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Research Article

Use of Different Parameters and Equations for Calculation of IC_{50} Values in Efflux Assays: Potential Sources of Variability in IC_{50} Determination

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Abstract. Drug interactions due to efflux transporters may result in one drug increasing or decreasing the systemic exposure of a second drug. The potential for *in vivo* drug interactions is estimated through *in vitro* cell assays. Variability in *in vitro* parameter determination (*e.g.*, IC_{50} values) among laboratories may lead to different conclusions in *in vivo* interaction predictions. The objective of this study was to investigate variability in *in vitro* inhibition potency determination that may be due to calculation methods. In a Caco-2 cell assay, the absorptive and secretive permeability of digoxin was measured in the presence of spironolactone, itraconazole and vardenafil. From the permeability data, the efflux ratio and net secretory flux where calculated for each inhibitor. IC_{50} values were then calculated using a variety of equations and software programs. All three drugs decreased the secretory transport of digoxin in a concentration-dependent manner while increasing digoxin's absorption to a lesser extent. The resulting IC_{50} values varied according to the parameter evaluated, whether percent inhibition or percent control was applied, and the computational IC_{50} equation. This study has shown that multiple methods used to quantitate the inhibition of drug efflux in a cell assay can result in different IC_{50} values. The variability in the results in this study points to a need to standardize any transporter assay and calculation methods within a laboratory and to validate the assay with a set of known inhibitors and non-inhibitors against a clinically relevant substrate.

KEY WORDS: Caco-2; drug interaction; IC_{50}; inhibition; P-glycoprotein.

INTRODUCTION

Recognizing the potential for drug–drug interactions (DDI) is an important factor in the development and regulatory review of a new drug (1–3). Transporter-based DDI may be due to competition for a transporter-binding site (by a competitive substrate or an inhibitor) or a change in level of transporter expression (from an inducer). Competition for the same transport pathways among coadministered drugs can result in significant changes in a drug's absorption, tissue distribution, metabolism, and excretion profiles (4). The objective of DDI studies is to determine the potential for clinical interactions between an investigational drug and other drugs that may be co-administered.

Early identification of compounds that are transporter substrates or inhibitors has become a routine task during the optimization and selection of drug candidate (5). An integrated *in vitro* to *in vivo* approach can aid in determining the need for *in vivo* drug interaction studies. Variability in *in vitro* parameter determination, such as IC_{50} or K_i values, among laboratories may lead to different conclusions for *in vivo* interaction projections using universal criteria such as those proposed in the FDA draft and EMA drug interaction guidances (2,3). Therefore, predictability of *in vitro* assays is important, as costly clinical studies might be initiated based solely on the *in vitro* results (1). Therefore, it is important to understand the sources of variability and to use standardized methods within a laboratory to minimize variability.

Bidirectional assays are the most direct and accepted models for evaluating the potential of new drugs as substrates or inhibitors of efflux transporters (2,5–7). *In vitro* transport assays utilizing the Caco-2 cell line with digoxin as the probe substrate is a well-established method to determine P-glycoprotein (P-gp) inhibition and mimics intestinal interactions (8,9). However, specific assay methodologies vary between laboratories (10,11) along with how the transport kinetics are calculated (1,12). There is no consensus on how to best calculate IC_{50} values (50% inhibition of substrate transport) from *in vitro* efflux assays (1,12–14). The calculation methods vary among laboratories which may lead to misinterpretation of the rank order of P-gp inhibitory potency (13). This lack of uniformity allows flexibility by investigators which may lead to potentially erroneous calculations and possibly erroneous interpretation of results (1).

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In this study with a Caco-2 cell assay, digoxin served as the probe P-gp substrate with spironolactone, itraconazole, and vardenafil as inhibitor compounds. Digoxin, a narrow therapeutic index drug, is a recommended probe substrate for *in vitro* and *in vivo* assays based on known clinical interactions (2,3,8,11,15–20). Digoxin is negligibly metabolized and renally eliminated unchanged, predominately through P-gp-mediated renal tubular secretion (21).

Spironolactone is a potassium-sparing diuretic that that has been shown to inhibit digoxin efflux in transfected cell lines (6,13,22). Spironolactone, given orally before and after digoxin, reduced digoxin renal and nonrenal clearances and prolonged digoxin's elimination half-life (23–25). Itraconazole is a triazole antifungal agent that is an inhibitor of several P-gp substrates in wild-type and transfected cell assays (6,13,19,26–28) and is considered to be a P-gp substrate (29,30). Clinically, itraconazole increases digoxin plasma levels and AUC (concentration-time curve) while decreasing digoxin's renal clearance (31,32). Vardenafil is a phosphodiesterase 5 inhibitor that is a potent inhibitor of P-gp *in vitro* as determined in cytotoxicity, accumulation and ATPase assays (33,34). Vardenafil is a substrate of the efflux transporters P-gp, breast cancer resistance protein, and multidrug resistant protein-2 in Caco-2 and transfected MDCK cells (35,36). However, vardenafil did not significantly alter the steady-state AUC or plasma concentration of digoxin *in vivo* (37).

