models are starting to converge, but the basolateral transporter is clearly needed to fit the data. Figure 2C shows that with 10 μM loperamide, the two models have converged substantially at early times, because the P-gp is nearly saturated, and most transport is through passive permeability. However, after 3 h, the data still are fitted somewhat better with the basolateral transporter. This is because the basolateral transporter causes the system to need a lower apical chamber concentration and reach its true steady state faster. The P-gp alone simulation now overestimates the data, because of the different binding constants needed to best fit the data without the basolateral transporter.

Although the fits looked good, we wanted to quantitate their statistical significance. We compared predictions separately for apical and basolateral compartments. In each case, predictions were significantly improved by adding the BT to the model with only the P-gp transporter ($p < 5 \times 10^{-7}$). For neither the apical nor the basolateral compartment did adding an AT to the P-gp plus BT model further improve the predicted concentrations of loperamide ($p > 0.7$).

We also fitted the transport data of loperamide in the A>B direction. The fits were just as good (data not shown). This finding
suggests that the basolateral transporter is bidirectional, as it was molded.

Figure 3 shows the B>A transport of 1 μM digoxin across the MDCKII-MDR1 confluent cell monolayers. Figure 3A shows that for the first 6 h the concentrations change roughly linearly with time, yielding no “fittable” data. The approach to the true steady-state is required to fit the kinetic parameters. When the cells were incubated for an extended period of time (>12 h), the transport curves showed toxic effects, e.g., lucifer yellow leakage or an A:B>A curve first increasing and then decreasing (data not shown).

To extend the time of the experiment we constructed a data stitching experiment. Figure 3B illustrates this construction. Once the concentrations of digoxin in the apical and basolateral chambers at the 6th h of A, we start the next experiment such that the initial concentration of digoxin in both chambers match as closely as possible. The concentration of digoxin in the apical chamber increases over time and nearly reaches a steady state. C, final stitched data. The fits for P-gp alone are shown by a dotted line (Acharya et al., 2006), whereas the solid line shows the fit from adding a basolateral facilitated transporter, with $V_{\text{max}}/K_m = 30 \text{ s}^{-1}$. All curves also contain the passive permeability ($P_{\text{gp}}$) contribution. P-gp alone clearly underestimates transport by at least 50%. The solid line shows the fit from adding a basolateral transporter with $V_{\text{max}}/K_m = 30 \text{ s}^{-1}$, in addition to P-gp. The solid line fits up to 12 h and beyond that, it predicts more transport than we actually measure. An apical facilitated transporter or active importer for digoxin, in addition to P-gp, would cause the final steady-state concentration for digoxin in the apical chamber to be smaller. This is shown by the dashed line, with $V_{\text{max}}/K_m = 2 \text{ s}^{-1}$ for the additional apical facilitator transporter. Statistical analysis of these data shows that the basolateral transporter is significant with $p < 0.01$ and the apical transporter is significant with $p < 0.05$, as explained in the text.
6th h are known, the next experiment begins with these initial concentrations of digoxin in the appropriate chambers. Using this approach, data are collected for consecutive stretches of 6 h and stitched together to create a time course up to 30 h. The only artifact in stitching is that for the next experiment the cytosol is initially empty of substrate, which it would not be at the end of the prior experiment. However, because the volume of the confluent cell monolayer cytosol is very small, the cells will fill within the first few minutes of substrate addition, which is insignificant on the time scale of these experiments (Acharya et al., 2006).

The kinetic fits for the digoxin concentration time curve are shown in Figure 3C. The fits are shown by a dotted line for P-gp alone (Acharya et al., 2006), a solid line when the basolateral facilitated transporter, denoted BT, is added and a dashed line when the basolateral facilitated transporter and the apical facilitated transporter, denoted AT, are added using eq. 2. In addition, all curves contain the predicted passive efflux, +GF120918. The P-gp alone model underestimates transport by at least 50% at all times. Adding a basolateral transporter yields a good fit up to 12 h when $V_{\text{max}}/K_m$ is 30 s$^{-1}$, and the parameters are given in Table 1. As mentioned above $V_{\text{max}}$ and $K_m$ were fitted independently, but only their ratio was constant. However, beyond 12 h it predicts too much transport, well away from the S.D. of the data. Addition of an apical facilitated transporter with a $V_{\text{max}}/K_m = 2$ s$^{-1}$ for digoxin, in addition to P-gp and the basolateral transporter, yields a lower true steady-state concentration for digoxin in the apical chamber and a good fit to the data.

