Derivation of a System-Independent K_i for P-glycoprotein Mediated Digoxin Transport from System-Dependent IC_{50} Data[§]

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ABSTRACT

It has been previously demonstrated that IC_{50} values for inhibition of digoxin transport across confluent polarized cell monolayers are system-dependent. Digoxin IC_{50} data from five laboratories participating in the P-glycoprotein (P-gp) IC_{50} Initiative, using Caco-2, MDCKII-hMDR1 or LLC-PK1-hMDR1 cells, were fitted by the structural mass action kinetic model for P-gp-mediated transport across confluent cell monolayers. We determined their efflux-active P-gp concentration $[T(0)]$, inhibitor elementary dissociation rate constant from P-gp (k_{rQ}), digoxin basolateral uptake clearance (k_B), and inhibitor binding affinity to the digoxin basolateral uptake transporter (K_{QB}). We also fitted the IC_{50} data for inhibition of digoxin transport through monolayers of primary human proximal tubule cells (HPTCs). All cell systems kinetically required a basolateral uptake transporter for digoxin, which also bound to all inhibitors. The

inhibitor k_{rQ} was cell system-independent, thereby allowing calculation of a system-independent K_i . The variability in efflux-active P-gp concentrations and basolateral uptake clearances in the five laboratories was about an order of magnitude. These laboratory-to-laboratory variabilities can explain more than 60% of the IC_{50} variability found in the principal component analysis plot in a previous study, supporting the hypothesis that the observed IC_{50} variability is primarily due to differences in expression levels of efflux-active P-gp and the basolateral digoxin uptake transporter. HPTCs had 10- to 100-fold lower efflux-active P-gp concentrations than the overexpressing cell lines, whereas their digoxin basolateral uptake clearances were similar. HPTC basolateral uptake of digoxin was inhibited 50% by 10 μ M ouabain, suggesting involvement of OATP4C1.

Introduction

The P-glycoprotein (P-gp) IC_{50} Initiative was established to assess interlaboratory variability in P-gp IC_{50} determinations. The results of this study were published by Bentz et al. (2013) and Ellens et al. (2013). The data showed significant laboratory-to-laboratory variability in the reported IC_{50} values, even for laboratories using the same cell line (for example, MDCKII-hMDR1 or Caco-2) and the same inhibitor. This result raised concerns about the utility of in vitro data for predicting in vivo digoxin drug-drug interaction (DDI) risk (Lee et al., 2014). Despite this variability, decision criteria could be derived by receiver operating characteristic analysis, which predicted the risk for a digoxin DDI with a low false-negative rate of 12% (Ellens et al., 2013). These papers made recommendations on the most robust way to determine a P-gp IC_{50} value, recommended refined decision criteria, and proposed that the decision criteria be specific for the P-gp probe substrate digoxin. These recommendations were accepted by the International Transporter Consortium (Lee et al., 2014).

Based upon previous studies (Acharya et al., 2008; Lumen et al., 2010; Agnani et al., 2011), Bentz et al. (2013) hypothesized that the IC_{50} variability was due to the intrinsic variability in the expression levels of efflux-active P-gp and the digoxin basolateral uptake transporter. There are several approaches to analyzing transport across confluent cell monolayers (Zamek-Gliszczynski et al., 2013). In this work, we used the structural mass action kinetic model (Bentz and Ellens, 2014) for P-gp to analyze a selected subset of data generated by the P-gp IC_{50} Initiative to investigate the reasons for the IC_{50} variability and whether a cell system-independent K_i can be extracted from these variable IC_{50} data, as proposed by Lumen et al. (2013). The selection criteria were focused on choosing data of a higher quality than imposed by Bentz et al. (2013) that could provide unambiguous answers to these questions and serve as the input to in vivo DDI predictions. The data from five laboratories were selected for this analysis. We also analyzed new data for digoxin transport across primary culture human proximal tubule cells (HPTCs) using the same inhibitors (Brown et al., 2008).

The structural mass action kinetic model (Bentz and Ellens, 2014) was developed to obtain elementary kinetic parameters for P-gp-mediated transport. Using this kinetic model for P-gp, we previously demonstrated that digoxin transport across MDCKII-hMDR1 and Caco-2 cells cannot be fitted by using only P-gp and digoxin passive permeability across the basolateral membrane (Acharya et al., 2008). The passive permeability

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ABBREVIATIONS: BT, basolateral digoxin uptake transporter; CV, coefficient of variation; DDI, drug-drug interaction; GF120918, *N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-; HPTC, human proximal tubule cell; NC, negative control; PC, positive control; PCA, principal component analysis; P-gp, P-glycoprotein; UWL, unstirred water layer.

alone, independently measured in the presence of *N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinoliny)ethyl]-phenyl)-9,10-(GF120918) (Tran et al., 2005), does not allow enough digoxin to enter the cell to permit the experimentally measured amount of digoxin effluxed to the receiver chamber by both P-gp and passive permeability. We have proposed that this is most likely due to a basolateral uptake transporter for digoxin (Acharya et al., 2008; Agnani et al., 2011; Lumen et al., 2013), although this putative transporter remains unidentified. For the purpose of the kinetic analysis presented here and in our previous work, it is not crucial whether this contributor to basolateral uptake transport is due to a basolateral uptake transporter or to “something else.” For convenience, we will refer to this contributor as the basolateral digoxin uptake transporter, or occasionally, BT, throughout this manuscript.

Previously, we have fitted the elementary kinetic rate constants of digoxin transport by P-gp (Agnani et al., 2011; Lumen et al., 2013). In this work, we used these kinetic rate constants of digoxin, as the probe substrate, for the fitting of the IC_{50} curves using eight P-gp inhibitors obtained from the P-gp IC_{50} Initiative to generate values for 1) the efflux-active P-gp concentration $[T(0)]$, 2) the clearance of digoxin by the basolateral uptake transporter (k_B), 3) the inhibitor dissociation rate constant (k_{rQ}) from P-gp, and 4) the inhibitor binding affinity to the digoxin uptake transporter (K_{QB}), as described previously (Lumen et al., 2013). Note that K_{QB} depends upon both uptake transporter surface density and its binding constant to the inhibitor. Thus, for a given inhibitor, the K_{QB} values for an inhibitor across different cell lines cannot be compared in any simple way.

The function of these kinetic parameters is illustrated in Fig. 1, where BT denotes the basolateral digoxin uptake transporter. The figure illustrates that all but one of the P-gp inhibitors used by Bentz et al. (2013) are shown here to also inhibit the digoxin uptake by BT in these cells (see Tables 4 and 6, as well as Supplemental Figs. 1–24 and Supplemental Tables 1 and 2). The data for amiodarone were too poor to analyze properly. Thus, all of the IC_{50} values in Bentz et al. (2013) are likely to be the product of the convolution of the inhibition of both P-gp and BT. This is a major contribution to the P-gp IC_{50} variability reported by Bentz et al. (2013).

The k_1 for association of substrates to P-gp is essentially the same for all compounds used for model validation (Lumen et al., 2013; Meng et al., 2017a), consistent with a large open binding site on P-gp (Li et al., 2014). The inhibitor dissociation constant $K_i = k_{rQ}/k_1$, with respect to the inhibitor concentration in the membrane, so the system independence of k_1 allows K_i to be calculated for each inhibitor from k_{rQ} alone. Relative to the cytosolic concentration, $K_i = (k_{rQ}/k_1) * K_{QPC}$, where K_{QPC} is the partition coefficient of inhibitor Q into a liposome mimic of the cytosolic inner lipid monolayer of these eukaryotic cells (Tran et al., 2005; Lumen et al., 2013).

The fitting of the values for $T(0)$, k_B , K_{QB} , and k_{rQ} for the inhibitors from the five laboratories used in this work allowed us to simulate IC_{50} curves for “virtual” cell lines, with individual kinetic parameters defined within the ranges of these parameters. This estimates how much IC_{50} variability the ranges of these parameters can create. The laboratory-to-laboratory variability in transporter expression levels for just these five laboratories can explain more than 60% of the IC_{50} variability found in the principal component analysis (PCA) plot in Bentz et al. (2013). This supports the hypothesis that the observed IC_{50} variability in Bentz et al. (2013) was primarily due to laboratory-to-laboratory differences in expression levels of P-gp and the basolateral digoxin uptake transporter.

Materials and Methods

Experimental. For the generation of IC_{50} data for the P-gp IC_{50} Initiative, the methods were reported by Bentz et al. (2013).

