Variability in P-Glycoprotein Inhibitory Potency (IC₅₀) Using Various in Vitro Experimental Systems: Implications for Universal Digoxin Drug-Drug Interaction Risk Assessment Decision Criteria[§]

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ABSTRACT

A P-glycoprotein (P-gp) IC₅₀ working group was established with 23 participating pharmaceutical and contract research laboratories and one academic institution to assess interlaboratory variability in P-gp IC₅₀ determinations. Each laboratory followed its in-house protocol to determine in vitro IC₅₀ values for 16 inhibitors using four different test systems: human colon adenocarcinoma cells (Caco-2; eleven laboratories), Madin-Darby canine kidney cells transfected with MDR1 cDNA (MDCKII-MDR1; six laboratories), and Lilly Laboratories Cells—Porcine Kidney Nr. 1 cells transfected with MDR1 cDNA (LLC-PK₁-MDR1; four laboratories), and membrane vesicles containing human P-glycoprotein (P-gp; five laboratories). For cell models, various equations to calculate remaining transport activity (e.g., efflux ratio, unidirectional flux, net-secretory-flux) were also evaluated. The difference in IC₅₀ values for each of the inhibitors across all test systems and equations ranged from a minimum of 20- and

24-fold between lowest and highest IC₅₀ values for sertraline and isradipine, to a maximum of 407- and 796-fold for telmisartan and verapamil, respectively. For telmisartan and verapamil, variability was greatly influenced by data from one laboratory in each case. Excluding these two data sets brings the range in IC₅₀ values for telmisartan and verapamil down to 69- and 159-fold. The efflux ratio-based equation generally resulted in severalfold lower IC₅₀ values compared with unidirectional or net-secretory-flux equations. Statistical analysis indicated that variability in IC₅₀ values was mainly due to interlaboratory variability, rather than an implicit systematic difference between test systems. Potential reasons for variability are discussed and the simplest, most robust experimental design for P-gp IC₅₀ determination proposed. The impact of these findings on drug-drug interaction risk assessment is discussed in the companion article (Ellens et al., 2013) and recommendations are provided.

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Introduction

In recent years, the role of membrane transporters in the absorption, disposition, and excretion of drugs has been increasingly recognized. In particular, P-glycoprotein (P-gp; encoded by the MDR1 or ABCB1 gene in human) has been shown to impact drug pharmacokinetics by limiting oral absorption, restricting central nervous system penetration,

and promoting excretion. For drugs that are transporter substrates and are not significantly metabolized, such as talinolol and digoxin, P-gp or other transporters play an important role in absorption and disposition. This may lead to drug-drug interactions (DDIs) when coadministered with other drugs that also interact with these transporters (Schwarz et al., 2000; Juan et al., 2007; Fenner et al., 2009; Shirasaka et al., 2010). Digoxin has a narrow therapeutic window; consequently, even slight changes in plasma exposure have been associated with adverse events. As a result, many examples of clinical digoxin DDI studies have been reported (Fenner et al., 2009) in which the mechanism of interaction is frequently ascribed to P-gp inhibition and sometimes to P-gp induction.

The recent DDI draft guidance from the FDA (US FDA/CDER, 2012; <http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm064982.htm>) provides decision criteria to assess the risk of a clinically significant DDI resulting from P-gp inhibition. A clinical DDI study with digoxin is recommended when the maximum total plasma (bound plus unbound) concentration of the investigational drug at steady state ($[I]_1$) divided by its in vitro P-gp inhibitory potency (IC_{50}) is greater than or equal to 0.1 or, for orally administered drugs, its nominal gut concentration ($[I]_2$) divided by its IC_{50} is greater than or equal to 10.

These decision criteria, originally proposed by Zhang et al. (2008) and reinforced by Agarwal et al. (2013), are based on in vitro P-gp IC_{50} data without regard to experimental system or remaining transport activity equation and where each IC_{50} value is generated by one single laboratory only. In both articles, the authors emphasized the need for standardization of in vitro methods to ensure that the most appropriate decision criteria are established. Two additional articles proposed different decision criteria that are based on IC_{50} values for multiple compounds generated in one single laboratory, using a single experimental system and a single transport activity equation. Cook et al. (2010) using human colon adenocarcinoma cells (Caco-2) cells proposed cut-off values for $[I]_1/IC_{50} > 0.1$ and for $[I]_2/IC_{50} > 5$ using the net-secretory-flux equation, while Sugimoto et al. (2011) using Lilly Laboratories Cells—Porcine Kidney Nr. 1 cells transfected with MDR1 cDNA (LLC-PK₁-MDR1) cells proposed a cut-off value for $[I]_2/IC_{50} > 30$, using an efflux ratio-based equation.

A variety of systems are available to measure P-gp activity including inhibition of efflux of probe substrate from P-gp-expressing cells, inhibition of unidirectional or bidirectional transport across a monolayer of P-gp-expressing polarized cells, and inhibition of uptake into inside-out membrane vesicles prepared from P-gp-expressing cells. The pharmaceutical industry frequently utilizes P-gp expressing polarized cell lines such as Caco-2, Madin-Darby canine kidney cells transfected with MDR1 cDNA (MDCKII-MDR1), and LLC-PK₁-MDR1 to measure P-gp mediated transport and to assess potential DDIs.

In addition to the use of various cellular and vesicular systems to evaluate inhibition of P-gp, equations used to calculate remaining P-gp transport activity as a function of inhibitor concentration also vary between laboratories and have been based on efflux ratio, net-secretory-flux, or unidirectional flux [Tang et al., 2002; Kalvass and Pollack, 2007; Balimane et al., 2008; and the FDA draft guidance for drug interactions (US FDA/CDER, 2006, 2012)]. To date, limited data are available to appropriately compare IC_{50} values generated using

different experimental systems and transport activity equations. Therefore a collaborative effort was initiated between 23 pharmaceutical and contract research laboratories and one academic institution to compare IC_{50} data generated using four in vitro systems (Caco-2, MDCKII-MDR1, LLC-PK₁-MDR1 cells, and MDR1-expressing membrane vesicles) and six transport activity equations. Multiple commercial non-linear regression software packages were employed to fit the inhibition curves. The objective of this collaboration was to determine in vitro IC_{50} values for 16 drugs for which clinical digoxin DDI data are available, to assess the extent of variability in IC_{50} values obtained in different systems and using different transport activity equations and to discern whether any one system or transport activity equation outperformed the others, both in terms of robustness of the assay result (IC_{50} value) and assessment of DDI risk. In this article, we focus on the variability in IC_{50} values. The DDI risk analysis and recommendation for new decision criteria is presented in the companion article.

Materials and Methods

Chemicals. Sixteen compounds evaluated as P-gp inhibitors (amiodarone, captopril, carvedilol, diltiazem, felodipine, isradipine, mibefradil, nicardipine, nifedipine, nitrendipine, quinidine, ranolazine, sertraline, telmisartan, troglitazone, and verapamil) were obtained from Sigma (St. Louis, MO). Other chemicals and reagents were obtained by each participating laboratory from local commercial sources.

Cell Lines and Culture. Caco-2 cells (ATCC HTB-37) were from the American Type Culture Collection (Rockville, MD) or INSERM U-505 (Paris, France). LLC-PK₁ cells expressing cDNAs encoding human MDR1 (LLC-PK₁-MDR1 cells) were obtained from the Netherlands Cancer Institute or from BD Gentest (Woburn, MA, cat. no. 450211). MDCKII cells expressing cDNAs encoding human MDR1 (MDCKII-MDR1 cells) were obtained from the Netherlands Cancer Institute, the National Cancer Institute at the US National Institutes of Health (Bethesda, MD), or were generated in-house at the participating laboratory. Table 1–3 indicates which cell line was used by each of the participants, as well as the origin of that cell line.

Culture conditions varied between participating laboratories (Tables 1, 2, 3) since participants were asked to generate IC_{50} values using the conditions as established and used in their own laboratories. In general, cells were seeded onto permeable filter supports of varying materials and formats, and grown until confluent, polarized monolayers were formed. Some of the laboratories have historical microscopic data to demonstrate formation of cell monolayers (Hidalgo et al., 1989; Tran et al., 2004; Xia et al., 2005). Most laboratories determined the optimal combination of seeding density and time in culture by measuring transepithelial electrical resistance (TEER) and/or paracellular permeability and transport of a P-gp substrate at various seeding densities and times after seeding when they implemented the assay in their company. The conditions were then standardized for subsequent experiments, typically to the earliest time after seeding when each of these controls performs well. The quality controls included in the current study were determination of digoxin bidirectional transport and mass balance (no issue for any of the participants), TEER values, and/or paracellular permeability (the latter two parameters recorded in Tables 1–3).

Membrane Vesicles. Membrane vesicles prepared from cells expressing P-gp were obtained from Solvo Biotechnology (Budapest, Hungary) following the method described by Keppler et al. (1998) or were generated in-house at the participating laboratory (Table 4) (Karlsson et al., 2010).

Bidirectional Transport Assays across Polarized Cell Monolayers

Assay conditions varied between participating laboratories (Tables 1–3). In general, bidirectional (apical-to-basolateral, or A>B, and basolateral-to-apical,

ABBREVIATIONS: A>B, apical-to-basolateral; AMP-PNP, 59-adenylylimidodiphosphate; B>A, basolateral-to-apical; Caco-2, human colon adenocarcinoma cells; DDI, drug-drug interaction; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; LC-MS/MS, liquid chromatography followed by tandem mass spectrometry; LLC-PK₁-MDR1, Lilly Laboratories Cells - Porcine Kidney Nr. 1 cells transfected with MDR1 cDNA; MDCKII-MDR1, Madin-Darby canine kidney cells transfected with MDR1 cDNA; P_{app} , apparent permeability; P-gp, P-glycoprotein, also often referred to as MDR1 or ABCB1; PCA, principal component analysis; TEER, transepithelial electrical resistance.

TABLE 1
Experimental details by company (Caco-2)

