articles

molecular pharmaceutics

Refining the In Vitro and In Vivo Critical Parameters for P-Glycoprotein, [I]/IC_{50} and [I_2]/IC_{50}, That Allow for the Exclusion of Drug Candidates from Clinical Digoxin Interaction Studies

Jack A. Cook,[†,||] Bo Feng,[‡,||] Katherine S. Fenner,[‡,⊥] Sarah Kempshall,[‡,⊥] Ray Liu,[§,#] Charles Rotter,[‡,||] Dennis A. Smith,[‡,⊥] Matthew D. Troutman,[‡,||] Mohammed Ullah,[‡,⊥] and Caroline A. Lee*[,‡,#]

Departments of Clinical Pharmacology, Pharmacokinetics, Dynamics & Metabolism, and Preclinical Statistics, Pfizer Global Research & Development, Groton/New London, Connecticut, Sandwich, U.K., and La Jolla, California

Received July 17, 2009; Revised Manuscript Received December 11, 2009; Accepted December 21, 2009

Abstract: The objective of this work was to further investigate the reasons for disconcordant clinical digoxin drug interactions (DDIs) particularly for false negative where in vitro data suggests no P-glycoprotein (P-gp) related DDI but a clinically relevant DDI is evident. Applying statistical analyses of binary classification and receiver operating characteristic (ROC), revised cutoff values for ratio of [I]/IC_{50} < 0.1 and [I_2]/IC_{50} < 5 were identified to minimize the error rate, a reduction of false negative rate to 9% from 36% (based on individual ratios). The steady state total C_{max} at highest dose of the inhibitor is defined as [I] and the ratio of the nominal maximal gastrointestinal concentration determined for highest dose per 250 mL volume defined [I_2]. We also investigated the reliability of the clinical data to see if recommendations can be made on values that would allow predictions of 25% change in digoxin exposure. The literature derived clinical digoxin interaction studies were statistically powered to detect relevant changes in exposure associated with digitalis toxicities. Our analysis identified that many co-meds administered with digoxin are cardiovascular (CV) agents. Moreover, our investigations also suggest that the presence of CV agents may alter cardiac output and/or kidney function that may act alone or are additional components to enhance digoxin exposure along with P-gp interaction. While we recommend digoxin as the probe substrate to define P-gp inhibitory potency for clinical assessment, we observed high concordance in P-gp inhibitory potency for calcein AM as a probe substrate.

Keywords: P-glycoprotein (P-gp); digoxin, calcein AM; receiver operating characteristic (ROC); pharmacokinetics; in vitro−in vivo correlation; IC_{50}; [I]/IC_{50}; [I_2]/IC_{50}

Introduction

P-glycoprotein (P-gp) is an efflux pump which is present in many organs including the gastrointestinal tract, kidney, liver and brain. It is involved in attenuating the permeability of drugs resulting in modulation of absorption, distribution and excretion. A large number of drugs are substrates of P-glycoprotein (P-gp) or inhibitors and the subject of numerous drug interaction evaluations. The most described P-gp related drug interactions which can result in adverse events are those involving digoxin, as it has a narrow safety margin, and toxic effects may occur if plasma levels are slightly elevated (greater than 33%) above therapeutic ones.[2−4] Digoxin is efficacious in treating atrial fibrillation[5] and heart failure,[6] and its small safety margin has led to a historical interest in the potential for interactions, well before

* Corresponding author: 10646 Science Center Drive, San Diego, CA 92121. Tel: 858-622-3080. Fax: 877-481-9465. E-mail address: caroline.lee01@pfizer.com.
† Departments of Clinical Pharmacology and Pharmacokinetics.
‡ Department of Dynamics & Metabolism.
§ Department of Preclinical Statistics.
|| Groton/New London, Connecticut.
⊥ Sandwich, U.K.
La Jolla, California.

10.1021/mp900174z
Published on Web 12/21/2009

the exact mechanisms were understood. Numerous digoxin clinical trials have been reported in the literature, and our previous work reviewed the clinical significance of P-gp related drug interactions.[7] Relatively few of these studies showed clinically relevant changes in digoxin exposure that would increase patient's risk of digitalis induced toxicities.

Many assays are available utilizing a variety of probe substrates and in vitro sources of P-gp activity to assess the potential liability that a drug may pose toward P-gp inhibition. The probe substrates include digoxin itself as well as others with technical advantages like fluorescence detection[8−10] such as calcein AM,[11,12] rhodiamine 123[13] and Hoechst 33342.[14] Two probe substrates have been used herein to generate data for direct comparison, digoxin in a lower throughput format and calcein AM for higher throughput fluorometric assay format.[11] The lower throughput assay utilizes the Caco-2 cell line, in a format similar to absorption assays but using radiolabeled digoxin as the substrate[15] which allows a direct in vitro−in vivo correlation of the impact of P-gp inhibition. Results from this assay can be manipulated in at least two ways to calculate the inhibition of P-gp by net secretory flux of the digoxin[15] or the change in the efflux ratio[16] of digoxin; both methods have been used herein. Calcein AM is a nonfluorescent, membrane permeable P-gp efflux substrate.[17] Upon entering the cell, cytosolic esterases quickly and irreversibly convert calcein AM into calcein. Calcein is highly fluorescent and nonpermeable and is not effluxed by P-gp. Therefore, the intracellular retention of calcein fluorescence is high in parent Madin-Darby canine kidney (MDCK) cells and low in MDCK cells expressing the human MDR1 gene (MDR1-MDCK). The high dynamic range of calcein fluorescence makes it particularly useful to rapidly characterize P-gp activity in MDR1-MDCK cells enabling the identification of P-gp inhibitors in relatively early stages of drug discovery. Identifying P-gp inhibitors early in drug discovery can avoid potential drug−transporter interactions that may occur later in development.

The values of [I] and $[I_2]$ are used to examine the possibility of systemic and gastrointestinal concentrations, respectively, that lead to drug−transporter interactions. [I], the steady state C_{max} of inhibitor, potentially defines distribution (brain) and clearance (excretion by kidney and liver) interactions while $[I_2]$ potentially defines absorption based interactions. The FDA draft guidance established an in vitro cutoff of $[I]/IC_{50}$ of 0.1 to define whether a new molecular entity (NME) may cause a clinical interaction with digoxin.[16] However, a high false negative rate was observed for $[I]/IC_{50}$ indicating that the cutoff ratio may be too large (in vitro cutoff predicts no clinical digoxin interaction, but there is a clinical interaction observed). Because P-gp is highly expressed in the gastrointestinal tract and interactions with P-gp can occur at this region, the systemic [I] may not fully reflect inhibitor concentration at the gastrointestinal tract. In a recent publication by authors from the FDA, the $[I_2]$ term was introduced and an additional cutoff of $[I_2]/IC_{50}$ of 10 was proposed to capture the interactions at the gastrointestinal site. By including the $[I_2]/IC_{50}$ ratio, the number of false negatives decreased.[18] In our previous evaluation,[7] in vitro and in vivo correlations (IVIVC) were based on the relation-

(1) Fawcett, T. An introduction to ROC analysis. *Pattern Recognit. Lett.* **2006**, *27*, 861–74.

(2) Tuncok, Y.; Hazan, E.; Oto, O.; Guven, H.; Catalyurek, H.; Kalkan, S. Relationship between high serum digoxin levels and toxicity. *Int. J. Clin. Pharmacol. Ther.* **1997**, *35* (9), 366–8.

(3) Piergies, A. A.; Worwag, E. M.; Atkinson, A. J., Jr. A concurrent audit of high digoxin plasma levels. *Clin. Pharmacol. Ther.* **1994**, *55* (3), 353–8.

(4) Bauman, J. L.; Didomenico, R. J.; Galanter, W. L. Mechanisms, manifestations, and management of digoxin toxicity in the modern era. *Am. J. Cardiovasc. Drugs* **2006**, *6* (2), 77–86.

(5) Wyse, D. G.; Waldo, A. L.; DiMarco, J. P.; Domanski, M. J.; Rosenberg, Y.; Schron, E. B.; Kellen, J. C.; Greene, H. L.; Mickel, M. C.; Dalquist, J. E.; Corley, S. D. A comparison of rate control and rhythm control in patients with atrial fibrillation. *N. Engl. J. Med.* **2002**, *347* (23), 1825–33.

(6) The Digitalis Investigation Group. The effect of digoxin on mortality and morbidity in patients with heart failure. *N. Engl. J. Med.* **1997**, *336*, (8), 525−33.

(7) Fenner, K. S.; Troutman, M. D.; Kempshall, S.; Cook, J. A.; Ware, J. A.; Smith, D. A.; Lee, C. A. Drug-drug interactions mediated through P-glycoprotein: clinical relevance and in vitro-in vivo correlation using digoxin as a probe drug. *Clin. Pharmacol. Ther.* **2009**, *85* (2), 173–81.

(8) Rautio, J.; Humphreys, J. E.; Webster, L. O.; Balakrishnan, A.; Keogh, J. P.; Kunta, J. R.; Serabjit-Singh, C. J.; Polli, J. W. In vitro p-glycoprotein inhibition assays for assessment of clinical drug interaction potential of new drug candidates: a recommendation for probe substrates. *Drug Metab. Dispos.* **2006**, *34* (5), 786–92.

(9) Ekins, S.; Kim, R. B.; Leake, B. F.; Dantzig, A. H.; Schuetz, E. G.; Lan, L. B.; Yasuda, K.; Shepard, R. L.; Winter, M. A.; Schuetz, J. D.; Wikel, J. H.; Wrighton, S. A. Three-dimensional quantitative structure-activity relationships of inhibitors of P-glycoprotein. *Mol. Pharmacol.* **2002**, *61* (5), 964–73.

(10) Tang, F.; Horie, K.; Borchardt, R. T. Are MDCK cells transfected with the human MDR1 gene a good model of the human intestinal mucosa. *Pharm. Res.* **2002**, *19* (6), 765–72.

