Extrapolation of elementary rate constants of P-glycoprotein mediated transport from MDCKII-hMDR1-NKI to Caco-2 cells

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Running Title: Extrapolation of P-gp kinetics to Caco-2 cells

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Number of text pages: 17
Number of tables: 3
Number of figures: 3
Number of reference: 22
Number of words in abstract: 248
Number of words in introduction: 814
Number of words in discussion: 1409
Non-standard Abbreviations:

DMEM: Dulbecco’s modified Eagle’s medium

GF120918: N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide

TEER: Transepithelial Electrical Resistance

UWL: unstirred water layer
Abstract

The best parameters for incorporation into mechanistic PBPK models for transporters are system independent kinetic parameters and active (not total) transporter levels. Previously, we determined the elementary rate constants for P-gp mediated transport (on- and off-rate constants from membrane to P-gp binding pocket and efflux rate constant into apical chamber) using the structural mass action kinetic model in confluent MDCKII-hMDR1-NKI cell monolayers. In the present work, we extended the kinetic analysis to Caco-2 cells for the first time and showed that the elementary rate constants are very similar compared to MDCKII-hMDR1-NKI cells, suggesting they primarily depend on the interaction of the compound with P-gp and are therefore mostly independent of the in vitro system used. The level of efflux active (not total) P-gp is also fitted by our model. The estimated level of efflux active P-gp was 5.0 ± 1.4 fold lower in Caco-2 cells than in MDCKII-hMDR1-NKI cells. We also kinetically identified the involvement of a basolateral uptake transporter for both digoxin and loperamide in Caco-2 cells, as found previously in MDCKII-hMDR1-NKI cells, due to their low passive permeability. This demonstrates the value of our P-gp structural model as a diagnostic tool in detecting the importance of other transporters, which cannot be unambiguously done by the Michaelis-Menten approach. The system independent elementary rate constants for P-gp obtained in vitro are more fundamental parameters than obtained using Michaelis-Menten steady-state equations. This suggests they will be more robust mechanistic parameters for incorporation into physiologically-based pharmacokinetic models for transporters.
Introduction

Numerous studies have shown that drug transporters play a significant role in drug absorption, distribution and elimination, and that their inhibition or induction can lead to sometimes serious drug-drug interactions (DDIs) (Giacomini et al., 2010; Zolk et al., 2011). Physiologically based pharmacokinetic (PBPK) modeling is gaining increased acceptance by Regulatory Agencies as a tool to address DDI questions. In some cases PBPK modeling can eliminate the need for a clinical DDI study (Huang et al., 2013). However, PBPK models for transporters often rely on compound specific scaling factors, the physical basis for which is not understood, thereby limiting the ability to extrapolate DDI results to other transporter substrates (Jones et al., 2014; Li et al., 2014; Varma et al., 2014).

P-glycoprotein (P-gp), a member of the ATP binding cassette (ABC) family of transporters, has been extensively studied due to its ability to efflux a broad range of drugs across biological membranes and its important role in DDIs (Giacomini et al., 2010). The most common experimental system to obtain in vitro kinetic parameters for incorporation into PBPK models is a polarized cell monolayer overexpressing P-gp. The study of P-gp kinetics is complicated by the fact that the substrate binding site on P-gp is located within the inner monolayer of the apical membrane (Aller et al., 2009; Li J. et al., 2014).

For study of P-gp efflux kinetics, the most commonly used mathematical models are compartmental models, which use initial rate data and steady state Michaelis-Menten assumptions to generate values for $K_m$ and $V_{max}$ (Zamek-Gliszczynski et al., 2013). However, a true $K_m$ value for binding to Pgp can only be derived in this way when the compounds have been demonstrated to interact with P-gp only and not with other transporters in the experimental in vitro system. Otherwise, $K_m$ will be a convolution of binding to P-gp and another transporter(s) and therefore dependent on the in vitro experimental system.
We have developed a structural mass action kinetic model for P-gp mediated transport (Tran et al., 2005; Acharya et al., 2008; Agnani et al., 2011; Lumen et al., 2013). The structural model uses the generally accepted mass action reactions for P-gp binding and efflux (equation 1), along with the known structural parameters for P-gp and the cells, then solves the non-linear differential equations of the mass-action reactions to fit the kinetic parameters. Unlike the Michaelis-Menten approach, there is no need for steady-state assumptions, since they are not required to execute this model (Bentz et al., 2005).

\[
T_0 + C_{PC} \xleftrightarrow[k_1]{k_r} T_1 \xrightarrow{k_2} T_0 + C_A
\]  

(1)

$T_0$ is the empty transporter, $C_{PC}$ is the substrate in the apical membrane inner monolayer, $T_1$ is the transporter bound by substrate and $C_A$ is the substrate after efflux into the apical chamber. $T(0)$ is the total concentration of efflux active P-gp which is given at all times by $T(0)=T_1(t)+T_0(t)$. The elementary rate constants are $k_1$ for association with P-gp, $k_r$ for dissociation from P-gp back into the membrane and $k_2$ for efflux out to the apical chamber. With this model, the elementary rate constants for several P-gp substrates as well as efflux active P-gp surface density, which mathematically define the $K_m$ and $V_{max}$, were successfully obtained in confluent MDCKII-hMDR1-NKI cells. Importantly, this model can detect kinetic involvement of additional uptake transporters for substrates with relatively low passive permeability, e.g. digoxin, loperamide and vinblastine (Lumen et al., 2013). The kinetic parameters fitted are specific to P-gp even in the presence of other transporters. Using the structural mass action kinetic model, it was also demonstrated mathematically that the IC$_{50}$ values for P-gp inhibition obtained using polarized P-gp expressing cell monolayers depended on efflux active P-gp level: higher P-gp levels result in higher IC$_{50}$ values (Lumen et al., 2013).
In this work we have investigated whether the elementary rate constants \( (k_1, k_r, \text{ and } k_2) \) obtained by our mass action kinetic model can be extrapolated to Caco-2 cells. We found that the elementary rate constants are very similar between the two cell lines. This suggests that these parameters are fairly system independent.