Fig. 1. Biologic mechanism of digoxin transport inhibition. The top portion (above the dashed line) shows digoxin transport in the absence of inhibitor, where the basolateral uptake transporter clearance is k_B (s^{-1}), making its fitted value a convolution of transporter surface density and the binding constant of digoxin to the transporter. Digoxin then diffuses within the plasma membrane (Tran et al., 2005), with an association rate constant to P-gp of k_1 ($M^{-1}s^{-1}$), and binds to P-gp with a binding constant of K_Q (M^{-1}). Digoxin is then either dissociated back into the bilayer, which is by far more frequent, or effluxed into the apical chamber with a rate constant k_2 (s^{-1}), which is rare. For digoxin, roughly 1×10^4 molecules bound to P-gp return to the apical bilayer for every one that is effluxed by P-gp into the apical chamber (Lumen et al., 2013). The bottom portion of the figure (below the dashed line) shows the case when there is also a P-gp inhibitor. If the inhibitor only binds to P-gp and not to the uptake transporter, then the IC_{50} is due solely to P-gp. However, we show in Supplemental Table 2 and Tables 4 and 6 that carvedilol, diltiazem, felodipine, isradipine, ketoconazole, mibefradil, nicardipine, nifedipine, nitrendipine, quinidine, ranolazine, sertraline, telmisartan, troglitazone, verapamil, and, of course, digoxin all bind to the basolateral uptake transporter, thereby inhibiting digoxin's uptake into the cells. It remains to be shown whether probe substrates such as loperamide and vinblastine, which also kinetically require a basolateral uptake transporter, would likewise be inhibited by all of these P-gp inhibitors (Lumen et al., 2013). Thus, for the P-gp IC_{50} Initiative data (Bentz et al., 2013), the fitted IC_{50} was commonly a convolution of the inhibition of P-gp and inhibition of the basolateral uptake transporter.

Materials and Methods for HPTCs. Cell culture reagents, including high-glucose Dulbecco's modified Eagle's medium, Ham's F-12 Nutrient Mixture, RPMI 1640 medium, fetal calf serum, penicillin, streptomycin, L-glutamine, trypsin (with 0.02% EDTA), and Dulbecco's phosphate-buffered saline, were obtained from Sigma-Aldrich, UK. SingleQuot kit renal epithelial growth medium supplements and growth factors were purchased from Lonza (Basel, Switzerland). Percoll was bought from GE Healthcare (UK), type 2 collagenase from Worthington Biochemicals (Lakewood, NJ), and 10X Hanks' balanced salt solution from Invitrogen (Carlsbad, CA). Radiolabeled substrates were sourced from Hartmann Analytics (Braunschweig, Germany) and (PerkinElmer, UK). All other chemicals were from Sigma-Aldrich, and were of the highest quality available.

HPTC Cell Culture. Primary HPTCs used in this study were isolated from human kidney donors that were not suitable for transplant. Informed consent and ethical approval for the use of human renal tissue for primary culture and drug safety studies was approved by the ethical review board of the tissue bank supplying the tissue. These kidneys were kept on ice after removal from the body and processed within 18 hours. All cell culture work was performed in a class II vertical laminar flow hood to ensure sterility. The protocol for human HPTC isolation was adapted from Brown et al. (2008). The procedure was as follows. Under sterile conditions, macroscopically normal tissue was decapsulated, and the cortex and outer stripe of the outer medulla (if present) were dissected, cut into pieces of about 1 mm³, and digested in collagenase solution (Worthington), with a final concentration 300 U/mg tissue in RMPI 1640 medium. The suspension was shaken vigorously for 2 hours at 37°C, then passed through a 120- μ m sieve. The resulting cell suspension was loaded on top of a discontinuous Percoll (Pharmacia) gradient made up in RMPI 1640 medium with densities of 1.04 and 1.07 g/ml. After centrifugation at 3000 rpm for 25 minutes in a 4 $\times$ 200-ml swing-out rotor, cells from the intersection were carefully aspirated, washed, and

brought into culture as a mixed population of proximal tubular and distal tubular and cortical collecting duct cells seeded directly onto 6.5-mm 0.4- μ m pore size polycarbonate Transwell filter supports (Costar) at a density of 50,000 cells/filter.

HPTC Transport Measurements. Transepithelial flux measurements of digoxin across monolayers of human tubular epithelial cells were measured essentially as previously described (Brown et al., 2008). Cell monolayers grown on permeable filter supports were extensively washed 4 $\times$ in a modified Krebs' buffer (millimoles per liter): NaCl 140, KCl 5.4, MgSO₄ 1.2, KH₂PO₄ 0.3, NaH₂PO₄ 0.3, CaCl₂ 2, glucose 5, and Hepes 10 buffered to pH 7.4 at 37°C with Tris base. Filters were then placed in 12-well plastic plates, each well containing 1 ml of prewarmed Krebs' or Krebs' plus inhibitor with a further 0.5 ml of identical solution added to the apical chamber. Monolayers were preincubated for 1 hour at 37°C. Basolateral to apical fluxes of digoxin and mannitol were measured in paired resistance-matched monolayers. Monolayers were paired according to their transepithelial resistance; additionally, monolayers were excluded if the transepithelial resistance of the monolayer corrected for the resistance of the filter was less than 60 Ω .cm². Flux was initiated by adding [³H]digoxin (1 μ Ci/ml) and [¹⁴C]mannitol (0.1 μ Ci/ml) to the basolateral chamber. A 250- μ l sample was removed from the apical chamber after a 60-minute flux period. [³H] or [¹⁴C] activity in the samples was determined by liquid scintillation spectrophotometry using a Beckman liquid scintillation counter. At the end of the flux period, the remaining solutions were aspirated off and the filters were washed 4 $\times$ in a 500-ml volume of ice-cold Krebs' buffer at pH 7.4 to remove extracellular isotope. The cell monolayers were then excised from the filter insert, and the cell-associated isotope was determined by liquid scintillation counting.

Kinetic Fitting for All Cells. Unless specifically noted, all calculations, including statistics, were performed using a 64-bit installation of MATLAB version 7.11 (release 2010b; MathWorks, Natick, MA). Logistic regressions (logistic fits), parameter, and S.E. estimates were fitted using nonlinear least-squares regression from MATLAB's statistics toolbox. S.E.s of log(IC₅₀) estimates were calculated as recommended by Lyles et al. (2008). Linear least-squares regressions were performed using MATLAB (Quinn and Keough 2002; Press et al., 2007). Analysis of variance and analysis of covariance were calculated via general linear models (Rao 1998; Quinn and Keough 2002). The transport kinetics fittings used a MATLAB program published by Agnani et al. (2011), and the program is freely available.

Calculation of K_i . The binding constant of the inhibitor Q in the plasma membrane to P-gp is defined as $K_i = k_{1Q}/k_{rQ}$, where k_{1Q} is the association rate constant of the inhibitor Q from the membrane to P-gp, and k_{rQ} is the dissociation rate constant of the inhibitor from P-gp back into the membrane. Based upon the system independence of k_1 , we set $k_{1Q} = k_1$ (Lumen et al., 2013). $K_i = K_{QPC}k_1/k_{rQ}$ is the system-independent dissociation constant of the inhibitor relative to the aqueous concentration of the inhibitor in the cytosol. We used a partition coefficient of $K_{QPC} = 350$ for all inhibitors used in this work, which is the value we measured for quinidine binding to 0.1 μ m of liposomes composed of a phosphatidylethanolamine/phosphatidylserine/cholesterol (1:1:1) mole ratio (Lumen et al., 2013). This lipid composition roughly mimics the cytosolic face of the plasma membrane (van Meer et al., 2008). Verapamil had a measured partition coefficient of 650, which would give a K_i roughly half as large (Lumen et al., 2013). None of the other inhibitors have known partition coefficients measured using this system. k_{1Q} has been measured for MDCKII-hMDR1-NKI cells for several P-gp substrates, including quinidine and verapamil, and was found to be well fitted as $1 \text{ e}^8 \text{ M}^{-1} \text{ s}^{-1}$ (Agnani et al., 2011; Lumen et al., 2013). The same value has been assumed for the LLC-PK1-hMDR1-NKI cells. However, for Caco-2 cells, Meng et al. (2017a) found that k_{1Q} was about 1.7-fold larger. This means that the K_i for an inhibitor with the Caco-2 cells would be 1.7-fold smaller than with MDCKII-hMDR1-NKI cells. Since mammalian plasma membranes appear to be similar with respect to lipid acyl chain composition (van Meer et al., 2008), the elementary rate constants of P-gp should not depend strongly on which plasma membrane it inhabits, which appears to be the case.

Unstirred Water Layer. There has been renewed interest in the unstirred water layer (UWL) enveloping the plasma membrane as a kinetic factor in total substrate permeation (Ghosh et al., 2014; Shibayama et al., 2015). The UWL is important when total transport is measured. Our kinetic model avoids this complication because the UWL contribution to transport is isolated to the passive permeability component, measured in the presence of GF120918 (Tran et al., 2005).