(11) Tiberghien, F.; Loor, F. Ranking of P-glycoprotein substrates and inhibitors by a calcein-AM fluorometry screening assay. *Anticancer Drugs* **1996**, *7* (5), 568–78.

(12) Polli, J. W.; Wring, S. A.; Humphreys, J. E.; Huang, L.; Morgan, J. B.; Webster, L. O.; Serabjit-Singh, C. S. Rational use of in vitro P-glycoprotein assays in drug discovery. *J. Pharmacol. Exp. Ther.* **2001**, *299* (2), 620–8.

(13) Wang, E.; Casciano, C. N.; Clement, R. P.; Johnson, W. W. The farnesyl protein transferase inhibitor SCH66336 is a potent inhibitor of MDR1 product P-glycoprotein. *Cancer Res.* **2001**, *61* (20), 7525–9.

(14) Muller, H.; Klinkhammer, W.; Globisch, C.; Kassack, M. U.; Pajeva, I. K.; Wiese, M. New functional assay of P-glycoprotein activity using Hoechst 33342. *Bioorg. Med. Chem.* **2007**, *15* (23), 7470–9.

(15) Choo, E. F.; Leake, B.; Wandel, C.; Imamura, H.; Wood, A. J.; Wilkinson, G. R.; Kim, R. B. Pharmacological inhibition of P-glycoprotein transport enhances the distribution of HIV-1 protease inhibitors into brain and testes. *Drug Metab. Dispos.* **2000**, *28* (6), 655–60.

(16) FDA, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf.

ship of in vitro cutoff ratios of $[I]/IC_{50}$[16] or $[I_2]/IC_{50}$[18] and digoxin area under the curve (AUC_i/AUC) in the presence (AUC_i) and absence (AUC) of a P-gp inhibitor derived from 26 digoxin clinical trials. Evaluating both in vitro $[I]/IC_{50}$ and $[I_2]/IC_{50}$ parameters separately to clinical digoxin AUC ratios, both parameters gave rise to discordance.

The objective of this work was to further investigate the reason for the discordance particularly where in vitro data suggests no P-gp related DDI but a clinically relevant DDI was evident (false negative) in order to better define appropriate in vitro cutoff values to safely predict which compounds can be excluded from a clinical DDI study. Key areas to examine are a statistical rationale to better define the in vitro cutoff values to predict when a clinical digoxin drug interaction study is needed, the variance in the two P-gp in vitro inhibition assays, calcein AM and digoxin, and the reliability of the clinical data to see if recommendations can be made on values that would allow predictions of 25% change in digoxin exposure.

Experimental Section

[^{3}H]-Digoxin was obtained from NEN (Hounslow, U.K.). Calcein AM was purchased from Invitrogen (USA). Mibefradil, amiodarone, quinidine, diltiazem, captopril, nicardipine, ranozaline, verapamil, cimetidine, isradipine, felodipine, clarithromycin, cyclosporin A, itraconazole, ketoconazole, digoxin and lucifer yellow were obtained from Sigma-Aldrich Ltd. (Poole, U.K., and St. Louis, MO). Talinolol, losartan, omeprazole, telmisartan, carvedilol, conivaptan, gemcabene, milameline, nifedipine, nitrendipine, troglitazone, paroxetine, sertraline, avasimibe, varenicline, sitagliptin, UK-343664, UK-369003 and UK-510569 were obtained from Pfizer Global Research and Development (Sandwich, U.K., and Milwaukee, WI). Ritonavir and saquinavir were obtained from Apin Chemicals (Abingdon, Oxon, U.K.). Dipyridamole, sparfloxacin, levofloxacin, citalopram, montelukast, pantoprazole, erythromycin, repaglinide, orlistat and meloxicam were purchased from Sequoia Research Products Ltd. (Oxford, U.K.). Caco-2 cells were obtained from ATCC (Rockville, MD), and MDR1-MDCK cells were obtained from Piet Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). Cell culture media, supplements and buffers were purchased from Invitrogen (Paisley, U.K.).

Inhibition Assay Using [^{3}H]-Digoxin as Probe Substrate. The digoxin probe substrate assay was described previously.[7] Briefly, Caco-2 cells were cultured at 37 °C in an atmosphere of 95% humidity and 5% CO_2 in MEM supplemented with 20% FCS, 1% NEAA, 2 mM L-glutamine and 2 mM sodium pyruvate. Cells were seeded onto 24-well HTS multiwell membrane inserts (Becton Dickinson, Cowley, U.K.), 1 μm pore size at 1.2×10^5 cells/cm^2. The medium was replaced 3 times per week, and cells were used 21 to 25 days after seeding.

Inhibition of [^{3}H]-digoxin (5 μM) efflux across Caco-2 cell monolayers was determined in the absence and presence of increasing concentrations of potential inhibitor in duplicate wells. Inhibitor was added to both apical and basolateral chambers along with the addition of the substrate to the donor chamber. Following two hour incubation at 37 °C and 5% CO_2 with 95% humidity, samples from both donor and acceptor were removed and added to liquid scintillant for counting on liquid scintillation counter. Digoxin IC_{50} values were calculated in two ways using Grafit v5 (Erithicus software limited). This curve fitting tool used either net secretory flux or efflux ratio data and inhibitor concentration to calculate IC_{50} values. Flux was defined as the amount of compound transported across the monolayer, and was calculated according to this equation:

$$\text{flux} = \delta Q/A \times \delta t$$

where δQ is the sampled concentration in the acceptor compartment, δt is incubation time and A is the area of the filter of the Transwell plate. Net secretory flux is the net amount of compound that is transported across the monolayer in the B to A direction and is calculated using the following equation:

$$\text{net secretory flux} = \text{flux}_{BA} - \text{flux}_{AB}$$

Efflux ratio (ER) was considered to be the ratio of digoxin flux in the B to A direction normalized by the flux in the A to B direction, and was determined from the following equation:

$$\text{ER} = \text{flux}_{BA}/\text{flux}_{AB}$$

It should be noted that the same efflux ratio would have been determined if apparent permeability (P_{app}) values were calculated and then used to determine efflux ratio.

IC_{50} values were determined using Grafit version 5 (Erithacus Software Ltd.). Inhibition data determined by an IC_{50} value with either net secretory flux or efflux ratio were fitted to the following equation:

$$y = \frac{\text{range}}{1 + \left(\frac{x}{IC_{50}}\right)^s} + \text{background}$$

For compounds with limited solubility, complete inhibition is achieved with a potent inhibitor (ketoconazole which achieves 100% inhibition) whose inhibitory profile is used to obtain the background value for the inhibitor allowing for the determination of an IC_{50} value.

The integrity of the Caco-2 cell monolayer was confirmed at the end of the experiment by a further incubation for 1 h with Lucifer yellow (100 μM) at 37 °C. Monolayers with P_{app} values less than 1×10^{-6} cm/s for Lucifer yellow were

(17) Homolya, L.; Hollo, Z.; Germann, U. A.; Pastan, I.; Gottesman, M. M.; Sarkadi, B. Fluorescent cellular indicators are extruded by the multidrug resistance protein. *J. Biol. Chem.* **1993**, *268* (29), 21493–6.

(18) Zhang, L.; Zhang, Y. D.; Strong, J. M.; Reynolds, K. S.; Huang, S. M. A regulatory viewpoint on transporter-based drug interactions. *Xenobiotica* **2008**, *38* (7−8), 709–24.

considered confluent. Ketoconazole, a known inhibitor of P-gp, was used as a marker of IC_{50} variability during the experimental period. The ketoconazole variability was small, a mean IC_{50} value of 4.16 μM ± 0.19 (n = 9), consistent with literature.[19]

Statistical Analysis for [I]/IC_{50} and [I_2]/IC_{50} Cutoff Values. A binary classification decision analysis, given a fixed discrimination threshold, results in four possible outcomes: true positive, in vitro data is in agreement with a digoxin DDI where AUC > 1.25-fold (TP); false negative, in vitro data is not in agreement with a digoxin DDI where AUC > 1.25-fold (FN); false positive, in vitro data is not in agreement with a digoxin DDI where AUC < 1.25-fold (FP); and true negative, in vitro data is in agreement with a digoxin DDI where AUC < 1.25-fold (TN). Performance metrics used in this analysis are defined as follows:

$$\text{accuracy} = \text{TP} + \text{TN}/(\text{total studies})$$

$$\text{true positive rate} = \text{TP}/P;\ \text{false negative rate} = \text{FN}/P$$

$$\text{true negative rate} = \text{TN}/N;\ \text{false positive rate} = \text{FP}/N$$

$$\text{sensitivity} = \text{true positive rate}$$

$$\text{specificity} = \text{TN}/N = 1 - \text{false positive rate} = \text{true negative rate}$$

		truth		
		+	−	total
prediction	+	true positive (TP)	false positive (FP)	P'
	−	false negative (FN)	true negative (TN)	N'
	total	P	N	

Relative operating characteristic (ROC) curve is a graphical method that plots the true positive rate (known as the sensitivity) against the false positive rate (known as 1 − specificity) for the different possible cutoff ([I]/IC_{50} or [I_2]/IC_{50}) values. The ROC method measures the area under the curve, and ROC measurement is denoted with a prime (AUC′). A classifier with higher statistical AUC′ value is preferred over a classifier with lower statistical AUC′ value. Therefore, the ROC curve is a measure of accuracy and overall performance, an AUC′ value of 1 represents a perfect score and a value of 0.5 represents a poor test or no correlation. The AUC′ also measures discrimination, that is, the ability of the in vitro cutoff ([I]/IC_{50} or [I_2]/IC_{50}) to correctly classify the interaction as being clinically significant. The optimal discrimination threshold is defined as the threshold that corresponds to the lowest classification error. The classification error is the combined probability of false positive and false negative predictions.

We have also assigned different penalties (weighing false negatives 2 to 5 times greater than false positives) to determine the most appropriate cutoff when using the combined in vitro parameters of [I]/IC_{50} and [I_2]/IC_{50} to achieve a negative rate less than 10%.

