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 ($k_{d}$), digoxin basolateral uptake clearance ($k_{a}$), and inhibitor binding affinity to the digoxin basolateral uptake transporter ($K_{OBI}$. 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 $k_{d}$ 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 μM 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

ABBREVIATIONS: BT, basolateral digoxin uptake transporter; CV, coefficient of variation; DDI, drug-drug interaction; GFT20918, N-[4-[[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isouquinolinyl)ethyl]-phenyl]-9,10-; HPTC, human proximal tubule cell; NC, negative control; PC, positive control; PCA, principal component analysis; P-gp, P-glycoprotein; UWL, unstimred water layer.
alone, independently measured in the presence of N-(4-[2-(1,2,3,4-
 tetrahydro-6,7-dimethoxy-2-isooquinolinyl)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 IC50 curves using eight P-gp inhibitors
obtained from the P-gp IC50 Initiative to generate values for 1) the
efflux-active P-gp concentration [T(0)], 2) the clearance of digoxin by the
basolateral uptake transporter (kQ), 3) the inhibitor dissociation rate constant (kQ/)
fourth the P-gp and, 4) the inhibitor binding affinity to the
digoxin uptake transporter (KQPC), as described previously (Lumen, et al.,
2013). Note that KQPC depends upon both uptake transporter surface
density and its binding constant to the inhibitor. Thus, for a given
inhibitor, the KQPC 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. 1–4 and
Supplemental Tables 1 and 2). The data for amiodarone were too poor to
analyze properly. Thus, all of the IC50 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 IC50 variability reported
by Bentz et al. (2013).

The kQ 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 kQ = kQ/kQ, with respect to the
inhibitor concentration in the membrane, so the system independence
of kQ allows kQ to be calculated for each inhibitor from kQ alone. Relative to
the cytosolic concentration, kQ = (kQ/kQ) KQPC, where KQPC is the partition
coefficient of inhibitor Q into a liposome mimic of the cytosolic inner
membrane of these eukaryotic cells (Tran et al., 2005; Lumen, et al.,
2013).

The fitting of the values for T(0), kQ, KQPC, and kQ for the inhibitors from the
five laboratories used in this work allowed us to simulate IC50 curves for “virtual” cell lines, with individual kinetic parameters defined
within the ranges of these parameters. This estimates how much IC50
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 IC50 variability found in
the principal component analysis (PCA) plot in Bentz et al. (2013). This
supports the hypothesis that the observed IC50 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 IC50 data for the P-gp IC50 Initiative, the
methods were reported by Bentz et al. (2013).

Biological mechanisms of digoxin transport inhibition

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 kQ (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 k1 (M−1s−1), and binds to P-gp with a
binding constant of Kc (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 k2 (s−1), which is rare. For digoxin, roughly 1 × 104 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 IC50 is due solely to P-gp. However,
we show in Supplemental Table 2 and Tables 4 and 6 that cerivastatin, alumenzum,
feofenazine, isradipine, ketoconazole, mibefradil, nifedipine, nitrendipine,
quindine, 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 IC50 Initiative data (Bentz et al., 2013), the fitted IC50 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 (Branschweig, 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
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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-μm 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× 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 h 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 μCi/ml) and [14C]mannitol (0.1 μCi/ml) to the basolateral chamber. A 250-μl 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× 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 including statistics, were performed using a 64-bit installation of 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 Aghani 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 \( K_i = k_i \frac{Q_P}{Q_B} \), where \( k_i \) is the association rate constant of the inhibitor Q from the membrane to P-gp, and \( k_{Q_P} \) is the dissociation rate constant of the inhibitor from P-gp back into the membrane. Based upon the system independence of \( k_i \), we set \( k_{Q_P} = k_i \) (Lumen et al., 2013). \( K_i = k_{Q_P} \frac{Q_B}{Q_P} \) 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 \( K_{Q_{PC}} = 350 \) for all inhibitors used in this work, which is the value we measured for quinidine binding to 0.1 μm 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. \( k_i \) 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 \( 1 \times 10^6 \text{M}^{-1} \text{s}^{-1} \) (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 \( k_i \) 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 inhabited, 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 μM GF120918 does not significantly affect the UWL, which is reasonable. This allows us to measure specifically the kinetic 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-actively 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. \( k_Q \) (s⁻¹) is the elementary dissociation rate constant of the inhibitor (Q) from P-gp back into the apical membrane. A smaller value of \( k_Q \) corresponds to a stronger binding of the inhibitor to P-gp.