In addition, our results provided kinetic support for the presence of basolateral uptake transporters for digoxin and loperamide in Caco-2 cells, as shown previously for MDCKII-hMDR1-NKI and MDCKII-hMDR1-NIH cells (Lumen et al, 2013). This means that for these compounds, the most essential assumption of the derivation of the Michalis-Menten steady state equations, that both substrate and inhibitor interact with only P-gp, is not met in these cell lines.

Finally, since our structural mass action kinetic model also derives efflux active P-gp levels. Our companion paper (Meng et al., 2016b) shows that there can be a substantial difference (~10 fold) between efflux active and total plasma membrane P-gp levels, depending on the morphology of the microvilli in the in vitro system. All of these simulations can be turned into a PBPK model within the MathWorks SimBiology program. SimBiology can also access all of the MATLAB functions needed to execute the calculations in our model.
Materials and Methods

Loperamide, quinidine and digoxin were from Sigma (St. Louis, MO); amprenavir and GF120918 were from GlaxoSmithKline. ^3^H-amprenavir (24Ci/mmol) and ^3^H-loperamide (11 Ci/mmol) were obtained from Amersham), ^3^H-digoxin (15-40 Ci/mmol) was from Perkin Elmer, ^3^H-quinidine (20Ci/mmol) was from American Radiolabeled Chemicals. SV 96 Total RNA Isolation System was from Promega. Oligo (dT)12-18 primer, SuperScript® III Reverse Transcriptase, TaqMan® Universal PCR Master Mix and Quant-iT™ RiboGreen® RNA Reagent were from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide and lucifer yellow were from Sigma-Aldrich (St. Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM) with 25 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) (HEPES) buffer, high glucose (4.5 g/L), L-glutamine, pyridoxine hydrochloride, without sodium pyruvate, and with phenol red was from Gibco (Grand island, NY). The same medium without phenol red was used for transport experiments. Fetal Bovine Serum, non-essential amino acids and penicillin-streptomycin were from Gibco (Grand Island, NY). Transwell 12-well plates with polycarbonate inserts (0.4 µM pore size and 12 mm in diameter) were obtained from Corning Life Sciences (Acton, MA). Epithelial Voltohmmeter (EVOM) and STX2 electrode set were from World Precision Instruments (Sarasota, FL).

Cell Culture

Caco-2 cells were maintained in culture media (DMEM supplemented with 10% Fetal Bovine Serum, 50 units/ml penicillin, 50 µg/ml streptomycin and 0.1mM MEM non-essential amino acids). Cells were split once a week in a ratio of 1:20 at 70%-80% confluency after 2 washes with PBS and trypsinization with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA). Cells were kept at 37° C in 5% CO2. All transport assays were performed with cells from passages 30-40.

Quantitative Real-time RT-PCR (qRT-PCR)
Total RNA from confluent MDCKII-hMDR1-NKI and Caco-2 cell monolayers was extracted using SV 96 Total RNA Isolation System according to the manufacturer’s instructions.

cDNA was prepared by performing reverse transcription using an oligo (dT)_{12-18} primer and catalyzed by Superscript II enzyme according to the manufacturer’s instructions. Gene expression of MDR1 was then evaluated by qPCR. Thermocycling was carried out in a final volume of 25 μL containing 12.5 μL Taqman ® Universal PCR Master Mix, 900 nM forward (5’-TATGGTGGTGAACTTTGGC-3’), and reverse (5’-CTGTCATTCTCCAAACACCAGC-3’) primers, 250 nM probe (5’-(FAM)-CCATGGGGCTGGACTTCCTCATGAT-(TAMRA)-3’) and 5 μL cDNA. Fluorescence was measured using the SDS software of the ABI Prism™ 7900HT Sequence Detection System (TaqMan™) starting with 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. Each sample was analyzed in triplicate. The targeted mRNA in MDCKII-hMDR1-NKI and Caco-2 cells was normalized to total RNA.

**Caco-2 Cell Monolayer Culture and Transport Study**

For the P-gp mediated transport experiments, cells were seeded on polycarbonate membrane inserts at a density of 2.5 × 10^5 / cm² and maintained for 21 days in complete medium; the medium was changed every 2-3 days. After 21 days the monolayer integrity was determined by measuring the TEER using an Epithelial Voltohmmeter, according to manufacturer’s instructions. The final values are expressed as Ω·cm² on the basis of the following equation: TEER = (R-R_a) × A, where R is the resistance of filter insert with cells, R_a is the resistance of the filter alone and A is the growth area of the filter. Mature Caco-2 cell monolayers exhibited a TEER > 600 Ω·cm² before used in transport experiments. Prior to the experiment, media was removed and cells were pre-incubated for 30 minutes with either transport medium alone or transport medium supplemented with 2 μM GF120918 to inhibit P-gp. Transport of a range of concentrations of ^3H-amprenavir, ^3H-loperamide, ^3H-digoxin or ^3H-quinidine
across the confluent cell monolayers over 6 hours 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. For incubations in the presence of GF120918, the inhibitor was added to both chambers. In addition, 100 µM lucifer yellow was added to donor chamber to monitor cell monolayer integrity. At the indicated time points, 25 µL samples were taken from both these chambers and counted by TopCount Model 19912. Lucifer yellow concentrations were determined using a SpectraMax Gemini fluorescence microplate reader at wavelengths of $\lambda_{ex}$ 430 nm and $\lambda_{em}$ 538 nm.