Inhibition Assay Using Calcein AM as Probe Substrate. For calcein AM assay, similar methods were used as previously described.[20] MDCK and MDR1-MDCK cells were grown and maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Cells were incubated at 37 °C in 10% CO_2 for 24 h before the study. Each cell line (50,000 cells per well) was plated into DB Falcon 353948 Optilux black 96-well plates with 100 μL of medium supplemented with 1% FBS and allowed to become confluent overnight. Test compounds between 0.4 μM and 100 μM were added to monolayers in 100 μL of Hanks balanced salt solution (HBSS) containing 1% DMSO as solvent. Plates were incubated at 37 °C for 30 min. Calcein AM was added in 10 μL of HBSS to yield a final concentration of 0.1 μM, and plates were incubated for an additional 60 min. Cells were then washed three times with ice-cold phosphate buffered saline (PBS), and fluorescence was measured using a Victor^2 fluorometer (Perkin-Elmer, Downers Grove, IL) at excitation and emission wavelengths of 485 and 535 nm, respectively. Cyclosporin A and Pfizer internal compound CP100356 were used as positive controls in the assay. The IC_{50} values were determined from the fluorescence in MDR1-MDCK vs concentration, using a data analysis program LabStats Excel Add-in (Pfizer Inc.).[21]

Digoxin Variability and Clinical Study Design. In order to determine the appropriate subject numbers, variability data were examined from 16 digoxin drug interaction studies conducted by Pfizer between June, 1989 through November, 2005 (Table 1). The studies employed between 8 and 24 subjects, all were crossover in nature, digoxin pharmacokinetics were measured after single and/or multiple doses, and either Lanoxin tablets or Lanoxicaps were administered at doses between 0.2 and 0.5 mg digoxin/day. Estimates of digoxin C_{max} and AUC variability were obtained by analyzing log-transformed values and an ANOVA model that contained terms for treatment, period, sequence, and subject-within-sequence. Variability estimates were reported as standard errors of the mean. Once the variability estimates were obtained, they were examined graphically for the influence of dose (0.20 mg Lanoxicap and 0.25 mg Lanoxin tablet vs 0.40 mg Lanoxicap and 0.50 mg Lanoxin tablet), dosing frequency (single dose or multiple dose), and formulation (Lanoxin tablet vs Lanoxicap). Central tendencies were estimated by median variability measurements. These central tendencies were then used to estimate the number of subjects

(19) Keogh, J. P.; Kunta, J. R. Development, validation and utility of an in vitro technique for assessment of potential clinical drug-drug interactions involving P-glycoprotein. *Eur. J. Pharm. Sci.* **2006**, *27* (5), 543–54.

(20) Feng, B.; Mills, J. B.; Davidson, R. E.; Mireles, R. J.; Janiszewski, J. S.; Troutman, M. D.; de Morais, S. M. In vitro P-glycoprotein assays to predict the in vivo interactions of P-glycoprotein with drugs in the central nervous system. *Drug Metab. Dispos.* **2008**, *36* (2), 268–75.

(21) Feng, B.; Xu, J. J.; Bi, Y. A.; Mireles, R.; Davidson, R.; Duignan, D. B.; Campbell, S.; Kostrubsky, V. E.; Dunn, M. C.; Smith, A. R.; Wang, H. F. Role of hepatic transporters in the disposition and hepatotoxicity of a HER2 tyrosine kinase inhibitor CP-724,714. *Toxicol. Sci.* **2009**, *108* (2), 492–500.

Table 1. Summary of Pfizer's Clinical Digoxin Trial Information[a]

drug	design (*N*)	digoxin dose[b] (mg)	RMSE intrasubject variability C_{max}	RMSE intrasubject variability AUC	RMSE total variability C_{max}	RMSE total variability AUC	ref
atorvastatin							
10 mg	MD (11)	0.25	0.1400	0.0871	0.3032	0.2748	44
80 mg	MD (11)	0.25	0.1575	0.1087	0.2043	0.1344	45
avasimibe	SD (11)	0.25	0.1231	0.1042	0.2776	0.3541	45
CI-1027	MD (12)	0.25	0.1403	0.1143	0.2376	0.3090	46
conivaptan	MD (12)	0.25	0.0942	0.0746	0.2092	0.2572	47
dofetilide	MD (8)	0.25	0.0600	0.0400	0.0740	0.1017	48
eplerenone	MD (24)	0.2	0.2013	0.2303	0.3154	0.3122	49
lasofoxifene	MD (11)	0.25	0.0653	0.0280	0.1693	0.1805	50
torcetrapib and atorvastatin	MD (14)	0.25	ND	0.0976	0.2462	0.1786	47
troglitazone	MD (14)	0.25	0.1994	0.1003	0.2366	0.2174	51
UK-369003	MD (48)	0.25	0.1104	0.0513	0.3108	0.2686	47
varenicline	MD (18)	0.2	0.0588	0.0609	0.2414	0.2270	52
voriconazole	MD (24)	0.25	0.0510	0.0390	0.1908	0.2150	53
median[c]			0.1167	0.0871	0.2376	0.2270	
milameline	SD (8)	0.5	0.1998	0.1812	0.3159	0.2719	47
pramipexole	SD (11)	0.4	0.2600	0.2264	0.4343	0.3128	47
UK-369003							
25 mg	MD (12)	0.5	0.1016	0.1416	0.3596	0.4545	47
100 mg	MD (12)	0.5	0.0792	0.1149	0.2818	0.4251	47
median[c]			0.1507	0.1614	0.3379	0.3698	

[a] MD = multiple dose; SD = single dose; RMSE = root mean square error of log transformed parameter values. [b] Digoxin tablets were used for doses of 0.25 and 0.5 mg. Lanoxicaps were used or doses of 0.2 and 0.4 mg. [c] Median values are presented for low (0.2 and 0.25 mg) and high (0.4 and 0.5 mg) digoxin doses.

needed to ensure no interaction given that the true ratio of mean C_{max} or AUC values was 1 or 1.1. In these instances, no interaction would be declared if 90% confidence intervals for the ratios were between 0.80 and 1.25. Additionally the central tendencies were used to estimate subject numbers for trials designed to show a statistical difference for two instances: (1) when the true ratio of mean values was 1.25 compared to a value of 1.0 and (2) when the true ratio of mean values was 1.5 compared to a value of 1.25. This represents cases where an investigator might want to power a study to establish that a compound was an inhibitor (1) and in this case when an investigator might want to establish that a compound was a clinically important inhibitor of digoxin. (2) Sample sizes were estimated for both parallel and crossover designed trials. Sample size estimates were determined for a power of 80%.

Results

Statistical Analysis Utilizing Receiver Operating Characteristic (ROC). The binary classification method and ROC graphical analysis were used to determine the appropriate cutoff for in vitro parameters to predict a clinical digoxin AUC change >25%. The method used digoxin in vitro IC_{50} data determined by net secretory flux and in vivo changes in digoxin AUC in the presence and absence of a P-gp inhibitor (Table 2), data previously published.[7] Taking the ROC approach, optimal cutoffs for the $[I]/IC_{50}$ and $[I_2]/IC_{50}$ ratios were 0.1 and 65, respectively, in order to predict a digoxin AUC ratio greater than 1.25 (Table 2). Areas under the ROC curves were 0.85 and 0.76 indicating that these cutoff values would rank a randomly chosen positive instance higher than a randomly chosen negative one 85% and 76% of the time for $[I]/IC_{50}$ and $[I_2]/IC_{50}$ ratios, respectively. Based on only the single cutoff of $[I]/IC_{50}$ to predict clinically significant digoxin AUC changes (AUC ratio >1.25), the false negative rate was 36% (Table 3A). Combining the cutoff values for $[I]/IC_{50}$ of 0.1 and the $[I_2]/IC_{50}$ of 10 based on the report of Zhang et al.,[18] the false negative rate declined to 18% (Table 3B) in comparison to $[I]/IC_{50}$ or $[I_2]/IC_{50}$ alone. Recognizing that the consequences of false negative classification are higher than those of a false positive classification, we assigned a weighting such that the significance of a false negative was evaluated over a range of 2 to 5 times higher than that of the false positive value. Based on this assumption, the ROC analysis gave optimal cutoff values of $[I]/IC_{50}$ of 0.1 and $[I_2]/IC_{50}$ of 5 that resulted in the false negative rate below 10% (Table 3C). ROC analysis was also conducted with the efflux ratio (ER) method to determine IC_{50}, and the AUC′ value from the ROC curve was similar to that from the NSF method and gave a similar false negative rate of 9%. The NSF method did have a slightly better false positive rate (60%) than the ER method (80%), but given the small sample size, this difference is likely not indicative of a truly better performance. For ease of presentation, results of the NSF method are used in the Discussion section. It should be noted that, in applying the ROC method

Table 2. Summary of In Vitro ($[I]/IC_{50}$ and $[I_2]/IC_{50}$) and In Vivo Digoxin Ratios for Compounds across Different Therapeutic Areas (Previously Reported by Fenner et al.[7])[a]

P-gp inhibitors	therapeutic class	AUC_i/AUC ratio	IC_{50} (μM)	$[I]/IC_{50}$	$[I_2]/IC_{50}$
quinidine	antiarrhythmics	2.65	21	0.243	352.28
quinidine	antiarrhythmics	1.76	21	0.169	161.76
amiodarone	antiarrhythmics	1.68	6	0.367	782.25
ranolazine	cardiovascular drugs	1.6	49	0.171	190.94
carvedilol	alpha/beta adrenergic antagonists	1.56	4	0.032	15.38
verapamil	calcium channel blockers	1.51	10	0.120	65.16
amiodarone	antiarrhythmics	1.63	6	0.417	391.17
diltiazem	calcium channel blockers	1.44	36	0.019	14.78
conivaptan	diuretics	1.43	39	0.030	8.23
captopril	angiotensin converting enzyme inhibitors	1.39	>1000	2×10^{-3}	0.23
mibefradil	calcium channel blockers	1.31	7.5	0.323	161.43
diltiazem	calcium channel blockers	1.24	36	0.019	22.17
carvedilol	alpha/beta adrenergic antagonists	1.24	4	0.032	15.38
nifedipine	calcium channel blockers	1.23	53	0.004	2.18
telmisartan	angiotensin II inhibitors	1.22	5	0.222	186.54
nifedipine	calcium channel blockers	1.21	53	0.002	1.09
nifedipine	calcium channel blockers	1.18	53	0.009	4.36
felodipine	calcium channel blockers	1.18	29	0.001	3.59
nitrendipine	calcium channel blockers	1.15	14	0.002	15.86
atorvastatin	HMG CoA reductase inhibitors	1.15	96	0.003	5.97
isradipine	calcium channel blockers	1.11	15	0.001	10.77
sertraline	selective serotonin reuptake inhibitors	1.1	92	0.004	28.40
nicardipine	calcium channel blockers	1.06	7.5	0.024	33.08
troglitazone	thiazolidinediones	1.04	31	0.117	116.89
atorvastatin	HMG CoA reductase inhibitors	1.03	96	2×10^{-3}	0.75
varenicline	cholinergic agonists	1.00	>300	1×10^{-3}	0.1

[a] For compounds with different $[I]/IC_{50}$ and $[I_2]/IC_{50}$, different doses were used in the study.