4. \( K_{OB} \) (M⁻¹) is the binding affinity of the inhibitor to the BT from the basolateral compartment. \( K_{OB} \) 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 \( k_Q \) and \( k_i \) (see Materials and Methods). We have found previously that \( k_i \) 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 \( t_{ab} > 3 \), 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 \( t_{ab} \) with \( t_p \) defined by O’Connor et al. (2015), which is simpler to calculate and a nearly perfect approximation for \( t_{ab} \).

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. \( t_p > 5 \), 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
CV < 20%. This is crucial because two of the four essential kinetic parameters, i.e., T(0) and κB, are specific to the cells and cannot depend on the inhibitor. Thus, two crucial parts of the IC50 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 IC50 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.

Table 2 shows the fitted values for the parameters that characterized these IC50 data. The IC50, κQ, laboratory average, τQ and β, and the IC50 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 κB for a particular cell system were fixed first by simultaneously fitting all qualified IC50 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 κB, 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 κB for each laboratory, but refitted the values for κQ and KQB for each inhibitor. Since there were four to eight qualified inhibitors for each chosen laboratory and five chosen laboratories, several independent κQ values were fitted at least twice (ranolazine) and up to five times (amiodarone). The other inhibitors had three to four independent fits for κQ.

Across all laboratories, the independent κQ values for each inhibitor were relatively clustered, indicating that this parameter appeared essentially cell-independent. These individual κQ values are shown in Supplemental Table 7. Table 3 shows the average, low, and high estimates for κQ at the 95% confidence level and the statistically significant one-digit consensus value for each κQ 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 κQ values for each inhibitor. Using this consensus κQ, 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 fits allowed T(0), κB, and KQB for each laboratory to adjust slightly to optimize the fit with the now fixed κQ 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 κQ values were used later to fit the IC50 data curve for the primary culture HPTCs. This provides strong support for the hypothesis that the elementary rate constant κQ is essentially system-independent.

Table 4 shows the final fits of the qualified data, together with the CV of the fit and the Kf. Of course, when the true partition coefficient KQPC of the inhibitors is measured, the Kf 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 IC50 Initiative, called Group 2 in their Table 9, that were lesser quality than the eight inhibitors in Tables 3 and 4 with respect to τQ values, as explained there. Data from selected laboratories, with τQ > 3 for six of these P-gp inhibitors (felodipine, nifedipine, nitenidipine, sertraline, telmisartan, and troglitazone), were fitted using their consensus κQ 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 IC50 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 IC50 curve for digoxin transport with a model that only contained P-gp, and not the basolateral digoxin
uption transporter, so \( k_b = 0 \) (Fig. 2). \( k_Q \) 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 e\(^{-3}\) M (Fig. 2A) to 1 e\(^{-5}\) M (Fig. 2B) or 1/10-fold to 1 e\(^{-3}\) 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. \( k_Q \) 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 \([T(0), k_b, k_Q, K_{Qb}]\) shown in Tables 4 and 6. PCA axis 1 is essentially the average of log\(_10\{IC_{50}\} (M)\) 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 \( k_Q \) values for each inhibitor, since no simulated experimental error was added.