**Kinetic Model of Transport across a Confluent Cell Monolayer**

The basic structural mass action kinetic model is depicted in Fig.1 and Eq 1, 2A and 2B. Important components of the model are: passive permeability across the lipid bilayers obtained in the presence of GF120918; partitioning of substrates into the membranes; active transport by P-gp and by other transporters (Tran et al., 2005). Active transport by P-gp occurs uni-directionally, with substrate binding to a site on P-gp within the apical membrane inner monolayer and with efflux into the apical chamber. The simplest Michaelis-Menten mass action reaction to model active P-gp transport is shown in Equation 1. In this “P-gp only” model, the elementary rate constants are simultaneously fitted to mass action equations for P-gp mediated transport and passive permeability. Importantly, the P-gp surface density is an explicit mass action variable and fitted along with other parameters. The elementary rate constants define the binding constant of the substrate to P-gp from the membrane ($K_C=k_1/k_r$), not the cytosol. The on rate constant, efflux active P-gp surface density, efflux rate constant and partition coefficient together give the molecular $K_m=((k_r+k_2)/k_1) * K_{PC}$, where $K_{PC}$ is the partition coefficient of the drug into the cytosolic lipid inner monolayer (Bentz et al., 2005) and $V_{max}=k_2T(0)$ for P-gp transport. Our kinetic analysis can use transport data from multiple initial drug...
concentrations and time points until the final steady state is approached, which is when concentrations in donor and receiver chamber no longer change, to fit the elementary rate constants for each new drug.

In addition, our model can kinetically detect the presence of other transporters contributing to the total P-gp mediated transport (Acharya et al., 2008). We used facilitated transporter models for additional transporters to allow the mass action kinetics a degree of freedom to determine transport directionality based on the fits. According to our previous data, the fits are consistent with a bidirectional facilitated transporter or an active (uni-directional) uptake transporter, although a bidirectional facilitated transporter fitted the data somewhat better (Agnani et al., 2011). Since the true identity of these transporters is unknown, each transporter was characterized by a single bidirectional first-order kinetic clearance ($k_B$). They are denoted as AT, for apical uptake transporter, and BT, for basolateral uptake transporter, Eq (2).

\[
\begin{align*}
C_A + AT & \overset{k_A}{\underset{\cdot}{\rightleftharpoons}} C_C + AT \\
C_B + BT & \overset{k_B}{\underset{\cdot}{\rightleftharpoons}} C_C + BT
\end{align*}
\]

(2A)

(2B)

It is possible that more than one transporter is involved at these membranes. In that case, we would be fitting some weighted average. Importantly, P-gp mediated transport was not estimated by simply subtracting the transport in the presence of GF120918 from the total transport, which does not take back flow into consideration (Tran et al., 2005). Instead, passive permeability coefficients over time were included within the mass action differential equations and fixed by the time-dependent experiments in the presence of GF120918.

Lipid bilayer partition coefficients are used to define the free concentrations within the aqueous compartment that is contained by the lipid monolayer (Tran et al., 2005; Lumen et al., 2013). Partition coefficients were fixed at the values shown in Table 1. The differential equations were solved in MATLAB 2014b using the ODE23s solver.
Data analysis using particle swarm

The particle swarm program was used to fit the elementary rate parameters for our data sets. All the applicable data is fitted simultaneously. All kinetic parameters were free to range during the fitting. Each fitted parameter is shown as the average and standard deviation of 6 independent fittings. The “goodness of fit” to a particular drug data set was quantified by the coefficient of variation (CV) calculated between model simulations and the experimentally measured kinetic data. T(0) and k₁ were fitted as a consensus for all the drug data, while kᵦ, k₂ and kᵦ were refitted as drug dependent (Agnani et al 2011; Lumen et al., 2013).
Results

P-gp mediated transport across Caco-2 cell monolayers was measured for amprenavir, quinidine, loperamide and digoxin over 6 hours in both B>A and A>B directions using a range of initial concentrations and both in the absence and presence of GF120918. For each drug, the number of initial concentrations varied: 6 data sets were generated for amprenavir, with initial concentrations between 0.2~50 μM (Supplemental Figure 1); 5 data sets for quinidine with concentrations between 0.1~30 μM (Supplemental Figure 2); 6 data sets for loperamide with concentrations between 0.1~30 μM (Supplemental Figure 3); and 5 data sets for digoxin with concentrations between 0.1~10 μM (Supplemental Figure 4). For each concentration and transport direction, concentration versus time curves were generated in triplicate for both donor and receiver chambers. The first time point taken was after 6 min and this time point was used as the starting point for fitting to eliminate the need to correct for initial transient effects (Tran et al., 2004). Each concentration versus time curve has 9 time points ending at 6 h: 6 min, 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h and 6 h. Therefore, each concentration yields 36 data points.