In developing and utilizing efflux transporter models, there are a number of variabilities in the assays that can affect the experimental outcomes. Sources of variabilities include the choice of cell line (11,38,39), culture conditions (*e.g.*, cell passage number and monolayer age) (40,41), control substrate or inhibitor specificity (17,42,43), level of transporter expression (44,45), and data analysis (13,19). The objective of this study is to focus on the variability in *in vitro* inhibition potency determination that may be caused by different calculation methods (*e.g.*, efflux parameters and software programs) from a bidirectional Caco-2 cell assay. Digoxin was utilized as a probe P-gp substrate in the efflux assay with spironolactone, itraconazole and vardenafil as selected inhibitor compounds. Utilizing several analysis methods for the data generated in the transporter assays, IC_{50} values were then calculated.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), nonessential amino acids, sodium pyruvate, penicillin, streptomycin, Hank's balanced salt solution (HBSS), and hydroxyethyl piperazineethanesulfonic acid (HEPES) were from Invitrogen/Life Technologies (Carlsbad, CA). 2-(N-Morpholino)ethanesulfonic acid (MES), spironolactone, itraconazole, digoxin, dimethyl sulfoxide (DMSO), and ethanol were purchased from Sigma-Aldrich (St. Louis, MO). Labeled [^{3}H]-digoxin was from Perkin-Elmer (Waltham, MA) and vardenafil was from Toronto Research Chemicals (Toronto, Canada).

The DMEM culture media contained 4.5 g/L glucose, 10% heat-inactivated FBS, 1% nonessential amino acids, sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin. The transport buffers were comprised of HBSS with either 25 mM HEPES (HBSS/HEPES, pH 7.4) or 10 mM 2-(*N-morpholino*)ethanesulfonic acid (HBSS/MES, pH 6.8). [^{3}H]-Digoxin (40 Ci/mmol) was diluted in ethanol to a concentration of 1,000 μCi/mL and stored at −80°C. It was then diluted to a working stock of 10 μCi/mL in ethanol for use in the transport assays. The stock solutions of unlabeled digoxin (1.0 mM), spironolactone (100 mM), vardenafil (100 mM), and itraconazole (10 mM) were prepared in DMSO, stored at 4°C, and diluted in distilled water to 10 times their final concentrations for use in the transport assays.

Caco-2 Monolayers

The Caco-2 cell monolayers were obtained from Absorption Systems L.P. (Exton, PA). The cells (CRL-2102, American Type Culture Collection, Manassas, VA) were cultured at 37°C with 5% CO_2 and plated for monolayer formation according to previously published reports (46,47). Caco-2 cells were seeded at 60,000 cells/cm^2 onto collagen-coated, polycarbonate membranes in 12-well Costar® Transwell® plates (1.13 cm^2 area, 0.4 μm pore size; Corning Life Sciences, Lowell, MA). The culture medium was changed 24 h after seeding and then changed every other day. The cells were incubated for up to 3 weeks to form confluent monolayers. The passage number of the Caco-2 cells ranged from 61 to 66 and the monolayer age at the time of the transport study was 23 or 24 days.

Caco-2 Transport Assay

The monolayers were removed from the incubator and the medium aspirated from the apical (AP) and basolateral (BL) chambers. Approximately 0.5 mL of HBSS/HEPES buffer was used to wash the cell monolayers. To the AP and BL chambers, 0.5 mL HBSS/MES and 1.5 mL HBSS/HEPES were added, respectively. The monolayers were incubated at 37°C, 5% CO_2 for 10–30 min. The TEER was measured in the cell wells and blanks with an epithelial voltohmmeter (EVOM^2; World Precision Instruments, Inc., Sarasota, FL). The average blank resistance (R_{blank}) measurement was subtracted from the cell monolayer insert (R_{sample}). TEER was calculated according to the following equation.

$$\text{TEER}\ (\Omega \times \text{cm}^2) = (R_{sample} - R_{blank}) \times 1.13\ \text{cm}^2 \quad (1)$$

For the Caco-2 cell monolayers, the TEER values were at least 250 Ω×cm^2.

The buffer was then removed from the chambers and replaced with HBSSS/MES (AP) or HBSSS/HEPES (BL) containing the inhibitor with control wells containing buffer only. The monolayers were pre-incubated for 30 min at 37°C with the inhibitor solution. The inhibitor solution from the donor chamber was then replaced with a digoxin solution containing the inhibitor. The amount of cold digoxin in each donor chamber was 5 μM with 0.1 μCi/well [^{3}H]-digoxin. During the transport experiment, the receiver chamber contained buffer with or without inhibitor solution and was replaced with the same buffer after a sample was removed.