Lab ID	Cell Source and Passage Number	Culture Medium	Seeding Density	Culture Time	Plate Format, Material, Pore Size, Vendor Cat. No.	Monolayer Integrity Acceptance Criteria	Substrate and Conc.	Assay Buffer and Solvent Content	Volumes AP/BL	Preincubation ^a / Assay time	Plate shaking	Positive control and Conc.
			<i>cells/well</i>	<i>days</i>			<i>nM</i>		μ l	<i>min</i>	<i>rpm</i>	μ M
16	ATCC p. 25-45	DMEM, 10% FBS, 1% NEAA, 3 mM L-glutamine	250,000	15-18	Coming no. 3401 12-well, PC, 0.4 μ m	Pre-TEER and post-TEER <30% change	³ H-Digoxin 13	HBSS pH 7.4 +25 mM HEPES + 5.56 mM Glucose <1% DMSO	500/1500	15/45	450	Ketoconazole 50
25	ATCC p. 23-35	DMEM, 10% FBS, 1% NEAA, 1% pen-strep, 1% L-glutamine	90,000	21-25	Coming no. 3401 12-well, PC, 0.4 μ m	Pre-TEER and post-TEER <50% change	³ H-Digoxin 100	HBSS pH 7.4 +10 mM HEPES <1% DMSO	400/1200	60/60	No	LY335979 5
17	ATCC p. 25-45	DMEM High Glucose, 10% FBS, 1% NEAA	40,000	21-25	BD Falcon cat. no. 351181 24-well, PET, 1.0 μ m	TEER > 400 Ω cm ² Lucifer Yellow: <1.0 $\times 10^{-6}$ cm/s	³ H-Digoxin 1,000	HBSS pH 7.4 +25 mM HEPES <0.5% DMSO	400/950	30/120	No	Ketoconazole 20
13	INSERM U505 p. 18-30	DMEM, 10% FBS, 1% pen-strep, 1% NEAA	100,000	7	Coming no. 3401 12-well, PC, 0.4 μ m	Lucifer Yellow: <1.0 $\times 10^{-6}$ cm/s	³ H-Digoxin 5000	HBSS pH 7.4 +20 mM HEPES <2% DMSO	800/800	30/120	No	Ketoconazole 10
24	ATCC p. 40-60	DMEM High Glucose, 10% FBS, 1% NEAA, 1X L-glutamine	100,000	21-25	Coming no. 3397 24-well, PC, 0.4 μ m	Lucifer Yellow: <1.0 $\times 10^{-6}$ cm/s	³ H-Digoxin 50	HBSS pH 7.4 +25 mM HEPES +10 mM Glucose <1% DMSO	200/1000	30/120	No	LY335979 10
12	ATCC p. 47-52	DMEM, 10% FBS	100,000	21-25	BD Falcon cat. no. 351181 24-well, PET, 1.0 μ m	TEER > 400 Ω cm ²	³ H-Digoxin 1000	HBSS pH 7.4 +10 mM HEPES <1% DMSO	200/1000	30/120	No	GFI20918 4
18	ATCC p. 25-35	DMEM+GlutaMax, 10% FBS, 1% NEAA, pyruvate	12,000	18-22	BD Falcon cat. no. 351131 96-well, PET, 1.0 μ m	TEER > 250 Ω cm ² Lucifer Yellow: <1.0 $\times 10^{-6}$ cm/s	Digoxin 10,000	HBSS pH 7.4 +10 mM HEPES <1% DMSO	75/250	30/120	~120 rpm	LY335979 1
19	ATCC p. 25-35	MEM + 20% FCS, 1% NEAA, 1% pyruvate, 1% L-glutamine	40,000	21-28	BD Falcon cat. no. 351181 24-well, PET, 1.0 μ m	Lucifer Yellow: <1% transport	³ H-Digoxin 5000	HBSS pH 7.4 +10 mM HEPES <1% DMSO	250/1000	0/120	150 rpm	Ketoconazole 20
15	ATCC p. 58-76	DMEM, 10% FBS, 1 mM sodium-pyruvate, 1% NEAA, 100 IU/ml penicillin, streptomycin 100 μ g/ml	67,200	21-28	Coming no. 3401 12-well, PC, 0.4 μ m	TEER 450-650 Ω cm ² Lucifer Yellow: $\leq 0.8 \times 10^{-6}$ cm/s	Digoxin 10,000	HBSS pH 7.4 +10 mM HEPES +15 mM Glucose <0.8% DMSO	500/1500	30/120	No	Cyclosporine A 5
14	ATCC p. 20-50	DMEM, 10% FBS, 1% NEAA, 1% L-glutamine, 100 IU/ml penicillin-G, streptomycin 100 μ g/ml	30,000	18-21	Coming no. 3396 24-well, PC, 0.4 μ m	TEER > 300 Ω cm ² Mannitol: <2.5 $\times 10^{-6}$ cm/s	³ H-Digoxin 5,000	HBSS pH 7.4 +10 mM HEPES <1% DMSO	200/600	0/120	No	Ketoconazole 50
08	ATCC no. HTB-37 p. 25-45	DMEM, 10% FBS, 5 mM HEPES, 0.4% glucose, 1% NEAA, 50 μ g/ml gentamycin	40,000	21-25	BD Falcon cat. no. 351181 24-well, PET, 1.0 μ m	TEER >250 Ω cm ² Lucifer Yellow: <2% transport	³ H-Digoxin 5000	HBSS pH 7.4 +10 mM HEPES <1% DMSO	100/600	0/120	~50	Ketoconazole 25

FBS, fetal bovine serum; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; MEM, modified Eagle's medium; NEAA, nonessential amino acids; pen-strep, penicillin-streptomycin.
^a Preincubation in presence of inhibitor.

TABLE 2
Experimental details by company (LLC-PK1-MDR1)

Lab ID	Cell Source and Passage Number	Culture Medium	Seeding Density	Culture Time	Plate Format, Material, Pore Size, Vendor Cat. No.	Monolayer Integrity Acceptance Criteria	Substrate and Conc.	Assay Buffer and Solvent Content	Volumes AP/BL	Pre-incubation ^a / Assay time	Plate shaking	Positive control and Conc.
09	Netherlands Cancer Institute p. 8-32	M199, 10% FCS, 1% 10,000 IU/ μ g per ml pen-strep, 100 ng/ml colchicine	cells/well 150,000	4	Coming no. 3399 24-well, PC, 3.0 μ m	TEER >300 Ω cm ² Lucifer Yellow: <2.5 $\times$ 10 ⁻⁶ cm/s	³ H-Digoxin 15	M199 <0.5% DMSO	300/700	0/120	No	GF120918 0.5 μ M
		ng/ml colchicine				Texas Red: <6% transport	³ H-Digoxin 100	HBSS pH 7.4 +10 mM HEPES <1% DMSO	500/500	0/180	No	CyclosporineA 10
10	Netherlands Cancer Institute p. 10-30	M199, 10% FBS, 1% PSG ^c	150,000	4	Millipore no. PSRP010R5 24-well, PET, 1.0 μ m							
08	BD Gentest cat. no. 450211 p. 14-31	M199, 7% FBS, 0.05 mg/ml gentamycin, 0.3 mg/ml L-glutamine, 0.1 mg/ml hygromycin B ^b	40,000	7	BD Falcon no. 351181 24-well, PET, 1.0 μ m	TEER >150 Ω cm ² Lucifer Yellow: <2% transport	³ H-Digoxin 5000	HBSS pH 7.4 +10 mM HEPES <1% DMSO	100/600	0/90	~50	Ketoconazole 25
		0.05 mg/ml gentamycin, 0.1 mg/ml hygromycin B ^b										
11	BD Gentest cat. no. 450211 p. 26-37	M199, 7% FBS, 0.05 mg/ml gentamycin, 0.1 mg/ml hygromycin B ^b	100,000	5-7	Coming no. 3401 12-well, PC, 0.4 μ m	TEER >400 Ω cm ² Mannitol: <2.0 $\times$ 10 ⁻⁶ cm/s	³ H-Digoxin 20	HBSS pH 7.4 +10 mM HEPES +25 mM Glucose <1% DMSO	500/1000	0/90	60-90	Verapamil 50

For additional abbreviations, see Table 1.
^a Preincubation in presence of inhibitor.
^b Hygromycin B added to culture flasks, but not monolayers.
^c Penicillin Glutamine Streptomycin.

TABLE 3
Experimental details by company (MDCKII-MDR1)

Lab ID	Cell Source and Passage Number	Culture Medium	Seeding Density <i>cells/well</i>	Culture Time <i>days</i>	Plate Format, Material, Pore Size, Vendor Cat. No.	Monolayer Integrity Acceptance Criteria	Substrate and Conc.	Assay Buffer and Solvent Content	Volumes AP/BL μ l	Preincubation/ Assay time <i>min</i>	Plate shaking <i>rpm</i>	Positive control and Conc.
07	Netherlands Cancer Institute p. 13-33	DMEM, 10% FBS, 1X NEAA, 1X L-glutamine, 1X Pen-Strep	214,000	3	BD Falcon no. 351181 24-well, PET, 1.0 μ m	TEER >300 Ω cm ²	³ H-Digoxin 39 nM	HBSS pH 7.4 +25 mM HEPES 1% DMSO	200/700	30/90	90	GF120918 2
04	Optivia p. 5-30	DMEM, 10% FBS, 1X Pen-Strep	150,000	3	Millipore no. PSRP010RS 24-well, PET, 1.0 μ m	TEER >90 Ω cm ²	³ H-Digoxin 100	HBSS pH 7.4 0.5% DMSO	250/500	30/120	60	Verapamil 100
06	National Cancer Institute p. X - 20	DMEM, 10% FBS + 1X L-glutamine, 1X pen-strep, Fungizone	175,000	7	Corning no. 3396 24-well, PC, 0.4 μ m	Lucifer Yellow: <5% transport	³ H-Digoxin 100	HBSS pH 7.4 +10 mM HEPES 1% DMSO	100/600	30/60	No	Verapamil 250
05	National Cancer Institute p. 29-36	DMEM, 10% FBS	52,000	4	Millipore no. PSRP010RS 24-well, PET, 1.0 μ m	Lucifer Yellow: <0.5 $\times$ 10 ⁻⁶ cm/s	Digoxin 5000	HBSS pH 7.4 +10 mM HEPES 1% DMSO	400/1000	0/60	No	GF120918 2
01/02	Netherlands Cancer Institute p. 36	DMEM + glutamax, 10% FBS, 50 IU/ml penicillin, 50 μ g/ml streptomycin	160,000	3-4	BD Falcon no. 351181 24-well, PET, 1.0 μ m	Lucifer Yellow: <5 $\times$ 10 ⁻⁶ cm/s	³ H-Digoxin 120	DMEM w/o pyruvate + L-glutamine + 25 mM HEPES + pyridoxine-HCL 1% DMSO	450/1300	30/90	~300	GF120918 2
03	Netherlands Cancer Institute p. 10 - 20	DMEM + glutamax, 10% FBS, 100 IU/ml penicillin, 100 μ g/ml streptomycin	450,000	3	Corning no. 3401 12-well, PC, 0.4 μ m	TEER >150 Ω cm ² Mannitol: <2.0 $\times$ 10 ⁻⁶ cm/s	³ H-Digoxin 50	HBSS pH 7.4 +25 mM HEPES <1% DMSO	500/1800	15-30/120	60	Ketoconazole 100

For abbreviations, see Table 1.
" Preincubation in presence of inhibitor.

TABLE 4
Experimental details by company (Vesicles)

Lab ID	Vesicle Source	Filtration Method (Vac. Manifold, Cell Harvester, etc.)	Protein/Well	Assay Temperature	Filter Plate Plus Cat. No.	Signal-to-Noise Acceptance Criteria	Substrate and Conc.	Assay Buffer and Solvent Content	Pre-incubation/ ^a Assay Time	Plate Shaking	Positive Control Inhibitor
23	In-house	Vacuum manifold (Millipore)	mg 50	37°C	Millipore, MSFBN6B50, MultiScreen HTS96-well, GF/B, 1.0 µm	>6-Fold difference between ATP+ and ATP-	³ H-NMQ ^a 1	250 mM sucrose, 10 mM TrisHCl, 10 mM MgCl ₂ pH 7.0, +/- 4 mM ATP, 1% DMSO	5/2	Yes	N/A
26	In-house and Solvo	96-Channel cell harvester (PerkinElmer)	20 Lilly 40 Solvo	37°C	PerkinElmer; 600517796-well GF/B, 1.0 µm	AMP-PNP value = inhibitor control	³ H Vinblastine 0.1	250 mM sucrose, 10 mM TrisHCl, 10 mM MgCl ₂ pH 7.4 with 4 mM ATP, ATP with 5 µM LSN335984, or 4 mM AMP-PNP, 0.5% DMSO	10/2.5	Yes	LSN335984, (5µM)
22	Solvo	Vacuum manifold (Millipore)	50	37°C	Millipore, MSFBN6B10, MultiScreen 96-well, GF/B, 1.0 µm	>3-Fold difference between ATP+ and ATP-	NMQ 2	250 mM sucrose, 10 mM TrisHCl, 10 mM MgCl ₂ pH 7.0, +/- 4 mM ATP, 1% DMSO	15/3	No	Ketoconazole 8 pt IC ₅₀
21	Solvo	Vacuum manifold (Millipore)	50	37°C	Millipore, MSFBN6B50, MultiScreen HTS 96-well, GF/B, 1.0 µm	>3-Fold difference between ATP+ and ATP-	³ H-NMQ 1	Assay buffer (and all other buffers) from PREDIVEZ Vesicular Transport Kit (SOLVO)	15/2	Yes	Vinblastine or erythromycin 4 pt IC ₅₀
20	Solvo	Vacuum manifold (Millipore)	50	37°C	Millipore, MSFBN6B50, MultiScreen HTS 96-well, GF/B, 1.0 µm	>6-Fold difference between ATP+ and ATP-	³ H-NMQ 2	+/- 4 mM ATP, 1% DMSO 250 mM sucrose, 10 mM TrisHCl, 10 mM MgCl ₂ pH 7.0, +/- 4 mM ATP, 1% DMSO	15/3	No	N/A

For abbreviations, see Table 1.
^a NMQ, N-methylquinidine.