In our model, we explicitly fit passive permeability coefficients at each time point when P-gp is fully inhibited by GF120918 and then use those parameters in the fitting of the total transport, when P-gp is active. This method is more accurate than simply subtracting the passive transport data curve from the total transport data curve since in the latter case possible backflow is ignored (Tran et al., 2005).

Regarding other transporters, it is possible that some of them are not inhibited by GF120918. In that case, the calculated passive permeability in the presence of GF120918 would include their contribution. The steady-state values for the passive permeability coefficients are shown in Table 1A.
Fitting the drug independent values: \( T(0) \) and \( k_1 \)

Previously, we found that the efflux active P-gp surface density \( T(0) \) in confluent MDCKII-hMDR1-NKI cell monolayers was independent of which drug was being transported. This is as expected since the same MDCK cell line was used to measure transport of each drug, and is a benchmark of our fitting approach (Tran et al., 2005; Agnani et al., 2011). The association rate constant \( k_1 \) was also drug independent, which was not required, and was on the order of the maximal diffusion rate of a drug like compound through a lipid bilayer (Tran et al., 2005). This is consistent with the fact that the P-gp binding site is large compared to the molecular size of the drugs we studied (Aller et al., 2009). The small standard deviations of the drug independent \( T(0) \) and \( k_1 \) values found here for Caco-2 cells (Table 1A) suggest that a good consensus fit was obtained for these parameters by simultaneously fitting transport data from the four drugs used in this work. As anticipated, \( T(0) \) is compound independent as the same Caco-2 cell line is used for each drug. The \( k_1 \) in Caco-2 cells was also drug independent and large, as was the case for the MDCK-MDR1-NKI cells, with an absolute value very close to that found in MDCK-hMDR1-NKI cells (Table 1B). The upper and lower bound for \( k_1 \) and efflux active P-gp surface density were the same as in Agnani et al. (2011).
Fitting the drug dependent values: $k_r$, $k_2$ and $k_B$.

We next fitted the drug dependent kinetic parameters $k_r$ and $k_2$. $T(0)$ and $k_1$ were fixed at their consensus values (Table 1A) and $k_r$ and $k_2$ were obtained in 6 independent replicate fits for each drug. For this first round of fitting, we assumed that no other transporter was involved in transcellular transport. Then the data sets were refitted to 3 scenarios, allowing a bidirectional basolateral transporter, a bidirectional apical transporter, or both in the model. The assessment of whether a basolateral or apical transporter plays a role is then based on improvement in the fits (Table 3). The goodness of fit is based on the coefficient of variation (CV) between data and fit. Our criterion for a good fit is a CV $\leq 0.03$ (Tran et al., 2005). If incorporation of an additional transporter results in a 25% improvement in the CV (regardless of the initial CV), then the additional transporter is considered to play a kinetically significant role. Amprenavir and quinidine transport data was well fitted with just $k_r$ and $k_2$. The CV for the fit to the amprenavir and quinidine data was 0.028 and 0.019, respectively (Table 3). No significant improvement was observed in the fits by adding additional uptake transporters in the model. These two compounds do not show any kinetic need for other uptake transporters. The symbols in Figure 2A and 2B show the experimental data for transport of 2µM amprenavir and 1µM quinidine in both A>B and B>A directions. The solid lines indicate the fits to the data, using the kinetic parameter values from Table 1A.

For digoxin and loperamide we found that the CV improved by 89% and 30% respectively when a basolateral uptake transporter was allowed in the model (Table 3). The symbols in Figure 3A show the experimental data for transport of 1 µM digoxin in the B>A direction. The dotted lines show the best fit to the data when only P-gp is incorporated in the model (kinetic values for P-gp from Table 1A). There is a substantial underestimate of the amount of digoxin transported. Without a basolateral uptake transporter the only way for drug to get into the cells according to the model is via passive permeability. It is clear from the fits that not enough loperamide and digoxin was able to enter the
cells by passive permeability alone to be adequately effluxed to the apical chamber to fit the data. With the added basolateral uptake transporter very good fits to the data were obtained.

It is worth noting that in contrast to the MDCKII-hMDR1-NKI cells, no significant improvement in CV was seen for digoxin with addition of an apical transporter in the current study (Table 3). This is likely due to the much shorter incubation time in this study (6 hours vs 18 hours stitched for the MDCKII-hMDR1-NKI cells) such that back transport from apical to basolateral compartment is insignificant here (Acharya et al., 2008; Lumen et al., 2013). Similar results were obtained for loperamide (Figure 3B), but the effect of the basolateral uptake transporter is smaller, for reasons that are unclear at this time.

The values for \( k_r \) and \( k_2 \) are very similar for Caco-2 cells (Table 1A) and MDCK-MDR1-NKI cells (Table 1B). In both cell lines, the “P-gp only” model is sufficient for amprenavir and quinidine, but incorporation of a basolateral uptake transporter was required for good fits with both digoxin and loperamide.

The efflux active P-gp surface density was \( 4.5 \pm 1.3 \) fold greater in the MDCK-MDR1-NKI cells than in Caco-2 cells. We measured P-gp mRNA expression in MDCKII-hMDR1-NKI and Caco-2 cells by quantitative RT-PCR. Table 2 shows that the P-gp mRNA expression level was about 7 fold higher in MDCKII-hMDR1-NKI than in Caco-2 cells. This is consistent with the higher efflux active P-gp surface density in these cells.
Discussion

Mechanistic PBPK models of transporter based DDI data ideally use transporter specific and system independent in vitro kinetic parameters for transport and transport inhibition as well as scaling factors for active transporter levels (Huang et al., 2013; Jones et al., 2015). In reality, however, these PBPK models currently often require compound specific scaling factors to generate a good fit to the data and thus lose mechanistic value. The physical nature of these compound specific scaling factors is generally not understood.