### Table 2

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<th>Cell(^a)</th>
<th>Laboratory(^b)</th>
<th>Inhibitor</th>
<th>IC(_{50})(^c)</th>
<th>( t_p )(^d)</th>
<th>Laboratory Average ( &lt;t_p &gt; )(^e)</th>
<th>( \beta )(^f)</th>
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\( ^{a} \)Cell and laboratory number as indicated in Bentz et al. (2013).  
\( ^{b} \)IC\(_{50}\) values are taken from Bentz et al. (2013).  
\( ^{c} \)IC\(_{50}\) was calculated as described by O’Connor et al. (2015) and replaces \( t_p \) 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 \( t_p > 5 \) for all data analyzed, a lower limit defined from preliminary analysis.  
\( ^{d} \)The laboratory average for \( t_p \) was calculated for the qualified inhibitors.  
\( ^{e} \)IC\(_{50}\) slope factor (also known as the Hill equation (Hill, 1913), calculated from the fit to the logistic equation (O’Connor et al., 2015).  
\( ^{f} \)This is the slope factor for the logistic IC\(_{50}\) curve, as analyzed in these data fits (see Discussion).
BT was not incorporated, shows the best fits for ketoconazole inhibition of digoxin transport without binding/inhibition by the inhibitor, not fitted at the NC. Figure 5B shows that when BT is added to the model 

\[ K_B = \frac{(k_{Qg})^2}{k_{Qc}} \]  

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 \( t_g \geq 3 \), except for ranolazine, where \( t_g = 2.4 \) (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 \( \beta \) was also smaller than those for the overexpressing cells. Although estimated \( \beta \) 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 \( \beta \) than 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 overexpressing cell lines used in the P-gp IC_{50} Initiative (Table 4). The fraction of total transport due to the basolateral digoxin uptake

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**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 \( K_Q \) 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, \( k_h = 0 \), 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}, \( k_h \), and the slope factor \( \beta \) 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 \( t_g \geq 3 \), except for ranolazine, where \( t_g = 2.4 \) (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).
transporter is greater (Fig. 5B), thus the inhibition of BT has a greater impact on the IC₅₀. The consensus values for kᵢ (Table 4) worked well with these HPTCs, expanding the system independence of this elementary kinetic parameter. Kᵢ 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 IC₅₀/Kᵢ ratio with carvedilol varied 25-fold between two kidney prep, which was entirely due to the variability in the IC₅₀ values (Table 5). Interestingly, this IC₅₀ variability was due more to the variability of kᵢ and Kᵢ rather than the efflux-active P-gp. The same is true for the ketoconazole, quinidine, and verapamil data. Nicardipine, on the other hand, shows IC₅₀ 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 IC₅₀. 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 μM of the OATP4C1 inhibitor ouabain. The inhibition followed a roughly linear inhibition curve versus log[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, Iₜₒₐᵤₒᵤₐₐ, was only about 20%–25% inhibited with 30 μM 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, 0–30 μM, was only somewhat reduced at and above 10 μM 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 Kᵢ values and 2) explore potential mechanistic factors that contribute to the variability in IC₅₀ values observed in the P-gp IC₅₀ Initiative (Bentz et al., 2013). A subset of the IC₅₀ data generated by the P-gp IC₅₀ 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 IC₅₀ curves of digoxin transport across confluent efflux surface active density of T(0) = 1 e⁻¹⁴ M, which remains poor. Clearly, altering the P-gp concentration, by itself, cannot significantly alter the fit to reach the NC.
Inhibitors used with the overexpressing cell lines were kinetically all overexpressing cell lines and the HPTCs, as well as six other P-gp lines used here. For a given inhibitor was found to be system-independent over the cell constant of the inhibitor from P-gp (obtained from Lumen et al. (2013). As shown in the parameters for the probe substrate, digoxin in this case, which were experimental error. Importantly, the P-gp to the basolateral uptake transporter (as opposed to just inhibiting P-gp. The same result verapamil inhibited digoxin transport through the basolateral digoxin uptake transporter, whereas amperanzavir, 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 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_Q), and the affinity of the inhibitor to the basolateral uptake transporter (K_B), 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_{QB} (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}(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) = 1 e^{-4} 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_50 < 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 amperanzavir, ketoconazole, quinidine, and verapamil did not (Lumen et al., 2013).
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 \( \mu \)M ouabain. T3 showed no significant reduction of digoxin uptake at 0–30 \( \mu \)M. In our study of ouabain, digoxin uptake into HPTCs was inhibited essentially linearly, not logistically, at ouabain concentrations between 0.1 and 30 \( \mu \)M, with an inhibition of about 50% at 10 \( \mu \)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 complex system of primary isolated cells. Given that GF120918 caused the basolateral uptake transporter clearance, ranging from 2 to 5 \( \times \) 10^{-3} M, \( k_d \), the basolateral uptake transporter clearance, ranging from 5 to 30 second^{-1}; and \( K_{d_{B}} \), the affinity constant of the inhibitor to the basolateral uptake transporter, ranging from 2 to 5 \( \times \) 10^{-3} M^{-1}. For each inhibitor, the consensus values of the inhibitor dissociation constant from P-gp, \( K_{d_{P}} \), from Table 4 were used. These combinations of these ranges were used to simulate IC50 data curves that were fit for the IC50 values that were then used to make the PCA plot. The color of the symbols denotes the value of \( k_d \), and their shape denotes the value of \( K_{d_{B}} \), 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) \( \geq 1 \times 10^{-3} \) M. The data points for the smaller T(0) values depicting the HPTCs lay between these extremes.