Table 3. Binary Classification of In Vitro and In Vivo Correlations

	$AUC_i/AUC > 1.25$	
	+	−
+	7	2
−	4	13
	(A) $[I]/IC_{50} > 0.1$[a]	
+	9	8
−	2	7
	(B) $[I]/IC_{50} > 0.1$ or $[I_2]/IC_{50} > 10$[b]	
+	10	9
−	1	6
	(C) $[I]/IC_{50} > 0.1$ or $[I_2]/IC_{50} > 5$[c]	

[a] False negative rate = 36%. [b] False negative rate = 18%. [c] False negative rate = 9%.

in this current work, the ROC definition of a false negative rate is based on the AUC ratio while, previously,[7] it was defined on the basis of the in vitro cutoff of 0.1 or 10. However, a compound defined as a false negative or false positive was consistent in either the past or present analysis.

P-gp Inhibition Measured by Digoxin Transwell Assay. Using digoxin as a probe substrate, 30 compounds were tested using the Transwell assay in Caco-2 cells at a maximum concentration range between 1 μM and 1000 μM, depending on solubility (Table 4). Concentration dependent inhibition was observed for 24 of the compounds tested, with the six others showing no inhibition at the maximum concentration. Eight compounds were identified as strong P-gp inhibitors based on IC_{50} value being less than 10 μM: amiodarone, carvediol, cyclosporin A, itraconazole, ketoconazole, mibefradil, nicardipine and telmisartan. The IC_{50} values were determined by either inhibition of the net secretory flux (NSF) or the efflux ratio (ER), and for most compounds the IC_{50} values were 2-fold more potent by the ER method. As an example, nitrendipine's IC_{50} value is 14 μM determined by the NSF method, while IC_{50} determined by ER method is 6 μM (Figure 1). Compounds that lack solubility or potency may not give a full inhibition profile. By running a potent inhibitor as a positive control, ketoconazole, the extremes of the IC_{50} curve can be defined and this information can aid the IC_{50} curve fit of the inhibitor (Figure 2).

P-gp Inhibition Measured by Calcein AM Inhibition Assay. The same set of compounds profiled in the digoxin assay described above were also tested up to 300 μM with calcein AM as the probe substrate in the MDR1-MDCK cell based P-gp inhibition assay (Table 4). Concentration dependent inhibition was observed for 25 of the 30 compounds, except for avasimibe, captopril, cimetidine, losartan and milameline. Six of the eight potent inhibitors ($IC_{50} < 10$ μM) observed in the digoxin assay were also potent in this assay,

Table 4. Summay of P-gp Inhibitory Potency Data

	IC_{50} (μM)		
probe substrate	[^{3}H]-digoxin	[^{3}H]-digoxin	calcein AM
cell line	Caco-2	Caco-2	MDCK-MDR1
assay type	Transwell (NSF)	Transwell (ER)	monolayer
amiodarone	6	4	11
avasimibe	>200	>200	>100
captopril	>1000	>1000	>100
carvedilol	4	2	17
cimetidine	>1000	>1000	>100
clarithromycin	66	34	57
conivaptan	39	25	22
cyclosporin A	3	2	3
diltiazem	36	14	30
felodipine	29	10	16
gemcabene	>1000	>1000	15
isradipine	15	6	31
itraconazole	6	2	0.6
ketoconazole	4.6	1.5	6.5
losartan	144	65	>100
mibefradil	8	4	6
milameline	>1000	>1000	>100
nicardipine	8[a]	3	4.2
nifedipine	53	22	47
nitrendipine	14	6	41
omeprazole	85[a]	19	54
paroxetine	>1000	>1000	61
quinidine	21	10	11
ranolazine	49	18	34
ritonavir	10	5	36
saquinavir	19	9	46
talinolol	294[a]	157	48
telmisartan	5	2	6
troglitazone	31	18	19
verapamil	10	4	30

[a] IC_{50} data generated using a potent inhibitor, ketoconazole, to define maximal inhibition of digoxin NSF.

except for amiodarone and carvediol, with values of 11 μM and 17 μM, respectively. In general, there was high concordance between the two assays with the exception of three compounds, gemcabene, talinolol and paroxetine, which were significantly more potent in the calcein AM−MDR1-MDCK assay than the digoxin Caco-2 assay.

Variability in In Vitro IC_{50} Values Obtained from Literature in Comparison with Values Generated Herein. The IC_{50} data were collected from literature reports encompassing several laboratories, cell lines and methods of IC_{50} calculations (Table 5). The variability in the IC_{50} ranged from small differences like talinolol (~2-fold) to high interlab differences as observed for verapamil (~30-fold). The plots depicted in Figures 3A and 3B show the individual IC_{50} data from each laboratory for a given inhibitor as well as showing the $[I]/IC_{50}$ and $[I_2]/IC_{50}$ cutoffs (cutoffs for nonshaded and shaded regions are <0.1 and >0.1 for $[I]/IC_{50}$ and <5 and >5 for $[I_2]/IC_{50}$, respectively). For most of these compounds, the $[I]/IC_{50}$ ratios fall into the same region despite the large interlab variability (Figure 3A). However, for verapamil and quinidine, the IC_{50} variation would generate $[I]/IC_{50}$ ratios that fall into either side of the cutoff ratio. For $[I_2]/IC_{50}$ ratios (Figure 3B), except for carvedilol, the various laboratory values fell into the same side of the $[I_2]/IC_{50}$ cutoff and the in vitro data was skewed to suggest that all inhibitors would trigger a clinical digoxin interaction study based on this data set.

Number of Clinical Subjects Included in Clinical Digoxin Interaction Studies. We have critically examined all the clinical studies in terms of their design to understand whether the apparently large clinical database for digoxin and P-glycoprotein (P-gp) interactions are robust. Recognizing that the number of studies available showing P-gp related drug interactions is relatively small, dose was the only covariate that appeared to influence variability. Variance appeared to be greater for higher doses (Table 1). Thus, the number of subjects required for the various study objectives is dependent on the dose, study design (crossover or parallel), study objective (detect or rule out an interaction) and assumed drug effect (Table 6). Given the various assumptions and designs, the number of subjects required to see small changes in digoxin AUC ranged from 3 to 106.

Evaluation of In Vitro Cutoff Values and Clinical Digoxin Exposure with Noncardiovascular Medicines. The co-meds administered with digoxin that led to clinically significant digoxin drug interaction (25% change in AUC ratio) were predominately in the cardiovascular therapeutic area (Table 2). All of these compounds were also cardiovascular agents. Ideally an ROC analysis for noncardiovascular compounds would have been conducted. However, removing the cardiovascular compounds from our database left only two (varenicline and troglitazone) compounds with a clinically meaningful interaction making an ROC analysis futile. In an attempt to determine if these medications might have biased our analysis, 11 noncardiovascular compounds were selected to be evaluated in the calcein AM assay. Prior to compound selection, there was no published information indicating that these compounds were P-gp inhibitors. An internal derived in silico P-gp substrate model indicated that these compounds were potential substrates for P-gp. The noncardiovascular compounds gave IC_{50} values (determined by the NSF method) that ranged from 8 to >300 μM (the maximum concentration evaluated) (Table 7). Montelukast and dipyridamole are highly to moderately potent P-gp inhibitors with IC_{50} of 8 and 26 μM, respectively, and both in vitro parameters $[I]/IC_{50}$ and $[I_2]/IC_{50}$ were greater than 0.1 and 5, respectively. However, these compounds did not show a corresponding change in digoxin AUC of clinical significance.