This assumes that 2 μ M GF120918 does not significantly affect the UWL, which is reasonable. This allows us to measure specifically the kinetics of P-gp and BT transport and inhibition.

Results

Acharya et al. (2008) and Lumen et al. (2013) previously showed that a basolateral digoxin uptake transporter was required in two of the three overexpressing cell lines analyzed here. Therefore, this uptake transporter is incorporated in the kinetic model used here (Fig. 1). If a data set did not kinetically require a basolateral digoxin uptake transporter, the fitted value of k_B would be zero.

The parameters required to fit the IC₅₀ curves for inhibition of probe-substrate transport in this model are as follows:

1. $T(0)$ [M, moles of P-gp per liter of acyl chains in the bilayer (Tran et al. 2005)] is the initial efflux-active concentration of P-gp prior to drug binding, which depends strongly on the microvilli morphology (Meng et al., 2017b). We fit the concentration of P-gp in the apical membrane needed to efflux the digoxin/probe substrate concentration into the apical chamber over time, which is the efflux-active concentration of P-gp prior to drug binding. Efflux from the rest of the P-gp in the apical membrane is reabsorbed back into the same or adjacent microvilli prior to reaching the apical chamber in a futile cycle.
2. k_B (s^{-1}) is the digoxin clearance rate constant across the basolateral membrane due to the BT. The identity of BT is currently not known, nor is its surface density.
3. k_{rQ} (s^{-1}) is the elementary dissociation rate constant of the inhibitor (Q) from P-gp back into the apical membrane. A smaller value of k_{rQ} corresponds to a stronger binding of the inhibitor to P-gp.
4. K_{QB} (M^{-1}) is the binding affinity of the inhibitor to the BT from the basolateral compartment. K_{QB} is a convolution of the surface density of BT and the binding constant for the inhibitor to BT. The term affinity, rather than binding constant, is used since the identity and the value of the surface density of BT is unknown.

These four kinetic parameters are necessary and sufficient to fit all of the IC₅₀ curves analyzed in this work, as shown later. We can calculate the inhibitor dissociation constant, K_i , from P-gp into the membrane from the inhibitor k_{rQ} and k_1 (see *Materials and Methods*). We have found previously that k_1 is essentially the same for all of the drugs we have studied in both MDCKII-hMDR1-NKI and Caco-2 cells (Agnani et al., 2011; Lumen et al., 2013; Meng et al., 2017a). %CV is the coefficient of variation as a percentage between the data and the fitted data points. It gives a quantitative rank order for the quality of the fit to the data.

Criteria for Choosing Data Sets to Fit. In Bentz et al. (2013) and Ellens et al. (2013), the sole qualifying criterion for a data set was that the t-statistic $t_{\alpha\beta} > 3$, which yields a 95% confidence that the measured IC₅₀ is within 4-fold of the true IC₅₀ (O'Connor et al., 2015). In this work, we have replaced $t_{\alpha\beta}$ with t_β defined by O'Connor et al., (2015), which is simpler to calculate and a nearly perfect approximation for $t_{\alpha\beta}$.

The choice of data sets from Bentz et al. (2013) used here for fitting the previously described four parameters from IC₅₀ curves in this work was based on more rigorous criteria derived from preliminary fittings focused on obtaining unambiguous data fits. At least four inhibitors out of the eight tested by the laboratory must satisfy the following two criteria:

1. $t_\beta > 5$, which implies a 95% confidence that fitted IC₅₀ is within 3-fold of the true IC₅₀ (O'Connor et al., 2015).
2. Laboratory average negative control (NC, no inhibitor) and positive control (PC, maximum inhibition) must both have

TABLE 1

Average values for NC and PC values in the different overexpressing cell systems for the qualified inhibitors with % CV < 20% for both NC and PC, and $t_{\beta} > 5$

Cells ^a	Laboratory ^a	NC or PC ^b	Laboratory Average ^b	Laboratory Average S.D. ^b	%CV ^c
		number of qualified inhibitors	nm/s	nm/s	
MDCK	2	NC (8)	198	16	8
		PC (8)	36	3	8
	7	NC (4)	179	12	7
		PC (4)	32	1	3
Caco-2	6	NC (7)	312	22	7
		PC (7)	119	7	6
	11	NC (6)	112	12	11
		PC (6)	27	4	15
LLC-PK	2	NC (4)	84	17	20
		PC (4)	33	4	12

^aMDCK, Caco-2, and LLC-PK stand for MDCKII-hMDR1-NKI, Caco-2 cells, and LLC-PK1-hMDR1-NKI cells, respectively. This shorthand is used in all tables herein. More specific descriptions of the cells and laboratory numbers were described by Bentz et al. (2013).

^bNC is the permeability coefficient of digoxin in the absence of inhibitor. PC is the permeability coefficient of digoxin with maximum inhibition of P-gp and BT by 2 μ M GF120918 (Tran et al., 2005; Acharya et al., 2008). S.D. is the standard deviation for the NC or PC of the qualified inhibitors.

^c%CV is the ratio of laboratory average NC or PC divided by the S.D., expressed as a percentage.

CV < 20%. This is crucial because two of the four essential kinetic parameters, i.e., $T(0)$ and k_B , are specific to the cells and cannot depend on the inhibitor. Thus, two crucial parts of the IC_{50} curve depend on the laboratory average value NC and PC for the cells grown in that laboratory. Variations of NC and/or PC across inhibitors, which was more common for the NC data in Bentz et al. (2013), suggest that the cells in that laboratory varied in transporter expression levels from experiment to experiment, so the IC_{50} fits would vary. Note that the value of NC will depend on the initial concentration of digoxin used, which varied widely across laboratories, ~ 100 -fold [see Supplemental Table 1 for examples or Bentz et al. (2013)].

Table 1 shows those laboratories that matched the quality criteria in terms of the laboratory average NC and PC controls for four or more inhibitors, and only these inhibitor data were fitted in this work. The average of NC and of PC, their S.D.s, and coefficients of variation are shown. It was important to have at least one LLC-PK1-hMDR1 laboratory in this analysis, which determined the maximum NC %CV allowed. The inhibitors left out for a laboratory did not show any consistent estimates for kinetic parameters.

Table 2 shows the fitted values for the parameters that characterized these IC_{50} data. The IC_{50} , t_{β} , laboratory average, t_{β} and β , and the IC_{50} fitted slope factor, which will be discussed later, are shown for each chosen laboratory and the chosen inhibitors for that laboratory.

Fitting Protocol. The values for $T(0)$ and k_B for a particular cell system were fixed first by simultaneously fitting all qualified IC_{50} data sets from the laboratory in question, as described previously, using an exhaustive fitting approach (Agnani et al., 2011; Lumen et al., 2013). Thus, for each laboratory, there is one consensus value for $T(0)$ and for k_B , both of which are only cell-dependent, not inhibitor-dependent. The second round of fits for each laboratory used these fixed values for $T(0)$ and k_B for each laboratory, but refitted the values for k_{rQ} and K_{QB} for each inhibitor. Since there were four to eight qualified inhibitors for each chosen laboratory and five chosen laboratories, several independent k_{rQ} values were fitted at least twice (mibefradil) and up to five times (ranolazine). The other inhibitors had three to four independent fits for k_{rQ} .

Across all laboratories, the independent k_{rQ} values for each inhibitor were relatively clustered, indicating that this parameter appeared essentially cell-independent. These individual k_{rQ} values are shown in Supplemental Table 7. Table 3 shows the average, low, and high

estimates for k_{rQ} at the 95% confidence level and the statistically significant one-digit consensus value for each k_{rQ} for all overexpressing cell lines, i.e., Caco-2, MDCKII-hMDR1-NKI and LLC-PK1-hMDR1-NKI.

A complete refit of the data was done using the fixed values of the consensus k_{rQ} values for each inhibitor. Using this consensus k_{rQ} , rather than the independently fit values, made little difference in the goodness of fit, as measured by the coefficient of variation for the fit relative to the data. This was because the refits allowed $T(0)$, k_B , and K_{QB} for each laboratory to adjust slightly to optimize the fit with the now fixed k_{rQ} value. As shown in more detail by Agnani et al. (2011), the global minima for these multiparameter kinetic fits lie within shallow “multidimensional valleys,” which makes mass action kinetic and evolutionary sense. These same consensus k_{rQ} values were used later to fit the IC_{50} data curve for the primary culture HPTCs. This provides strong support for the hypothesis that the elementary rate constant k_{rQ} is essentially system-independent.

Table 4 shows the final fits of the qualified data, together with the CV of the fit and the K_i . Of course, when the true partition coefficient K_{QPC} of the inhibitors is measured, the K_i values for each inhibitor may change somewhat from the values shown in Table 4.