For absorptive (AP-BL) permeability studies, the buffer solution was aspirated from the AP chamber of the

monolayers and replaced with digoxin solution in HBSS/MES, with or without the inhibitor, using four wells for each group. The plates were returned to a 37°C, 5% CO_2 incubator on a plate shaker for agitation during the transport experiment. At 30, 60, 90, and 120 min, the insert was transferred to a new well containing HBSS/HEPES buffer, with or without inhibitor. A sample was taken from the donor chamber at 120 min and all receiver and donor samples were stored at −20°C in labeled vials until analysis by liquid scintillation counting (LSC).

For secretive (BL-AP) permeability studies, the buffer was aspirated from the BL chamber of the monolayers and replaced with digoxin solution in HBSS/HEPES, with or without the inhibitor, using four wells for each group. The plates were returned to the incubator on a plate shaker for agitation during the transport experiment. At the time points of 30, 60, 90, and 120 min, 0.5 mL samples were collected from the AP chamber and replenished with an equal volume of HBSS/MES buffer, with or without inhibitor. A sample was taken from the donor chamber at 120 min, and all receiver and donor samples are stored at −20°C in labeled vials until analysis by LSC.

To determine the amount of digoxin in the assay samples, 100 μL was added to 5 mL of scintillation fluid (3a70B™, Research Products International, Corp., Mount Prospect, IL). The samples were analysed in a LS 650 scintillation counter (Beckman Coulter™ Inc., Fullerton, CA) following an internal instrument calibration. The disintegration counts per minute (dpm) were converted to digoxin concentrations (micromolars) taking into account the sample size (100 μL) and volume of the AP (0.5 mL) or BL (1.5 mL) chamber.

Calculations

The apparent permeability (P_{app}) values were calculated for digoxin in both AP-BL and BL-AP directions in the absence or presence of the inhibitors. P_{app} ($\times 10^{-6}$ cm/s) was calculated from the following equation:

$$P_{app} = \frac{V_R}{(A \times C_0)} \times \frac{dC}{dt} \tag{2}$$

where V_R was the volume in the receiver chamber, A the filter surface area (1.13 cm^2), C_0 the initial digoxin concentration in the donor chamber, and dC/dt was the slope of the linear portion of the concentration *vs.* time curve. In comparing the P_{app} results, a two-sample Student's t test assuming unequal variances was calculated for the rate values with an Excel spreadsheet (Office 2003, Microsoft, Redmond, WA).

The efflux ratio (ER) of digoxin was calculated from the bidirectional P_{app} values in the BL-AP and AP-BL directions.

$$ER = \frac{P_{app,BL\text{-}AP}}{P_{app,AP\text{-}BL}} \tag{3}$$

The net secretory flux (NSF; centimeters per second), calculated by subtracting the absorptive from the secretory permeability, measured the net amount of digoxin transported across the monolayers in the BL-AP direction (17,19):

$$NSF = P_{app,BL\text{-}AP} - P_{app,AP\text{-}BL} \tag{4}$$

The parameters $P_{app,BL\text{-}AP}$, ER, and NSF for digoxin were calculated for the experiments in the presence and absence of each inhibitor and designated as original, or non-normalized, data. From these data, percent control and percent inhibition values were calculated for digoxin in the presence of the inhibitors. The percent control was calculated for each inhibitor concentration according to the following equation:

$$\%\,\text{Control} = \frac{\text{Parameter with inhibitor}}{\text{Parameter without inhibitor}} \times 100 \tag{5}$$

The inhibition of digoxin transport in the presence of the inhibitors was calculated based on the parameter in the absence (negative control) or presence of the inhibitor (1,7,17).

$$\%\ \text{Inhibition} = \left(\frac{(\text{Parameter without inhibitor} - \text{Parameter with inhibitor})}{(\text{Parameter without inhibitor})}\right) \times 100 \tag{6}$$

IC_{50} calculations for $P_{app,\ BL\text{-}AP}$, ER, and NSF data with modified Hill equations were completed with Phoenix® WinNonlin® software (version 6.1, Pharsight®, St. Louis, MO), GraphPad Prism® (version 5.0, La Jolla, CA), and SigmaPlot® (version 12.0, Systat Software, Inc., San Jose, CA) software programs. With the WinNonlin® analysis, Eq. 7 was used for the original and %control data and Eq. 8 for the %inhibition data.

$$E = E_0 + \left(\frac{E_{max} \times C^{\gamma}}{EC_{50}{}^{\gamma} + C^{\gamma}}\right) \tag{7}$$

$$E = E_0 \times \left(1 - \frac{E_{max} \times C^{\gamma}}{EC_{50}{}^{\gamma} + C^{\gamma}}\right) \tag{8}$$

In the equations, E is the effect (original data, %control, or %inhibition), E_0 is the baseline value, E_{max} is the maximal effect, C is the inhibitor concentration, EC_{50} is the concentration at 50% maximal value, and γ is the sigmoidicity factor. The assumptions are that baseline value (E_0) is 0% in the case of %inhibition, and E_0 is 100% for the %control data.