TABLE 5
Equations used to calculate remaining transport activity in presence of inhibitor in cell models

Eq. No.	Name	Equation ^b	Reference
A1 ^a	ER	$1 - [(BA0 / AB0) - (BAi / ABi)] / [(BA0 / AB0) - (BAp / ABp)]$ can be written as: $[(BAi / ABi) - (BAp / ABp)] / [(BA0 / AB0) - (BAp / ABp)]$	Balimane et al. (2008)
A2	ER (FDA)	$(BAi / ABi - 1) / (BA0 / AB0 - 1)$	US FDA/CDER (2006)
B1	Unidirectional flux (B>A)	$(BAi - BAp) / (BA0 - BAp)$	Tang et al. (2002)
B2	Unidirectional flux (A>B)	$(ABi - ABp) / (AB0 - ABp)$	Tang et al. (2002)
C1	KP (A>B)	$(ABi / AB0)[(ABi - ABp) / (AB0 - ABp)]$	Kalvass and Pollack (2007)
C2	KP (B>A)	$(BA0 / BAi)[(BAi - BAp) / (BA0 - BAp)]$	Adapted from Kalvass and Pollack (2007)
D	Net-secretory-flux	$(ABi - BAi) / (AB0 - BA0)$	Balimane et al. (2008)

ER, efflux ratio.

^a Equation A2 is the FDA-recommended equation (US FDA/CDER, 2006). For this particular initiative, due to the limited number of inhibitor concentrations in the cell line experiments, the participants were asked to fit data to eq. A1 so that a no inhibitor control and positive control inhibitor could be included as data points (see *Materials and Methods* section).

^b ABi Receiver A>B with inhibitor; BAi Receiver B>A with inhibitor; AB0 Receiver A>B, without inhibitor; BA0 Receiver B>A, without inhibitor; ABp Receiver A>B with positive control; BAp Receiver B>A with positive control.

or B>A) transport of the P-gp probe substrate digoxin across polarized Caco-2, LLC-PK₁-MDR1, or MDCKII-MDR1 cell monolayers was assessed in the absence and presence of six increasing concentrations of various inhibitors. Digoxin concentrations used in these systems were 13 nM to 10 μ M. A positive control inhibitor was included in each assay at a single concentration. Assay buffer containing digoxin and inhibitor (if applicable) was added to the donor compartment, and assay buffer containing inhibitor (if applicable) was added to the receiver compartment. Monolayers were then incubated at 37°C for 45–180 minutes, at which point samples were taken from both donor and receiver compartments. Digoxin concentrations in each sample were determined by scintillation counting or liquid chromatography followed by tandem mass spectrometry (LC-MS/MS).

Vesicle Uptake Assays

Assay conditions varied between participating laboratories (Table 4). Briefly, the ATP-dependent uptake of either 1–2 μ M *N*-methylquinidine or 0.1 μ M vinblastine into inside-out membrane vesicles was assessed in the absence and presence of seven different concentrations of inhibitors listed above. The reaction mixtures without ATP or in the presence of 5'-adenylylimidodiphosphate (AMP-PNP, a non-hydrolyzable analog of ATP) were used as a negative ATP control (no ATP-dependent transport activity). The reaction mixtures were incubated at 37°C for 2–3 minutes. The reaction was then stopped by addition of ice-cold sucrose buffer and vesicles were collected onto a filter plate. The substrate concentration in each sample was determined by either scintillation counting or LC-MS/MS. Digoxin was not used as a P-gp probe substrate in vesicles due to a low signal-to-background ratio potentially resulting from binding of digoxin to the extracellular domain of the Na⁺/K⁺ ATPase (the pharmacological target for digoxin) in right-side-out membrane vesicles.

Data Analysis

Since previous inhibition data were available in the literature, the number of inhibitor concentrations used for the cell-based experiments was limited to six. Two additional data points were provided by the “positive control inhibitor” and the “no inhibitor” controls. For the vesicle-based experiments seven inhibitor concentrations were used. Each participant was provided with an Excel template for standardized data collection and automated calculation of the fraction transport activity remaining as a function of increasing inhibitor concentration using several different published transport activity equations for the cell lines (Table 5) and a single equation for the vesicles. The cell line template also generated an interpolated IC₅₀ value for immediate assessment of the data.

Data points associated with inhibitor concentrations that showed limited solubility in the assay buffer (as evident by visual inspection) and/or toxicity to the cells (as evident by failing a monolayer integrity test such as elevated permeability of a paracellular low permeability marker) were excluded from the analysis.

Probe Substrate Transport. For the cell monolayer experiments, digoxin transport was expressed as P_{app} (apparent permeability) and calculated as follows (Tran et al., 2004):

$$P_{app} = \frac{V_R C_R(t)}{A t C_D} \quad (1)$$

where:

P_{app} is the apparent permeability in cm/s

V_R is the volume of the receiver compartment (ml)

C_R is the concentration in the receiver solution at the end of the incubation (μ M)

C_D is the initial concentration in the donor solution (μ M)

A is the surface area of insert filter membrane (cm²)

t is the time interval over which permeability is measured (s).

For the vesicle experiments, the ATP-dependent uptake of *N*-methylquinidine or vinblastine was determined by subtracting probe substrate uptake in incubations without ATP or with AMP-PNP (nonhydrolyzable analog of ATP) from that in the presence of ATP. Probe substrate uptake was expressed as pmol/min per milligram vesicle protein or as ATP-dependent uptake clearance (μ l/min per milligram).

Inhibition of Probe Substrate Transport. For cell monolayer data, the fraction of transport activity remaining in the presence of inhibitor was initially determined using six different transport activity equations discussed in the recent literature (Table 5, eqs. A1, B1, B2, C1, C2, and D). The final analysis was performed using eqs. A1, B1, C1, and D only. For membrane vesicle data, the fraction of transport activity remaining in the presence of inhibitor was determined as the ratio of ATP-dependent uptake in the presence of inhibitor divided by that in the absence of inhibitor.

IC₅₀ Determinations. IC₅₀ calculations were not performed if a compound did not inhibit probe substrate transport, or if inhibition did not exceed 50% at the highest inhibitor concentration tested. As a first analysis step, IC₅₀ values for cell monolayer data were determined by linear interpolation in Excel as follows:

$$IC_{50}(\text{interpolated}) = \frac{x_1(0.5 - y_2) - x_2(0.5 - y_1)}{y_1 - y_2} \quad (2.1)$$

where:

y_1 is relative P-gp inhibition calculated by a particular equation when the inhibitor concentration is x_1 (μ M)

y_2 is relative P-gp inhibition calculated by a particular equation when the inhibitor concentration is x_2 (μ M)

and $y_1 > 0.5 > y_2$ and chosen to be closest to 0.5.

This linear interpolation control was put in place to have an easy check on the IC₅₀ values obtained by nonlinear regressions submitted by each of the participants.

Nonlinear regression analysis was performed by participants using various commercially available software packages, including Grafit (Erithacus Software Ltd., East Grinstead, UK), GraphPad Prism (GraphPad Software Inc., La Jolla, CA), Origin (OriginLabs Co, Northampton, MA), Sigma Plot (SPSS Inc., Chicago, IL), WinNonlin (Pharsight Corporation, Raleigh-Durham, NC), Kaleida Graph (Synergy Software, Reading, PA), and XLfit (ID Business Solutions Ltd., Guildford, UK). All data (fraction of transport activity remaining in the presence of inhibitor) were fitted to the standard IC₅₀ equation or Hill equation:

$$A([I]) = A(\infty) + \frac{A(0) - A(\infty)}{1 + \left(\frac{[I]}{IC_{50}}\right)^\beta} \quad (2.2)$$

where:

$A([I])$ is the activity measured when the inhibitor concentration is $[I]$

$A(\infty)$ is remaining transport when P-gp is completely inhibited by the positive control inhibitor, e.g., GF120918 or in membrane vesicles represents uptake activity in the absence of ATP or presence of AMP-PNP.

$A(0)$ is the activity measured in absence of inhibitor (the negative control)

IC_{50} is the inhibitor concentration at which $A([IC_{50}]) = [A(0) + A(\infty)]/2$

β is slope factor or Hill coefficient.

For a 4-parameter fit, IC_{50} , β , $A(0)$, and $A(\infty)$ were independently and simultaneously fitted. For a 3-parameter fit, the positive control $A(\infty)$ was fixed and IC_{50} , β , $A(0)$ were independently and simultaneously fitted. For a 2-parameter fit $A(0)$ and $A(\infty)$ were fixed and IC_{50} and β were independently and simultaneously fitted.

Statistical Analysis

Variance Component Analysis of Digoxin Transport in the Absence of Inhibitor. The purpose of this analysis was to evaluate variability between cell lines and laboratories with respect to transport of digoxin in the $A > B$ and $B > A$ directions, as well as digoxin efflux ratios. For the variance component analysis of digoxin transport, an analysis was performed using cell lines and laboratories as random effects. Each of the experiments, which encompassed differences in experimental protocols (Table 1–3), was included as a fixed effect. In this analysis, random effects for interactions between cell lines by experiment and laboratories by experiment were also investigated. Replicates, which included well-to-well variability and measurement variability, were used to determine the overall error in the model. Each response was evaluated using this model separately.

Evaluation of the Quality of the IC₅₀ Fit. Data submitted by participants were of variable quality. To ensure that only robust IC₅₀ values were used for further analysis, an objective statistical test designated as the “t-statistic” was developed to select data sets as follows. IC₅₀ values for all $B > A$ and $A > B$ transport inhibition data and vesicle uptake inhibition data were fitted by one of the participants (without prior data transformation to calculate remaining transport activity, Table 5) to a different version of eq. 2.2, namely eq. 2.3, using 2-, 3-, and 4-parameter fits.

$$A([I]) = A(\infty) + \frac{A(0) - A(\infty)}{1 + \exp\{\alpha + \beta \ln\{[I]\}\}} \quad (2.3)$$

$$IC_{50} = \exp\{-\alpha/\beta\}$$

The slope factor β is defined by the slope of the inhibition curve at the IC_{50} and α is defined as the IC_{50} “locator”. We show this version of eq. 2.2 because the t-statistic developed to score the quality of the fitted data utilizes β , α , and their corresponding SEs.

All replicates were used as input data, rather than just the average transport at each inhibitor concentration. Each company’s positive and negative control data (e.g., transport in the presence of a prototypical P-gp inhibitor to define maximal inhibition, and transport in the absence of inhibitor to define no inhibition, respectively) were incorporated as data points in the fits. The incorporation of the positive and negative controls into the fits was performed because a significant number of inhibition plots lacked well defined upper and lower plateaus (i.e., sigmoidicity), using only the six or seven inhibitor

concentrations without the positive and negative controls. Nearly all of the data used inhibitor concentration ranges with roughly 3-fold differences between concentrations. Empirically, it was found that placing the negative control at 3-fold below the lowest inhibitor concentration and the positive control at 9-fold above the highest inhibitor concentration gave fits that did not bias the IC₅₀ values fitted, i.e., the controls did not exert undue influence over the IC₅₀ estimates. If the negative or positive control was placed too close to the nearest inhibitor concentration (<3-fold lower or 9-fold higher, respectively), the fitting routine did not recognize the controls as defining the appropriate plateau. If the negative or positive control was placed too far from the nearest inhibitor concentration (>3-fold lower or 9-fold higher, respectively), the fitted parameters depended strongly on the exact placement of the negative and positive controls. Thus, the placement of the negative and positive controls, if not done properly, could influence the IC₅₀ fits of those data sets lacking an inhibitor concentration defined plateau. Further justification for this empirical treatment was provided by a core panel of 10 participants (described below, in this same section). This panel did not include the single participant who performed all the fits described in this paragraph.