Discussion

From a broad drug interaction perspective, P-gp related inhibition is not likely to result in significant pharmacokinetic changes in drug exposure (AUC or C_{max}),[7] unless P-gp clearance systems (e.g., CYP450). With the exception of

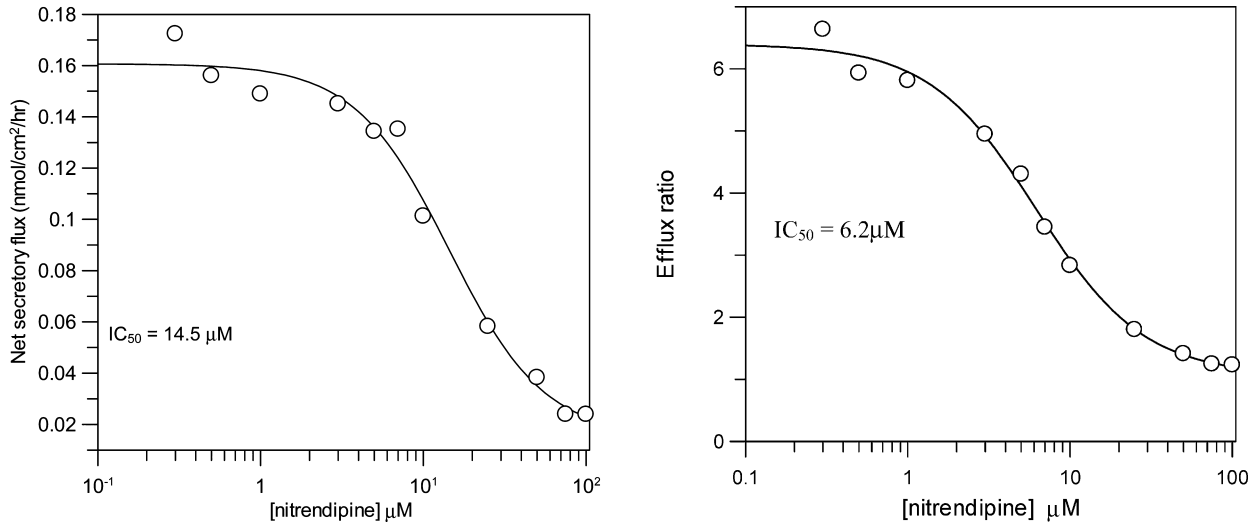

Figure 1. Examples of IC_{50} curves plotted using either the net secretory flux data or efflux ratio.

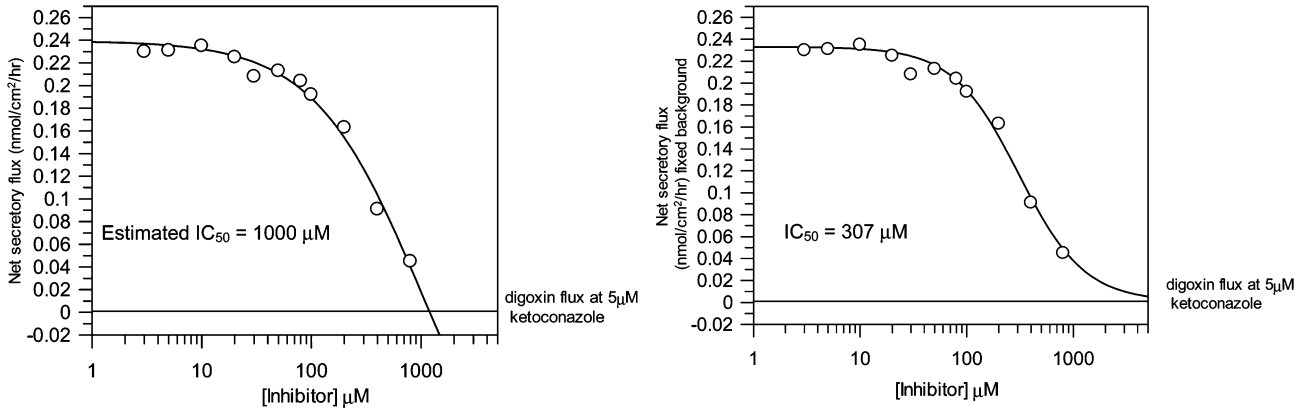

Figure 2. Comparison plots to exemplify the use of a potent inhibitor (ketoconazole) to define complete inhibition of [^{3}H]-digoxin net secretory flux in Caco-2 cells.

digoxin (and potentially similar low therapeutic ratio compounds with P-gp involvement in absorption and clearance), small changes in drug exposure caused by P-gp inhibition do not trigger untoward clinical side effects or require any form of dose adjustment. We have therefore focused our analysis on digoxin.

In our previous analysis of in vitro and in vivo digoxin drug interaction data,[7] we observed a higher than acceptable rate of false negative and false positive outcomes when comparing the FDA guidance parameter of $[I]/IC_{50}$ and $[I_2]/IC_{50}$, the more recently added parameter, to clinical digoxin AUC ratio changes in the presence and absence of P-gp inhibitor. Herein, we have investigated the factors that may potentially influence digoxin's in vitro and in vivo correlation (IVIVC). Our findings show that, by applying the binary statistical ROC approach, we could reduce the false negative rate to 9% by refining the in vitro cutoff values of $[I]/IC_{50}$ to 0.1 and $[I_2]/IC_{50}$ to 5. Moreover, we concluded that confounding factors related to cardiovascular disease state or therapeutic agent induced cardiovascular or renal physiological changes may also contribute to increase in digoxin exposure which should be considered along with altered P-gp activity.

Receiver operating characteristic (ROC) curves were applied to obtain estimates of the optimal cutoff levels for $[I]/IC_{50}$ and $[I_2]/IC_{50}$ to define the digoxin clinical significance criterion of AUC ratio >1.25. The receiver operating characteristic (ROC) curve is a statistical tool that describes the performance of given test. The in vitro and in vivo data are examples of binary data that can give rise to two potential errors: false positive, where the test shows positive values for data that are in fact negative, and false negative, where the test shows negative values for data that are in fact positive. To our knowledge, in the 26 clinical digoxin studies utilized in this analysis, patients were dosed with the Lanoxin formulation in which intestinal and kidney P-gp interactions are in scope to define the $[I]/IC_{50}$ and $[I_2]/IC_{50}$ cutoff ratios. Also, all clinical digoxin studies selected for in vitro and in vivo correlation (IVIVC) analyses were powered correctly based on our analysis to see small changes in digoxin AUC changes in the presence of P-gp co-meds. We made an assumption that the consequences of making a false negative error was more severe than the consequences of making a false positive error as the former may result in unexpected digitalis toxicity in a patient taking digoxin and was therefore weighted greater than the false positive error. However, it

Table 5. Comparison of In Vitro Inhibitory Potency Data Generated with Numerous Probe Substrate and Cell Lines[a]

	IC_{50} (μM)											
substrate	[^{3}H]-digoxin	[^{3}H]-digoxin	[^{3}H]-digoxin	[^{3}H]-digoxin	[^{3}H]-digoxin	[^{3}H]-digoxin	calcein AM	calcein AM	calcein AM	H 33342	R 123
cell line	Caco-2	Caco-2	Caco-2	MDCK-MDR1	MDCK-MDR1	LLC-GA5-COL150-MDR1	MDCK-MDR1	MDCK-MDR1	NIH-3T3-G185	A2780adr	NIH-3T3-G185
data source	in-house	in-house	Tang[10]	Ekins[9]	Tang[10]	Rautio[8]	Takara	in-house	Rautio[8]	Wang[13]	Muller[14]	Wang[13]
assay type	Transwell (NSF)	Transwell (ER)	K_i (B−A)	Transwell (NSF)	K_i (B−A)	Transwell (B−A)	Transwell (NSF)	monolayer	monolayer	FACS		FACS
amiodarone	6	4						11			6.6	
carvediol	4	2						17				
clarithromycin	66	34						57		56.1		15.1
cyclosporin A	3	2	0.46		2.18	1.6		3	2.2	3.2	1.4	1.7
diltiazem	36	14					77.7	30			62	
ketoconazole	4.6	1.5		1.2		3.07		6.5	10.1	4.5		53.4
nicardipine	8	3				4.2	4.54	4.2		4.2	5.1	>25.8
quinidine	21	10	3.23	2.2	8.59	14.9		11	55.5	9.7		33.9
verapamil	10	4	8.11	2.1	15.1	10.7	13.2	30	60.9	2.2	6.6	6.5

[a] Talinolol data source was Pfizer generated. Values in Caco-2 (digoxin) were 387 μM (NSF) and 157 μM (ER). Bibliographic reference next to the data source name is the reference for the literature report.

is noted that the choice of weighting between false positives and negatives is not simple. Reducing false negatives will increase false positives. However, given the high level of safety that the public demands[22] it seems reasonable to trade the cost of a clinical study for the increased assurance of safety brought about by weighting false negatives more heavily. To reflect the emphasis on safety, a false negative rate of no more than 10% was considered desirable. Allowing the false negative weighting to range from 2- to 5-fold greater than false positives, the ROC approach resulted in $[I]/IC_{50}$ of 0.1 and $[I_2]/IC_{50}$ of 5 as the cutoff ratios when these parameters were combined to predict a digoxin AUC ratio >1.25. Additionally, the highest degree of accuracy resulted in a reduction of the false negative rate to 9% from 36% when only the single cutoff ratio of $[I]/IC_{50}$ was utilized. Thus, the ROC analysis provided statistical confirmation that the $[I]/IC_{50}$ ratio of 0.1 is an appropriate cutoff, although its origin was leverage from the P450 DDIs as noted in the FDA draft DDI guidance[16] and refined the $[I_2]/IC_{50}$ to 5. Only one co-med (captopril) accounted for the false negatives (based on 11 studies). The false negative result with captopril is not related to P-gp inhibition but is attributed to changes in the kidney that will be discussed later, when we discuss direct pharmacological effects of compounds. Thus, applying the ROC analysis to our data set the cutoff ratios are very similar to those reported by Zhang et al.,[18] with the only difference in the cutoff ratio for $[I_2]/IC_{50.}$

As a test case of the newer in vitro criteria, felodipine provides the most compelling data set. Felodipine, a relatively potent inhibitor of P-gp with in vitro IC_{50} of 29 μM, is administered as a low dose (10 mg), and plasma concentrations are low, which would suggest no systemic effects on P-gp as the $[I]/IC_{50}$ is 0.001[7] and no gastrointestinal effects as $[I_2]/IC_{50}$ ratio is 3. In a double blind, parallel group, placebo controlled study with 23 heart failure patients, findings showed that felodipine (a plain tablet) resulted in a small increase in digoxin C_{max} (15%) with no change in the 6 h or trough concentrations.[23] Such a finding could reflect changes in the rate of absorption rather than changes in overall bioavailability of digoxin. Because the pharmacodynamics of digoxin are dominated by its slow offset from the receptor, such a small elevation in C_{max} with no effects on trough is clinically insignificant. When felodipine was given as an extended release tablet (double blind, placebo controlled crossover study in 14 heart failure patients) no alterations in digoxin pharmacokinetics were observed.[23] In the latter study, an 11% increase in C_{max} was noted for open administration of the plain tablet. Taken together their small increases in C_{max} and the lack of clinical significance would suggest that the in vitro ratios classified felodipine correctly.

