Application of Receiver Operating Characteristic (ROC) Analysis to Refine the Prediction of Potential Digoxin Drug Interactions


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ABSTRACT

In the 2012 FDA draft guidance on drug-drug interactions (DDIs), a new molecular entity that inhibits P-glycoprotein (P-gp) may need a clinical DDI study with a P-gp substrate such as digoxin when $\frac{[I_1]}{IC_{50}} \geq 0.1$ or $\frac{[I_2]}{IC_{50}} \geq 10$. In this manuscript, refined criteria are presented, determined by receiver operating characteristic (ROC) analysis, utilizing $IC_{50}$ values generated by 23 laboratories. P-gp probe substrates were digoxin for polarized cell-lines and N-methyl quinidine or vinblastine for MDR1 over-expressed vesicles. Inhibition of probe substrate transport was evaluated using 15 known P-gp inhibitors. Importantly, the criteria derived in this manuscript take into account variability in $IC_{50}$ values. Moreover, they are statistically derived based on the highest degree of accuracy in predicting true positive and true negative digoxin DDI results. The refined criteria of $(\frac{[I_1]}{IC_{50}} > 0.03$ and $\frac{[I_2]}{IC_{50}} > 45)$ and FDA criteria were applied to a test set of 101 in vitro-in vivo digoxin DDI pairs collated from the literature. The number of false negatives (none predicted but DDI observed) were similar, 10 and 12%, while the number of false positives (DDI predicted but not observed) substantially decreased, from 51% to 40%, relative to the FDA criteria. Based on estimated overall variability in $IC_{50}$ values a theoretical 95% confidence interval calculation was developed for single laboratory $IC_{50}$’s, translating into a range of $\frac{[I_1]}{IC_{50}}$ and $\frac{[I_2]}{IC_{50}}$ values. The extent by which this range falls above the criteria is a measure of risk associated with the decision, due to variability in $IC_{50}$ values.
INTRODUCTION

Digoxin is a widely prescribed medicine to treat heart failure and atrial fibrillation (The Digitalis Investigation Group, 1997; Wyse et al., 2002). Digoxin has a narrow therapeutic range, and toxic effects may occur when plasma levels are elevated (e.g. > 2 ng/mL). Plasma concentrations of digoxin have been shown to increase in the presence of concomitantly administered drugs such as quinidine, verapamil, or itraconazole (Muller and Fromm, 2011). Concomitant administration of drugs associated with P-glycoprotein (P-gp) inhibition act to increase intestinal absorption or decrease biliary and renal elimination of digoxin resulting in elevated digoxin plasma levels (Benet et al., 1999; Greiner et al., 1999; Kusuhara and Sugiyama, 2002; Susanto and Benet, 2002; Chan et al., 2004). Many pharmaceutical companies currently perform an in vitro P-gp inhibition study to assess the risk of a clinically significant P-gp-mediated DDI between a candidate drug and digoxin. The decision to perform a clinical digoxin DDI study is based on decision criteria published in the FDA (Food and Drug Administration) draft guidance on DDIs. A clinical drug interaction study with digoxin is warranted if \([I_1]/IC_{50} > 0.1\) or \([I_2]/IC_{50} > 10\), where \([I_1]\) represents the maximum blood/plasma concentration of inhibitor at steady state, \([I_2]\) represents the concentration of inhibitor in the gastrointestinal tract based on highest approved dose dissolved in 250 mL and IC_{50} represents the concentration of inhibitor resulting in 50% inhibition of in vitro P-gp mediated transport (CDER, 2012). This guidance however, does not take into account potential lab-to-lab or system-to-system variability in the IC_{50} measurement (Bentz et al., under review).