In the GraphPad Prism® equation, log-transformed concentration values and the effect data were fitted to a four-parameter logistic equation. The original, %control, or %inhibition data are represented by Y along with their minimal (min) and maximal (max) values. The inhibitor concentration is represented by X, IC_{50} is the concentration at 50% maximal value, and HillSlope is the slope factor.

$$Y = \min + \left(\frac{(\max - \min)}{1 + 10^{((X \ - \ \log IC_{50}) \times \text{Hill slope})}} \right) \quad (9)$$

For the SigmaPlot® plot analysis, the same four-parameter logistic nonlinear regression equation, without baseline, was utilized. The original, %control, or %inhibition data are represented by Y along with their minimal (min) and maximal (max) values. The inhibitor concentration is represented by X, IC_{50} is the concentration at 50% maximal value, and HillSlope is the slope factor.

$$Y = \frac{(\max - \min)}{1 + 10^{((\log EC_{50} - X) \times \text{Hill slope})}} \quad (10)$$

Data fitting for the IC_{50} values included %coefficient of variance (%CV) and R^2 values (see Electronic Supplementary Material).

RESULTS

The average TEER value for the monolayers in the three experiments was 577±56 Ω×cm^2. In the experiments, $P_{app,\ AP\text{-}BL}$ and $P_{app,\ BL\text{-}AP}$ for digoxin in the absence of an inhibitor was 0.850±0.214 and 8.972±0.404×10^{-6} cm/s, respectively, demonstrating significant efflux through the Caco-2 cell monolayers (p=0.00004). The ER and NSF for digoxin ranged from 8.60 to 12.67 (10.89±2.06) and 8.00 to 8.34 cm/s (8.12±0.19), respectively, in the absence of the inhibitors (Figs. 1, 2, and 3). All three drugs decreased the $P_{app,\ BL\text{-}AP}$, ER, and NSF values for digoxin in a concentration-dependent manner. The drugs increased digoxin's absorptive permeability with increasing concentrations, although to a lesser extent than their reduction in digoxin's secretive permeability.

The IC_{50} values for the inhibitors was highly dependent upon the parameter ($P_{app,\ BL\text{-}AP}$, ER, NSF) used in the data analysis along with inclusion or exclusion of the control (absence of inhibitor). When using the four-parameter logistic equation to estimate the IC_{50} parameter, the inhibitor concentrations are log transformed, and as a result, the zero inhibitor concentration (control) is omitted from the analysis. By contrast, the Hill equation fits untransformed concentration data, inclusive of the baseline effect. Comparing the Hill equation *vs.* the four-parameter logistic equation, there is significant difference in the estimate of IC_{50} when the zero concentration of the inhibitor is either included or excluded in the analysis (data not shown). When the zero concentration is included, there was a difference in the IC_{50} value estimate and goodness of fit measures between the Hill and four-parameter logistic equations as the fit is for a truncated dataset for the four-parameter logistic equation. When the zero concentration was excluded, the Hill and four-parameter logistic equations yielded very similar IC_{50} estimates and goodness of fit measures.

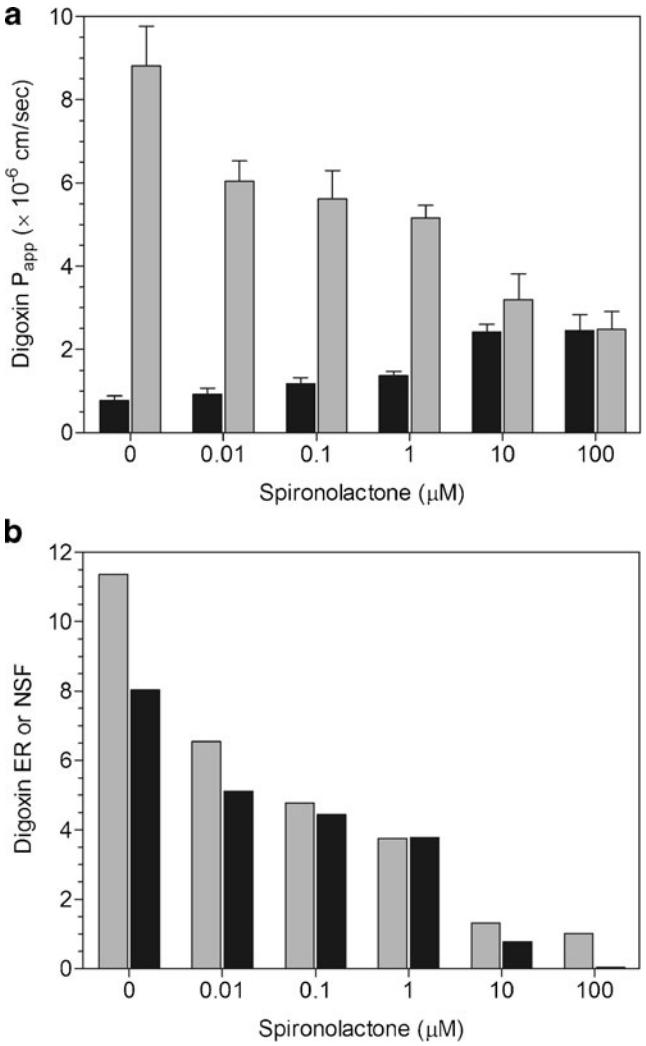

Fig. 1. **a** Inhibition of digoxin $P_{app,\ AP\text{-}BL}$ (*black bar*) and $P_{app,\ BL\text{-}AP}$ (*gray bar*) by spironolactone. Mean±SD of four wells. **b** Inhibition of digoxin ER (*gray bar*) and NSF (*black bar*) by spironolactone