In the Michaelis-Menten steady state analysis, the mass action kinetic equation for transport can be solved algebraically when considering initial rate data only. In the structurally based mass action kinetic model we developed for P-gp transport, the kinetic analysis is not limited to initial rates, but in fact relies on approaching a final steady state for transport, i.e. until the concentrations of drug in donor and receiver chamber no longer change. In this case the mass action kinetic equations for transport are solved by numerical integration coupled with Global Optimization to obtain the elementary rate constants governing P-gp transport as well as the efflux active P-gp surface density. This model has been validated using transport data across confluent MDCKII-hMDR1-NKI cells (Agnani et al., 2011).

When Michaelis-Menten steady state kinetics are applied to P-gp mediated transport across polarized cells it is typically assumed that P-gp is the only transporter involved in the transport process. The structural mass action model for Pgp-mediated transport and transport inhibition can serve as a diagnostic tool to detect involvement of other transporters in the in vitro transport process (Lumen et al., 2013). Using this model, it was demonstrated that digoxin, loperamide and vinblastine transport across MDCKII-hMDR1-NKI cells involves an uptake transporter, as well as P-gp. If another transporter is involved, then the Michaelis-Menten $K_m$ becomes a convolution of both transport...
processes. In other words, the $K_m$ value becomes system dependent and translation to in vivo is unclear, unless the contribution of the other transport process is explicitly recognized. Our ultimate goal is to obtain robust system-independent kinetic parameters for P-gp mediated drug transport in vitro, which can be used in PBPK models, in combination with scaled values for efflux active (not total) transporter levels to predict drug concentrations in tissues.

In order to determine if the fitted elementary rate constants from the structural model can be directly extrapolated to other systems, we determined these rate constants in a second cell line, Caco-2. As is shown in Tables 1A and 1B, the P-gp specific elementary rate constants ($k_1$, $k_r$ and $k_2$) fitted for transport of amprenavir, quinidine, loperamide and digoxin across Caco-2 cells are very similar to those obtained in MDCKII-hMDR1-NKI cells. Since MDCKII-hMDR1-NKI and Caco-2 cells are both mammalian cells which very likely have similar plasma membrane composition (van Meer et al., 2008), it is not surprising that the association rate constant to P-gp, which depends on lateral diffusion of the substrate in the lipid bilayer and the size of the binding pocket on P-gp, is the same in the two cell lines.

P-gp is an ATP-dependent efflux pump. To date, the maximal ATP hydrolysis rate found for lipid reconstituted or detergent solubilized P-gp is on the order of 10-20 s$^{-1}$ (Loo et al., 2000; Urbatsch et al., 2003). The highest efflux rate constant in Caco-2 and MDCKII-hMDR1-NKI cells was fitted for amprenavir and ranged from 10 s$^{-1}$ in Caco-2 cells to 30 s$^{-1}$ in MDCK cells, in the range of these biochemically measured ATP hydrolysis rates. The dissociation rate constants are very similar in two cell lines, resulting in low binding constants in both cell lines, consistent with broad substrate specificity.

As for MDCKII-hMDR1-NKI cells, Caco-2 cells showed a kinetic requirement for a basolateral uptake transporter for digoxin and loperamide (Acharya et al., 2008; Agnani et al., 2011). Since the identity of these uptake transporters and their expression levels is not known, a comparison of the first order
rate constants of the uptake clearance values for digoxin and loperamide between the two cell lines is meaningless.

It has been claimed recently that the unstirred water layer (UWL) should be accounted for when measuring transport kinetics using cultured cell systems. Otherwise, the apparent affinity of the substrate to the transporters may be underestimated (Shibayama et al., 2015). In our model, since the passive permeability was fitted when P-gp and the basolateral uptake transporter are fully inhibited by GF120918 (Tran et al., 2005), any effect of UWL will be included in the passive permeability coefficient. As a result, our fitted elementary rate constants for P-gp active transport or BT clearance will not be biased due to the effect of UWL.

An important difference between the structural model and the compartmental models based on Michaelis-Menten analysis is that in the structural model efflux active P-gp is treated as an explicit chemical species, rather than being embedded in the \( V_{\text{max}} \) or represented by a rate-constant between compartments. The efflux active P-gp surface density is a very important variable of the model, fitted along with the elementary rate constants. The term “efflux active” indicates that our kinetic analysis does not measure total plasma membrane P-gp, but only measures those P-gp molecules that successfully efflux drug into apical chamber, which is where we measure it and which is the physiologically significant value.

Several papers have found that MDCKII-hMDR1-NIH (Tang et al., 2002) and MDCKII-hMDR1-NKI cells (Taub et al., 2005) have a higher level of total P-gp than Caco-2 cells. Our fitted efflux active P-gp surface density \( T(0) \) in Caco-2 cells is 4.5± 1.3 -fold lower compared to MDCKII-hMDR1-NKI cells. This result was expected since a similar fold difference between MDCKII-hMDR1-NIH and a different Caco-2 cell line was observed in Lumen et al (2013). This difference could be due to a lower total P-gp expression level in the Caco-2 cells. This explanation was reasonable at first, as a 7 fold
lower P-gp mRNA expression was observed in Caco-2 cells compared to MDCKII-hMDR1-NKI cells (Meng et al., 2016b).