To estimate the statistical significance of these fits, we compared model predictions to measurements separately for apical and basolateral compartments. In all cases, model predictions were highly correlated with measured amounts of digoxin in each compartment ($r^2 > 0.90$ for linear regressions). Correlations increased and RMS prediction errors decreased, in general, when a BT and then an AT were added to the models ($r^2 > 0.995$, RMS error $< 0.03$ for full models). Adding an apical transporter to the P-gp plus BT model neither increased the correlation nor decreased the prediction error for the loss of digoxin from the basolateral chamber.

Regression measured basolateral compartment digoxin data versus model predictions yielded slopes significantly $>1.0$ for the P-gp alone model (slope = 1.86, $p < 0.01$). Both the P-gp plus BT model and the P-gp plus BT plus AT model yielded virtually indistinguishable prediction with slopes (0.974 and 0.984, respectively) not different from 1.0 ($p > 0.05$). Regressions of apical compartment digoxin on predictions gave slopes of 1.58, 0.822, and 0.966 for the P-gp alone model, P-gp plus BT model, and P-gp plus BT plus AT model, respectively. Only the P-gp plus BT plus AT model slope was indistinguishable from 1.0 at $p = 0.05$.

Analyses of the curvilinearity of relationships gave similar results. For basal compartment digoxin, the P-gp alone model regression was strongly curved ($p < 0.01$), but the other two models had no significant curvature ($p > 0.05$) to the regressions. For apical compartment digoxin, both the P-gp and P-gp plus BT model regressions were strongly curved ($p < 0.0001$). The P-gp plus BT plus AT model regression was significantly curved ($p = 0.01$) but with a curvature so small that it did not affect the biological predictions.

An attempt was made to identify the canine kidney transporters involved with digoxin and loperamide transport. By preincubating cells with a “high” concentration of a substrate/inhibitor for the basolateral transporter, the transport of digoxin should decrease to the levels predicted by the P-gp efflux alone curves (e.g., a 50% reduction in digoxin transport). This makes the kinetic analysis a very sensitive instrument for determining substrates or inhibitors of the basolateral transporter. Discovering a substrate/inhibitor of the apical digoxin transporter would be more difficult, given its smaller effect on total transport.

Inhibition studies were completed with a variety of prototypical substrates or inhibitors for OAT, OATP, OCT, and MRP transporters (Table 2). The compounds in Table 2 were selected because they cover a broad range of possibilities for known transporters on the basolateral or apical membranes of human and mouse kidney cells. To evaluate whether these compounds have any effect on loperamide or digoxin transport, we used concentrations at or higher than the maximum concentration used for the experiments in the respective references. Clearly the human isoform of a particular transporter could have a substrate or inhibitor not shared by its canine isoform. So this strategy need not succeed, but it is certainly the obvious first step.

Except for loperamide, incubations with the substrates/inhibitors failed to inhibit digoxin transport, B>A or A>B, within 3 h; the canine transporters do not interact with these substrates/inhibitors. The inhibition of digoxin transport by loperamide and vice versa certainly involves P-gp, but to determine whether they share a single basolateral transporter we will need a protocol for assaying digoxin transport kinetic parameters more practical than the cumbersome “stitching” approach used here.

We did find that 100 and 200 μM benzobromarone inhibited 1 μM digoxin transport in the B>A direction by 20 and 50%, respectively (data not shown), but these concentrations also showed toxic effects on the cells, e.g., the transport curves stopped abruptly after a few hours and the cell monolayer became leaky. These results led us to perform the cell concentration exclusion assay using 1 μM benzobromarone with the aim to determine whether it actually caused less accumulation of digoxin in the cells. The amount of radioactivity from digoxin associated with the cells was measured in the presence and absence of benzobromarone. Presumably if benzobromarone inhibits the basolateral and/or apical transporter(s), then less digoxin would be accumulated within the cells.