Spironolactone (0.01–100 μM) caused a concentration-dependent decrease in digoxin $P_{app,\ BL\text{-}AP}$ while increasing $P_{app,\ AP\text{-}BL}$ at 10 and 100 μM (Fig. 1a). At 100 μM, there were equivalent AP-BL and BL-AP permeabilities, resulting in ER and NSF values of 1.01 and 0.04, respectively (Fig. 1b). Itraconazole (0.01–50 μM) produced a concentration-dependent decrease in digoxin efflux while increasing $P_{app,\ AP\text{-}BL}$ at 10 and 50 μM (Fig. 2a). The ER and NSF values for itraconazole were 1.59 and 1.30, respectively at 50 μM (Fig. 2b). Vardenafil (0.0001–100 μM) caused a concentration-dependent decrease in digoxin $P_{app,\ BL\text{-}AP}$ while increasing $P_{app,\ AP\text{-}BL}$ at 0.1–100 μM (Fig. 3a). There was unity at 100 μM with equivalent AP-BL and BL-AP permeabilities, generating ER and NSF values of 1.04 and 0.10, respectively (Fig. 3b).

The IC_{50} values for the drugs varied depending upon which parameter was considered ($P_{app,\ BL\text{-}AP}$, ER, and NSF), whether the data was original (non-normalized), %control or %inhibition, and which IC_{50} software program was utilized. The IC_{50} values for spironolactone and vardenafil were similar with GraphPad® and SigmaPlot® with values generally lower when estimated with WinNonlin®. For itraconazole, the IC_{50} values were similar with all three programs.

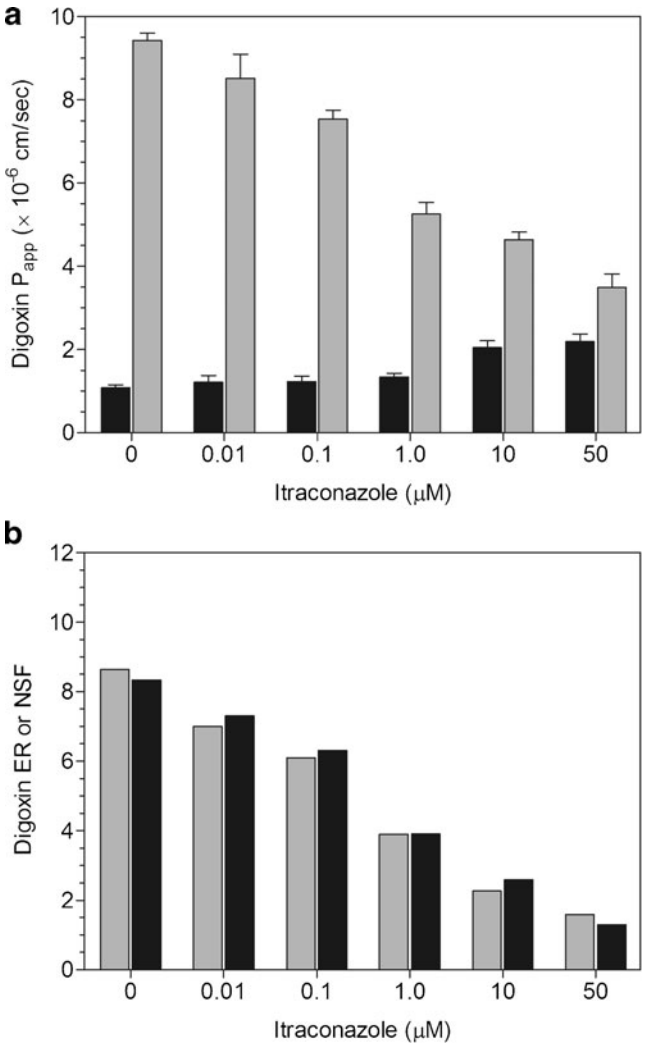

Fig. 2. **a** Inhibition of digoxin $P_{app,\ AP\text{-}BL}$ (*black bar*) and $P_{app,\ BL\text{-}AP}$ (*gray bar*) by itraconazole. Mean±SD of 4 wells. **b** Inhibition of digoxin ER (*gray bar*) and NSF (*black bar*) by itraconazole