Finally, P-gp vesicles have been suggested as a simpler and possibly superior system for determination of P-gp IC50 values. However, the vesicle IC50 data generated by the P-gp IC50 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.
Conclusions

We have previously demonstrated that the elementary rate constants for P-gp–mediated transport \( (k_1, k_2, \text{ and } k_r) \) 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 \( t_b \), \( 5 \). 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 \( k_1, k_2, \text{ and } k_B \) 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

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

IC50 values were calculated as described by O’Connor et al. (2015).

\( t_b \) 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 \( t_b > 3 \), as in Bentz et al. (2013).

\( \beta \) 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.
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

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aT(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.
bk_Qb is the consensus dissociation constant of inhibitor from P-gp (Table 3).
cK_Qm is the affinity constant of the inhibitor binding to the uptake transporter.
d%CV is the coefficient of variation for the fit to the IC50 curve expressed as a percentage.
eK_i = (k_Qb/K_Qm) × 10² (µM). Defined in Table 4.

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.
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, 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, Valligyi, 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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Bentz J, O’Connor MP, Bednarczyk D, Coleman J, Lee C, Jain P, Pak YA, Perloff ES, Reynier E, Balimane P, et al. (2013) Variability in P-glycoprotein inhibitory potency (IC50) using various transporters OATP1A2, OATP1B1, OATP1B3, and OATP2B1 but is a substrate for a sodium-dependent transporter expressed in HEK293 cells. Drug Metab Dispos 41:1347–1356.

Address correspondence to: Dr. Joe Bentz, Drexel University, Biological Department, 3245 Chestnut Street, Philadelphia, PA 19104. E-mail: bentzj@drexel.edu
Supplemental Data for Drug Metabolism and Disposition (2018):

Derivation of a system-independent Ki for Pgp-mediated digoxin transport from system-dependent IC50 data.