The IC_{50} is defined by both α and β (eq. 2.3), while the Hill coefficient is defined by β . The SE of α and β will depend on the spread in the data as well as the number of data points on the upper plateau (no inhibition), lower plateau (complete inhibition), and on the slope. The statistical analysis was developed based on the Student t-statistic for each fitted parameter (α and β) (eqs. 3.1 and 3.2):

$$t_\alpha = \frac{\alpha}{SE_\alpha} \quad (3.1)$$

$$t_\beta = \frac{\beta}{SE_\beta} \quad (3.2)$$

The SE and the t-statistic are inversely related; the smaller the SE, the larger the t-statistic for α and β , which corresponds to a better fit. Since the goodness of the IC₅₀ fit depends on both α and β terms, a joint statistic is defined as their geometric average

$$t_{\alpha\beta} = \sqrt[2]{(t_\alpha t_\beta)} \quad (3.3)$$

During development of the t-statistic it was found that the optimal statistical analysis was based on the combined t-statistics of a 2- and 3-parameter fit of the same data set (eq. 2.3). The respective t-statistics are denoted as $t_{\alpha\beta}(2p)$ or $t_{\alpha\beta}(3p)$. A joint combined t-statistic for the 2- and 3-parameter fits was defined as:

$$t_{\alpha\beta}(2p*3p) = \sqrt{[t_{\alpha\beta}(2p)][t_{\alpha\beta}(3p)]} \quad (3.4)$$

The t-statistic ($t_{\alpha\beta}(2p)$, $t_{\alpha\beta}(3p)$ or $t_{\alpha\beta}(2p*3p)$) is a descriptor of the quality of the curve fit; that is, the more sigmoidal the curve (i.e., clearly defined plateaus) and the smaller the standard error between replicates, the larger the t-statistic, and correspondingly, the better the fit. Since there was no clearly determined statistically significant “P value” for this novel statistic, a cut off value was established by group consensus as follows: statistical $t_{\alpha\beta}$ values for the 2- and 3-parameter fits, denoted as $t_{\alpha\beta}(2p)$ and $t_{\alpha\beta}(3p)$, respectively (eq. 3.3), as well as the combined statistic for the 2- and 3-parameter fits denoted as $t_{\alpha\beta}(2p*3p)$ (eq. 3.4) were generated for each fit. A core panel of ten participants evaluated the amassed best-fit IC₅₀ curves and identified a cut-off value of $t_{\alpha\beta}(2p*3p) > 3$ for datasets of sufficient quality for final evaluation. Data sets for which the $t_{\alpha\beta}(2p*3p) < 3$ were excluded from further analysis. This panel evaluation and calibration of the t-statistic justifies the empirical treatment of inclusion and placement of negative and positive controls at one-third of the lowest inhibitor concentration and at nine times the highest inhibitor concentration, respectively. In cases where this placement of the controls was not in accord with the six or seven inhibitor concentrations and the controls exerted undue influence over the IC₅₀ fits, the resulting t-statistic is low (poor quality fit).

After this t-statistic was derived and used in Matlab (MATLAB Version 7.11.0.584 (R2010b)), we found that obtaining α and SE_α from the common fitting software packages was difficult. Obtaining SE_β was easy and direct, since β is the slope factor. Subsequently it was shown that a t-statistic defined as

$$t_{\beta\beta}(2p*3p) = \sqrt{[t_{\beta\beta}(2p)][t_{\beta\beta}(3p)]} \quad (3.5)$$

gave essentially the same values as $t_{\alpha\beta}(2p3p)$ and can be used to assess data quality. Here β is used as a surrogate for α . The terms $t_{\beta\beta}(2p)$ and $t_{\beta\beta}(3p)$ are surrogates for $t_{\alpha\beta}(2p)$ and $t_{\alpha\beta}(3p)$ in eq. 3.4. We found only a few cases in all of the curated data where $t_{\alpha\beta}(2p*3p) < 3 < t_{\beta\beta}(2p*3p)$.

The t-statistic based on SE_{β} is straightforward to implement. It is easily available in common software packages used for logistic regression and is estimated similarly in different software packages, unlike the standard error of the IC₅₀ estimate. Additionally t_{β} (as well as t_{α}) should be (nearly) unaffected by data transformations including those used to perform 2-, 3-, and 4-parameter logistic fits—unlike the root-mean-square error (RMSE) and other measures of residual error. The t-statistics are strongly, though not necessarily linearly, related to other measures of fit quality such as the standard errors of α , β , and $\log(IC_{50})$, and measures of residual error such as RMSE (root-mean-square error) and r^2 . A detailed analysis of this relationship will be presented in a future publication (O'Connor et al., personal communication).

There were several cases where the $IC_{50} \approx 1 \mu M$, in which case $\ln\{IC_{50}\} \approx 0$, which caused the t-statistic to be artifactually small. This was alleviated by fitting the data using units of molar (M), rather than μM .

Variance Component Model Analysis of IC₅₀ Values. The variance component analysis represents a nested design, in which the laboratories are completely nested within the systems (that is each laboratory used only one single P-gp expression system). Furthermore, each laboratory contributed a single IC₅₀ value, but for multiple inhibitors. For the variance component analysis of the IC₅₀ data, an analysis was performed using cell lines and laboratories as random effects and experiment (each time an IC₅₀ value was determined) as a fixed effect. This is a commonly used analysis for determining the components of variability. In this analysis, experiment encompassed differences in the experimental protocols, as well as the additional variability due to the use of multiple inhibitors. For the IC₅₀ data, only a single value was calculated and reported for each cell, laboratory, and inhibitor combination. No further tests could be done on interactions. Replicates included random effects due to cell-by-inhibitor interactions, laboratory-by-inhibitor interactions, well-to-well variability, and measurement variability. Each response was evaluated using this model separately. Values below 1 and above 1000 were omitted from the analysis, and the IC₅₀ values were logged using a natural log transformation as the raw data did not appear normally distributed. A similar analysis was also performed to determine the source of variability when considering all systems together, cell lines, and vesicles.

Principal Component Analysis. Principal component analysis (PCA) identifies patterns in complex data sets and expresses the data highlighting similarities and differences in the underlying components. The PCA is like a least squares fit in multiple dimensions. In a least squares regression the straight line fitted is chosen to maximally explain the variance of the data. Similarly, the PCA chooses the first axis through a “cloud” of data points in the direction that explains the largest variance in the data. The second axis is orthogonal to the first and chosen to explain most of the residual variance in the data. For the robust PCA analysis a complete set of IC₅₀ values is needed from each participant. Therefore, unlike the variance component analysis, the PCA analysis was limited to IC₅₀ values from eight inhibitors for a total of 24 data sets, using cell line-based as well as vesicle-based IC₅₀ values. PCA analysis was performed using the algorithm for PCA via singular value decomposition (SVD) provided by Press et al. (2007) and run in Matlab. The results were compared against PCA performed in standard programs like SAS and R with identical results.

Results

The process of data generation and analysis by the “P-gp inhibition working group” is illustrated in Fig. 1. Experimental data for P-gp inhibition by 16 compounds reported to be inhibitors of P-gp, were generated by all participating laboratories using their respective established methods (Tables 1–4). In the cell-based experiments, each participant used digoxin as the probe substrate, whereas *N*-methylquinidine or vinblastine was used in the vesicle-based experiments. The inhibition experiments were performed in a protein-free assay buffer.

The primary difference in methodology between the laboratories was the P-gp expression system used, i.e., three different cell lines and inverted membrane vesicles. There were also differences in other experimental conditions: 1) those potentially affecting the properties of the cells, such as cell line origin (in case of the MDCKII-MDR1 and LLC-PK₁-MDR1 cells), cell maintenance procedures, cell passage number, seeding density, growth media, and time in culture, or the properties of the vesicles, such as cell source and procedure for vesicle preparation; and 2) those conditions applying to the inhibition experiment, such as assay buffer, probe substrate and probe substrate concentration, pre-incubation with inhibitor, and plate shaking. Since captopril did not inhibit P-gp in any of the systems used it was not included in the analysis of P-gp IC₅₀ variability.

Probe substrate transport data (P_{app} values for cell models and uptake values for vesicles) were entered into a standardized Excel spreadsheet to calculate fraction P-gp activity remaining as a function of inhibitor concentration. The use of a standardized Excel spreadsheet to capture raw data proved invaluable in the data checking and analysis process. The P-gp activity in the respective cell lines was calculated using various equations from the literature that were incorporated in the spreadsheet (Table 5). Subsequently, the inhibition data were subjected to a statistical test (t-statistics) to ensure data were sufficiently robust for IC₅₀ fitting. Final IC₅₀ values were then determined using nonlinear regression by each of the participants for each of the transport activity equations. The standardized Excel sheet also estimated an initial IC₅₀ value for each transport activity equation by linear interpolation of the inhibition data, providing a means to verify the fitted IC₅₀ value derived from each of the transport activity equations. Detailed results of each step are outlined below.

Transport of Probe Substrates. Table 6 summarizes digoxin P_{app} values in the B>A and A>B transport direction, in the absence and presence of a positive control P-gp inhibitor, across the three cell lines in the various participating laboratories. Donor chamber digoxin concentrations varied between participants, from 13 nM to 10 μM . Initial experiments performed by several participants demonstrated that the P_{app} value for digoxin was independent of donor concentration in the range used here (unpublished data). Substantial interlaboratory variability was observed in the Caco-2-derived data. Variability in TEER values, paracellular permeability, and transporter expression in Caco-2 cells has been noted previously (Walter and Kissel, 1994, 1995) and has been ascribed to cell source, passage number, and culture conditions (Behrens and Kissel, 2003; Hayashi et al., 2008; Volpe, 2008; Miliotis et al., 2011). Less variability in digoxin transport was observed across MDCKII-MDR1, and even less with the LLC-PK₁-MDR1 cell lines. There were also fewer participants in the MDCKII-MDR1 and LLC-PK₁-MDR1 groups compared with the Caco-2 group. Variance component analysis using cell lines and laboratories as random effects and each experiment measuring digoxin transport as fixed effect shows that the largest source of variability in A>B and B>A transport is the laboratory-to-laboratory variability (~80%), rather than a difference between the three cell lines ($\leq 9\%$) (Table 7). This is true for efflux ratios as well, with 62% of the variance ascribed to laboratory-to-laboratory variability and $\leq 8\%$ to a difference between the cell lines (Table 7). Table 8 summarizes uptake of the P-gp probe substrates *N*-methyl quinidine or vinblastine into P-gp-expressing vesicles in the presence and absence of ATP. The vesicles also show substantial variability in uptake of probe substrate. This may be due in part to differences in P-gp expression systems used for vesicle preparation (e.g., differences in P-gp expression levels, membrane composition) or differences in the proportion of inside-out to right-side-out vesicles. It is also possible that differences in probe substrates (*N*-methylquinidine versus vinblastine) and differences in

Fig. 1. Flowchart illustrating the process of data generation and analysis by the P-gp inhibition working group. Since one of the 16 compounds (captopril) did not inhibit P-gp in any of the systems used it was not included in the analysis of P-gp IC₅₀ variability. Therefore the variability analysis was performed on 15 compounds.

postincubation vesicle collection and washing procedures contribute to the observed variability in ATP-dependent uptake clearance, as may differences in the thawing and handling of the vesicles prior to the assay.

Transport Inhibition. The purpose of the current study was to investigate whether this intrinsic interlaboratory difference in transport of probe substrates also results in an inter-laboratory difference in inhibition of transport, expressed as the IC₅₀ values. The 16 inhibitors used in this study were selected based on the availability of robust clinical DDI data with digoxin (Fenner et al., 2009; Cook et al., 2010) and are listed in the *Materials and Methods* section.

Data Quality. Initial visual inspection of the transport inhibition curves (~1300) generated by participants (facilitated by the standardized spreadsheets) revealed data quality aspects that needed to be addressed before further data analysis. Two common observations were 1) the “no inhibition” plateau on the left of the inhibition curve was not consistently well defined as illustrated by the transport inhibition of digoxin across Caco-2 cells by carvedilol and troglitazone in Fig. 2, A and B, respectively and 2) the “complete inhibition” plateau on the right of the inhibition curve was defined often by only the positive control inhibitor as illustrated by transport inhibition across LLC-PK₁-MDR1 cells by ranolazine and across MDCKII-MDR1 cells by verapamil in Fig. 2, C and D, respectively. The data point at the extreme left of the curve represents the digoxin P_{app} value in the absence of inhibitor, while the data point at the extreme right represents the digoxin P_{app} value in the presence of the positive control inhibitor (see *Materials and Methods*). Another issue sometimes encountered was substantial variability in the triplicate data points as shown in Fig. 2, B and C. For these reasons, the

authors implemented an objective statistical analysis (t-statistic), based on 2 and 3-parameter fits to the data using a logistic equation, eq. 2.3, that identified data sets that gave robust IC₅₀ values, as described in the *Materials and Methods* section. The t-statistics for the data shown in Fig. 2 will be discussed below, after a description of the implementation of this statistical analysis.

When scrutinizing the resulting fits to eq. 2.3, the authors found that the same primary data could result in the same or different IC₅₀ values depending on the data quality and fitting algorithm, i.e., a 2-, 3 or 4-parameter fit. An example of this is provided in Fig. 3. Two and four parameter fits (eq. 2.3) of three representative data sets of acceptable quality are shown. (A) and (B) show the inhibition of digoxin transport through MDCKII-MDR1 cells by verapamil. While the no inhibition plateau on the left is well defined and there are several points along the slope, the complete inhibition plateau is only defined by the positive control inhibitor itself. However, since the positive inhibitor control value was in accord with all the other points, the IC₅₀ values of the 2- and 4-parameter fits are quite close. (C) and (D) show the inhibition of digoxin transport in Caco-2 cells by diltiazem. This curve (and others for diltiazem) was very broad, suggesting that inhibition does not occur at either a single-binding site or a single transporter, in contrast to, for example, verapamil (Fig. 3, A and B). However, also for diltiazem, the IC₅₀ values of the 2 and 4-parameter fits are quite close (Fig. 3, C and D). (E) and (F) show the inhibition of vinblastine uptake in vesicles by quinidine. The data are nearly a straight line and the IC₅₀ values generated by a 2 and 4-parameter fit are about 2-fold different due to the uncertainty in the plateaus and absence of sigmoidicity.