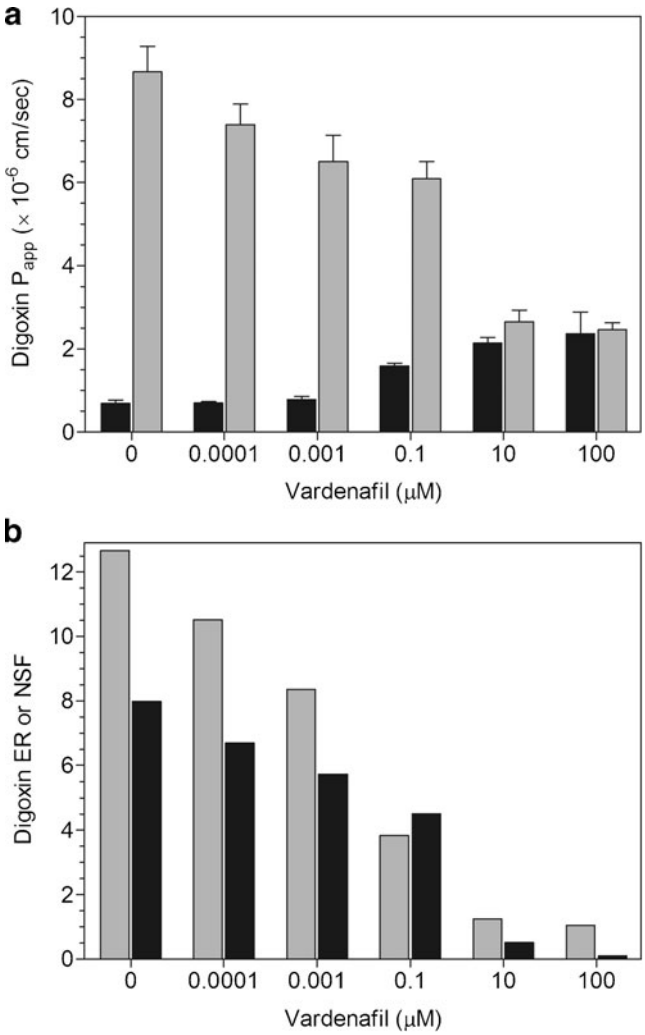

Fig. 3. **a** Inhibition of digoxin $P_{app,\ AP\text{-}BL}$ (*black bar*) and $P_{app,\ BL\text{-}AP}$ (*gray bar*) by vardenafil. Mean±SD of four wells. **b** Inhibition of digoxin ER (*gray bar*) and NSF (*black bar*) by vardenafil

For spironolactone, more variable IC_{50} values were noted with WinNonlin® than GraphPad® and SigmaPlot® software (Table I). Overall, IC_{50} values from the ER data were lower than those from $P_{app,\ BL\text{-}AP}$ or NSF data. Utilizing the GraphPad® and SigmaPlot® programs, the IC_{50} values were the same whether derived from the original, %control or %inhibition data with a rank order for spironolactone IC_{50} values of ER<$P_{app,\ BL\text{-}AP}$<NSF.

For itraconazole, all three programs had similar IC_{50} values with a rank order of $P_{app,\ BL\text{-}AP}$<ER<NSF (Table II). In the WinNonlin® analyses, IC_{50} values based on the NSF and $P_{app,\ BL\text{-}AP}$ data were the same for all calculation sets. There was some variability with the ER IC_{50} values based on original, %control, or %inhibition data. Utilizing the GraphPad® program, IC_{50} values for itraconazole using the different datasets were comparable with each parameter. With the SigmaPlot® analysis, the IC_{50} values were the same whether derived from the original, %control or %inhibition data.

For vardenafil, GraphPad® and SigmaPlot® had similar IC_{50} values while those calculated with WinNonlin® had more variability (Table III). In the WinNonlin® analyses, the IC_{50} values for the NSF and $P_{app,\ BL\text{-}AP}$ parameters was higher than the other calculation method. In the SigmaPlot® and GraphPad® analyses, the IC_{50} values were the same whether derived from the original, %control, or %inhibition data for each parameter. Overall, the rank of vardenafil IC_{50} values with the three programs was ER<NSF<$P_{app,\ BL\text{-}AP}$.

DISCUSSION

There is interest in predicting potential clinical interactions between an investigational new drug and other coadministered drugs during drug development and regulatory review (2,3,20). A bidirectional assay in Caco-2 or transporter overexpressed cell (*e.g.*, MDCK or LLC-PK_1) lines is a preferred method for *in vitro* evaluation of a new drug as an efflux substrate or inhibitor. Besides differences in test systems with P-gp-expressing cell lines, differing methodology and data processing approaches play a role in the interlaboratory variability of P-gp inhibition data (48,49).