Aqsa Chaudhry, Git Chung, Adam Lynn, Akshata Yalvigi, Colin Brown, Harma Ellens,
Michael O’Connor, Caroline Lee and Joe Bentz

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Newcastle University, Institute for Cell and Molecular Biosciences, United Kingdom (GC, CB)
GlaxoSmithKline Pharmaceuticals, Drug Metabolism and Pharmacokinetics, King of Prussia, PA (HE)
Drexel University, Department of Biodiversity, Ecology and Earth Sciences, Philadelphia, PA, 19104 (MO)
Ardea Biosciences Inc., Translational Sciences, San Diego, CA 92121 (CL)
Supplemental Data Contents:

1. Parameters and figures for the Group 2 P-gp inhibitors
   a. Table S1: IC_{50} values, t_β-statistic values for inhibitors and IC_{50} slope factor β
   b. Table S2: P-gp Efflux Active Concentration and Elementary Kinetic Parameters
   c. Table S3: Legends for Figs. S1-S24
   d. Supplemental Data Figures S1-S24
2. Figures for the data of Tables 2 and 4 in the main paper
   a. Table S4: Legends for Figs. S25-S53
   b. Supplemental Data Figures S25-S53
3. Figures for the data of Tables 6A and 6B in the main paper
   a. Table S5: Legends for Figs. S54-S67
   b. Supplemental Data Figures S54-S67
4. Figures for the HPTC data of T3 inhibition of digoxin uptake and J_{B→A} transport in the main paper
   a. Table S6: Legends for Figs. S68-S69
   b. Supplemental Data Figures S68-S69
5. Supplemental Data Table S7 for Individual Lab fits for log_{10}\{k_rQ\} for Main Table 3.
Supplemental Data Table S1. IC₅₀ parameters for the 7LI (Bentz et al., 2013)

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Footnotes to Table

* T(0) is the P-gp efflux active surface density and kₜ is the basolateral uptake clearance as fitted for the 8 better inhibitors in Table 5 of the main paper, since these parameters should not depend on inhibitor.
\( k_{Q} \) is the consensus inhibitor P-gp dissociation constant for all labs shown. \( K_{QB} \) is the affinity for the basolateral uptake transporter for the inhibitor for each lab.

\( \%CV \) is the coefficient of variation between the data and the fit.

\([DGX]\) is the initial concentration of digoxin in the basolateral chamber. This explains why the NC values for digoxin vary widely for the different labs.

MDCK refers to the MDCKII-hMDR1-NKI cells and LLCPK refers to LLC-PK1-hMDR1-NKI cells for the labs shown.
**Supplemental Data Table S2. Best fits for the 7L1 (Bentz et al., 2013)**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Cell Line</th>
<th>Lab</th>
<th>T(0) (M)</th>
<th>( k_B ) (s(^{-1}))</th>
<th>( k_{QB} ) (s(^{-1}))</th>
<th>( K_{QB} ) (M(^{-1}))</th>
<th>%CV fit</th>
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<td>MDCK(^d)</td>
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<td>1e-3</td>
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</table>

Footnotes to Table

\(^a\) T(0) is the P-gp efflux active surface density and \( k_B \) is the basolateral uptake clearance as fitted for the 8 better inhibitors in Table 5 main paper, since these parameters should not depend on inhibitor.

\(^b\) \( k_{QB} \) is the consensus inhibitor P-gp dissociation constant for all labs shown. \( K_{QB} \) is the affinity for the basolateral uptake transporter inhibitor for each lab.

\(^c\) %CV is the coefficient of variation between the data and the fit.

\(^d\) MDCK refers to the MDCKII-hMDR1-NKI cells for the labs shown and LLCPK refers to LLC-PK1-hMDR1-NKI cells for the labs shown.
Supplementary Data Table S3. Figure Legends for Tables S1 and S2. (symbols show the B>A digoxin transport data, error bars show the data standard deviation and the line shows the fit to the data)