The companion manuscript (Bentz et al., under review) evaluated and characterized the interaction of drugs with human P-gp in various cell lines and expression systems routinely employed across the pharmaceutical industry (Burton et al., 1993; Anderle et al., 1998; Gao et
al., 2001; Hooiveld et al., 2002; Giacomini et al., 2010). A total of twenty-three laboratories determined IC$_{50}$ values for fifteen inhibitors where each laboratory used their in-house established experimental system (cell lines or P-gp over-expressing membrane vesicles). Substantial lab-to-lab variability was observed, indicating that the current universal decision criteria applied to a single laboratory IC$_{50}$ value might not be optimal.

It has recently been demonstrated that digoxin is not a P-gp specific probe substrate, but is also a substrate of uptake transporters (Bentz et al., 2005; Kimoto et al., 2011; Taub et al., 2011). These uptake transporters may be present in the experimental systems used to measure IC$_{50}$ values. Acharya et al., provided kinetic evidence of an apical and basolateral digoxin uptake transporter in MDCKII-MDR1 and Caco-2 cells (Acharya et al., 2008; Lumen/Bentz, personal communication), while Taub et al., demonstrated active sodium-dependent digoxin uptake in a human embryonic kidney cell line (Taub et al., 2011). The in vitro IC$_{50}$ values determined in the currently used cell lines with digoxin as a probe substrate may therefore not be entirely due to inhibition of P-gp, but may represent inhibition of both P-gp and an uptake transporter. The current FDA P-gp decision tree recommends digoxin as a probe substrate of P-gp and does not account for interaction of digoxin and inhibitors with other potential transporters. This should be kept in mind when extrapolating either in vitro “P-gp” inhibition data, or in vivo digoxin clinical DDI data to other P-gp substrates.

This paper describes the application of Receiver Operating Characteristic (ROC) analysis as a statistical tool to generate improved decision criteria for assessment of a drug candidate’s propensity to increase digoxin clinical exposure. The ROC analysis was utilized to derive refined decision criteria that incorporated in vitro P-gp IC$_{50}$ values determined by twenty-three laboratories, thereby accounting for IC$_{50}$ variability. The refined ROC derived decision criteria
were then assessed utilizing a test set consisting of *in vitro* P-gp IC₅₀ values and *in vivo* digoxin DDI data obtained from literature publications.

The primary objectives of the work herein were to: (1) refine the current P-gp DDI decision tree criteria by using a large data set of P-gp IC₅₀ values generated by twenty three laboratories for fifteen compounds for which clinical digoxin DDI data was available and (2) apply the ROC-derived criteria to a test set of 101 *in vitro-in vivo* pairs to characterize the rate of false negatives (FNs) and false positives (FPs) and compare the FN and FP results generated when applying the FDA proposed criteria. Furthermore, because the decision criteria were developed specifically for digoxin and digoxin may not be a P-gp specific probe substrate, it is proposed herein that the “P-gp” decision tree be referred to as a “digoxin” decision tree.
Materials and Methods

Chemicals and Reagents

Fifteen drugs for which in vivo interaction data with digoxin is available (amiodarone, carvedilol, diltiazem, felodipine, isradipine, mibefradil, nicardipine, nifedipine, nitrendipine, quinidine, ranolazine, sertraline, telmisartan, troglitazone, verapamil) were obtained from Sigma (US and UK). Other chemicals and reagents were obtained by each participating lab from local commercial sources. For details on the cell lines, cell culture procedures, membrane vesicles, bidirectional transport assays across polarized cell monolayers, and the vesicle uptake assays, the reader is referred to the companion paper (Bentz et al., under review).