(22) Eichler, H. G.; Abadie, E.; Raine, J. M.; Salmonson, T. Safe drugs and the cost of good intentions. *N. Engl. J. Med.* **2009**, *360* (14), 1378–80.

(23) Kirch, W.; Laskowski, M.; Ohnhaus, E. E.; Aberg, J. Effects of felodipine on plasma digoxin levels and haemodynamics in patients with heart failure. *J. Intern. Med.* **1989**, *225* (4), 237–9.

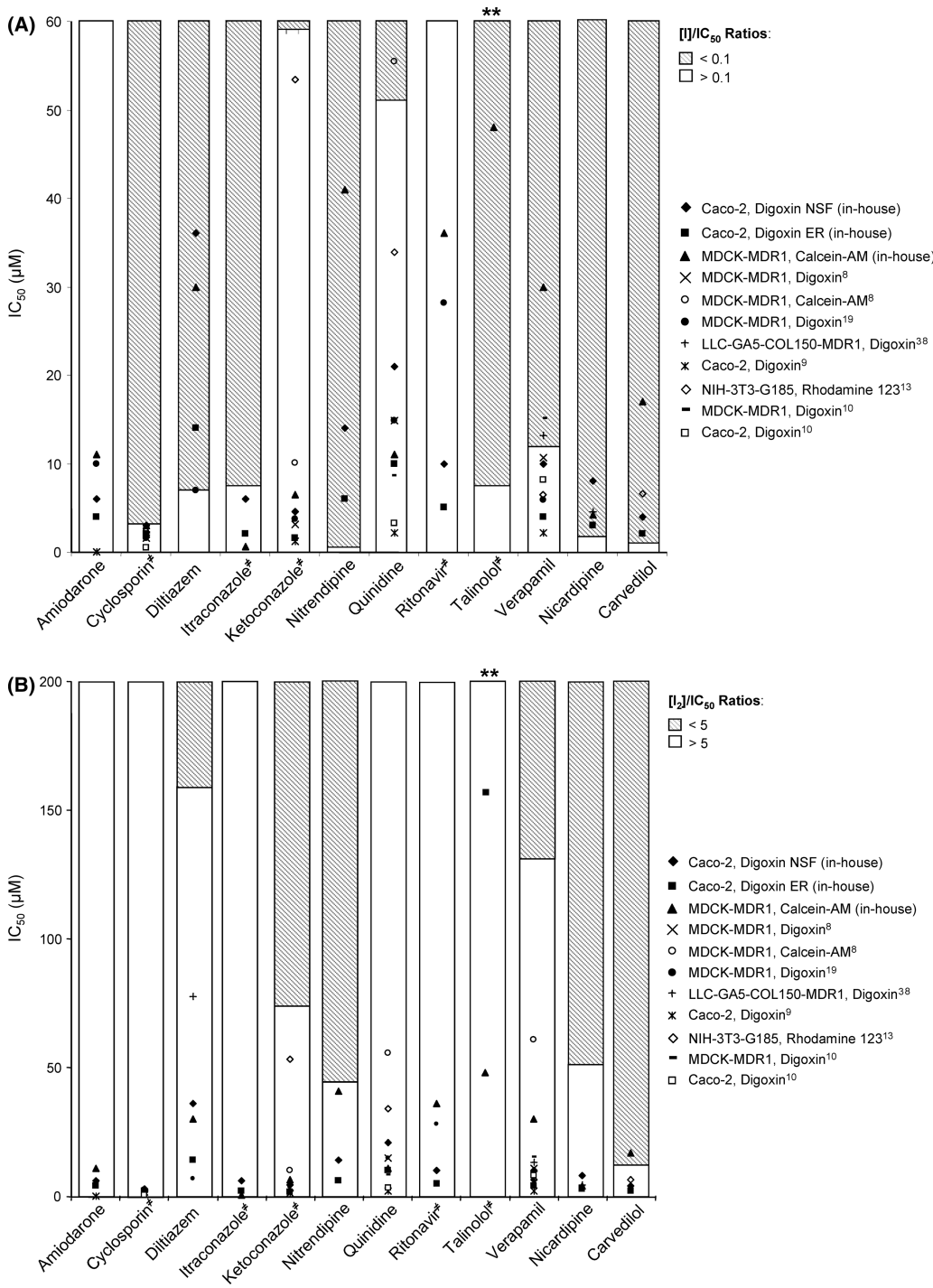

Figure 3. (A) Comparison of in vitro inhibitory potency values (IC_{50}) from both in-house and literature data sources. (**For talinolol there are two IC_{50} values that lie beyond the *Y* axis scale.) The hatched region defines the $[I]/IC_{50}$ ratio <0.1, and the open region defines $[I]/IC_{50}$ ratio >0.1 where [I] is the reported total C_{max} value from clinical data (Table 2). Digoxin data in LLC-GA5-Col150-MDR1 was reported by Takara et al.[38] ‡Total C_{max} values are reported in the literature for cyclosporin A,[39] itraconazole,[40] ketoconazole,[41] ritonavir[42] and talinolol.[43] (B) Comparison of in vitro inhibitory potency values (IC_{50}) from both in-house and literature data with cutoff of $[I_2]/IC_{50}$ set to 5. The hatched region defines the $[I_2]/IC_{50}$ ratio <5, and the open region defines $[I_2]/IC_{50}$ ratio >5 where $[I_2]$ is defined as the projected gastrointestinal concentrations following the clinical human dose (Table 2). Digoxin data in LLC-GA5-Col150-MDR1 was reported by Takara et al.[38] ‡Clinical doses are reported in the literature for cyclosporin A,[39] itraconazole,[40] ketoconazole,[41] ritonavir[42] and talinolol.[43]

While felodipine example appears to support the new cutoff values, the analysis of evaluating the theoretical $[I_2]/IC_{50}$ over a range in IC_{50} values (Figure 3B) raises concern of the benefit of including the $[I_2]/IC_{50}$ term as essentially

Table 6. Sample Size Estimates for Digoxin Interaction Studies

parameter	study design	RMSE[a]	sample size for 80% power: no effect given ratio of 1	no effect given ratio of 1.1	effect different from ratio of 1 given ratio of 1.25	effect different from ratio of 1.25 given ratio of 1.5
			Digoxin Dose = 0.25 mg			
C_{max}	crossover	0.1167	7	13	5	6
C_{max}	parallel	0.2376	21	45	15	22
AUC	crossover	0.0871	5	8	3	4
AUC	parallel	0.2270	20	41	14	20
			Digoxin Dose = 0.5 mg			
C_{max}	crossover	0.1507	10	19	7	10
C_{max}	parallel	0.3379	41	88	30	44
AUC	crossover	0.1614	11	22	8	11
AUC	parallel	0.3698	49	106	35	52

[a] RMSE = root mean square error of log transformed parameter values.

Table 7. Summary of In Vitro ($[I]/IC_{50}$ and $[I_2]/IC_{50}$) and In Vivo Digoxin Ratios for Noncardiovascular Compounds[a]

precipitant	therapeutic class	AUC_i/AUC ratio	$C_{max,i,ss}/C_{max,ss}$ ratio	in vitro IC_{50} (μM)	inhibitor concn (μM)	$[I]/IC_{50}$	hypothetical inhibitor intestinal concn (μM)	$[I_2]/IC_{50}$
dipyridamole	anticlot	1.08	1.23	26	3.70	0.140	792.7	30.0
sparfloxacin[b]	antibiotics	1.02	0.92	300	2.50	0.008	4077.5	13.6
levofloxacin[b]	antibiotics	1.01	0.99	500	12.50	0.025	5534.5	11.1
Citalopram	SSRI-postpartum depression	1.01	0.96	58	0.23	0.004	493.2	8.5
montelukast[c]	antiasthma	1.09	1.00	8	0.89	0.111	68.2	8.5
pantoprazole	antagonist	1.11	1.10	69	5.78	0.084	370.0	5.4
erythromycin[d]	antibiotics	0.96	1.00	394	4.70	0.012	1362.5	3.5
sitagliptin[b]	antidiabetic DPP4 inh	1.11	1.18	300	0.94	0.003	764.4	2.5
repaglinide	antidiabetes	1.03	1.03	17	0.10	0.006	35.4	2.0
orlistat[b]	antiobesity	0.99	0.95	500	0.007	0.000	968.2	1.9
meloxicam[b]	NSAID	1.03	1.03	500	5.41	0.011	170.8	0.3

[a] In vitro probe substrate: calcein. [b] Denotes IC_{50} value set at highest concentration evaluated as inhibition did not reach 50%. [c] Digoxin single dose oral. [d] Digoxin administered iv.

any compound with a propensity to inhibit P-gp will require a clinical digoxin investigation despite the fact that the addition of this term lowered the false negative rate to 9%. A caveat to bear in mind is that the application of these cutoff ratios assumes that P-gp is the primary mechanism governing digoxin's pharmacokinetics. Additionally, the utility of a theoretical optimal $[I_2]$ concentration assumes complete dissolution of the highest approvable dose normalized by intestinal fluid volume of 250 mL. Some drugs have very low solubilities and are orally absorbed due to high permeability. Incorporation of solubility data, in a conservative "worst-case" form, should be explored to determine a realistic $[I_2]/IC_{50}$ value that represents the in vivo situation. In a similar vein drug formulations that modify the release rate should also adjust the $[I_2]$ values to correspond to the situation. We believe that further work should focus on more accurate methods to determine the intestinal concentration that would increase the accuracy of the in vitro cutoff values and to assess whether an in vivo digoxin evaluation is necessary.