Figure 4 shows cellular digoxin radioactivity by itself and in the presence of quinidine (a P-gp substrate inhibitor), GF120918 (inhibits P-gp and the additional basolateral and apical transporters), and benzobromarone. In the presence of 20 μM quinidine, P-gp is relatively saturated (Acharya et al., 2006), and there is more digoxin present within the cells compared with digoxin alone. In the presence of 2 μM GF120918, P-gp and the additional transporters are completely inactive, and there is even more digoxin present within the cells as a result of the lack of P-gp efflux of digoxin. In contrast, in presence of 1 μM benzobromarone there is significantly less digoxin accumulation, suggesting inhibition of a basolateral and/or apical uptake transporter(s), whereas P-gp could actively efflux digoxin.

Surprisingly, 1 μM benzobromarone had no effect on digoxin transport. With 1 μM benzobromarone the monolayer was nonleaky to 4 h, i.e., the total flux of lucifer yellow remained $<10$ nm/s. Therefore, 1 μM benzobromarone did not have a toxic effect on the cells. There is no significant effect of 1 μM benzobromarone on digoxin transport ($p < 0.05$) for both B>A and A>B directions based on linear regression analysis (data not shown).

**Discussion**

The rigorous kinetic analysis of P-gp-mediated transport through the confluent MDCKII-hMDR1 cell monolayer has provided a quantitative understanding of P-gp function, because the elementary rate constants so clearly define the function of P-gp and the selective pressure on it (Tran et al., 2005; Acharya et al., 2006). The transports of amprenavir and quinidine across the MDCKII-hMDR1 confluent cell monolayer are quantitatively fitted by P-gp and passive permeability alone. However, for loperamide and digoxin the kinetic mod-
ning shows other novel transporter(s) in the MDCKII cells that facilitate passage through the basolateral membrane and also through the apical membrane for digoxin.

In Tran et al. (2005) and Acharya et al. (2006) it was noted that loperamide transport, especially at low concentrations, was being affected by another process. Adding a facilitated transporter on the basolateral membrane allows loperamide to enter the cytosol from the basolateral chamber more rapidly than predicted by the +GF120918 passive permeation coefficients, i.e., the loperamide basolateral transporter is inhibited by GF120918. We obtained good fits from 12 nM to 30 μM loperamide with the transporter over a 3 order of magnitude range in concentration (Table 1). Statistical analysis supported the existence of the basolateral transporter ($p < 0.000001$). There is no statistical evidence for an apical loperamide transporter. Because we fitted both B>A and A>B transport data for loperamide using the basolateral transporter, this result suggests that transport is bidirectional.

This addition to the kinetic model for loperamide has changed the estimate for the binding constant of loperamide to P-gp from the apical membrane, $K_c$, from 4000 $M^{-1}$ (Acharya et al., 2006) to 20,000 $M^{-1}$ (Table 1). The binding constant is fit primarily from A>B transport experiment (Tran et al., 2005), and the basolateral transporter for loperamide speeds up permeation to the basolateral chamber. Thus, a stronger binding constant to P-gp is required to fit the same A>B data.

For digoxin, the passive permeability was so slow that transport was linear with time for the first 6 h. Without some degree of curvature, i.e., approaching steady state, fitting cannot be done. We applied a stitching method to approximate a 30-h transport experiment. The end of one 6-h experiment became the starting point for the next 6-h experiment. Both a basolateral transporter and an apical transporter were needed to fit this data. We did not attempt the A>B experiment, because the transport in this direction would be much smaller, thereby making the stitching approach more problematic. We are currently working on a new protocol under which steady state should be reached faster. This will be required to determine whether digoxin and loperamide use the same basolateral transporter or not. They partially inhibit each other’s transport, but we cannot yet prove whether or not this inhibition is beyond what would be predicted by inhibition of P-gp alone.

For digoxin, there is a basolateral importer and an apical importer that allow its reentry into the cells after efflux into the apical chamber.