P-gp inhibition experiments using polarized cell lines or vesicles

Bidirectional transport assays across polarized cell monolayers were conducted as described in (Bentz et al., under review). Briefly, basolateral-to-apical (B>A) and apical-to basolateral (A>B) transport of the P-gp probe substrate digoxin across polarized Caco-2, LLC-PK1-MDR1, or MDCKII-MDR1 cell monolayers were assessed in the absence and presence of six increasing concentrations of the inhibitors listed above. 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 receiver compartment. Monolayers were then incubated at 37°C for 45 to 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 LC-MS/MS. P-gp inhibition studies using membrane vesicles are also described in (Bentz et al., under review). Briefly, P-gp dependent uptake of either N-methylquinidine or vinblastine into P-gp over-
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expressing vesicles was assessed in the absence and presence of seven increasing concentrations of the inhibitors listed above. The reaction mixtures without ATP or in the presence of AMP-PNP (non-hydrolysable 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 to 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 due to binding of digoxin to the extracellular domain of the Na+/K+-ATPase (the pharmacological target for digoxin) in P-gp over-expressing membrane vesicles.

IC$_{50}$ calculations

Only IC$_{50}$ values obtained with probe substrate transport in the B-to-A direction across polarized cell monolayers or into P-gp over-expressing membrane vesicles were utilized in the ROC analysis. The IC$_{50}$ values were fitted with the logistic equation using a 2 parameter fit with lower and upper bounds constrained to no inhibition and complete inhibition, respectively (Lyles et al., 2008; Bentz et al., under review):

$$A(I_1) = A(\infty) + \frac{A(0) - A(\infty)}{1 + \exp\left(\alpha + \beta \ln\left[I_1\right]\right)}$$  
Eqn 1

$$IC_{50} = \exp\left[-\alpha / \beta\right]$$

Where:

A([I$_1$]) is the transport activity measured when the inhibitor concentration is [I$_1$]
A(∞) is remaining transport activity 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 transport activity measured in absence of inhibitor (the negative control).

IC$_{50}$ is the inhibitor concentration at which \((A(0)+A(∞))/2\).

β is slope factor or Hill coefficient.

Establishment of decision criteria by Receiver Operating Characteristic (ROC) analysis

The objective is to establish \([I_1]/IC_{50}\) and \([I_2]/IC_{50}\) binary classification or decision criteria to predict a binary outcome of the clinical digoxin PK ratio (AUC or C$_{max}$ ratio) in the presence and absence of inhibitor. The optimal discrimination criteria corresponds to the lowest classification error, i.e. the lowest combined probability of false positive and false negative predictions. A positive clinical outcome occurs when the PK ratio is 1.25 or greater and a negative outcome occurs when the PK ratio is < 1.25. A positive prediction (a clinical digoxin DDI will occur) is made when \([I_1]/IC_{50} > c_1\) or \([I_2]/IC_{50} > c_2\), where c1 and c2 represent the discrimination criteria for \([I_1]/IC_{50}\) and \([I_2]/IC_{50}\). The prediction obtained from the \([I_1]/IC_{50}\) and \([I_2]/IC_{50}\) discrimination criteria can then be compared with the true in vivo outcome and a 2 x 2 table constructed to estimate the accuracy of the prediction and select the optimal values of c1 and c2 as shown below in Table 1.
The TP, FP, FN and TN are the number of true positives, false positives, false negatives and true negatives, respectively. The total number of positive predictions (P) and the total number of negative predictions (N) are also listed in Table 1. The true positive rate (TPR) is defined as TP/P and the false positive rate (FPR) is defined as FP/N. The TP and FP are then calculated for a wide range of possible combinations of c1 and c2, with c1 ranging from 0.0001 to 0.1 and c2 from 1 to 1000. The value for the combination of c1 and c2 is then selected based on the highest accuracy (TP+TN)/(P+N) achieved. A ROC curve can then be drawn by plotting the TPR (sensitivity) against the FPR (1-specificity). A ROC curve with a higher AUC’ value (area under the ROC curve) is preferred over a curve with a lower value, an AUC’ value of 1 representing perfect predictability and a value of 0.5 indicates no predictability. Therefore the AUC’ value is also a measure of discrimination, or the likelihood that the criteria ([I1]/IC50 > c1 or [I2]/IC50 > c2) correctly classifies the DDI as being clinically significant.