The $[I]/IC_{50}$ and $[I_2]/IC_{50}$ really require unbound intracellular concentrations to be known (assuming that the P-gp active site is in equilibrium with intracellular concentration) to be scientifically rigorous. Clearly these are not available at present. For $[I_2]/IC_{50,}$ we believe that, during the absorption phase, the net flux of a compound across the gastrointestinal tract can be viewed as drug in solution and that no protein binding correction is needed, however solubility of the dose in 250 mL or effects of formulation should be factored for poorly soluble drugs. For $[I]/IC_{50,}$ a more complex situation arises where the intracellular concentrations for many P-gp substrates and inhibitors reflect transport by uptake systems. Under these situations a pragmatic solution is the only option until further advances allow this complex interplay to be unraveled. The unbound concentrations of compounds within the cell, following uptake transport, will exceed those of the unbound drug, probably by a significant margin. Affinity for uptake proteins and plasma protein binding correlates broadly with lipophilicity so total drug may represent the best surrogate of intracellular unbound concentration. This convergence of properties means that the more highly bound a drug is the greater the concentration gradient between the unbound drug in plasma and the unbound intracellular concentration. Although intrinsic membrane permeability is obviously a factor, the net effect of the convergence is a

canceling out of the effects of plasma protein binding and free fraction. A concentration gradient does indeed exist as demonstrated by inspection of Table 4 which reveals that the $[I]/IC_{50}$ values are low even when systemic inhibition of P-gp is suspected (e.g., quinidine, a drug likely to be a substrate for uptake transporters). Correcting for the unbound fraction would lower some of these values further (4-fold for quinidine) compressing the dynamic range and the ability to discriminate drugs. For these reasons, we recommend that $[I]/IC_{50}$ and $[I_2]/IC_{50}$ should utilize total drug for both values.

While it is recognized that patient safety is of utmost concern, our work suggests that co-meds may increase digoxin's exposure due to inhibition of P-gp and non P-gp related mechanisms. The in vitro studies to determine IC_{50} measure only P-gp inhibition, and false positives may arise if the co-med is an inducer of P-gp such that the two effects are compensated in vivo. Another possibility for false positives is the challenge to measure small changes in digoxin exposure; however this was dismissed in our analysis of study design which showed that the studies were powered correctly to see small changes in digoxin AUC in the presence of P-gp co-meds. On the other hand, the P-gp related in vitro studies may lead to false negatives if other transporters are involved in digoxin's uptake. It has been shown that digoxin is a substrate for OATP1B3,[24] a liver uptake transporter, and OATP4C1,[25] a kidney uptake transporter. If a co-med inhibits these OATPs, the change in digoxin AUC would be similar to that observed for P-gp inhibition resulting in false negatives. Moreover, non transporter related interactions have been shown to contribute to elevated digoxin levels. Tetracycline and other antibiotics have been shown to elevate digoxin levels in some individuals.[26] It is believed that, in a subset of patients, a significant fraction of digoxin dose undergoes colonic metabolism, via reduction of the lactam ring, by gut flora, in particular the common anaerobe *Eubacterium lentum*.[27] In these individuals there can be a marked increase in digoxin levels if an appropriate antibiotic is given. All cases of colonic metabolism were observed with tablet formulations (e.g., Lanoxin) and do not appear to occur with digoxin formulated as a gel suspension (Lanoxicaps), indicating the complete absorption of the capsule formulation from the upper gastrointestinal tract.[27]

Disease state or drug induced pharmacodynamic actions can lead to digoxin pharmacokinetic changes that are not related to P-gp. Cardiovascular effects have been shown to modulate renal clearance.[28] An example is captopril, which does not inhibit P-gp. In patients suffering from mild cardiac failure (CHF), captopril has little effect on digoxin; however, an increase in digoxin AUC by 39% has been observed in patients with severe CHF (NYHA class 4).[29] This effect is pharmacologically mediated and reflects suppression of angiotensin II by captopril. Angiotensin II maintains glomerular hydrostatic pressure and GFR when renal pressure falls as in the case of severe CHF. Even for known P-gp inhibitors it is important to consider if the pharmacological effect of the drug could alter digoxin clearance. Another example is with conivaptan, a vasopressin V1a/V2 receptor antagonist which acts on receptors in the kidney to reduce urine osmolality, increase electrolyte-free water excretion, and raise serum sodium concentration which is classified as a cardiovascular agent. The clinical dose is 40 mg, and the $[I_2]/IC_{50}$ is ~8, which suggests a possible gastrointestinal effect. The $[I]/IC_{50}$ value is below what would be expected for a systemic effect on clearance (0.03), yet the magnitude of change suggests (digoxin AUC increases by 43%) a potential effect on renal clearance. It is likely that conivaptan does not increase digoxin PK parameters via a P-gp related interaction but rather conivaptan modulates kidney physiology that relates directly to a lowering of digoxin tubular transport and a reduction in digoxin clearance. Upon closer evaluation of the interactions are listed in Table 2, many of the digoxin drug interactions comedicated with cardiovascular agents, the exceptions are troglitazone, atorvastatin and varenicline. Interestly, these three compounds do not cause a clinically significant change in digoxin exposure.

To further examine if digoxin PK changes are related to pharmacodynamic effects associated with the mechanism of action of cardiovascular co-med, we selected eleven non-cardiovascular agents to better understand if P-gp inhibition alone could lead to a clinically significant change in digoxin exposure. In this evaluation, the IC_{50} values for the eleven compounds were determined with calcein AM as the probe substrate. We demonstrated that there is high conconcordance; ~80% of compounds evaluated as inhibitors of calcein and digoxin were placed in the same category when data were binned to the following categories of IC_{50}: less than 10 μM, between 10 μM and 100 μM and greater than 100 μM. Of the eleven compounds, montelukast and dipyridamole were the most potent with IC_{50} values of 8 and 26 μM, respectively. The computed in vitro parameter cutoff

(24) Kullak-Ublick, G. A.; Ismair, M. G.; Stieger, B.; Landmann, L.; Huber, R.; Pizzagalli, F.; Fattinger, K.; Meier, P. J.; Hagenbuch, B. Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. *Gastroenterology* **2001**, *120* (2), 525–33.

(25) Mikkaichi, T.; Suzuki, T.; Onogawa, T.; Tanemoto, M.; Mizutamari, H.; Okada, M.; Chaki, T.; Masuda, S.; Tokui, T.; Eto, N.; Abe, M.; Satoh, F.; Unno, M.; Hishinuma, T.; Inui, K.; Ito, S.; Goto, J.; Abe, T. Isolation and characterization of a digoxin transporter and its rat homologue expressed in the kidney. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (10), 3569–74.

(26) Rodin, S. M.; Johnson, B. F.; Wilson, J.; Ritchie, P.; Johnson, J. Comparative effects of verapamil and isradipine on steady-state digoxin kinetics. *Clin. Pharmacol. Ther.* **1988**, *43* (6), 668–72.

(27) Lindenbaum, J.; Rund, D. G.; Butler, V. P., Jr.; Tse-Eng, D.; Saha, J. R. Inactivation of digoxin by the gut flora: reversal by antibiotic therapy. *N. Engl. J. Med.* **1981**, *305* (14), 789–94.

(28) Dupont, A. G. Renal haemodynamics in hypertension: effects of antihypertensive drugs. *J. Hum. Hypertens.* **1993**, *7* (Suppl. 1), S42–5.

(29) Kirimli, O.; Kalkan, S.; Guneri, S.; Tuncok, Y.; Akdeniz, B.; Ozdamar, M.; Guven, H. The effects of captopril on serum digoxin levels in patients with severe congestive heart failure. *Int. J. Clin. Pharmacol. Ther.* **2001**, *39* (7), 311–4.

values indicate that these two inhibitors should result in an AUC change consistent with a clinical digoxin interaction, but the clinical data is not supportive. Thus, with a limited data set, it appears that P-gp inhibition alone is not always sufficient to elevate digoxin exposure and that the pharmacodynamic effects associated with cardiovascular medicines may be an additive factor.

While concordance is high for calcein AM and digoxin assessments of P-gp potency, three compounds, gemcabene, paroxetine and talinolol, did exhibit significant nonconcordance in IC_{50} values between the two in vitro assays. Possible explanations for the discrepancy are the basolateral uptake transporters for influx of digoxin into MDR-MDCK and Caco-2 cells, composition differences between the two cell lines and multiple binding sites in P-gp. The need for uptake transporters on the basolateral membrane has been discussed previously for rhodamine 123, loperamide and digoxin[30,31] in both MDCK-MDR1 and Caco-2 cells. Inhibition of this influx transporter would lead to decreased B−A transport of digoxin, and the measured IC_{50} value for certain compounds would be against this uptake process and not P-gp mediated efflux. However due to the high passive permeability of calcein AM when this is used as a substrate, influx transporters are not required. Second, composition differences between MDR1-MDCK and Caco-2 may contribute to the discrepancy, but it is not known what endogenous factors are involved. Third, although concordance was high, ~80%, it is postulated that these substrates may bind to different sites in the P-gp active site,[32−34] thus resulting in differential inhibition by the inhibitors. While calcein AM assay affords fast, inexpensive results and the ability to profile large compound sets in discovery, it is recommended that digoxin based assay be utilized in development to guide decisions to conduct a clinical digoxin interaction study.