TABLE 6
Digoxin transport in cell models in presence and absence of inhibitor

Lab	P _{app}				ER = (B>A/A>B)	
	A>B w/o Positive Control Inhibitor	A>B with Positive Control Inhibitor	B>A w/o Positive Control Inhibitor	B>A with Positive Control Inhibitor	ER w/o Inh	ER with Inh
	nm/s	nm/s	nm/s	nm/s	nm/s	
M01	4.6	29.6	211.7	35.3	46.1	1.2
M02	3.2	26.1	195.4	34.3	60.5	1.3
M03	5.9	28.1	106.7	24.4	18.1	0.9
M06	4.6	12.7	101.7	15.0	21.9	1.2
M07	7.0	25.4	151.3	31.5	21.7	1.2
M04	9.6	23.0	81.9	42.8	8.5	1.9
M05	0.9	4.2	50.7	15.8	59.2	3.8
Average	5 ± 4 ^a	21 ± 11 ^a	128 ± 63 ^a	28 ± 12 ^a	25 ± 18 ^b	1.3 ± 1 ^b
L08	6.3	26.5	102.6	46.3	16.4	1.7
L09	6.2	34.3	86.4	35.4	14.0	1.0
L10	5.9	35.1	90.3	48.2	15.4	1.4
L11	9.7	26.7	58.9	57.2	6.1	2.1
Average	7 ± 4 ^a	31 ± 6 ^a	85 ± 21 ^a	47 ± 13 ^a	12 ± 7 ^b	1.5 ± 0.5 ^b
C08	10.7	34.2	104.9	54.2	9.8	1.6
C15	8.8	32.1	154.8	43.8	17.6	1.4
C17	12.8	37.9	106.8	45.9	8.3	1.2
C18	11	44.1	106.1	45.2	9.7	1.0
C24	2.0	11.4	65.9	15.2	32.6	1.3
C25	10.6	24.4	116.3	26.6	11.0	1.1
C12	5.8	33.3	90.0	36.9	15.7	1.1
C13	10.2	35.5	102.8	47.1	10.0	1.3
C14	1.5	37.0	153.0	50.0	102.0	1.4
C16	36.0	113.4	310.2	125.0	8.6	1.1
C19	16.0	40.9	121.3	44.3	7.6	1.1
Average	11 ± 10 ^a	37 ± 29 ^a	120 ± 74 ^a	45 ± 31 ^a	11 ± 11 ^b	1.2 ± 1 ^b

^a SD for a single reported result (average of three replicates) at a single laboratory using variance components model.

^b Estimated SD for a single reported result (ratio of the average of three replicates) at a single laboratory (Casella and Berger, 2001). <http://www.math.umt.edu/patterson/549/Delta.pdf>.

To avoid introducing variability associated with the fitting algorithm, a 2-parameter fit was applied to all data as the upper and lower plateaus were constrained to no inhibition and complete inhibition. Since in this case upper and lower plateaus are defined rather than fitted, the resulting fits are more constrained and therefore less susceptible to variation.

Quality of the IC₅₀ Curves (Implementation of t-Statistic). To objectively determine which data sets yielded robust data for final data analysis, a statistical metric (known as the t-statistic) was developed as described in the *Materials and Methods* section. The t-statistics (t_{α} , t_{β} , $t_{\alpha\beta}$) (eqs. 3.1, 3.2, 3.3, 3.4) were used to assess the sigmoidicity of the

IC₅₀ curve fit. The statistics on α and β were selected because these coefficients were directly estimated as part of the logistic regression analysis, and were used to calculate the derived estimate of IC₅₀ values. A t-statistic $t_{\alpha\beta}(2p*3p) < 3$ cut-off was selected for exclusion of data sets.

Some examples of the application of the t-statistics are shown using Figs. 2 and 3. Fig. 2A shows the inhibition of B>A digoxin transport across Caco-2 cells by increasing concentrations of carvedilol. The t-statistic $t_{\alpha\beta}(2p*3p)$ for this data set is 2 and therefore these data are excluded from further analysis. For the 2 and 3-parameter fits of these

TABLE 7
Variance component analyses (% of the variability by source)

Response	Cell Line Data Only								All Systems
	A>B w/o Inhibitor	B>A w/o Inhibitor	Log ER ¹ w/o Inhibitor	A>B with Inhibitor	B>A with Inhibitor	Log ER ^a with Inhibitor	Log IC ₅₀ A>B ^a	Log IC ₅₀ B>A ^a	Log IC ₅₀ B>A or Uptake into Inside-Out Vesicle ^a
Cells/systems	9	0	8	7	8	0	13	14	28
Labs	77	82	62	78	79	45	35	35	29
Cell/system by experiment ^b	0	0	0	0	0	0	Not modeled	Not modeled	Not modeled
Lab by experiment ^c	9	14	20	6	8	29	Not modeled	Not modeled	Not modeled
Error	5	4	10	8	5	25	52 ^d	51 ^d	43 ^d
Total	100	100	100	100	100	100	100	100	100

^a Log ER and log IC₅₀ were used in variance component analysis since data are log normally distributed. ER, efflux ratio.

^b Interaction between experiment and cell/system (for each time the experiment was performed, this is the variability in probe substrate transport that can be ascribed to differences between the three cell lines/four experimental systems).

^c Interaction between experiment and laboratory (for each time the experiment was performed, this is the variability in probe substrate transport that can be ascribed to differences between the laboratories).

^d Variability due to replication, which in this case constitutes interactions between experiment and cell/system, experiment and laboratory, well-to-well variability and measurement variability that could not be further deconvoluted because only a single IC₅₀ value for each inhibitor was used. While the vesicle laboratories generated two sets of IC₅₀ values for most compounds, only day 1 vesicle-IC₅₀ values were included for consistency with the cell-based systems.

TABLE 8
Substrate uptake in vesicles in presence and absence of ATP

Laboratory	Vesicle source	Probe Substrate	Substrate Concentration	ATP-Dependent Uptake Rate	ATP-Dependent Uptake Clearance	Uptake Ratio +ATP/-ATP
			μM	pmol/min per milligram	$\mu\text{L/min}$ per milligram	
20	Solvo	$^3\text{H-N-Methylquinidine}$	2	514 ± 104	257 ± 52	9.4
21	Solvo	$^3\text{H-N-Methylquinidine}$	1	52 ± 31	52 ± 31	23.9
22	Solvo	N-Methylquinidine	2	6.7 ± 1.9	3.3 ± 0.93	5.9
23	Internal	$^3\text{H-N-Methylquinidine}$	1	226 ± 58	226 ± 58	15.6
26-1	Internal	$^3\text{H-Vinblastine}$	0.1	4.3 ± 2.0	43 ± 20	4.7
26-2	Solvo	$^3\text{H-Xinblastine}$	0.1	2.9 ± 1.2	29 ± 12	5.7

data the individual t-statistics were $t_{\alpha\beta}(2p) = 5.7$ and $t_{\alpha\beta}(3p) = 0.9$. The 2-parameter fit was more tolerant of the poorly defined no-inhibitor plateau on the left than the 3-parameter fit, resulting in the poor combined t-statistic. Since in a 2-parameter fit the no-inhibitor and complete-inhibition plateaus are fixed, the t-statistic for a 2-parameter fit will be equal to or greater than that for a 3-parameter fit.

As described in the *Materials and Methods* section, after data analysis was completed a slightly different t-statistic, $t_{\beta\beta}(2p*3p)$, was tested. The later statistic is more easily obtained from commonly used software packages and therefore of greater general utility. It was found that there were a few cases in our data set where $t_{\alpha\beta}(2p*3p) < 3 < t_{\beta\beta}(2p*3p)$. One of these is the carvedilol example shown in Fig. 2A. Here the $t_{\beta\beta}(2p*3p) = 4.1$, while $t_{\alpha\beta}(2p*3p) = 2$.

Fig. 2B shows inhibition of B>A digoxin transport by troglitazone across Caco-2 cells. The combined $t_{\alpha\beta}(2p*3p) = 3.7$ and $t_{\beta\beta}(2p*3p) = 3.7$ results in the inclusion of this data set for IC_{50} variability assessment. Fig. 2C shows inhibition of B>A digoxin transport by ranolazine (LLC-PK₁-MDR1 cells). The combined $t_{\alpha\beta}(2p*3p) = 5.0$ and $t_{\beta\beta}(2p*3p) = 4.9$ results in acceptance of this data set. Fig. 2D shows inhibition of B>A digoxin transport by verapamil (MDCKII-MDR1 cells). The combined $t_{\alpha\beta}(2p*3p) = 18.6$ and $t_{\beta\beta}(2p*3p) = 18.6$ was one of the highest found for the cell data, despite the fact

that the complete-inhibition plateau on the right was defined by the positive control inhibitor. This was because the replicates were very close and the well-described no inhibition plateau on the left largely defined the IC_{50} value. The amplitude of the positive control inhibitor on the right agreed with the rest of the fit.

Fig. 3 illustrates the practical meaning of the t-statistic cut-off for verapamil as measured by two laboratories in MDCK II-MDR1 cells (Figs. 3, A and B), for diltiazem measured across Caco-2 cells (Figs. 3, C and D) and for quinidine using the vesicle assay (Figs. 3, E and F).

Fig. 3, A and B data are of high quality, as judged by $t_{\alpha\beta}(2p*3p) = 18.6$ and $t_{\beta\beta}(2p*3p) = 18.6$. The IC_{50} values for both 2- and 4-parameter fits are essentially identical. The data shown in Fig. 3, C and D are acceptable with $t_{\alpha\beta}(2p*3p) = 5.5$ and $t_{\beta\beta}(2p*3p) = 5.4$. The IC_{50} values for both fits are about 1.7-fold different. The data shown in Fig. 3, E and F are also acceptable with $t_{\alpha\beta}(2p*3p) = 4.9$ and $t_{\beta\beta}(2p*3p) = 4.2$. The IC_{50} values for both fits are about 1.8-fold different, but there is a $5.8 \mu\text{M}$ difference in the absolute values. The individual statistics for the 2 and 4-parameter fits were $t_{\beta}(2p) = 6.2$ and $t_{\beta}(4p) = 0.2$, showing lesser confidence in the 4-parameter fit accounting for the different estimates of the IC_{50} values. These examples illustrate the utility of the t-statistic. The larger its value above 3, the smaller the probability that the fitted IC_{50} value is significantly wrong. While

Fig. 2. Inhibition of digoxin transport. The symbols in (A), (B), (C), and (D) are triplicate data points of inhibition of B>A digoxin transport by increasing concentrations of carvedilol (Caco-2 cells), troglitazone (Caco-2 cells), ranolazine (LLC-PK₁-MDR1 cells), and verapamil (MDCKII-MDR1 cells), respectively. The line is the 3-parameter logistic fit to the data.

Fig. 3. Example data set illustrating the impact of the selected regression model (2- or 4-parameter logistic fit) on the resulting IC₅₀ value. Symbols represent experimental data, lines represent nonlinear regression results for 2-parameter [(A), (C), (E)] or 4-parameter [(B), (D), (F)] fits. (A) and (B) show inhibition of B>A digoxin transport by increasing concentrations of verapamil (MDCKII-MDR1 cells) with $t_{\alpha\beta}$ (2p*3p) = 18.6 and $t_{\beta\beta}$ (2p*3p) = 18.6. (C) and (D) show inhibition of B>A digoxin transport by increasing concentrations of diltiazem (Caco-2 cells) with $t_{\alpha\beta}$ (2p*3p) = 5.5 and $t_{\beta\beta}$ (2p*3p) = 5.4. (E) and (F) show inhibition of vinblastine uptake by increasing concentrations of quinidine (vesicles) with $t_{\alpha\beta}$ (2p*3p) = 4.9 and $t_{\beta\beta}$ (2p*3p) = 4.2.

the data quality was assessed by the combined t-statistic on a 2- and 3-parameter fit, all of the subsequent data analysis of the curated data was performed using the IC₅₀ values obtained by a 2-parameter fit, as mentioned above.