Fig. S1. Inhibition by felodipine with Caco-2 cells from L07.
Fig. S2. Inhibition by felodipine with Caco-2 cells from L11.
Fig. S3. Inhibition by felodipine with MDCK-hMDR1-NKI cells from L02.
Fig. S4. Inhibition by felodipine with MDCK-hMDR1-NKI cells from L03.
Fig. S5. Inhibition by felodipine with LLC-PK1-hMDR1-NKI cells from L01.
Fig. S6. Inhibition by nifedipine with Caco-2 cells from L06.
Fig. S7. Inhibition by nifedipine with Caco-2 cells from L07.
Fig. S8. Inhibition by nifedipine with Caco-2 cells from L11.
Fig. S9. Inhibition by nitrendipine with Caco-2 cells from L06.
Fig. S10. Inhibition by nitrendipine with Caco-2 cells from L07.
Fig. S11. Inhibition by nitrendipine with Caco-2 cells from L08.
Fig. S12. Inhibition by nitrendipine with Caco-2 cells from L11.
Fig. S13. Inhibition by nitrendipine with MDCK-hMDR1-NKI cells from L02.
Fig. S14. Inhibition by nitrendipine with MDCK-hMDR1-NKI cells from L03.
Fig. S15. Inhibition by sertraline with MDCK-hMDR1-NKI cells from L01.
Fig. S16. Inhibition by telmisartan with Caco-2 cells from L02.
Fig. S17. Inhibition by telmisartan with Caco-2 cells from L10.
Fig. S18. Inhibition by telmisartan with LLC-PK1-hMDR1-NKI cells from L02.
Fig. S19. Inhibition by troglitazone with Caco-2 cells from L02.
Fig. S20. Inhibition by troglitazone with Caco-2 cells from L06.
Fig. S21. Inhibition by troglitazone with Caco-2 cells from L07.
Fig. S22. Inhibition by troglitazone with Caco-2 cells from L10.
Fig. S23. Inhibition by troglitazone with Caco-2 cells from L11.
Fig. S24. Inhibition by troglitazone with LLC-PK1-hMDR1-NKI cells from L02.
SuppData Fig. S3

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
SuppData Fig. S5

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
SuppData Fig. S6

Digoxin Concentration A:B>A (µM) vs. Inhibitor Concentration (µM)
SuppData Fig. S7

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
SuppData Fig. S8

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
SuppData Fig. S9

Digoxin Concentration A:B>A (µM) vs. Inhibitor Concentration (µM)
SuppData Fig. S10

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
SuppData Fig. S11

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)

SuppData Fig. S12
SuppData Fig. S13

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
SuppData Fig. S14

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
SuppData Fig. S15

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
SuppData Fig. S16

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
SuppData Fig. S17

**Digoxin Concentration A:B>A (µM)**

**Inhibitor Concentration (µM)**
SuppData Fig. S18

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
SuppData Fig. S20

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
SuppData Fig. S21
SuppData Fig. S22

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)

1.E-03 1.E-02 1.E-01 1.E+00 1.E+01 1.E+02 1.E+03 1.E+04
SuppData Fig. S23

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
SuppData Fig. S24

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Supplemental Data Table S4. Figure Legends for Tables 2 and 4 in the main paper. For all figures, symbols show the B>A digoxin transport data, error bars show the data standard deviation and the line shows the fit to the data.