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**TABLE 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Transporter*</th>
<th>Drug Used (1 μM)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzbromarone</td>
<td>hMRP1–6, hURAT1</td>
<td>Not tested</td>
<td>N.A.</td>
</tr>
<tr>
<td>Digoxin</td>
<td>oat1a4,4c1,1b3,1b1, oatp2,</td>
<td>moderate inhibition with 10 μM DGX</td>
<td>Enomoto and Endou, 2005; Iwanga et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Oat-K2</td>
<td></td>
<td>Dresser et al. 2001</td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>hOAT3, roat3,</td>
<td>300 μM inhibits after 4 h</td>
<td>Iwanga et al., 2005</td>
</tr>
<tr>
<td>Glycyrrhizic acid</td>
<td>hOATP1B1,1B3, roatp1a1, 1a4, 1b2</td>
<td>No effect with 200 μM</td>
<td>Tahara et al., 2006</td>
</tr>
<tr>
<td>Indocyanine green</td>
<td>roat2, 3, rhOATP2</td>
<td>Not tested</td>
<td>Ismair et al., 2003</td>
</tr>
<tr>
<td>Loperamide</td>
<td>MRP1, BCRP</td>
<td>No effect with 25 μM</td>
<td>Morrow et al., 2006; Pan and Elmqvist, 2007</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>Not tested</td>
<td>No effect with 10 μM</td>
<td>Morita et al., 2001</td>
</tr>
<tr>
<td>Ouabain</td>
<td>No effect with 25 μM</td>
<td>No effect</td>
<td>Dresser et al., 2001</td>
</tr>
<tr>
<td>PAH</td>
<td>m5r Oat1, hOAT1, roat2,3</td>
<td>No effect with 100 μM</td>
<td>Dresser et al., 2001; Kikuchi et al., 2004</td>
</tr>
<tr>
<td>Probenecid</td>
<td>Mr5r Oat1,3,4, roat-K2</td>
<td>No effect with 200 μM</td>
<td>Dresser et al., 2001; Sugiyama et al., 2001; Honikawa et al., 2002</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Not tested</td>
<td>No effect with 3 μM</td>
<td>Dresser et al., 2001; Vavricka et al., 2002</td>
</tr>
<tr>
<td>Taurocholic acid</td>
<td>Bile acid/salt transporters ASBT, BSEP, NTCP</td>
<td>No effect with 200 μM</td>
<td>Harris et al., 2004; Mita et al., 2006</td>
</tr>
<tr>
<td>Tetraethylammonium</td>
<td>Many OCTs</td>
<td>No effect</td>
<td>Dresser et al., 2001</td>
</tr>
</tbody>
</table>

N.A., not applicable; URAT, uric acid transporter; PAH, para-aminophenylurate.

*The compounds used here are substrates and/or inhibitors of known mammalian transporters. Names of all human (h) transporters (OATs, OATPs, and MRPs) are in upper case. Transporters in mice (m) or rats (r) are in all lower case (e.g., oatp) or start with an upper case letter and the rest are in lower case (e.g., Oat).

†No effect means lack of significant (>10%) inhibition by 2 h.

‡Benzbromarone at 100 and 200 μM was tested, but these concentrations had toxic effects on cells.

§Digoxin, loperamide, and fexofenadine are P-gp substrates.

¶Mitoxantrone is an inhibitor of bcrp1/ABC2G.

‖The best inhibition is seen when there is digoxin transport in the presence of ouabain without any preincubation; 30 min of preincubation with ouabain produced a jump at 4 h in two separate experiments.

‖‖Rifampicin is a P-gp inducer.

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**FIG. 4.** Digoxin cell concentration exclusion by benzbromarone. This figure shows radioactivity in disintegrations per minute from radiolabeled digoxin (DGX) in MDCKII-hMDR1 cells by itself, in the presence of 20 μM quinidine (QND), 2 μM GF120918 (918), and 1 μM benzbromarone (BBZ).
Both were modeled as bidirectional, but only B>A data were fitted, so only import was kinetically important. Statistical analyses have shown that the basolateral transporter was required to precisely predict the amount of digoxin remaining in the basolateral compartment \((p < 0.01)\). Both the basolateral and apical transporters were needed to accurately predict the amount of digoxin transported to the apical compartment \((p < 0.05)\).

The magnitude of the increased flux of digoxin and loperamide into the cells \((-\text{GF120918})\) proves that the basolateral transporter(s) are inhibited by GF120918. For the transport of loperamide from the basolateral chamber into the cytosol, 60% is due to passive permeability \((-\text{GF120918})\) and 40% is due to the transporter \((-\text{GF120918})\) (Table 1). For the transport of digoxin from the basolateral chamber into the cytosol, 30% is due to passive permeability \((-\text{GF120918})\) and 70% is due to the basolateral transporter \((-\text{GF120918})\) (Table 1).