However, in our companion paper, we found that amounts of P-gp protein per transwell were effectively the same for both cell lines (Meng et al., 2016b). It was possible that the level of total P-gp is the same in both cell lines, but that a greater fraction of Pgp on MDCKII-hMDR1-NKI cells is efflux active due to a difference in morphology of microvilli between the two cell lines (Tran et al., 2005).

By electron microscopy, Caco-2 cells possess longer and more densely packed microvilli than MDCK cells (Butor et al., 1992; Gill et al., 2008; Nicolaou et al., 2012). We have speculated previously that for the packed microvilli, like Caco-2 cells, the efflux into the apical chamber happens only from P-gp at the tips of the microvilli (Tran et al., 2005). Drug molecules released from P-gp at the base of a microvillus would randomly diffuse through the aqueous space between microvilli, until most partition again into the apical membrane, as though the molecule had never been effluxed. In this case the efflux active P-gp is a fraction of the total plasma membrane P-gp. The fraction of efflux active Pgp will depend on the morphology of the microvilli and be greater on cells with shorter or less densely packed microvilli, such as MDCKII-hMDR1-NKI cells. However, to prove any of these assertions, given the range of all parameters found in Bentz et al. (2013), is was necessary to perform all experiments with the same cells in one lab. Otherwise one can’t explain the causes of the differences.

**Conclusion**

The best parameters for incorporation into mechanistic PBPK models for P-gp are system independent kinetic parameters and efflux active transporter levels. The elementary rate constants for transport by P-gp (on-, off- and efflux rate constants, k_1, k_r, and k_2, respectively) are very similar for P-gp expressed in Caco-2 cells versus MDCK-hMDR1-NKI cells and largely independent of the in vitro system in which they are generated. Lumen et al. (2013) showed by simulations for probe substrates with a
passive permeability of less than 300-350 nm/s that our kinetic analysis could identify the kinetic need for a basolateral uptake transporter. For compounds with lower passive permeabilities, we have kinetically identified that a basolateral uptake transporter is contributing to the overall transport kinetics of several P-gp substrates in MDCKII-hMDR1-NKI, MDCKII-hMDR1-NIH and two Caco-2 cell lines (Lumen et. al, 2013; this work). Thus for these compounds the Michaelis-Menten $K_m$ is a convolution of binding to two transporters and is therefore system dependent.
Acknowledgements

The authors would also like to thank Dr. Michael O’Connor (Biodiversity, Ecology and Earth Sciences, Drexel University) for statistical advice. The authors would also like to thank the following individuals for their expert assistance with the experiments: Philip Clark and Cory Watson (Drug Metabolism and Pharmacokinetics, GlaxoSmithKline).
Authorship Contributions

Participated in research design: Meng, Ellens and Bentz

Conducted experiments: Meng

Contributed new reagents or analytic tools: NA

Performed data analysis: Meng, Ellens and Bentz

Wrote or contributed to the writing of the manuscript: Meng, Ellens and Bentz
References:


Footnotes

This work was previously presented as a meeting abstract in Biophysical Society 57th Annual Meeting and in Zhou Meng’s PhD thesis “Identification of System Independent and Dependent Parameters for Reliable in vitro-in vivo Extrapolation of P-glycoprotein using a Mass Action Kinetic Model”.

DMD # 72140
Figure Legends

Figure 1
Cartoon of polarized cells, with the apical membrane on top and the basolateral membrane on the bottom. Passive permeability occurs in both directions. P-gp expressed on the apical membrane transports substrate from the inner apical membrane monolayer into the apical chamber. Substrate binds to P-gp with an association rate constant $k_1$, dissociates from P-gp back into apical membrane with an off rate constant $k_r$, and effluxes from membrane to apical chamber with an efflux rate constant $k_2$. $k_A$ and $k_B$ are the first order clearance values for other transporters in apical and basolateral membrane, respectively.

Figure 2
Transport data of amprenavir and quinidine over 6 hours and their fitted curves across Caco-2 cell monolayers. Figure 2A, 2 µM amprenavir; Figure 2B, 1 µM quinidine. The symbols show the data points with error bars showing the standard deviation of triplicate measurements. The lines show the best fits for amprenavir and quinidine transport assuming there are no other transporters. A:B>A denotes the substrate concentration in the apical chamber when the basolateral chamber is the donor, while B:B>A denotes the substrate concentration remaining in the donor basolateral chamber. The A:B>A transport is high because P-gp actively effluxes drug into the apical chamber. The B:A>B denotes the substrate concentration in the basolateral chamber when apical chamber is the donor, while A:A>B denotes the substrate concentration remaining in the donor apical chamber. Triangle symbols show A>B transport and square symbols show B>A transport. Red shows the concentration of drug in the apical chamber and blue shows the concentration of drug in the basolateral chamber.
Figure 3

Digoxin and loperamide B>A transport data in Caco-2 cells fitted with and without a basolateral transporter. Fig. 3A, 1 µM digoxin. Fig. 3B, 0.3 µM loperamide. The symbols show the data points with error bars showing the standard deviation of triplicate measurements. The fits for P-gp alone are shown by a dashed line, whereas the solid line shows the effect of adding a basolateral uptake transporter modeled with first-order clearance. Both digoxin and loperamide show an underestimate of the data for P-gp alone. (double black arrow).
Table 1A: Fitted Consensus Kinetic Values for Caco-2 Confluent Cell Monolayers