Fig. S25. Inhibition by carvedilol with MDCK-hMDR1-NKI cells from L02.
Fig. S26. Inhibition by diltiazem with MDCK-hMDR1-NKI cells from L02.
Fig. S27. Inhibition by isradipine with MDCK-hMDR1-NKI cells from L02.
Fig. S28. Inhibition by mibebradil with MDCK-hMDR1-NKI cells from L02.
Fig. S29. Inhibition by nicardipine with MDCK-hMDR1-NKI cells from L02.
Fig. S30. Inhibition by quinidine with MDCK-hMDR1-NKI cells from L02.
Fig. S31. Inhibition by ranolazine with MDCK-hMDR1-NKI cells from L02.
Fig. S32. Inhibition by verapamil with MDCK-hMDR1-NKI cells from L02.
Fig. S33. Inhibition by carvedilol with MDCK-hMDR1-NKI cells from L07.
Fig. S34. Inhibition by nicardipine with MDCK-hMDR1-NKI cells from L07.
Fig. S35. Inhibition by ranolazine with MDCK-hMDR1-NKI cells from L07.
Fig. S36. Inhibition by verapamil with MDCK-hMDR1-NKI cells from L07.
Fig. S37. Inhibition by carvedilol with Caco-2 cells from L06.
Fig. S38. Inhibition by diltiazem with Caco-2 cells from L06.
Fig. S39. Inhibition by isradipine with Caco-2 cells from L06.
Fig. S40. Inhibition by nicardipine with Caco-2 cells from L06.
Fig. S41. Inhibition by quinidine with Caco-2 cells from L06.
Fig. S42. Inhibition by ranolazine with Caco-2 cells from L06.
Fig. S43. Inhibition by verapamil with Caco-2 cells from L06.
Fig. S44. Inhibition by carvedilol with Caco-2 cells from L11.
Fig. S45. Inhibition by diltiazem with Caco-2 cells from L11.
Fig. S46. Inhibition by isradipine with Caco-2 cells from L11.
Fig. S47. Inhibition by nicardipine with Caco-2 cells from L11.
Fig. S48. Inhibition by quinidine with Caco-2 cells from L11.
Fig. S49. Inhibition by ranolazine with Caco-2 cells from L11.
Fig. S50. Inhibition by mibebradil with LLC-PK1-hMDR1-NKI cells from L02.
Fig. S51. Inhibition by quinidine with LLC-PK1-hMDR1-NKI cells from L02.
Fig. S52. Inhibition by ranolazine with LLC-PK1-hMDR1-NK1 cells from L02.
Fig. S53. Inhibition by verapamil with LLC-PK1-hMDR1-NK1 cells from L02.
Fig. S25

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Fig. S26
Fig. S27

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Fig. S28

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Fig. S29

Digoxin Concentration A:B>A (µM) vs. Inhibitor Concentration (µM)

[Graph showing data points and a curve]
Fig. S30

Digoxin Concentration A:B>A (µM) vs. Inhibitor Concentration (µM)
Fig. S31

Graph showing the relationship between Digoxin Concentration A:B>A (µM) and Inhibitor Concentration (µM). The graph indicates a decrease in Digoxin concentration as the Inhibitor concentration increases.
Fig. S32

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Fig. S33

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Fig. S34

Digoxin Concentration A:B>A (µM) vs. Inhibitor Concentration (µM)
Fig. S35

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)

Fig. S36
Fig. S37

Digoxin Concentration A:B>A (µM) vs. Inhibitor Concentration (µM)
Fig. S38

Digoxin Concentration A:B>A (µM) vs. Inhibitor Concentration (µM)
Fig. S39

![Graph showing the concentration of Digoxin A:B>A (µM) as a function of Inhibitor Concentration (µM)].

- **Y-axis**: Digoxin Concentration A:B>A (µM)
- **X-axis**: Inhibitor Concentration (µM)

The graph illustrates a decrease in Digoxin Concentration A:B>A with increasing Inhibitor Concentration.
Fig. S40

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)

Fig. S41
Fig. S42

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Fig. S43

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Fig. S44

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)

Fig. S45
Fig. S46

Digoxin Concentration A>B>A (µM) vs. Inhibitor Concentration (µM)

- Concentration values range from 1E-04 to 1E+04 µM.
- Data points with error bars indicating variability.
- The graph shows a decreasing trend in Digoxin Concentration as the Inhibitor Concentration increases.
Fig. S47

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Fig. S48

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Fig. S49

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Fig. S50

Digoxin Concentration A:B>A (µM) vs. Inhibitor Concentration (µM)

- X-axis: Inhibitor Concentration (µM)
- Y-axis: Digoxin Concentration A:B>A (µM)

The graph shows a decrease in Digoxin Concentration A:B>A as the Inhibitor Concentration increases.
Fig. S51

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Fig. S52

Digoxin Concentration A:B>A (µM) vs. Inhibitor Concentration (µM)
Fig. S53

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Supplemental Data Table S5. Figure Legends for Tables 5A and 5B in the main paper. For all figures, symbols show the B>A digoxin transport data, error bars show the data standard deviation and the line shows the fit to the data.