Bentz et al. (2013) also obtained data from seven other P-gp inhibitors used in the P-gp IC_{50} Initiative, called Group 2 in their Table 9, that were lesser quality than the eight inhibitors in Tables 3 and 4 with respect to t_{β} values, as explained there. Data from selected laboratories, with $t_{\beta} > 3$ for six of these P-gp inhibitors (felodipine, nifedipine, nitrendipine, sertraline, telmisartan, and troglitazone), were fitted using their consensus k_{rQ} values. Initial fittings of these data showed that using their consensus values for each of these inhibitors, calculated as shown in Table 3, as opposed to individual fits, made no significant difference in the quality of the fits. All fits and fitted kinetic parameters are shown in Supplemental Figs. 1–24 and Supplemental Tables 1–3. All B > A amiodarone data from Bentz et al. (2013) were too poor to fit for unknown reasons.

All Four Kinetic Parameters Are Required to Fit the IC_{50} Curves. We performed fitting studies to test whether all of these fitted parameters are necessary and sufficient to fit the data. The best fits (line) for carvedilol inhibition of digoxin transport across a confluent cell monolayer of MDCKII-hMDR1-NKI cells are shown in Figs. 2 and 3. Figure 2A shows the fit to the carvedilol IC_{50} curve for digoxin transport with a model that only contained P-gp, and not the basolateral digoxin

TABLE 2
P-gp-overexpressing cell IC_{50} values, t_{β} -statistic values for inhibitors, and IC_{50} slope factor β

Cell ^a	Laboratory ^a	Inhibitor	IC_{50} ^b	t_{β} ^c	Laboratory Average $<t_{\beta}>$ ^d	β ^e
MDCK	2	Carvedilol	6.8	11.5	9.8	1.0
	2	Diltiazem	53.9	7.4		0.7
	2	Isradipine	35.4	7.0		1.0
	2	Mibefradil	7.3	11.0		1.0
	2	Nicardipine	2.6	7.7		1.0
	2	Quinidine	8.4	9.0		1.6
	2	Ranolazine	68.2	10.7		1.3
	2	Verapamil	11.8	14.9	12.7	1.2
	7	Carvedilol	8.1	11.2		1.5
	7	Nicardipine	5.5	14.5		1.1
	7	Ranolazine	114.0	5.3		1.8
	7	Verapamil	32.2	19.7		1.1
Caco-2	6	Carvedilol	0.7	10.7	12.6	0.9
	6	Diltiazem	8.3	17.3		1.2
	6	Isradipine	7.2	14.8		0.9
	6	Nicardipine	1.0	6.8		1.1
	6	Quinidine	2.3	14.8		1.0
	6	Ranolazine	9.8	10.7		1.0
	6	Verapamil	1.8	13.0		1.0
	11	Carvedilol	1.4	7.0	9.6	1.1
	11	Diltiazem	5.7	10.5		0.9
	11	Isradipine	2.6	8.6		0.6
	11	Nicardipine	1.8	8.5		1.4
	11	Quinidine	2.3	7.5		0.9
	11	Ranolazine	15.3	15.3		1.6
LLC-PK	2	Mibefradil	4.5	8.5	6.8	1.2
	2	Quinidine	15.7	6.9		1.2
	2	Ranolazine	55.4	5.7		1.2
	2	Verapamil	8.6	6.1		1.0

^aCell and laboratory number as indicated in Bentz et al. (2013).

^b IC_{50} values are taken from Bentz et al. (2013).

^c t_{β} was calculated as described by O'Connor et al. (2015) and replaces $t_{\alpha\beta}$ used by Bentz et al. (2013). This data quality statistic measures the goodness of fit of the experimental IC_{50} data to a logistic curve, the canonical shape of an IC_{50} curve. The present work required that $t_{\beta} > 5$ for all data analyzed, a lower limit defined from preliminary analysis.

^d $<t_{\beta}>$, the laboratory average for t_{β} , was calculated for the qualified inhibitors.

^e β is the slope factor for the logistic IC_{50} curve, also known as the Hill equation (Hill, 1913), calculated from the fit to the logistic equation (O'Connor et al., 2015). It is used in all commercial software fitting programs for IC_{50} plots as the slope factor estimate of the IC_{50} curve as it passes through the estimated IC_{50} . Any other interpretations of its meaning, e.g., binding cooperativity, are inapplicable to P-gp as analyzed in these data fits (see Discussion).

uptake transporter, so $k_B = 0$ (Fig. 2). k_{rQ} was fixed at the value shown in Table 4. This model clearly does not fit the data, especially at the NC. Changing the P-gp efflux-active concentration 10-fold from $1 \text{ e}^{-3} \text{ M}$ (Fig. 2A) to $1 \text{ e}^{-2} \text{ M}$ (Fig. 2B) or 1/10-fold to $1 \text{ e}^{-4} \text{ M}$ (Fig. 2C) did not improve the fit of digoxin transport kinetics. Therefore, P-gp efflux-active concentration does not significantly affect the fit to NC.

When the basolateral digoxin uptake transporter was added to the model, the predicted curve for carvedilol inhibition of digoxin transport kinetics fit much better. Figure 3A shows that the NC, where inhibitor concentration goes to zero, can now be fitted. That is the primary contribution of k_B to these fits, i.e., allowing enough digoxin uptake into the cells for the measured amount of P-gp-mediated efflux to occur in the absence of inhibitor. At high inhibitor concentrations, the complete inhibition of P-gp efflux is inadequate to fit the full extent of inhibition of digoxin reaching the apical chamber. BT allows digoxin into the cell faster than would passive permeability. Allowing the inhibitor to bind to the BT with the affinity K_{QB} allows the inhibitor to inhibit both P-gp and BT. This gives a good fit at the PC, as shown in Fig. 3B.

Carvedilol required all four kinetic parameters to fit the digoxin inhibition IC_{50} curve. The same was true for all other inhibitors. All final fits are shown in Supplemental Figs. 25–53 and Supplemental Table 4.

PCA Analysis of IC_{50} Variability. We examined the variability of $T(0)$, k_B , and K_{QB} measured here as potential causes for the variability in IC_{50} values across the chosen five laboratories from the P-gp IC_{50} Initiative and the HPTCs. k_{rQ} was fixed at the consensus value for each inhibitor (Table 4) and does not contribute significant variability in this

calculation. Figure 4 shows the PCA plot for IC_{50} data simulated for virtual cell lines based on the ranges of kinetic parameters [$T(0)$, k_B , k_{rQ} , and K_{QB}] shown in Tables 4 and 6. PCA axis 1 is essentially the average of $\log_{10}\{IC_{50}(\text{M})\}$ over all qualified inhibitors within each laboratory, as was the case in Bentz et al. (2013). The amplitude of the second axis of this PCA is very small since, in the simulation, the only remaining variabilities are the fixed k_{rQ} values for each inhibitor, since no simulated experimental error was added.

TABLE 3
Average fitted values for k_{rQ} for dissociation from P-gp

Drug	$<k_{rQ}>$ ^a	Lo (95% CI) ^b	Hi (95% CI) ^b	Consensus k_{rQ} ^c
Carvedilol	9.6E+03	1.8E+03	2.7E+04	1E+04
Diltiazem	6.5E+04	3.1E+02	6.8E+05	7E+04
Isradipine	5.9E+04	6.9E+02	4.9E+05	6E+04
Mibefradil	8.2E+03	1.0E+03	1.5E+04	8E+03
Nicardipine	6.7E+03	1.4E+02	2.3E+04	7E+03
Quinidine	1.3E+04	2.8E+03	3.2E+04	1E+04
Ranolazine	5.1E+04	4.2E+03	9.7E+04	5E+04
Verapamil	2.2E+04	7.4E+03	5.0E+04	2E+04

^aFits were performed over $\log_{10}\{k_{rQ}\}$ fits, and for each inhibitor, these $\log_{10}$ values were averaged and transformed (10^x) to $<k_{rQ}>$. The individual k_{rQ} values are shown in Supplemental Table 7.

^b k_{rQ} was treated as log-normally distributed. Hence statistics including confidence intervals were calculated on $\log_{10}\{k_{rQ}\}$. 95% Confidence interval bounds on k_{rQ} were determined by inverse transformation (10^x) of the intervals on $\log_{10}\{k_{rQ}\}$. The lower (Lo) and upper (Hi) 95% confidence interval bounds are shown.

^cConsensus k_{rQ} is the one-digit estimate to $<k_{rQ}>$.