In a Caco-2 cell assay with digoxin as the substrate, secretory permeability ($P_{app,\ BL\text{-}AP}$), ER, and NSF were used as parameters to calculate P-gp inhibitor IC_{50} values with a

Table I. Spironolactone IC_{50} (micromolars) Values

Program	Parameter	Original	%Control	%Inhibition
WinNonlin®	$P_{app,\ BL\text{-}AP}$	9.277	3.280	1.371
	ER	0.077	0.448	0.705
	NSF	9.817	2.759	0.024
GraphPad®	$P_{app,\ BL\text{-}AP}$	3.279	3.308	3.308
	ER	0.455	0.377	0.377
	NSF	2.760	2.840	2.840
SigmaPlot®	$P_{app,\ BL\text{-}AP}$	3.280	3.280	3.280
	ER	0.445	0.445	0.445
	NSF	2.761	2.761	2.761

number of equations in commercial software packages. Overall, the drugs' IC_{50} values for each parameter were the same no matter if original, %control, or %inhibition data were used in the SigmaPlot® analyses. More variability was noted with the WinNonlin® calculation within data sets, especially with spironolactone. For vardenafil and spironolactone, the IC_{50} values from SigmaPlot® and GraphPad® were similar and usually higher than those with WinNonlin®. With itraconazole, the IC_{50} values with each parameter were essentially the same for the three programs. In looking at the different parameters, the ER data yielded the lowest IC_{50} values for spironolactone and vardenafil whereas $P_{app,\ BL\text{-}AP}$ had the lowest values for itraconazole.

Literature reports on the inhibition of P-gp by compounds have utilized a variety of equations and software packages to calculate IC_{50} values. These include programs for Excel™ (1,48,50,51), GraphPad® (12,13,39,52–54), WinNonlin® (12,49,55), GraFit (6,12,15–19,56), and SigmaPlot® (12,14). The calculations in these publications were some modification of the Hill equation that included an effect (%inhibition or %control), maximal effect or range of effect, inhibitor concentration, EC_{50} or IC_{50}, and a Hill coefficient or factor. The effect of an inhibitor on a P-gp substrate was measured based upon the $P_{app,\ BL\text{-}AP}$ (1,6,12,13,18,57), ER (1,12–14,19,48–51,57), or NSF (1,12,14,17,19,52,53).

Variability in IC_{50} determinations results in challenges to use an "universal" cutoff criteria as prosposed in the FDA's draft DDI guidance (2) to project *in vivo* interaction potential based on *in vitro* inhibition data. Comparisons have been made in publications as to how the different paramaeters affect the IC_{50} calculations. Different conclusions concerning the inhibitory potential of drug could be derived from the same experimental dataset if different calculation methods are utilized (1,12). For example, a multi-laboratory study with several cell systems with digoxin as the substrate found great variability in IC_{50} values with equations based on either P_{app}, NSF or ER (12). IC_{50} values for 16 inhibitors derived from ER or $P_{app,\ AP\text{-}BL}$ data were lower than those from $P_{app,BL\text{-}AP}$ or NSF data (12). Perloff *et al.* used ER and NSF parameters in their calculation of IC_{50} values for digoxin transport in Caco-2 and MDR1-LLC-PK1 cells with 16 inhibitor compounds (51). The IC_{50} values based on NSF were on an average 2.5-fold higher than corresponding values based upon ER (51). Lin *et al.* calculated IC_{50} values for ketoconazole with two P-gp substrates, with those based on ER less than those from P_{app} calculations (57).

Balimane *et al.* examined different %inhibition calculation methods in a Caco-2 cell P-gp inhibition assay using the parameters $P_{app,\ AP\text{-}BL}$, $P_{app,\ BL\text{-}AP}$, and ER in the presence and absence of inhibitors (1). For the over 50 tested compounds, the %inhibition results notably differed depending on the calculation method used (1). When the IC_{50} values were compared from the different %inhibition equations, there was a 4-fold variation based on the calculation method used, with significantly lower values observed with the ER calculation method (1). The commonly used calculation method of ER requires studies in bidirectional mode and may lead to results that are oversensitive in the "low" inhibition range which can potentially lead to false positives (1).

Sugimoto *et al.* found that the IC_{50} values generated for over 20 drugs from $P_{app,\ BL\text{-}AP}$ were larger than those from ER for compounds tested, and there was a positive correlation between these IC_{50} values (13). Cook *et al.* found that for the majority of the 30 compounds they tested in a Caco-2 cell assay, IC_{50} values were 2-fold more potent by the ER method (ER<NSF) (19). For example, the IC_{50} value for itraconazole was 6 and 2 μM based upon NSF and ER, respectively (19).

The calculation of IC_{50} values based on $P_{app,\ BL\text{-}AP}$ provides a simple experimental design with confluent cell monolayers in the presence of increasing inhibitor concentrations (12). It is recommended that multiple inhibitor concentrations are assayed to define both the upper and lower plateaus of the response curve (12). However, it should be noted that a drug's aqueous solubility may limit the lower plateau (maximal effect) and a positive control inhibitor can be used to define complete inhibition of P-gp activity in the cells (12,56).