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Association to P-gp</th>
<th>Efflux active P-gp</th>
<th>Dissociation from P-gp into apical membrane</th>
<th>Efflux from P-gp into apical chamber</th>
<th>Partition coefficient into inner cytoplasmic monolayer</th>
<th>Passive permeability coefficient at steady state (nm/s)</th>
<th>Other bidirectional transporter (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k₁ (M⁻¹s⁻¹) ¹</td>
<td>T(0) P-gp/pm² ²</td>
<td>T(0) pmol/ well ³</td>
<td>k₂ (s⁻¹) ⁴</td>
<td>Kₚₐ ⁵</td>
<td>Pₐₕ ⁶</td>
<td>Pₕₐ ⁶</td>
</tr>
<tr>
<td>Amprenavir</td>
<td>(1.7±0.5)x10⁻⁸ ⁸</td>
<td>176±28 ⁹</td>
<td>0.1±0.02</td>
<td>(5.9±1.6)x10⁻⁴</td>
<td>10±0.02</td>
<td>100±2</td>
<td>437±54</td>
</tr>
<tr>
<td>Quinidine</td>
<td>Same as above ¹ ⁰</td>
<td>Same as above ¹ ¹</td>
<td>Same as above ¹ ¹</td>
<td>Same as above ¹ ¹</td>
<td>(1.1±0.01)x10⁻⁴</td>
<td>1.1±0.01</td>
<td>350±40</td>
</tr>
<tr>
<td>Loperamide</td>
<td>Same as above ¹ ¹</td>
<td>Same as above ¹ ¹</td>
<td>Same as above ¹ ¹</td>
<td>Same as above ¹ ¹</td>
<td>(6.0±0.02)x10⁻⁴</td>
<td>0.26±0.01</td>
<td>1500±250</td>
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<td>(n=7)</td>
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<td>(n=5)</td>
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</tr>
<tr>
<td>Digoxin</td>
<td>Same as above ¹ ¹</td>
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<td></td>
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<tr>
<td>(n=5)</td>
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</tbody>
</table>

¹k₁ and T(0) are fitted simultaneously using the data for all drugs, therefore noted by “same as above”.

The units P-gp/μm² can be converted to μmols P-gp per liter of inner apical membrane simply by dividing by 0.8 (Tran et al., 2005).

²kᵢ is the dissociation rate constant from the P-gp binding site into the apical bilayer.

³k₂ is the efflux rate constant from the P-gp binding site into the apical chamber.

⁴The partition coefficient between the cytosol and the inner plasma/apical monolayer, Kₚₑ. Cell membrane partition coefficients were estimated using data from 0.1 μm extruded unilamellar liposomes (LUV) whose lipid compositions mimic roughly the lipid compositions of the respective membrane monolayers: inner cytosolic PS/PE/ chol (1:1:1); apical outer, PC/SM/chol; and basolateral outer, PC/chol (2:1) (Lumen et al., 2013). Only the inner cytosolic monolayer partition coefficient, Kₚₑ, is shown in this table.

⁵Pₐₕ and Pₕₐ refers to the steady-state passive permeability coefficient in the presence of GF120918, B>A and A>B respectively.
$k_B$ and $k_A$ refer to the 1$^{st}$ order clearance rates ($s^{-1}$) for transport modeled as a bidirectional update transporter (Agnani et al., 2011).
Table 1B: Fitted Consensus Kinetic Values for MDCK-hMDR1-NKI Confluent Cell Monolayers\(^a\)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Association to P-gp</th>
<th>Efflux active P-gp</th>
<th>Dissociation from P-gp into apical membrane</th>
<th>Efflux from P-gp into apical chamber</th>
<th>Partition coefficient into inner cytoplasmic monolayer</th>
<th>Passive permeability coefficient at steady state</th>
<th>Other bidirectional transporter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(k_1) (M(^{-1})s(^{-1}))</td>
<td>(T(0)) P-gp/(\mu)m(^2)</td>
<td>(T(0)) pmol/well</td>
<td>(k_3) (s(^{-1}))</td>
<td>(K_{NC})</td>
<td>(P_{BA})</td>
<td>(P_{AB})</td>
</tr>
<tr>
<td>Amprenavir</td>
<td>(1±0.4)x10(^{+8})</td>
<td>800±200</td>
<td>0.45±0.11</td>
<td>(7±3)x10(^{+4})</td>
<td>30±8</td>
<td>100±2</td>
<td>420±50</td>
</tr>
<tr>
<td>(n=7)</td>
<td>Same as above</td>
<td>Same as above</td>
<td>Same as above</td>
<td>(4±2)x10(^{+4})</td>
<td>3±0.4</td>
<td>350±40</td>
<td>670±50</td>
</tr>
<tr>
<td>Quinidine</td>
<td>Same as above</td>
<td>Same as above</td>
<td>Same as above</td>
<td>(2±1)x10(^{+4})</td>
<td>0.4±0.08</td>
<td>1500±250</td>
<td>320±90</td>
</tr>
<tr>
<td>(n=5)</td>
<td>Same as above</td>
<td>Same as above</td>
<td>Same as above</td>
<td>(3±1)x10(^{+4})</td>
<td>3±1</td>
<td>100±11</td>
<td>50±10</td>
</tr>
<tr>
<td>Loperamide</td>
<td>Same as above</td>
<td>Same as above</td>
<td>Same as above</td>
<td>(2±1)x10(^{+4})</td>
<td>3±1</td>
<td>100±11</td>
<td>50±10</td>
</tr>
<tr>
<td>(n=7)</td>
<td>Same as above</td>
<td>Same as above</td>
<td>Same as above</td>
<td>(3±1)x10(^{+4})</td>
<td>3±1</td>
<td>100±11</td>
<td>50±10</td>
</tr>
<tr>
<td>Digoxin</td>
<td>Same as above</td>
<td>Same as above</td>
<td>Same as above</td>
<td>(2±1)x10(^{+4})</td>
<td>3±1</td>
<td>100±11</td>
<td>50±10</td>
</tr>
<tr>
<td>(n=5)</td>
<td>Same as above</td>
<td>Same as above</td>
<td>Same as above</td>
<td>(3±1)x10(^{+4})</td>
<td>3±1</td>
<td>100±11</td>
<td>50±10</td>
</tr>
</tbody>
</table>