Fig. S54. Inhibition by carvedilol with HPTC cells from K06.
Fig. S55. Inhibition by carvedilol with HPTC cells from K10.
Fig. S56. Inhibition by diltiazem with HPTC cells from K13.
Fig. S57. Inhibition by isradipine with HPTC cells from K08.
Fig. S58. Inhibition by ketoconazole with HPTC cells from K02.
Fig. S59. Inhibition by ketoconazole with HPTC cells from K11.
Fig. S60. Inhibition by mibefradil with HPTC cells from K07.
Fig. S61. Inhibition by nicardipine with HPTC cells from K04.
Fig. S62. Inhibition by nicardipine with HPTC cells from K13.
Fig. S63. Inhibition by quinidine with HPTC cells from K01.
Fig. S64. Inhibition by quinidine with HPTC cells from K02.
Fig. S65. Inhibition by ranolazine with HPTC cells from K12.
Fig. S66. Inhibition by verapamil with HPTC cells from K01.
Fig. S67. Inhibition by verapamil with HPTC cells from K10.
Fig. S56

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Fig. S57

Digoxin Concentration A:B>A (µM) vs. Inhibitor Concentration (µM)
Fig. S58

Digoxin Concentration A:B>A (µM) vs. Inhibitor Concentration (µM)
Digoxin Concentration A:B>A (µM) vs. Inhibitor Concentration (µM)

Fig. S59
Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)

Fig. S60
Fig. S61

Digoxin Concentration A:B>A (µM) vs Inhibitor Concentration (µM)
Fig. S62

Digoxin Concentration A:B>A (µM) vs. Inhibitor Concentration (µM)
Fig. S63
Fig. S64

Digoxin Concentration A:B>A (µM) vs. Inhibitor Concentration (µM)

- The x-axis represents the inhibitor concentration ranging from 1E-04 to 1E+04 µM.
- The y-axis represents the digoxin concentration A:B>A ranging from 0.00 to 0.05 µM.
- The graph shows a decreasing trend in digoxin concentration as the inhibitor concentration increases.
- The data points are shown with error bars indicating variability in the measurements.
- The red curve is a fit to the data, suggesting a possible concentration effect.

Overall, the graph illustrates the relationship between the inhibitor concentration and the digoxin concentration A:B>A.
Fig. S65

Digoxin Concentration A:B>A (µM) vs. Inhibitor Concentration (µM)
Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)

Fig. S66
Fig. S67

Digoxin Concentration A:B>A (µM)

Inhibitor Concentration (µM)
Supplemental Data Table S6. Figure Legends for HPTC digoxin in the main paper. For all figures, S54-S67, symbols show the B>A digoxin transport data, error bars show the data standard deviation and the line shows the fit to the data.

Fig. S68. Inhibition by T3 of digoxin uptake into HPTC cells.
Fig. S69. Inhibition by T3 of digoxin transport, J_{B>A}, through HPTC cells.
Supplemental Data Table S7. Individual Lab fits for log_{10}(k_{rQ}) for Main Table 3

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<th>k_{rQ} c</th>
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Footnotes for Supplemental Data Table S7:

a Cells 1, 2 and 3 are MDCKII-hMDR1-NKI, Caco-2 and LLC-PK1-hMDR1-NKI, respectively.

b Inhibitors: CRV (carvedilol); DLT (Diltiazem); IZR (Isradipine); MBF (Mibepradil); NCD (Nicardipine); QND (Quinidine); RNO (Ranolazine); VRP (Verapamil).

c $\log_{10}\{k_{rQ}\}$ is the fitted value.

d $<\log_{10}\{k_{rQ}\}>$ is the inhibitor average over the fitted labs. $<k_{rQ}> = 10^<\log_{10}\{k_{rQ}\}>$. 