TABLE 4
P-gp-overexpressing cells, P-gp efflux-active concentration, and kinetic parameters for the qualified inhibitors

Cell	Laboratory	Inhibitor	$T(0)^a$	k_B^a	k_{rQ}^b	K_{QB}^c	$\%CV^c$	K_i^d	$IC'_{50}K_i^e$
			M	s^{-1}	s^{-1}	M^{-1}		μM	
MDCK	2	Carvedilol	1E-03	30	1E+04	1E+05	0.17	0.3	24
	2	Diltiazem	"	"	7E+04	1E+04	0.32	2.0	27
	2	Isradipine	"	"	6E+04	7E+04	0.42	2.0	21
	2	Mibefradil	"	"	8E+03	7E+03	0.15	0.2	32
	2	Nicardipine	"	"	7E+03	2E+04	0.28	0.2	13
	2	Quinidine	"	"	1E+04	2E+04	0.23	0.3	29
	2	Ranolazine	"	"	5E+04	5E+03	0.30	1.0	48
	2	Verapamil	"	"	2E+04	3E+03	0.22	0.6	21
	7	Carvedilol	2E-03	27	1E+04	1E+05	0.50	0.3	28
	7	Nicardipine	"	"	7E+03	5E+04	0.25	0.2	28
	7	Ranolazine	"	"	5E+04	3E+03	0.44	1.0	80
	7	Verapamil	"	"	2E+04	3E+03	0.48	0.6	56
	6	Carvedilol	3E-04	30	1E+04	2E+05	0.29	0.2	4
	6	Diltiazem	"	"	7E+04	2E+04	0.22	1.0	7
Caco-2	6	Isradipine	"	"	6E+04	1E+04	0.12	1.0	7
	6	Nicardipine	"	"	7E+03	5E+04	0.26	0.1	9
	6	Quinidine	"	"	1E+04	1E+04	0.58	0.2	14
	6	Ranolazine	"	"	5E+04	1E+04	0.29	0.8	12
	6	Verapamil	"	"	2E+04	2E+04	0.22	0.3	5
	11	Carvedilol	4E-04	15	1E+04	2E+05	0.57	0.2	8
	11	Diltiazem	"	"	7E+04	1E+05	0.31	1.0	5
	11	Isradipine	"	"	6E+04	1E+05	0.40	1.0	3
	11	Nicardipine	"	"	7E+03	5E+04	0.23	0.1	15
	11	Quinidine	"	"	1E+04	1E+04	0.31	0.2	14
	11	Ranolazine	"	"	5E+04	1E+05	0.29	0.8	18
LLC-PK	2	Mibefradil	1E-03	5	8E+03	5E+05	0.17	0.2	20
	2	Quinidine	"	"	1E+04	5E+04	0.40	0.3	55
	2	Ranolazine	"	"	5E+04	2E+04	0.16	1.0	39
	2	Verapamil	"	"	2E+04	1E+04	0.22	0.6	15

^a $T(0)$, the P-gp efflux-active concentration (moles P-gp per liter of apical membrane), and k_B , the basolateral plasma membrane uptake transporter clearance for digoxin (s^{-1}), are inhibitor-independent, i.e., they are fitted simultaneously for all qualified inhibitors for each laboratory.

^b k_{rQ} is the consensus dissociation constant of inhibitor from P-gp from Table 3, and K_{QB} is the affinity constant of the inhibitor binding to the BT.

^c $\%CV$ is the coefficient of variation for the fit versus the data expressed as a percentage.

^d $K_i = (k_{rQ}/k_i) \times K_{QPC} \times 10^6$ (micromolar) is the system-independent dissociation constant of the inhibitor measured relative to the aqueous concentration of the inhibitor in the cytosol. Most of the inhibitors we used do not have a partition coefficient, K_{QPC} , measured by the technique we used (Tran et al., 2005; Lumen et al., 2013). So we used the value of 350 for all inhibitors, which is the value we measured for quinidine binding to 0.1 μM liposomes composed of a phosphatidylethanolamine/phosphatidylserine/cholesterol (1:1:1) mole ratio (Lumen et al., 2013). This roughly mimics the cytosolic face of the plasma membrane (Lumen et al., 2013). Verapamil had a measured partition coefficient of 650, which would give a K_i roughly half as large as that shown in this table (Lumen et al., 2013). None of the other inhibitors in this work have known partition coefficients measured using this uniform system. k_{rQ} has been measured for MDCKII-hMDR1-NKI cells for several P-gp substrates, including quinidine and verapamil, and was found to be well fitted as $1 e^8 M^{-1}s^{-1}$ (Agnani et al., 2011; Lumen et al., 2013). The same value has been assumed for the LLC-PK1-hMDR1-NKI cells. However, for Caco-2 cells, Meng et al. (2017a) found that k_{rQ} was about 1.7-fold larger. This means that the K_i for an inhibitor with the Caco-2 cells would be 1.7-fold smaller than with MDCKII-hMDR1-NKI cells.

^e IC'_{50}/K_i is the IC_{50} divided by the dissociation constant of the inhibitor to P-gp, both in micromolar units, so it is dimensionless.

Fitting Transport Kinetics for Primary Cell Culture Monolayers of HPTCs. The inhibition of digoxin transport through confluent cell monolayers composed of primary HPTCs using the same inhibitors was fitted as well, using the fixed consensus k_{rQ} values in Table 3. Figure 5 shows the best fits for ketoconazole inhibition of digoxin transport across a confluent monolayer of HPTCs. Figure 5A shows that when a BT was not incorporated, $k_B = 0$, the fit was poor, as digoxin transport is not fitted at the NC. Figure 5B shows that when BT is added to the model without binding/inhibition by the inhibitor, $K_{QB} = 0$, digoxin can be fitted at the smaller inhibitor concentrations, but not the data at the high inhibitor concentrations. Figure 5C shows that when inhibition of BT is accounted for by K_{QB} , then the observed data are fit well by the model. K_{QB} for ketoconazole is responsible for about a 50% inhibition of digoxin transport. The primary culture HPTCs, with smaller efflux-active P-gp concentrations, show a much greater role of inhibitor binding to BT on transport than in the P-gp-overexpressing cell lines (see Fig. 5, B and C).

The kinetic parameters of inhibition of digoxin transport conducted in HPTCs are shown in Tables 5 and 6. Table 5 shows the IC_{50} , t_β , and the slope factor β for these data, which came from 10 out of a total of

13 kidneys evaluated. These data were not filtered through the same quality criteria used for the overexpressing cells, but instead just used $t_\beta > 3$, except for ranolazine, where $t_\beta = 2.4$ (Table 5). These ranolazine data were included because they were the only acceptable data for this inhibitor for the HPTCs. The IC_{50} values were typically lower than the values observed with the overexpressing cells, mostly due to the lower efflux-active P-gp concentration in these primary cells, which correlates with the IC_{50} (Lumen et al., 2010).

The slope factor β was also smaller than those for the overexpressing cells. Although estimated β values for the HPTCs include a few values near or greater than 1, the mean of the estimates is approximately 0.71 (S.E. = 0.062), and the 95% confidence interval for the values (0.58–0.85) does not include 1.0. Thus, the HPTCs have a lower average $\beta < 1$ estimate than the cultured cells. A hypothesis for this behavior is given in the *Discussion*.

Table 6 shows this final fitting of the qualified HPTC data. These cells have roughly 10- to 100-fold less efflux-active P-gp than the over-expressing cell lines used in the P-gp IC_{50} Initiative (Table 4). The fraction of total transport due to the basolateral digoxin uptake

Fig. 2. Inhibition of digoxin transport through a confluent cell monolayer of MDCKII-hMDR1-NKI cells by carvedilol without a basolateral digoxin uptake transporter. The squares are the data points with S.D.s. The lines are the fits to the data. All figures use the same format. The inhibitor dissociation constant from P-gp, k_{rQ} (s^{-1}), is fixed at the consensus value found for all cells used (Table 4). (A) $[T(0) = 1 \text{ e}^{-3} \text{ M}$, $k_B = 0 \text{ second}^{-1}$, $k_{rQ} = 1 \text{ e}^4 \text{ (s}^{-1}\text{)}$, and $K_{QB} = 0 \text{ M}^{-1}$] The best fit without the basolateral uptake transporter, which is very poor. The NC, with little or no inhibitor, cannot be reached. Can changing the efflux-active P-gp concentration change this fit? (B) The fit with a higher P-gp efflux-active concentration of $T(0) = 1 \text{ e}^{-2} \text{ M}$, which is the value for closely packed P-gp, i.e., the maximum possible (Tran et al., 2005; Agnani et al., 2011); the other kinetic parameters are as in (A). The best fit is poor. (C) A lower P-gp

transporter is greater (Fig. 5B), thus the inhibition of BT has a greater impact on the IC_{50} . The consensus values for k_{rQ} (Table 4) worked well with these HPTCs, expanding the system independence of this elementary kinetic parameter. K_{QB} values were similar to those shown in Table 4 for the overexpressing cells.