Table 9 summarizes the result of the statistical analysis of the data quality. Overall, the data quality obtained with the vesicles was higher than with the cells. For the cells, the B>A data are more robust than the A>B data, which is probably related to the relatively low transport of digoxin in this direction and the higher associated variability.

Variability in IC₅₀ Values. The IC₅₀ values for each of the fifteen compounds in each of the four experimental systems are shown in Fig. 4 (see Supplemental Data for individual IC₅₀ values). As mentioned above, one of the 16 compounds was not included in the variability analysis (captopril) because it did not inhibit P-gp in any of the systems used. For the cell line-derived data, IC₅₀ values based on remaining transport activity eqs. A1, B1, C1 and D are included in this figure. Since a substantial fraction of the data were excluded due to unacceptable $t_{\alpha\beta}$ (2p*3p) values, the number of IC₅₀ values for each compound, system and transport activity equation in Fig. 4 is not the same. For the efflux ratio based eqs. A1 and B1, IC₅₀ values were included in the analysis only if A>B as well as B>A data were of acceptable quality ($t_{\alpha\beta}$ (2p*3p) > 3). The reason for this is the substantial influence of the A>B data on the efflux ratio. For equation D, IC₅₀ values were included if B>A data were of acceptable quality, without consideration of the quality of the A>B data. The reason for this is that the effect of the A>B data in equation D is minimal (subtraction of a small number from a large number) when digoxin is the probe substrate (see also section on equation comparison below).

As mentioned above, participants used digoxin concentrations between 13 nM and 10 μ M for the cell-based experiments. One of the participants determined IC₅₀ values in MDCKII-MDR1 cells for GF120918 and verapamil at digoxin concentrations of 30 nM, 500 nM and 10 μ M and found no difference in the resulting IC₅₀ values (data not shown).

The lowest variability in IC₅₀ values was observed for isradipine (Inhibitor 5) and sertraline (Inhibitor 12) (24- and 20-fold difference between lowest and highest IC₅₀ values) and the highest for verapamil (Inhibitor 15) and telmisartan (Inhibitor 13) (796- and 407-fold range). In these two extreme cases, the variability was greatly influenced by data from one contributor, but a different one in each case. For telmisartan, a very similar value was reproduced by the company that generated the extremely low IC₅₀ value. Ignoring these two extreme data points brings the verapamil and telmisartan variability down to 159- and 69-fold, respectively.

Transport Activity Equation Comparison. Fig. 5A shows that transport activity eqs. A1 and A2 (efflux ratio-based equations) are essentially the same. Equation A1 includes a positive and negative control value in the calculation of transport activity, whereas eq. A2 does not. Equation A2 is the FDA recommended equation (US FDA/CDER, 2006, 2012). For this particular initiative, due to the limited number of inhibitor concentrations in the cell line experiments, the participants were asked to fit data to eq. A1 so that a no inhibitor control and positive control inhibitor could be included as data points (see *Materials and Methods* section). Note in Fig. 4 that the lowest cell-based IC₅₀ values for all compounds are based on transport activity eqs. A1 (blue diamonds) and C1 (red triangles) and the highest

on eqs. B1 (green squares) and D (black circles), with the exception of Inhibitor 15 (verapamil). It has been noted previously for MDCKII-MDR1 cells as well as LLC-PK₁-MDR1 cells (Lumen et al., 2010, Sugimoto et al., 2011) that an IC₅₀ based on transport activity expressed as an efflux ratio (eq. A1 or A2) is typically lower than one based on B>A transport (B1). It is shown in Fig. 5, B and C that transport activity eqs. A1 and C1 result in very similar IC₅₀ values, as do eqs. B1 and D. By contrast, eqs. B1 and D typically give higher values than eqs. A1 and C1 (Fig. 5D, only comparison shown is for eqs. A1 and B1). Equation B1 typically results in an IC₅₀ value which is on average 3-fold higher than the IC₅₀ value resulting from eq. A1. In 23% of the cases eq. B1 generates an IC₅₀ value which is at least 6-fold higher than the eq. A1 IC₅₀ value. Therefore, some of the variability in IC₅₀ values shown in Fig. 4 is due to the use of different equations to calculate remaining transport activity. For example the highest nicardipine cell-based IC₅₀ value (based on eqs. A1, B1, C1 and D combined) was 46 and the lowest 0.3 μM, resulting in a 148-fold difference. Considering only eq. B1 the highest and lowest IC₅₀ values were 21.4 and 0.8 μM, a 27-fold difference. Another example is quinidine, with highest and lowest cell-based IC₅₀ values (based on eqs. A1, B1, C1 and D) of 78 and 0.8 μM (98-fold difference), whereas eq. B1 resulted in highest and lowest IC₅₀ values of 73 and 2.2 μM, a 33-fold difference. Taking vesicle IC₅₀ values into account as well as eq. A1, B1, C1 and D-based IC₅₀ values, the least variability was observed for sertraline, with a 20-fold difference between lowest and highest IC₅₀ values and the greatest variability with verapamil (796-fold difference). Considering vesicle IC₅₀ values and only eq. B1-based IC₅₀ values, the least variability of 6-fold was observed with troglitazone and the greatest of 406-fold with telmisartan. Hence, standardizing the cell-based IC₅₀ determination to one transport activity eq. (B1) reduces the lowestfold variability across cell- and vesicle-based assays from 20-fold (sertraline) to 6-fold (troglitazone) and the greatestfold variability from 796-fold (verapamil) to 406-fold (telmisartan). However, substantial variability remains.

Equation B1 (based on B>A transport) declines in magnitude as inhibitor is added causing the B>A([I]) flux to decline. As inhibitor

TABLE 9

Percentage of data accepted and average statistics for accepted compounds

	Group 1		Group 2	
	Carvedilol, Diltiazem, Isradipine, Mibefradil, Nicardipine, Quinidine, Ranolazine, Verapamil ^b		Amiodarone, Felodipine, Nifedipine, Nitrendipine, Sertraline, Telmisartan, Troglitazone	
	B>A	A>B	B>A	A>B
Cell-based				
% $t_{\alpha\beta}(2p*3p) > 3$	73	52	56	40
Average $t_{\alpha\beta}(2p*3p)^a$	6.7 ± 3.1	5.6 ± 2.3	5.6 ± 2.7	5.9 ± 3.3
Vesicle-based				
% $t_{\alpha\beta}(2p*3p) > 3$	88		80	
Average $t_{\alpha\beta}(2p*3p)^a$	10.5 ± 6.5		7.5 ± 4.5	

^a Average ± standard deviation for those compounds with $t_{\alpha\beta}(2p*3p) > 3$.
^b For the PCA analysis a complete set of IC₅₀ values is needed from each participant. Therefore, the PCA analysis was limited to IC₅₀ values from 8 inhibitors (group1) for a total of 24 data sets, using cell line-based as well as vesicle-based IC₅₀ values. Group 2 comprises the remaining seven compounds

is added, eq. A1 (based on efflux ratio) declines in magnitude as the B>A([I]) flux declines in the numerator, like eq. B1, but additionally declines as the A>B([I]) flux increases in the denominator. Therefore eq. A1 is declining faster than eq. B1, resulting in a lower IC₅₀ value. The smaller IC₅₀ generated by eq. A1 relative to the value resulting from eq. B1 is simply algebraic. Equation B1 (based on B>A transport) and D (based on net flux: B>A minus A>B transport) result in similar IC₅₀ values. This may not be the case for all P-gp probe substrates, but for the probe substrate digoxin this is likely due to the fact that the absolute decline of digoxin transport in the B>A direction is greater than the absolute increase in transport in the A>B direction for any given inhibitor concentration (exemplified in Table 6 for the positive control inhibitor). This minimizes the effect of the increase in A>B transport on the decline in the B>A direction and thus on the IC₅₀ value obtained for the net flux equation.

For further statistical analyses (variance component analyses and principal component analysis) we used IC₅₀ values obtained without

Fig. 4. IC₅₀ values for 15 compounds in the four experimental systems. For cell-based data, IC₅₀ values were based on eqs. A1, B1, C1, and D to calculate P-gp transport activity. The numbers on the x-axis refer to inhibitors as follows: amiodarone (1), carvedilol (2), diltiazem (3), felodipine (4), isradipine (5), mibefradil (6), nicardipine (7), nifedipine (8), nitrendipine (9), quinidine (10), ranolazine (11), sertraline (12), telmisartan (13), troglitazone (14), and verapamil (15). Captopril was not included because it did not inhibit P-gp in any of the systems used. Blue diamonds, cell eq. A1, green squares cell eq. B1, red triangles, cell eq. C1, black circles, cell eq. D1, black stars, vesicles. Note: each of the inhibitors has a different number of IC₅₀ values for each of the four experimental systems and for each of the four data transformation equations applied to the cell-based data. As seen in the Supplemental Data, a considerable amount of data is missing due to unacceptable t-statistics.

Fig. 5. Comparisons between IC₅₀ values for eqs. A1 and A2 [(A): since IC₅₀ values were not fitted for eq. A2, interpolated values are used for comparison with eq. A1), A1 and C1 (B), B1 and D (C) and A1 and B1 (D). The blue circles represent IC₅₀ values obtained using Caco-2 cells, the red squares MDCKII-MDR1 cells, and the red triangles LLC-PK₁-MDR1.

prior data transformation as in the transport activity eqs. A1, B1, C1 and D. This data transformation results in propagation of error (when subtracting or dividing two numbers, each with associated error), which can be avoided by simply applying the IC₅₀ fitting algorithm to untransformed data (Papp values in presence of inhibitor with positive control inhibitor and no inhibition controls included as data points in the fits), as described in the materials and methods section under “Evaluation of the Quality of the IC₅₀ Fit.” This particular data treatment and IC₅₀ fitting protocol results in the largest, most robust IC₅₀ data set. The resulting IC₅₀ values will be most similar to those based on unidirectional flux transport activity equations, the only difference being data normalization prior to IC₅₀ fitting. Standardizing the cell-based IC₅₀ determination to eq. 2.3 applied to untransformed data reduces the lowestfold variability across cell- and vesicle-based assays from 20-fold (sertraline) to 7-fold (troglitazone) and the greatestfold variability from 796-fold (verapamil) to 354-fold (telmisartan).

Variance Component Analysis. The variance component analysis represents a nested design, where the laboratories are completely

nested within the systems. Experimental system and laboratory were used as random effects. The effect of inhibitor was accounted for as a fixed effect. Each laboratory was asked to complete testing on all inhibitors. This is a commonly used analysis for determining the components of variability. In Figs. 6, A and B, the large blue rectangles represent the variability across systems and the small red rectangles represent variability across laboratories. Therefore, it is easy to see that systems, especially the cell-based systems contribute little variability, whereas laboratory-to-laboratory variability, even within the same system, is higher.

For cell-based IC₅₀ values obtained for transport inhibition in both A>B and B>A directions the largest source of variability was interlaboratory differences, 35% in either direction (Table 7 and Fig. 6, A and B). The variability due to the use of different cell lines is substantially smaller at 13% and 14%, respectively. The remaining variability (51–52%) was due to replication, which in this case constitutes interactions between experiment and cell, experiment and laboratory, well-to-well variability, and measurement variability,

Fig. 6. Variability plot of cell line-derived Ln IC_{50} values calculated for transport inhibition in the A>B direction (A) and of cell line and vesicle-derived Ln IC_{50} values for transport inhibition in the B>A (or in the case of vesicles B>A equivalent) direction (B). All IC_{50} values were calculated by eq. 2.2. Each of the data points in the graphs represents a Ln IC_{50} value. The range of values within each system is represented by the blue outline. The range of values within each laboratory is represented by the red outline. The large blue rectangles represent the variability across systems and the small red rectangles represent variability across laboratories.

and could not be further deconvoluted because only one IC_{50} value is reported by each company for each inhibitor. In addition to the laboratory-to-laboratory variability in absolute IC_{50} values, the rank-ordering of inhibitor potency also varied from laboratory to laboratory (Fig. 6, A and B). When vesicle- and cell-based (B>A only) IC_{50} values were combined, variance component analysis demonstrated that laboratory-to-laboratory variability remained high at 29% and was approximately equal to system variability at 28%, while variability due to replication was 43% (Table 7 and Fig. 6B).