There are numerous reported methods to determine percent inhibition of P-gp activity;[15,35−37] the work presented herein focused on two methods, ER and net secretory flux (NSF). Within the FDA draft guidance, it is suggested that efflux ratio (ER) for substrate in the absence and presence of inhibitor be used to calculate IC_{50}. While IC_{50} values determined from efflux ratio data often appear more potent, this is a mathematical artifact as ER can never numerically achieve zero (see Figure 1). By utilizing the NSF method to determine IC_{50} values, the inhibitory effect on P-gp is calculated as a net amount of compound permeating across a monolayer and this movement is considered only once in the calculation. For the ER method, the presence of an inhibitor affects compound moving across the apical to basolateral side (A to B) and the basolateral to apical side (B to A). In the ER calculation, the effect of the inhibitor is accounted twice, A to B and B to A directions. Moreover, the observation that ER yields greater P-gp inhibition is consistent with the findings of Balimane et al.,[32] in which they also compared these two methods. Whether this moves a compound from a position of noninteraction to interaction is also dependent on the dose[I_2] or C_{max} [I] of that particular compound. Based on our ROC analysis herein, P-gp activity measured by the NSF or ER methods gave similar false

(30) Acharya, P.; O'Connor, M. P.; Polli, J. W.; Ayrton, A.; Ellens, H.; Bentz, J. 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.* **2008**, *36* (2), 452–60.

(31) Troutman, M. D.; Thakker, D. R. Rhodamine 123 requires carrier-mediated influx for its activity as a P-glycoprotein substrate in Caco-2 cells. *Pharm. Res.* **2003**, *20* (8), 1192–9.

(32) Shapiro, A. B.; Corder, A. B.; Ling, V. P-glycoprotein-mediated Hoechst 33342 transport out of the lipid bilayer. *Eur. J. Biochem.* **1997**, *250* (1), 115–21.

(33) Scala, S.; Akhmed, N.; Rao, U. S.; Paull, K.; Lan, L. B.; Dickstein, B.; Lee, J. S.; Elgemeie, G. H.; Stein, W. D.; Bates, S. E. P-glycoprotein substrates and antagonists cluster into two distinct groups. *Mol. Pharmacol.* **1997**, *51* (6), 1024–33.

(34) Dey, S.; Ramachandra, M.; Pastan, I.; Gottesman, M. M.; Ambudkar, S. V. Evidence for two nonidentical drug-interaction sites in the human P-glycoprotein. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94* (20), 10594–9.

(35) Balimane, P. V.; Marino, A.; Chong, S. P-gp inhibition potential in cell-based models: which "calculation" method is the most accurate. *AAPS J.* **2008**, *10* (4), 577–86.

(36) Kalvass, J. C.; Pollack, G. M. Kinetic considerations for the quantitative assessment of efflux activity and inhibition: implications for understanding and predicting the effects of efflux inhibition. *Pharm. Res.* **2007**, *24* (2), 265–76.

(37) Tran, T. T.; Mittal, A.; Aldinger, T.; Polli, J. W.; Ayrton, A.; Ellens, H.; Bentz, J. The elementary mass action rate constants of P-gp transport for a confluent monolayer of MDCKII-hMDR1 cells. *Biophys. J.* **2005**, *88* (1), 715–38.

(38) Takara, K.; Tsujimoto, M.; Ohnishi, N.; Yokoyama, T. Digoxin up-regulates MDR1 in human colon carcinoma Caco-2 cells. *Biochem. Biophys. Res. Commun.* **2002**, *292* (1), 190–4.

(39) Dorian, P.; Strauss, M.; Cardella, C.; David, T.; East, S.; Ogilvie, R. Digoxin-cyclosporine interaction: severe digitalis toxicity after cyclosporine treatment. *Clin. Invest. Med.* **1988**, *11* (2), 108–12.

(40) Jalava, K. M.; Partanen, J.; Neuvonen, P. J. Itraconazole decreases renal clearance of digoxin. *Ther. Drug Monit.* **1997**, *19* (6), 609–13.

(41) Bottiger, Y.; Tybring, G.; Gotharson, E.; Bertilsson, L. Inhibition of the sulfoxidation of omeprazole by ketoconazole in poor and extensive metabolizers of S-mephenytoin. *Clin. Pharmacol. Ther.* **1997**, *62* (4), 384–91.

(42) Ding, R.; Tayrouz, Y.; Riedel, K. D.; Burhenne, J.; Weiss, J.; Mikus, G.; Haefeli, W. E. Substantial pharmacokinetic interaction between digoxin and ritonavir in healthy volunteers. *Clin. Pharmacol. Ther.* **2004**, *76* (1), 73–84.

(43) Westphal, K.; Weinbrenner, A.; Giessmann, T.; Stuhr, M.; Franke, G.; Zschiesche, M.; Oertel, R.; Terhaag, B.; Kroemer, H. K.; Siegmund, W. Oral bioavailability of digoxin is enhanced by talinolol: evidence for involvement of intestinal P-glycoprotein. *Clin. Pharmacol. Ther.* **2000**, *68* (1), 6–12.

(44) Boyd, R. A.; Stern, R. H.; Stewart, B. H.; Wu, X.; Reyner, E. L.; Zegarac, E. A.; Randinitis, E. J.; Whitfield, L. Atorvastatin coadministration may increase digoxin concentrations by inhibition of intestinal P-glycoprotein-mediated secretion. *J. Clin. Pharmacol.* **2000**, *40* (1), 91–8.

(45) Sahi, J.; Milad, M. A.; Zheng, X.; Rose, K. A.; Wang, H.; Stilgenbauer, L.; Gilbert, D.; Jolley, S.; Stern, R. H.; LeCluyse, E. L. Avasimibe induces CYP3A4 and multiple drug resistance protein 1 gene expression through activation of the pregnane X receptor. *J. Pharmacol. Exp. Ther.* **2003**, *306* (3), 1027–34.

negative rates, but false positive rates were lower by the NSF method. However, it should be noted that, due to the small sample size consisting of 26 clinical digoxin DDIs, this difference might not be meaningful.

In conclusion, our analysis gives rise to four principal recommendations:

1. Based on the ROC analysis, we recommend that the in vitro guidance be set to $[I]/IC_{50}$ and $[I_2]/IC_{50}$ ratios of <0.1 and <5 to predict an AUC_i/AUC ratio <1.25, and $[I]/IC_{50} > 0.1$ or $[I_2]/IC_{50} > 5$ to predict in vivo $AUC_i/AUC > 1.25$. However, the cutoff ratios for $[I]/IC_{50}$ and $[I_2]/IC_{50}$ will continue to evolve as more digoxin P-gp related interaction studies are conducted to improve this guidance. In addition, the $[I_2]/IC_{50}$ parameter should not be used in isolation to predict likelihood of clinical digoxin interaction given the assumptions made in estimation of $[I_2]$. Weighing the different variables by which inhibitor concentration can be derived, we recommend that $[I]/IC_{50}$ and $[I_2]/IC_{50}$ should utilize total drug concentrations for both values.

2. Careful consideration should be given to the study design of clinical digoxin interaction studies in order to assess clinically significant outcomes, given the relatively small changes in digoxin plasma levels (typically <2-fold for most interactions). Our analysis suggests that the number of subjects required to detect small changes in digoxin levels need be as few as 3 for crossover design but at least 14 per group for parallel group design. In addition, higher number of subjects are required at the higher digoxin dose of 0.5 mg.

3. Non P-gp related factors, such as attenuation of active uptake of digoxin, pharmacological action of co-meds on kidney, and effect on colonic absorption of digoxin, can contribute to elevation of digoxin levels in humans. Our analysis suggests that additional criteria should be considered with respect to pharmacological activity of co-meds which could give rise to clinically significant interactions with digoxin. In particular drugs which could have pharmacological effects influencing kidney blood flow or urine osmolarity could well perturb digoxin renal excretion by a non P-gp mechanism. In the vast majority of cases, these drugs would be antihypertensives or diuretics, typical medicines in the cardiovascular therapeutic area. With such agents, a clinical digoxin study whether in volunteers or as part of a larger patient study (sparse sampling) may be desirable due to their influence on kidney physiology and the high frequency of polypharmacy of these medicines with digoxin.

4. High concordance was observed between digoxin and calcein AM in vitro assays with respect to IC_{50} data. Our analysis suggests that P-gp activity measured by the NSF and ER method were comparable based on false negative rates but NSF is slightly favorable due to the lower false positive rates to predict clinical digoxin drug interactions. Therefore, we recommend that the NSF be adopted as a standard practice to measure digoxin in vitro P-gp activity.

Acknowledgment. The authors thank Drs. Paul Morgan, Bill Smith and Xiang Gao for their thoughtful review and suggestions of our work.

MP900174Z

(46) Data on file. Pfizer Global Research and Development, Pfizer Inc., New London, CT.

(47) Vaprisol package insert. Astella Pharma US, Inc.: Deer Field, IL, 2007.

(48) Kleinermans, D.; Nichols, D. J.; Dalrymple, I. Effect of dofetillide on the pharmacokinetics of digoxin. *Am. J. Cardiol.* **2001**, *87* (2), 248–50, A9−10.

(49) Tolbert, D. S., Reid, S. E., Gillespie, B. K., Quian, J. Roniker, B. Pharmacokinetics and safety of eplerenone and digoxin coadministration. *AAPS Pharm. Sci.* **2002**, *4* (Abstract T2265).

(50) Roman, D.; Bramson, C.; Ouellet, D.; Randinitis, E.; Gardner, M. Effect of lasofoxifene on the pharmacokinetics of digoxin in healthy postmenopausal women. *J. Clin. Pharmacol.* **2005**, *45* (12), 1407–12.

(51) Loi, C. M.; Knowlton, P. W.; Stern, R.; Randinitis, E. J.; Vassos, A. B.; Koup, J. R.; Sedman, A. J. Effect of troglitazone on steady-state pharmacokinetics of digoxin. *J. Clin. Pharmacol.* **1998**, *38* (2), 178–83.

(52) Faessel, H. M.; Gibbs, M. A.; Clark, D. J.; Rohrbacher, K.; Stolar, M.; Burstein, A. H. Multiple-dose pharmacokinetics of the selective nicotinic receptor partial agonist, varenicline, in healthy smokers. *J. Clin. Pharmacol.* **2006**, *46* (12), 1439–48.

(53) Purkins, L.; Wood, N.; Kleinermans, D.; Nichols, D. Voriconazole does not affect the steady-state pharmacokinetics of digoxin. *Br. J. Clin. Pharmacol.* **2003**, *56* (Suppl. 1), 45–50.