We note that the kinetic parameters can vary significantly between different kidney samples for the same inhibitor. For example, the IC_{50}/K_i ratio with carvedilol varied 25-fold between two kidney preps, which was entirely due to the variability in the IC_{50} values (Table 5). Interestingly, this IC_{50} variability was due more to the variability of k_B and K_{QB} rather than the efflux-active P-gp. The same is true for the ketoconazole, quinidine, and verapamil data. Nicardipine, on the other hand, shows IC_{50} variability between kidney samples due to differences in efflux-active P-gp concentrations. This highlights the importance of the basolateral digoxin uptake transporter and its inhibition in any in vivo DDI predictions and the need to know which parameters are the primary drivers of the IC_{50} . All fits are shown in Supplemental Figs. 54–67 and Supplemental Table 5.

Potential Involvement of OATP4C1 in Basolateral Uptake of Digoxin in HPTC Monolayers. Figure 6A shows that digoxin uptake into the HPTCs was 50% inhibited with about $10 \mu\text{M}$ of the OATP4C1 inhibitor ouabain. The inhibition followed a roughly linear inhibition curve versus $\log[\text{inhibitor concentration}]$, i.e., not a logistical curve as observed for the other inhibitors studied here. Digoxin uptake into the cells is significantly greater in the presence of GF120918, where P-gp is fully inhibited, than in the control cells, where P-gp is fully active. Figure 6B shows that digoxin transport across the HPTCs, $J_{B \rightarrow A}$, was only about 20%–25% inhibited with $30 \mu\text{M}$ OATP4C1 inhibitor ouabain, also with a roughly linear, not logistic, inhibition curve.

The inhibition of digoxin uptake into and transport through the HPTCs by T3, $0\text{--}30 \mu\text{M}$, was only somewhat reduced at and above $10 \mu\text{M}$ T3. This was similar to the uptake results by Mikkaichi et al. (2004). The data for T3 inhibition of digoxin uptake and digoxin transport across the HPTC confluent cell monolayer are shown in Supplemental Figs. 68–69 and Supplemental Table 6.

Discussion

We have used the structural mass action kinetic model for digoxin transport through a confluent monolayer of P-gp–overexpressing polarized cells to 1) derive system-independent P-gp inhibitor dissociation rate constants for calculation of system-independent K_i values and 2) explore potential mechanistic factors that contribute to the variability in IC_{50} values observed in the P-gp IC_{50} Initiative (Bentz et al., 2013). A subset of the IC_{50} data generated by the P-gp IC_{50} Initiative participants was selected for this work, based on data quality criteria described in the Results section. We used the data from two Caco-2 laboratories, two MDCKII-hMDR1-NKI laboratories, and one LLC-PK1-hMDR1-NKI laboratory. Newly generated data for digoxin transport inhibition across primary HPTC monolayers were also included in this analysis using the same inhibitors, as well as ouabain and T3.

The structural mass action kinetics model for P-gp–mediated transport has been extensively validated as a reliable diagnostic tool using several cell lines to determine the efflux-active P-gp concentrations and to identify kinetically required uptake transporters for the transport of P-gp substrates across confluent cell monolayers (Acharya et al., 2008; Agnani et al., 2011; Lumen et al., 2013; Bentz and Ellens, 2014; Meng et al., 2017a,b). The IC_{50} curves of digoxin transport across confluent

efflux surface active density of $T(0) = 1 \text{ e}^{-4} \text{ M}$, which remains poor. Clearly, altering the P-gp concentration, by itself, cannot significantly alter the fit to reach the NC.

Fig. 3. Transport of digoxin through a confluent cell monolayer of MDCKII-hMDR1-NKI cells with a basolateral uptake transporter, without and with inhibitor binding to the uptake transporter. The same data as in Fig. 2 are fitted for the basolateral uptake clearance, k_B (s^{-1}). (A) $[T(0) = 1 \text{ e}^{-3} \text{ M}$, $k_B = 30 \text{ second}^{-1}$, $k_{rQ} = 1 \text{ e}^4 \text{ (s}^{-1}\text{)}$, and $K_{QB} = 0 \text{ M}^{-1}$] A good fit to NC, but a poor fit to the PC plateau. The PC fit without binding of the inhibitor to the uptake transporter is about 15% too high. (B) $[K_{QB} = 1 \text{ e}^5 \text{ M}^{-1}$ for inhibitor binding to the BT and all other parameters the same as in (A)] A good fit to PC. K_{QB} is called an affinity constant since it is a convolution of the uptake transporter surface density and the inhibitor's binding constant to the uptake transporter. Although the PC correction may not look large, that smallness is largely due to these cells overexpressing P-gp (see Fig. 5 for the HPTCs).

cell monolayers studied here were analyzed using this model. The kinetic parameters needed to fit the data shown in Figs. 2, 3, and 5 were the efflux-active P-gp concentration $[T(0)]$, uptake clearance of digoxin by the basolateral digoxin uptake transporter (k_B), the dissociation constant of the inhibitor from P-gp (k_{rQ}), and the affinity of the inhibitor to the basolateral uptake transporter (K_{QB}), as well as the relevant kinetic parameters for the probe substrate, digoxin in this case, which were obtained from Lumen et al. (2013). As shown in the *Results* section, these kinetic parameters were necessary and sufficient to fit IC_{50} data in P-gp-overexpressing cell lines and the primary culture HPTCs to within experimental error. Importantly, the k_{rQ} (and therefore, the calculated K_i) for a given inhibitor was found to be system-independent over the cell lines used here.

Our kinetic analysis found that the eight P-gp inhibitors used here in all overexpressing cell lines and the HPTCs, as well as six other P-gp inhibitors used with the overexpressing cell lines were kinetically

required to bind to and inhibit the digoxin basolateral uptake transporter (see Tables 4 and 6 and Supplemental Tables 1–3). This means that any IC_{50} value reported by Bentz et al. (2013) could be due to inhibitor binding to P-gp, or inhibitor binding to the digoxin basolateral uptake transporter, or more likely a complex convolution of both binding events.

Bentz et al. (2013) used PCA to show that the largest variability was due essentially to the differences in the average $\log_{10}\{IC_{50} \text{ (M)}\}$ over the inhibitors between the different laboratories, which was PCA axis 1 in Fig. 7 of that paper. This result raised concerns about the utility of in vitro data for predicting in vivo digoxin DDI risk (Lee et al., 2014). Here, IC_{50} curves were simulated using all combinations of efflux-active P-gp and the kinetic parameters from within the ranges shown in Tables 4 and 6. Figure 4 shows the PCA analysis on these simulated curves, which had no added random error in the data. Here, PCA axis 1 was essentially the average of the $\log_{10}\{IC_{50}\}$, the same as in Bentz et al. (2013). The range for PCA axis 1, i.e., the minimum and maximum values, is determined by the data for $T(0) \geq 1 \text{ e}^{-4} \text{ M}$. The data points for the smaller $T(0)$ values depicting the HPTC lay between these extremes. This range covers more than 60% of the range of axis 1 found in Bentz et al. (2013). Thus, most of the IC_{50} variability found in Bentz et al. (2013) can be explained by the cells in each laboratory, regardless of origin, expressing a range of efflux-active P-gp concentrations and expression levels of basolateral digoxin uptake transporter. The remaining variability in Bentz et al. (2013) is most likely due to a combination of experimental error, laboratories with $3 < t_\beta \leq 5$ with NC and PC variations in excess of $\%CV < 20\%$, and finally, the convolution of P-gp and BT inhibition within the fitting of the inhibition data to a single logistic IC_{50} curve.

The kinetic need for a basolateral uptake transporter to explain transport of P-gp substrates across a P-gp-expressing polarized cell monolayer is not unique to digoxin. We have found that both loperamide and vinblastine kinetically require a basolateral uptake transporter in MDCKII-hMDR1-NKI, MDCKII-hMDR1-NIH, and Caco-2 cells (Acharya et al., 2008; Lumen et al., 2013). If a P-gp substrate uses a BT, then how can that be shown? Lumen et al. (2013) showed by simulations that a basolateral uptake transporter could be observed when the passive permeability, in the presence of 2 μM GF120918, was less than about 320 nm/s . When this is so, the experimental probe substrate reaching the apical chamber is significantly greater than what can be fitted by the kinetic model without a mechanism for increasing the permeation of the probe substrate through the basolateral membrane beyond the passive permeability through the bilayer. When the passive permeability is larger than this threshold of 320 nm/s , then the model fitted deficit due to the absence of BT becomes insignificant compared with the total probe-substrate transport. This passive permeability threshold matched our findings that digoxin, loperamide, and vinblastine kinetically required the basolateral uptake transporter, whereas amprenavir, ketoconazole, quinidine, and verapamil did not (Lumen et al., 2013).