On the other hand, for the digoxin transporter from the apical chamber into the cytosol, 95% of the flux is due to passive permeability \((-\text{GF120918})\) and 5% is due to the apical transporter \((-\text{GF120918})\). Five percent digoxin transport by the apical transporter is too small to determine whether GF120918 inhibits this transporter, which will require performing the A>B transport experiment with an improved experimental protocol.

As a compromise between computational cost and rigor, we modeled the transporters by the Michaelis-Menten equation, with both \(V_{\text{max}}\) and \(K_m\) independently fitted. For all loperamide concentrations, the best fits for the basolateral transporter yielded 1 to 2 order of magnitude ranges for \(K_m\) and \(V_{\text{max}}\), but their ratio was constant for each fit. This result means that the fitting predicted a first-order rate constant for this transporter, just like a passive permeability. So there was no evidence for saturation of the basolateral transporter up to 10 \(\mu\text{M}\) loperamide. The convergence between the P-gp alone curve and the P-gp plus basolateral transporter curve is due to the saturation of P-gp at a high substrate concentration.

We have tried to identify these transporters by adding prototypical substrates/inhibitors of known transporters. If we found one, then the loperamide or digoxin transport curves would drop to the levels predicted by the “P-gp only” curves (Figs. 2 and 3). The change in the predicted curves would be large, making the kinetic analysis an extremely sensitive assay for discovering functional transporters in the confluent cell monolayer. Although this approach is reasonable, it is always possible that the human or mouse transporter would not share a substrate/inhibitor with its canine counterpart.

None of the compounds tested in Table 2 showed simple inhibition of transport of digoxin or loperamide during the first 3 h, except loperamide and digoxin, respectively. There were some changes noted after 3 h, but these appeared to be related to toxicity. Because the other transporter(s) face the basolateral or apical chambers, there is no reason that simple inhibition would require more than 3 h (Acharya et al., 2006).

There may be several reasons for the lack of an inhibitory effect by the P-gp substrate fexofenadine. Fexofenadine has a very low passive permeability through Caco-2 cells (Petri et al., 2004). We have shown that at “low” concentrations of probe substrate and of inhibitor substrate, there is no inhibition of probe transport because P-gp has more than one efflux pathway (Acharya et al., 2006). Also, it may be that the binding constant of fexofenadine is much smaller than that of digoxin, so a lower digoxin concentration may be needed.

A cell concentration exclusion assay was also tried, wherein the concentration of digoxin within the confluent cell monolayer was assayed as a function of benz bromarone concentration. Addition of benz bromarone resulted in a very significant reduction of digoxin accumulation within the cell monolayer, as expected for a substrate/inhibitor of the basolateral digoxin transporter. However, benz bromarone had no significant effect on digoxin B>A or A>B transport.

The reduction in cell-associated digoxin by benz bromarone is due to another mechanism besides inhibition of the basolateral or apical transporter. The simplest hypothesis is that benz bromarone reduces the cytosolic volume of the cell, so less digoxin can accumulate. This reduction would cause no predicted change in the transport of digoxin, because the kinetic model predicts that nearly all of it diffuses along the inner plasma membrane (Tran et al., 2005). Thus, cell concentration exclusion assays must be carefully controlled when they are used with amphipathic compounds effluxed from a transporter whose binding sites are within the plasma membrane, such as P-gp (Lugo and Sharom, 2005).

In conclusion, we have used a rigorous kinetic analysis to detect the presence of novel transporters of loperamide and digoxin in the basolateral membrane of this cell line, as well as a digoxin transporter in the apical membrane. Based on the current findings, one could question whether digoxin and loperamide are robust substrates for screening compounds for P-gp interactions in the MDCKII-hMDR cell line. The worst case scenario in using them would be a false-positive result if the novel compound did indeed inhibit the basolateral transporter but not P-gp. If a compound inhibits the efflux of digoxin, a follow-up experiment with amineprin or quinidine would identify the source of the inhibition. Quinidine and amineprin appear to be robust probe substrates, as their transport across the MDCKII-hMDR1 cell monolayer depends only upon passive permeability and P-gp. Use of another cell line, e.g., LLC-hMDR1 or Caco-2, with digoxin or loperamide does not avoid the problem, because the same kinetic analysis as shown here is required to determine which compounds are robust P-gp probe substrates for individual cell lines.

References


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