Bentz *et al.* suggest that the differences in IC_{50} values for ER *vs.* $P_{app,\ BL\text{-}AP}$ for digoxin is likely due to the decline of $P_{app,\ BL\text{-}AP}$

Table II. Itraconazole IC_{50} (micromolars) Values

Program	Parameter	Original	%Control	%Inhibition
WinNonlin®	$P_{app,\ BL\text{-}AP}$	0.376	0.378	0.378
	ER	0.572	0.686	0.629
	NSF	0.859	0.861	0.860
GraphPad®	$P_{app,\ BL\text{-}AP}$	0.376	0.408	0.408
	ER	0.687	0.701	0.701
	NSF	0.862	0.797	0.797
SigmaPlot®	$P_{app,\ BL\text{-}AP}$	0.376	0.376	0.376
	ER	0.686	0.686	0.686
	NSF	0.862	0.862	0.862

Table III. Vardenafil IC_{50} (micromolars) Values

Program	Parameter	Original	%Control	%Inhibition
WinNonlin®	$P_{app,\ BL\text{-}AP}$	9.864	0.485	0.540
	ER	0.005	0.004	0.005
	NSF	1.619	0.415	0.396
GraphPad®	$P_{app,\ BL\text{-}AP}$	0.485	0.539	0.539
	ER	0.004	0.004	0.004
	NSF	0.416	0.396	0.396
SigmaPlot®	$P_{app,\ BL\text{-}AP}$	0.485	0.485	0.485
	ER	0.004	0.004	0.004
	NSF	0.416	0.416	0.416

is greater than the increase in $P_{app,\ AP\text{-}BL}$ for any given inhibitor concentration (12). This minimizes the effect of the increase in $P_{app,\ AP\text{-}BL}$ on the decline in $P_{app,\ BL\text{-}AP}$ and thus on the IC_{50} value obtained for the net flux equation (12). We also found that the change in $P_{app,\ BL\text{-}AP}$ was greater that for $P_{app,\ AP\text{-}BL}$ with the three inhibitors (Figs. 1, 2, and 3). IC_{50} values determined from ER data often appear more potent, which may be due to a mathematical artifact as ER can never numerically achieve zero (19).

Additionally, the difference between the IC_{50} values generated from ER and $P_{app,\ BL\text{-}AP}$ may be attributed to the fact that the inhibitory rate based on $P_{app,\ BL\text{-}AP}$ does not disregard the influence of passive permeability of the substrate (13). Using loperamide as the P-gp substrate, Taur *et al.* speculated that the ER approach may overestimate the P-gp inhibition potency since ER was calculated from the ratio of $P_{app,\ BL\text{-}AP}$ normalized by $P_{app,\ AP\text{-}BL}$, thus the inhibition effect of inhibitors may have been accounted twice under the calculation (14).

Besides the sources of variability as discussed above, from our study we found that there is some degree of variability in the parameter estimates among different software programs, such as those obtained via WinNonlin® in comparison to SigmaPlot® and GraphPad® (Tables I, II, and III) because of the nature of the software's fitting equation. SigmaPlot® and GraphPad® utilize a four-parameter logistic equation, while the data was fitted to a Hill equation with cooperativity coefficient in WinNonlin®. If there is a perfectly sigmoidal curve, the software will not matter since the estimate of the IC_{50} is highly dependent on the plateau regions (both at low response levels and saturation of response). While the fits are expected to be essentially identical for a well characterized sigmoidal dose response, high variability in estimates are often observed with a dose response curve that is not perfectly sigmoidal. In cases where the baseline effect is quite distinct from the effect of the lowest concentration, IC_{50} estimates are greatly influenced by the omission of the baseline effect. Conversely, this influence is minimal when the data assumes a complete sigmoid shape (*i.e.*, low concentrations of inhibitor have an effect that is similar to baseline). In addition, the log transformation also changes the assumption of normal distribution of error and may result in smaller erroneous estimates. Therefore, it may be appropriate to use a Hill equation rather than a four-parameter logistic equation, especially when the shape of response curve is not perfectly sigmoidal.

Based upon this limited data set, suggestions for the calculation of IC_{50} values include the use of original (non-normalized) data and the fitting of nontransformed data. The use of original data results in fewer assumptions, *i.e.*, 100% (maximum) or 0% (minimum) activity, which could affect the E_0 or E_{max} estimate, and IC_{50} by extension. Fitting of untransformed data does not violate the assumption that errors are normally distributed as transformation alters this distribution.

CONCLUSIONS

This study highlights the factors that investigators need to consider when using software programs to calculate IC_{50} values based on the shape of their inhibition curve. The variability in the IC_{50} results in this study reinforces the need to standardize any transporter assay and calculation methods within a laboratory (1,12). The assay should be validated with acceptance criteria for a set of known inhibitors and non-inhibitors against a clinically relevant substrate. From such a study, a single parameter (*e.g.*, $P_{app,\ BL\text{-}AP}$ or ER) and a Hill-type IC_{50} equation ought to be utilized to determine whether a new drug is a transporter inhibitor and confidently predict if a clinical DDI study is necessary for development and regulatory purposes.

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