Mechanistically, it is very interesting that Lumen et al. (2013) showed for Caco-2 and MDCKII-hMDR1-NIH cells that ketoconazole and verapamil inhibited digoxin transport through the basolateral digoxin uptake transporter, as opposed to just inhibiting P-gp. The same result was found here in Tables 4 and 6. Thus, although these two P-gp inhibitors were not kinetically required to use a basolateral uptake transporter for their own transport, this does not mean that they do not bind to that transporter and, thereby, inhibit the transport of another P-gp substrate.

The identity of the basolateral digoxin uptake transporter in all these cells remains unknown. The possibility of a digoxin transporter in Caco-2 cells has been reported (Lowes et al., 2003). Taub et al. (2011) showed

Fig. 4. PCA plot for the variability of the IC_{50} values with simulated data for “virtual” cells using the kinetic parameters from the five laboratories from the P-gp IC_{50} Initiative (Table 4) and the HPTC data (Table 6). $T(0)$, the efflux-active P-gp concentration, ranged from 1 e^{-5} to $5 \text{ e}^{-3} \text{ M}$; k_B , the basolateral uptake transporter clearance, ranged from 5 to 30 second^{-1} ; and K_{QB} , the affinity constant of the inhibitor to the basolateral uptake transporter, ranged from 2 e^4 to $1 \text{ e}^6 \text{ M}^{-1}$. For each inhibitor, the consensus values of the inhibitor dissociation constant from P-gp, k_{iQ} , from Table 4 were used. These combinations of these ranges were used to simulate IC_{50} data curves that were fit for the IC_{50} values that were then used to make the PCA plot. The color of the symbols denotes the value of k_B , and their shape denotes the value of K_{QB} , as indicated on the right-hand legend. The value for $T(0)$ was not indicated, as a third element embedded into the symbols makes the plot unintelligible. The important point for PCA axis 1 is that its range, i.e., the minimum and maximum values, is determined by the data for $T(0) \geq 1 \text{ e}^{-4} \text{ M}$. The data points for the smaller $T(0)$ values depicting the HPTCs lay between these extremes.

that digoxin is not a substrate of OATP1A2, 1B1, 1B3, and 2B1, but was a substrate of a sodium-dependent transporter endogenously expressed in HEK293 cells. The kinetic modeling of the basolateral digoxin uptake transporter in MDCKII-hMDR1-NKI cells by Agnani et al. (2011) found that digoxin's uptake was somewhat better fitted as a bidirectional passive transporter as compared with an active importer.

In Mikkaichi et al. (2004), MDCK cells whose “endogenous expression of OATP4C1 in MDCK cell was not detected” were transfected with human OATP4C1. Confluent monolayers of these cells were used to examine digoxin uptake into and the digoxin flux across these monolayers as a function of the OATP4C1 inhibitors ouabain and T3. Their Fig. 5A showed that digoxin uptake into the cells was inhibited logistically to about 50% at about $0.4 \mu\text{M}$ ouabain. T3 showed no significant reduction of digoxin uptake at $0\text{--}30 \mu\text{M}$ (Mikkaichi et al., 2004). In our study of ouabain, digoxin uptake into HPTCs was inhibited essentially linearly, not logistically, at ouabain concentrations between 0.1 and $30 \mu\text{M}$, with an inhibition of about 50% at $10 \mu\text{M}$ ouabain. Thus, OATP4C1 may be a basolateral digoxin uptake transporter in this system.

The fact that ouabain's inhibition curve of basolateral uptake in the HPTCs is basically linear, rather than logistical, suggests that there could be a second basolateral digoxin uptake transporter involved in this complex system of primary isolated cells. Given that GF120918 caused the digoxin uptake into the HPTCs to be larger than found without ouabain (Fig. 6A), whereas digoxin's $J_{B \rightarrow A}$ flux was smaller than that caused by ouabain (Fig. 6B), suggests that one of the hypothesized two (or more) basolateral digoxin uptake transporters in HPTCs is not sensitive to GF120918 inhibition. The basolateral digoxin uptake transporter in the overexpressing cells used here is completely inhibited by $2 \mu\text{M}$ GF120918 (Tran et al., 2005; Acharya et al., 2008).

Whether ouabain directly affects P-gp transport function has not been reported. It has been reported that ouabain had no effect on P-gp ATPase activity (Shapiro and Ling, 1994). Brouillard et al. (2001) reported that ouabain induced P-gp expression, but that is unlikely to effect the immediate inhibition of digoxin transport by P-gp in this work.

The β in the IC_{50} logistic or Hill equation (Hill, 1913) is called the slope factor, because it is fitted to the slope of the IC_{50} curve at the IC_{50} (O'Connor et al., 2015). Historically, $\beta > 1$ has also been considered an indicator of cooperativity in substrate binding to a protein, e.g., hemoglobin (Hill, 1913). The slope factor is used in all commercial IC_{50} fitting packages, so it is part of every published IC_{50} fit, yet it is

rarely reported or discussed. In Bentz et al. (2013), the β values averaged near 1, but there was a wide range between the minimum (0.3) and maximum (3.6) in Table 7 here. For the higher-quality data sets used in this work, with roughly 4-fold fewer fitted data points, the fitted values were still centered on $\beta = 1$, but the ranges were significantly smaller, ranging from 0.6 to 1.8 in Table 7.

There have been very many papers on the cooperativity of substrate binding to P-gp, mostly based upon the ATPase activity, but there has been no clear consensus (Lumen, 2011). There are several citations to works that claim multiple substrates being bound simultaneously to P-gp (Lumen, 2011), but there is no evidence that all of these binding sites actually efflux substrate. The structural mass action kinetic model has only a single substrate binding site per P-gp, explained in detail by Tran et al. (2005), so this kinetic model cannot support cooperativity. To date, no kinetic need for efflux cooperativity has been observed using this kinetic model for P-gp efflux kinetics. Together, these data suggest that the fitted β values have little, if anything, to do with the number of cooperative binding sites in P-gp. The slope factor β does provide an additional degree of freedom for fitting IC_{50} data, without any mechanistic control for β values greater than 1.

On the other hand, for the HPTCs, the average of the slope factor β is significantly less than 1, with a mean of the estimates of $\langle \beta \rangle = 0.71$ and a 95% confidence interval of 0.58–0.85 (Table 6). The simplest hypothesis that explains this difference from the overexpressing cells is that the HPTCs express two different basolateral digoxin uptake transporters. The interaction of their respective binding sites would reduce the slope of the inhibition curve at the IC_{50} , i.e., yielding $\beta < 1$. This speculation is also consistent with the linear, rather than logistical, inhibition curve of digoxin uptake by ouabain in HPTCs, described above (Fig. 6A).

Finally, P-gp vesicles have been suggested as a simpler and possibly superior system for determination of P-gp IC_{50} values. However, the vesicle IC_{50} data generated by the P-gp IC_{50} Initiative using *N*-methylquinidine as a probe substrate were also very variable from laboratory to laboratory as the confluent cell monolayer data (Bentz et al., 2013). Currently, these vesicles are typically derived from mammalian or insect cell membranes, which contain a variety of endogenous transporters, just like the polarized cell systems, and must be assumed to vary from laboratory to laboratory in expression levels of P-gp and other transporters, just as the polarized cell monolayers.

TABLE 5

HPTC IC₅₀ values, t_β statistic values for inhibitors, and IC₅₀ slope factor β

Cell	Kidney Number ^a	Inhibitor	IC ₅₀ ^b	t_β ^c	β ^d
HPTC	6	Carvedilol	1.4	4.8	0.6
	10	"	0.05	7.7	0.9
	13	Diltiazem	1.1	5.0	0.8
	8	Isradipine	8.7	6.7	0.4
	2	Ketoconazole	9.8	4.4	0.5
	3	"	0.7	3.1	0.9
	11	"	2.8	5.9	0.4
	7	Mibefradil	4.2	9.1	0.8
	4	Nicardipine	0.8	5.0	1.2
	13	"	0.3	5.2	0.8
	1	Quinidine	0.7	7.5	0.6
	2	"	3.1	3.7	1.1
	12	Ranolazine	1.8	2.4	2.8
	1	Verapamil	1.1	4.5	0.5
	10	"	0.16	4.9	0.6

^aNumbers give the chronological order of receipt of the human kidneys as a simple means to identify the source of the data.

^bIC₅₀ values were calculated as described by O'Connor et al. (2015).