\(^a\) Data from Lumen et al., 2013 PLoS Open License. All parameters here have the same definition as in Table 1A.
Table 2: Ratio of efflux active P-gp surface density and mRNA levels in MDCKII-hMDR1 and Caco-2 cells

<table>
<thead>
<tr>
<th></th>
<th>Efflux Active P-gp Surface Density</th>
<th>mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-gp/ µm²</td>
<td>Copies of P-gp mRNA /ng RNA</td>
</tr>
<tr>
<td>MDCKII-hMDR1</td>
<td>800±200</td>
<td>6748±653</td>
</tr>
<tr>
<td>Caco-2</td>
<td>176±28</td>
<td>918±78</td>
</tr>
<tr>
<td>Ratio MDCK/Caco</td>
<td>4.5± 1.3</td>
<td>7.4± 0.9</td>
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Table 3: Coefficients of variation for the fits

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Amprenavir</th>
<th>Quinidine</th>
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<tr>
<td>Transporters</td>
<td>P-gp only</td>
<td>AT</td>
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<tr>
<td>CV comparison of fits with different uptake transporters&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>CV&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.028</td>
<td>0.028</td>
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<tr>
<td>Pgp/um²</td>
<td>176</td>
<td>176</td>
</tr>
<tr>
<td>k&lt;sub&gt;i&lt;/sub&gt;(M&lt;sup&gt;-1&lt;/sup&gt;s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>1.7 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>1.7 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>k&lt;sub&gt;e&lt;/sub&gt;(s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>5.9 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>5.9 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
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<tr>
<td>k&lt;sub&gt;f1&lt;/sub&gt;(s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>10.0</td>
<td>10.0</td>
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<tr>
<td>k&lt;sub&gt;f2&lt;/sub&gt;(s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>14.6</td>
<td>10.4</td>
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<tr>
<td>k&lt;sub&gt;f3&lt;/sub&gt;(s&lt;sup&gt;-1&lt;/sup&gt;)</td>
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<td>1.1</td>
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</table>

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<tr>
<th>Drugs</th>
<th>Loperamide</th>
<th>Digoxin</th>
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<tr>
<td>Transporters</td>
<td>P-gp only</td>
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</tr>
<tr>
<td>CV comparison of fits with different uptake transporters</td>
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<tr>
<td>CV&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.017</td>
<td>0.017</td>
</tr>
<tr>
<td>Pgp/um²</td>
<td>176</td>
<td>176</td>
</tr>
<tr>
<td>k&lt;sub&gt;i&lt;/sub&gt;(M&lt;sup&gt;-1&lt;/sup&gt;s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>1.7 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>1.7 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>k&lt;sub&gt;e&lt;/sub&gt;(s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>5.9 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>5.9 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>k&lt;sub&gt;f1&lt;/sub&gt;(s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.5</td>
<td>0.5</td>
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<tr>
<td>k&lt;sub&gt;f2&lt;/sub&gt;(s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>k&lt;sub&gt;f3&lt;/sub&gt;(s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>19.6</td>
<td>19.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>CV is the value of the coefficient of variation for fits with P-gp only, with an apical uptake transporter (AT), with a basolateral uptake transporter (BT) and with both basolateral and apical uptake transporters (AT + BT).

<sup>b</sup>CV comparisons show the relative percent change in the CV with respect to the previous condition. AT shows the comparison with respect to just P-gp. BT shows the comparison with respect to AT, with P-gp in both cases. AT+BT shows the comparison with respect to BT, with P-gp in both cases. When the comparison with AT + BT is small compared with BT, this shows that adding AT to BT make no significant improvement in the fit, i.e. AT is not kinetically required for the measured transport. Agnani et. al. (2011) found that an CV comparison exceeding 25% was sufficient to show that a uptake transporter was kinetically required. The Table shows that...
BT was kinetically required for the measured transport of loperamide and dixoxin in Caco-2 cells, as has been shown for hMDR1-MDCKII-NKI cells (Agnani et al. (2011). Amprenavir and quinidine show no kinetic need for an uptake transporter in either cell line.
Figure 1

- **P-gp**
  - $k_1$
  - $k_r$
  - Other transporters

- **Substrate**
  - $k_2$

- **Apical Membrane**
  - $k_A$

- **Intracellular**

- **Basolateral Membrane**
  - $k_B$

- **Passive Permeability**
  - Other transporters
Figure 2
Figure 3

A

B

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