Principal Component Analysis. As mentioned in the *Materials and Methods* section, for a robust PCA analysis a complete set of IC_{50}

values is needed from each participant. Consequently, the PCA analysis performed here did not include all inhibitors. Furthermore, the PCA analysis was only performed for IC_{50} values based on inhibition of B>A transport. Table 10 shows axes 1–8 for the PCA performed on eight compounds evaluated in four systems (Caco-2, MDCKII-MDR1, LLC-PK₁-MDR1 cells, and vesicles) with IC_{50} values contributed from all but two laboratories. Axis 1 explains 54% of the variance in the data, axis 2 explains 14%, and axis 3 explains 13%, i.e., the first three axes explain greater than 80% of the variance. The remaining axes each explain <10% of the variance in the data. Notably in axis 1, the weights (i.e., the values of the eigenvectors) of

TABLE 10
Principal Component Analysis

Axis	1	2	3	4	5	6	7	8
Eigenvalue	1.0429	0.28	0.2554	0.1336	0.0989	0.0579	0.0516	0.0289
Fraction variance	0.535	0.1437	0.131	0.0685	0.0507	0.0297	0.0265	0.0149
Cumulative fraction variance	0.535	0.6787	0.8097	0.8783	0.929	0.9587	0.9851	1
Drug	Eigenvectors							
Carvedilol	0.383	-0.093	0.184	-0.072	0.241	-0.473	-0.252	-0.678
Diltiazem	0.361	0.102	-0.776	0.113	-0.424	-0.03	0.136	-0.212
Isradipine	0.279	0.072	-0.115	-0.871	0.103	-0.152	0.068	0.327
Mibefradil	0.243	0.583	0.5	-0.094	-0.515	0.223	0.055	-0.156
Nicardipine	0.347	-0.701	0.15	-0.109	-0.264	0.495	-0.195	-0.023
Quinidine	0.421	0.177	0	0.335	0.042	-0.163	-0.606	0.532
Ranolazine	0.299	0.285	-0.135	0.058	0.638	0.612	0.077	-0.142
Verapamil	0.446	-0.178	0.243	0.295	0.09	-0.236	0.706	0.246

the eight drugs are all approximately 0.3, indicating that the bulk of the variance is due to the difference in the average $\log_{10}\{\text{IC}_{50}\}$ value for the eight compounds generated by each participant in one of the four systems. For axis 2, most of the weights are close to 0, apart from the maximum weight of 0.58 for mibefradil and the minimum weight of -0.70 for nicardipine. For axis 3, most of the weights are close to 0, apart from the maximum weight of 0.5 for mibefradil and the minimum weight of -0.78 for diltiazem. This indicates that the second and third largest variability observed are derived from the IC_{50} values for mibefradil, nicardipine, and diltiazem, suggesting that these compounds interact quite differently in some systems.

Basically, the PCA created a two-dimensional map of the “relatedness” between the cells according to their aggregate IC_{50} values for the eight inhibitors. Fig. 7 shows the graphical display of the PCA for axis 1 and 2. Of the four systems, the MDCKII-MDR1 and LLC-PK₁-MDR1 cell lines are closely related with respect to the average $\log_{10}\{\text{IC}_{50}\}$ values for the eight inhibitors multiplied by their Eigen values (Table 9), i.e., axis 1. The Caco-2 cell lines varied much more widely, with some Caco-2 laboratories having average $\log_{10}\{\text{IC}_{50}\}$ values similar to the MDCKII-MDR1 and LLC-PK₁-MDR1 cells and other laboratories having much lower average $\log_{10}\{\text{IC}_{50}\}$ values. The vesicles ranged broadly with the Caco-2 cells,

with the highest average $\log_{10}\{\text{IC}_{50}\}$ value just below the smallest average $\log_{10}\{\text{IC}_{50}\}$ value for the MDCKII-MDR1 and LLC-PK₁-MDR1 cell lines. While the variance component analysis quantifies the overall variation in IC_{50} values, it does not show this “relatedness” between the various systems with respect to their IC_{50} values. To gain further insight into the reason for the variation, the PCA points out which systems and which inhibitors to examine more closely.

Discussion

Given the importance of P-gp in the absorption, disposition, and excretion of drugs, it is essential to have in vitro assays that assess potential DDIs with P-gp during drug development. Currently, a variety of biologic systems are used to assess the potential for these DDIs, including membrane vesicles and nonpolarized and polarized cells expressing P-gp (either endogenously or by expression of transfected constructs encoding P-gp). The International Transporter Consortium (ITC) publication (International Transporter Consortium et al., 2010) and FDA’s 2012 draft Drug Interaction Guidance suggested the use of polarized cells, such as Caco-2 or P-gp transfected cell lines (e.g., MDCKII-MDR1), for the assessment of inhibitor interaction potential and established cut-off values for identifying when a clinical

Fig. 7. Principal component analysis. Closed blue circles, Caco-2; closed red squares, MDCKII-MDR1; open red triangles, LLC-PK₁-MDR1; open blue stars, vesicles. Laboratory numbers (also shown in Tables 1–4) are associated with each data point in the graph. Axis 1 represents the average $\log_{10}\{\text{IC}_{50}\}$ value for the eight inhibitors in group 1 (Table 9) multiplied by their eigenvalues. Axis 2 represents the second largest variance in the IC_{50} values which depends largely on mibefradil and nicardipine (Table 10).

DDI study for P-gp is warranted. However, it has not been established whether different biologic systems, or even biologic systems of similar type, such as polarized cell lines, yield similar IC_{50} values across different laboratories. Additionally, given the abundance of transport activity equations used in the literature to determine an IC_{50} value for P-gp, there has been little apparent consensus regarding how to calculate an IC_{50} value for efflux transporters expressed in polarized cell systems and whether the existing equations for doing so generate similar IC_{50} values. The potential concern created by the current decision trees for P-gp is that the variability of IC_{50} values can impact the DDI risk assessment, i.e., some biologic systems and equations may suitably identify molecules that necessitate an *in vivo* DDI study, whereas other systems may either over- or underestimate the need for conducting such a study, generating an undesirable number of false positives or false negatives. This study systemically evaluated both the biologic systems and the transport activity equations used for the *in vitro* determination of the IC_{50} values against P-gp through collaborative work among 23 companies and an academic institution studying a common set of known P-gp inhibitors. The companion article (Ellens et al., 2013) describes the derivation of new decision criteria based on a receiver operating characteristic analysis using the IC_{50} values described in this article. The companion article (Ellens et al., 2013) also describes how to incorporate P-gp IC_{50} variability in the DDI risk assessment to optimize decision making.

Substantial interlaboratory variability was observed in the probe substrate transport data as well as the transport inhibition data (IC_{50} values) across all systems and equations. Caco-2 cells are derived from a human colon carcinoma and are known to express many transporters in addition to P-gp (Lowe et al., 2003; Hilgendorf et al., 2007; Hayashi et al., 2008). MDCKII-MDR1 and LLC-PK₁-MDR1 cell lines are derived from canine and porcine kidney, respectively. Limited information is available on transporter expression in the latter two (for example Di et al., 2011). We are not aware of studies comparing expression levels of multiple transporters across all three cell lines.

The least variability in IC_{50} values submitted by the participants was seen with sertraline and the greatest with verapamil, with a 20- and 796-fold difference between lowest and highest IC_{50} values, respectively, clearly indicating the need for standardization, as suggested by Zhang et al. (2008). Standardizing the cell-based IC_{50} determination to eq. 2.3 applied to untransformed data reduces the lowestfold variability across cell- and vesicle-based assays from 20-fold (sertraline) to 7-fold (troglitazone) and the greatestfold variability from 796-fold (verapamil) to 354-fold (telmisartan). This still leaves substantial variability across cell- and vesicle-based IC_{50} values, with 13 of the 15 inhibitors showing a difference between lowest and highest IC_{50} values of at least 20-fold, and only two inhibitors showing less than a 20-fold difference.

The variance component analysis of the variability in probe substrate transport and IC_{50} values shows that much of the variance observed was due to laboratory-to-laboratory differences rather than due to implicit systematic differences between the P-gp expression systems. The PCA analysis is consistent with this observation and, more importantly, provides insight into the relationship between the various systems with respect to their aggregate IC_{50} values, as well as into which inhibitors contribute most to the observed variability. This insight will help guide further investigations into the reasons for the variability.

In the cell systems only, the laboratory-to-laboratory differences constitute differences in properties of the cells as maintained in each laboratory, differences in the monolayers on the Transwell inserts as prepared in each laboratory, and differences in experimental protocols to measure inhibition. When vesicles are added to the analysis, differences in the expression systems used for vesicle preparation and

vesicle preparation procedures, as well as differences in inhibition study protocols, contribute to the laboratory-to-laboratory differences, as well as the overall variability. Each of the factors and their potential contribution to variability are discussed below.

The IC_{50} value for P-gp in confluent monolayers is a complicated function of substrate and inhibitor efflux and influx kinetics (Lumen et al., 2010). For example, consider two cell lines identical in all respects except that the P-gp surface density in cell line 2 is greater than that of cell line 1. In the absence of inhibitor, cell line 2 will have a smaller intracellular probe substrate concentration than cell line 1, so the fraction of P-gp with substrate bound to it in the absence of inhibitor will be smaller in cell line 2. This means that more inhibitor will have to be added to cell line 2 to reduce the fraction of P-gp with substrate bound to it by 50% (Lumen et al., 2010). Thus, the IC_{50} value for cell line 2 will be higher than the IC_{50} value for cell line 1. This result is simply a consequence of the fact that the smaller the fraction of substrate bound to any site, the larger the amount of inhibitor required to reduce the bound substrate by 50%. If, on the other hand, cell line 2 only differed from cell line 1 by having a larger substrate passive permeability, with identical P-gp surface densities, then cell line 2 would have a lower IC_{50} value than cell line 1. Therefore, differences between cell monolayer preparations in surface density of P-gp or passive permeability for digoxin are likely to be important contributors to the observed variability in IC_{50} values derived from cell lines.

The fact that the MDCKII-MDR1 cell line has a basolateral digoxin uptake transporter that is inhibited by GF120918 (Acharya et al., 2008) adds a separate layer of complexity to the interpretation of an IC_{50} value for P-gp. Any inhibitor that can bind to this digoxin uptake transporter would decrease digoxin efflux and thereby affect the measured IC_{50} value such that this value is not a pure P-gp IC_{50} . Hence the presence of digoxin uptake transporters and differences in their expression levels may also contribute to variability in IC_{50} values. The contribution of several different factors to the value of the IC_{50} in cell lines might explain not only the laboratory-to-laboratory variability in the absolute value of the IC_{50} , but also the laboratory-to-laboratory differences in inhibitor potency rank-ordering.

In the current study, participants used digoxin concentrations between 13 nM and 10 μ M. Simulations using the model published in Agnani et al. (2011) with quinidine and verapamil as inhibitors have shown that the IC_{50} value in a given system is affected by digoxin concentration, but at digoxin concentrations > 10 μ M only (unpublished data). IC_{50} values determined in two different cell lines with different P-gp surface densities will be strongly dependent on P-gp surface density, but not dependent on digoxin concentrations of ≤ 10 μ M. In the current study only two laboratories used 10 μ M digoxin. Hence the differences in digoxin concentrations used in this study are not likely to contribute a great deal to the observed IC_{50} variability. Moreover, removing data from laboratories using digoxin concentrations ≥ 5 μ M did not affect our conclusion (removing laboratories 5, 8, 15, 18, and 19 from Fig. 7). Similarly, preincubation and plate-shaking conditions did not explain laboratory-to-laboratory differences.

Overall, while this variability analysis did not identify the source of the variability, it appears likely that the substantial variability in cell-based IC_{50} values is largely due to cell source, passage number and culture conditions, as previously concluded for differences between laboratories in TEER values, paracellular permeability and transporter expression in Caco-2 cells (Hayashi et al., 2008), rather than to an implicit difference between cell lines. Standardizing cell type, cell source, culture conditions and passage number could reduce interlaboratory variability in cell-based IC_{50} values for inhibition of digoxin transport. Sambuy et al. (2005) performed an interlaboratory

study of variability in TEER, mannitol permeability, and alkaline phosphatase expression in Caco-2 cells with the aim to establish a standardized protocol allowing a meaningful comparison of results obtained in different laboratories. Such a study has not yet been performed for transporter expression. While this might be a very worthwhile effort, it is beyond the scope of the current investigation. Some general recommendations can be made with respect to cell culture practices. When first establishing the assay, it is recommended that each laboratory determines the optimal conditions to ensure properly formed tight monolayers by monitoring TEER values, paracellular permeability and efflux transport as a function of seeding density and time. Existence of monolayers should be confirmed by microscopy. The assay should then be performed soon after formation of properly functioning monolayers. Each time the assay is performed quality controls should be included, such as measurement of TEER values, paracellular permeability, and transport activity.