^c t_β was calculated as described by O'Connor et al. (2015). This data quality statistic measures the sigmoidicity of the experimental IC₅₀ curve, the expected shape of an IC₅₀ curve. Because these were primary cells, the criteria for qualification was set at $t_\beta > 3$, as in Bentz et al. (2013).

^d β is the slope factor for the IC₅₀ curve in the Hill equation used in all commercial software fitting programs, which is the slope of the IC₅₀ curve as it passes through the IC₅₀.

Conclusions

We have previously demonstrated that the elementary rate constants for P-gp-mediated transport (k_1 , k_2 , and k_r) obtained using our structural mass action kinetics model are essentially the same in both MDCKII-hMDR1-NKI and Caco-2 cells for amprenavir, quinidine, loperamide, and digoxin, i.e., they are essentially system-independent. Here, we have shown that k_{rQ} is also system-independent for the cells used, including with the primary cell culture HPTCs. We can therefore calculate a system-independent P-gp-specific K_i using highly variable IC₅₀ data, even when another transporter or two are involved in the transport of the probe substrate.

Here, the criteria for selecting qualified data sets were based upon having IC₅₀ data curves from multiple laboratories for multiple inhibitors. These criteria can be adapted to a single lead compound. It requires at least three completely independent IC₅₀ data curves, e.g., for different cell preparations, for the inhibitor of interest. For these curves, the average of the negative and of the positive controls must each have a %CV < 20%. Each of the IC₅₀ curves must have $t_\beta > 5$. It is best to simultaneously fit all data curves to obtain the consensus elementary rate constants, but they can be fitted separately and then averaged, as was done for k_{rQ} in Table 3. The k_1 , k_r , k_2 , and k_B for the probe substrate used must be known, since they are part of the IC₅₀ data-fitting program. These are already available for amprenavir, digoxin, ketoconazole, loperamide, quinidine, verapamil, and vinblastine (Lumen et al., 2013).

For the purpose of the kinetic analysis presented here, and in our previous work, it is not crucial whether the basolateral uptake clearance of the probe substrate is due to a basolateral uptake transporter or to "something else." However, any proposed "nontransporter" mechanism for this enhanced basolateral membrane permeability of the probe substrate must have a plausible mechanism for the inhibition of this enhanced permeability as a function of increased inhibitor concentration to account for the significant effect of K_{QB} on the fit.

The final point is how to use the structural mass action kinetic model to obtain in vivo DDI predictions. Basically, our MATLAB program can replace the linearized Michaelis-Menten programs within existing in vivo Physiologically Based Pharmacokinetics (PBPK) computer programs. At present, this appears most easily accomplished within the MATLAB SimBiology framework, which has access to other required

Fig. 5. Digoxin transport through a confluent cell monolayer of primary HPTCs with inhibition by ketoconazole. (A) [$T(0) = 1.5 \times 10^{-5}$ M, $k_B = 0 \text{ second}^{-1}$, $k_rQ = 3 \times 10^4 \text{ (s}^{-1}\text{)}$, and $K_{QB} = 0 \text{ M}^{-1}$] Fitted with just P-gp, i.e., no basolateral uptake clearance. The fit is very poor, as in Fig. 2. (B) [$k_B = 45 \text{ s}^{-1}$ and all other parameters the same as (A)] A good fit to NC, but a poor fit to PC, notably worse than Fig. 3A. The PC fit without binding of the inhibitor to BT is about 50% too high. (C) [$K_{QB} = 2 \times 10^6 \text{ M}^{-1}$ and all other parameters the same as (B)] A good fit to PC.

TABLE 6
HPTC P-gp efflux-active concentration and elementary kinetic parameters for the data in Table 5

Cells	Kidney Number ^a	Inhibitor	$T(0)^a$ M	k_B^a s^{-1}	k_{rQ}^b s^{-1}	K_{QB}^c M^{-1}	%CV ^d	K_i^e μM	IC_{50}/K_i
HPTC	6	Carvedilol	3E-05	17	1E+04	1E+05	0.13	0.3	4.9
	10	"	1E-05	2	1E+04	7E+06	0.04	0.3	0.2
	13	Diltiazem	3E-05	15	7E+04	2E+06	0.09	2.0	0.6
	8	Isradipine	2E-04	8	6E+04	1E+04	0.18	1.7	5.1
	2	Ketoconazole	3E-05	20	3E+04	1E+04	0.19	0.9	11.4
	3	"	2E-05	45	3E+04	2E+06	0.09	0.9	0.8
	11	"	1E-05	40	3E+04	1E+05	0.08	0.9	3.3
	7	Mibefradil	2E-04	15	8E+03	5E+03	0.08	0.2	18.4
	4	Nicardipine	1E-05	20	7E+03	5E+05	0.05	0.2	4.0
	13	"	3E-05	15	7E+03	5E+05	0.08	0.2	1.5
	1	Quinidine	2E-05	9	1E+04	1E+04	0.04	0.3	2.5
	2	"	3E-05	20	1E+04	2E+05	0.16	0.3	10.9
	12	Ranolazine	3E-05	9	5E+04	4E+05	0.08	1.4	1.3
	1	Verapamil	2E-05	9	2E+04	3E+05	0.08	0.6	1.9
	10	"	1E-05	2	2E+04	3E+03	0.04	0.6	0.3

^a $T(0)$, the P-gp efflux-active concentration (moles P-gp per liter of membrane), and k_B , the basolateral plasma membrane uptake transporter clearance (s^{-1}), are fitted independently for each kidney.

^b k_{rQ} is the consensus dissociation constant of inhibitor from P-gp (Table 3).

^c K_{QB} is the affinity constant of the inhibitor binding to the uptake transporter.

^d%CV is the coefficient of variation for the fit to the IC_{50} curve expressed as a percentage.

^e $K_i = (k_{rQ}/k_i) \times K_{QPC} \times 10^6$ (μM). Defined in Table 4.

MATLAB programs and which can run the Particle Swarm global optimization program we use, but other options are possible. Our analysis provides the necessary elementary rate constants from in vitro

experiments, all of which appear system-independent. Although there are good biophysical reasons for expecting the elementary rate constants for P-gp transport to be essentially system-independent, that is not the

Fig. 6. Digoxin uptake in and transport through a confluent cell monolayer of primary HPTCs inhibited by ouabain. (A) The inhibition of digoxin uptake into the HPTCs by ouabain. Ouabain (10 μM) inhibits digoxin uptake by about 50%. The decreased transport is mostly linear rather than logistic, as discussed. (B) The ouabain inhibition of digoxin transport, $J_B > A$, through the HPTCs. Ouabain (30 μM) only inhibits digoxin $B > A$ transport by 20%–25%. The decreased transport is mostly linear rather than logistic, as discussed.

TABLE 7
Effect of data quality on the slope factor β

	Number of Fits	β (Average) ^a	%CV ^b	Min ^c	Max ^c
IC ₅₀ fits from all laboratories for all data sets with $t_\beta > 3$ (Bentz et al., 2013) ^a					
MDCK	48	1.3	50	0.3	3.6
Caco-2	70	1.1	31	0.6	2.4
LLC-PK	13	1.4	64	0.6	3.3
IC ₅₀ fits from those laboratories which qualified in this work, e.g., $t_\beta > 5$ ^d					
MDCK	12	1.2	26	0.7	1.8
Caco-2	13	1.0	24	0.6	1.6
LLC-PK	4	1.2	9	1.0	1.2

^aTotal number of fitted data sets ($t_\beta > 3$) for the slope factor β from Bentz et al. (2013) (Supplemental Data 2 of that paper).

^bCoefficient of variation for fitted β expressed as a percentage.

^cMinimum and maximum values for β .

^dThese data are calculated from Table 2.

case for the microvilli morphology-dependent efflux-active P-gp and the as-yet unidentified basolateral and apical uptake transporter(s) kinetic parameters. The need for in vivo data fitting is seen clearly here in the HPTC data, with large kidney-to-kidney variability in IC₅₀ values for the same inhibitor that was due mostly to variability in the basolateral digoxin uptake transporter clearance and inhibition, not P-gp activity. Therefore, using the in vitro-derived elementary rate constants, the in vivo PBPK model can fit in vivo data for the efflux-active P-gp, the basolateral and apical uptake transporter clearances, and the inhibitor affinities for these uptake transporters. These fits would allow the formulation of in vivo DDI predictions.

Authorship Contributions

Participated in research design: Brown, O'Connor, Lee, Ellens, Bentz.
Conducted experiments: Chung.
Contributed new reagents or analytic tools: O'Connor, Bentz.
Performed data analysis: Chaudhry, Chung, Lynn, Yalvigi, Brown, O'Connor, Lee, Ellens, Bentz.
Wrote or contributed to the writing of the manuscript: Chung, Brown, O'Connor, Lee, Ellens, Bentz.

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