The variability in uptake of probe substrate and IC₅₀ values obtained with vesicles from the same source was surprising given the relatively straightforward assay setup and format. It is recommended to ensure that commercially obtained vesicles perform according to the quality control data provided by the vendor.

The multitude of transport activity equations used in the estimation of P-gp IC₅₀ values in cell lines is a consequence of the asymmetry of the experimental system, i.e., the ability to access P-gp from two directions, A>B and B>A. For the commonly used probe substrate digoxin, flux in the B>A direction is much greater than that observed in the A>B direction; additionally, there is a substantial transport component not inhibited. P-gp transports substrates from the inner leaflet of the plasma membrane to the outside of the cell. If the probe substrate only interacts with P-gp and if inhibitor is added to both donor and receiver chambers, the IC₅₀ value measured in the B>A direction should be the same as that in the A>B direction. To assess the transport activity of P-gp as a function of inhibitor concentration, there are several equations that incorporate transepithelial flux in both directions. However, the rationale for favoring bidirectional flux activity equations (A1, A2, and D) rather than unidirectional flux activity equations (B1, B2, C1) is typically not provided. Our analysis demonstrates that algebraic differences between equations result in different IC₅₀ values.

The simplest experimental design recommended to determine P-gp IC₅₀ values when using digoxin as probe substrate in confluent cell monolayers is the measurement of B>A flux in the presence of increasing inhibitor concentrations. In addition, eight to 12 inhibitor concentrations of equal spacing (e.g., half-log dilutions) are recommended with a wide enough range to define both the upper and lower plateaus, with at least three points in the linear range of the IC₅₀ curve. However, depending on compound solubility, if the lower plateau cannot be realized, a “true” positive control inhibitor, with complete inhibition of P-gp, must be used to define the plateau. The logistic eq. 2.2 is recommended to fit the IC₅₀ value as data transformation in transport activity equations A1, A2, B1, and D contributes error to the IC₅₀ value and standard statistical analyses are inappropriate (examined in greater detail in a future publication by O'Connor et al.). Furthermore, if sigmoidicity is questionable, the application of the $t_{\beta\beta}(2p*3p)$ statistic to judge the quality of the fit is also recommended.

In summary, substantial variability was observed in the participant submitted IC₅₀ values for the 15 compounds examined, for cell lines as well as vesicles. This difference was to a large extent due to interlaboratory differences in behavior of the P-gp expression systems. The IC₅₀ variability likely derives from the properties of the P-gp expression system used in a particular laboratory (e.g., P-gp surface

density and involvement of other transporters), even when the same cell line is used in different laboratories. The feasibility of standardizing to a single expression system, with a consistent behavior over time, has not yet been demonstrated. The use of different transport activity equations fractionally contributed to the variability observed with the cell lines.

The authors recommend: 1) using best cell culture practices to establish a tight monolayer of polarized cells, 2) ensuring digoxin concentration is sufficiently below the (apparent) K_m for the system such that it does not affect the IC₅₀ value, 3) standardization to a single experimental design (digoxin B>A transport only) and IC₅₀ fitting routine (logistic eq. 2.2 or 2.3 applied to transport inhibition data without prior data transformation). When using commercially obtained vesicles it is recommended these are treated as recommended by the vendor and perform according to the quality control data provided.

Finally, the analysis performed here does not identify a P-gp expression system that outperforms the others, but it does point out that the decision criteria as currently derived may not be optimal. More appropriate DDI risk assessment criteria are presented in the companion article (Ellens et al., 2013).

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References

- Acharya P, O'Connor MP, Polli JW, Ayrton A, Ellens H, and Bentz J (2008) Kinetic identification of membrane transporters that assist P-glycoprotein-mediated transport of digoxin and loperamide through a confluent monolayer of MDCKII-hMDR1 cells. *Drug Metab Dispos* **36**: 452–460.
- Agarwal S, Arya V, and Zhang L (2013). Review of P-gp inhibition data in recently approved new drug applications: utility of the proposed $[I_1]/IC_{50}$ and $[I_2]/IC_{50}$ criteria in the P-gp decision tree. *J Clin Pharmacol* **53**:228–233.
- Agnani D, Acharya P, Martinez E, Tran TT, Abraham F, Tobin F, Ellens H, and Bentz J (2011) Fitting the elementary rate constants of the P-gp transporter network in the hMDR1-MDCK confluent cell monolayer using a particle swarm algorithm. *PLoS ONE* **6**:e25086.
- Balimane PV, Marino A, and Chong S (2008) P-gp inhibition potential in cell-based models: which “calculation” method is the most accurate? *AAPS J* **10**:577–586.
- Behrens I and Kissel T (2003) Do cell culture conditions influence the carrier-mediated transport of peptides in Caco-2 cell monolayers? *Eur J Pharm Sci* **19**:433–442.
- Casella, G and Berger, R (2001) *Statistical Inference*, 2nd ed. Cengage Learning, Kentucky.
- Cook JA, Feng B, Fenner KS, Kempshall S, Liu R, Rotter C, Smith DA, Troutman MD, Ullah M, and Lee CA (2010) Refining the in vitro and in vivo critical parameters for P-glycoprotein, $[I_1]/IC_{50}$ and $[I_2]/IC_{50}$, that allow for the exclusion of drug candidates from clinical digoxin interaction studies. *Mol Pharm* **7**:398–411.
- Di L, Whitney-Pickett C, Umland JP, Zhang H, Zhang X, Gebhard DF, Lai Y, Federico JJ, 3rd, Davidson RE, and Smith R, et al. (2011) Development of a new permeability assay using low-efflux MDCKII cells. *J Pharm Sci* **100**:4974–4985.
- Ellens H, Deng S, Coleman J, Bentz J, Taub ME, Ragueneau-Majlessi I, Chung SP, Herédi-Szabó K, Neuhoff S, Palm J, et al. (2013) Application of receiver operating characteristic analysis to refine the prediction of potential digoxin drug interactions. *Drug Metab Dispos* **41**:1367–1374.

- Fenner KS, Troutman MD, Kempshall S, Cook JA, Ware JA, Smith DA, and Lee CA (2009) Drug-drug interactions mediated through P-glycoprotein: clinical relevance and in vitro-in vivo correlation using digoxin as a probe drug. *Clin Pharmacol Ther* **85**:173–181.
- Hayeshi R, Hilgendorf C, Artursson P, Augustijns P, Brodin B, Dehertogh P, Fisher K, Fossati L, Hovenkamp E, and Korjamo T, et al. (2008) Comparison of drug transporter gene expression and functionality in Caco-2 cells from 10 different laboratories. *Eur J Pharm Sci* **35**:383–396.
- Hidalgo JJ, Raub TJ, and Borchardt RT (1989) Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* **96**:736–749.
- Hilgendorf C, Ahlin G, Seithel A, Artursson P, Ungell AL, and Karlsson J (2007) Expression of thirty-six drug transporter genes in human intestine, liver, kidney, and organotypic cell lines. *Drug Metab Dispos* **35**:1333–1340.
- International Transporter Consortium, Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, Dahlin A, Evers R, Fischer V, and Hillgren KM, et al. (2010) Membrane transporters in drug development. *Nat Rev Drug Discov* **9**:215–236.
- Juan H, Terhaag B, Cong Z, Bi-Kui Z, Rong-Hua Z, Feng W, Fen-Li S, Juan S, Jing T, and Wen-Xing P (2007) Unexpected effect of concomitantly administered curcumin on the pharmacokinetics of talinolol in healthy Chinese volunteers. *Eur J Clin Pharmacol* **63**:663–668.
- Kalvass JC and Pollack GM (2007) Kinetic considerations for the quantitative assessment of efflux activity and inhibition: implications for understanding and predicting the effects of efflux inhibition. *Pharm Res* **24**:265–276.
- Karlsson JE, Heddl C, Rozkov A, Rotticci-Mulder J, Tuveson O, Hilgendorf C, and Andersson TB (2010) High-activity p-glycoprotein, multidrug resistance protein 2, and breast cancer resistance protein membrane vesicles prepared from transiently transfected human embryonic kidney 293-epstein-barr virus nuclear antigen cells. *Drug Metab Dispos* **38**:705–714.
- Keppler D, Jedlitschky G, and Leier I (1998) Transport function and substrate specificity of multidrug resistance protein. *Methods Enzymol* **292**:607–616.
- Lowes S, Cavet ME, and Simmons NL (2003) Evidence for a non-MDR1 component in digoxin secretion by human intestinal Caco-2 epithelial layers. *Eur J Pharmacol* **458**:49–56.
- Lumen AA, Acharya P, Polli JW, Ayrton A, Ellens H, and Bentz J (2010) If the K_i is defined by the free energy of binding to P-glycoprotein, which kinetic parameters define the IC_{50} for the Madin-Darby canine kidney II cell line overexpressing human multidrug resistance 1 confluent cell monolayer? *Drug Metab Dispos* **38**:260–269.
- Miliotis T, Ali L, Palm JE, Lundqvist AJ, Ahnoff M, Andersson TB, and Hilgendorf C (2011) Development of a highly sensitive method using liquid chromatography-multiple reaction monitoring to quantify membrane P-glycoprotein in biological matrices and relationship to transport function. *Drug Metab Dispos* **39**:2440–2449.
- Press WH, Teukolsky SA, Vetterling WT, and Flannery BP (2007) *Numerical Recipes: The Art of Scientific Computing*, 3rd ed., Cambridge University Press, Cambridge, UK.
- Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A, and Zucco F (2005) The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biology and Toxicology* **21**:1–26.
- Schwarz UI, Gramatté T, Krappweis J, Oertel R, and Kirch W (2000) P-glycoprotein inhibitor erythromycin increases oral bioavailability of talinolol in humans. *Int J Clin Pharmacol Ther* **38**:161–167.
- Shirasaka Y, Kuraoka E, Spahn-Langguth H, Nakanishi T, Langguth P, and Tamai I (2010) Species difference in the effect of grapefruit juice on intestinal absorption of talinolol between human and rat. *J Pharmacol Exp Ther* **332**:181–189.
- Sugimoto H, Matsumoto SI, Tachibana M, Niwa SI, Hirabayashi H, Amano N, and Moriwaki T (2011) Establishment of in vitro P-glycoprotein inhibition assay and its exclusion criteria to assess the risk of drug-drug interaction at the drug discovery stage. *J Pharm Sci* **100**:4013–4023.
- Tang F, Horie K, and Borchardt RT (2002) Are MDCK cells transfected with the human MDR1 gene a good model of the human intestinal mucosa? *Pharm Res* **19**:765–772.
- Tran TT, Mittal A, Gales T, Maleeff B, Aldinger T, Polli JW, Ayrton A, Ellens H, and Bentz J (2004) Exact kinetic analysis of passive transport across a polarized confluent MDCK cell monolayer modeled as a single barrier. *J Pharm Sci* **93**:2108–2123.
- US FDA/CDER (2006, 2012). Drug Interaction Studies—Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations, Draft Guidance.
- Volpe DA (2008) Variability in Caco-2 and MDCK cell-based intestinal permeability assays. *J Pharm Sci* **97**:712–725.
- Walter E and Kissel T (1994) Transepithelial transport and metabolism of thyrotropin-releasing hormone (TRH) in monolayers of a human intestinal cell line (Caco-2): evidence for an active transport component? *Pharm Res* **11**:1575–1580.
- Walter E and Kissel T (1995) Heterogeneity in the human intestinal cell line Caco-2 leads to differences in transepithelial transport. *Eur J Pharm Sci* **3**:215–230.
- Xia CQ, Liu N, Yang D, Miwa G, and Gan L-S (2005) Expression, localization, and functional characteristics of breast cancer resistance protein in Caco-2 cells. *Drug Metab Dispos* **33**:637–643.
- Zhang L, Zhang YD, Strong JM, Reynolds KS, and Huang SM (2008) A regulatory viewpoint on transporter-based drug interactions. *Xenobiotica* **38**:709–724.

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