This ROC analysis was performed using IC50 values from each of the four experimental systems (Caco-2, LLC-PK1-MDR1, MDCKII-MDR1 and P-gp over-expressing vesicles) combined. The IC50 values can be found in supplemental Table 4.

The ROC derived criteria of ([I1]/IC50 > c1 and [I2]/IC50 > c2) were optimized together based on highest degree of accuracy in predicting TP and TN to obtained new criteria. All analysis was conducted in SAS V9.2 (SAS Institute, Inc, Cary, NC).

**Assembling digoxin DDI set to test the decision criteria: The test set**

The refined ROC derived decision criteria were tested with an assembled test set consisting of *in vitro* P-gp IC50 values and *in vivo* digoxin DDI data obtained from literature publications. This P-gp inhibitor test set was distinct from the fifteen inhibitors used to generate
the refined ROC decision criteria described above. Clinical digoxin DDI studies and *in vitro* IC$_{50}$ values for perpetrators were collected by searching the Drug Interaction Database (University of Washington) and PubMed. Perpetrators with both clinical digoxin DDI and IC$_{50}$ values were collated and paired. If more than two *in vitro* IC$_{50}$ values were reported, the lowest and highest values were selected to avoid over-populating the database with a single perpetrator. Among the *in vitro* perpetrators, twenty-two had two IC$_{50}$ values and twenty-five had only one value. The AUC and C$_{max}$ ratios were extracted from 70 clinical DDI studies. In total, 101 pairs of [I$_1$]/IC$_{50}$ and [I$_2$]/IC$_{50}$ versus clinical AUC or C$_{max}$ ratios were created to form the data set.

**95% Confidence Interval determination for single IC$_{50}$ values**

Typically, a single lab will determine an IC$_{50}$ value for a drug candidate using a single experimental system. In all likelihood, if that IC$_{50}$ was measured in several different labs, substantially different IC$_{50}$ values might be generated. Therefore the authors have developed 95% confidence interval calculations for individual IC$_{50}$ values to determine the range of plausible IC$_{50}$ values, based on the variance in IC$_{50}$ values observed in the companion paper (Bentz *et al.*, under review).

The estimate of an IC$_{50}$ value does not follow a normal distribution. However, an approximate 95% confidence interval for the true population IC$_{50}$ can be determined by first considering the natural logarithm (ln) of the estimated IC$_{50}$. The standard deviation (SD) of ln IC$_{50}$ is then approximated by the total variance found in the IC$_{50}$ values in the companion paper and the 95% confidence interval for the ln IC$_{50}$ of a new drug candidate determined in a single lab can be defined as:
(ln IC\textsubscript{50} - 1.96 * SD [ln IC\textsubscript{50}]) to (ln IC\textsubscript{50} + 1.96 * SD [ln IC\textsubscript{50}])  Eqn 2

The SD of ln IC\textsubscript{50} (SD[ln IC\textsubscript{50}]) is the square root of the total variance based on all IC\textsubscript{50} values reported in the companion manuscript (Bentz \textit{et al.}, under review), which is 1.08. This value represents the SD of the typical variability seen in generating IC\textsubscript{50} values regardless of the protocol, system or lab.

The 95% confidence intervals were calculated for several individual compounds from the test set to determine the range of plausible IC\textsubscript{50} values. For example, the ln IC\textsubscript{50} for maraviroc is 5.21 (ln(183)) and the SD of ln IC\textsubscript{50} is 1.08. To obtain the 95% confidence interval for the IC\textsubscript{50} of maraviroc, the antilog of the upper and lower limits are determined. The 95% confidence interval for the population of IC\textsubscript{50} values for maraviroc is expressed as exp \textsuperscript{3.09} to exp \textsuperscript{7.32}. The mean IC\textsubscript{50} for maraviroc is likely to range from 22 to 1521. This interval gives a wide range of possible values for the IC\textsubscript{50} due to the large variability in IC\textsubscript{50} measurements. This range in IC\textsubscript{50} values can then be utilized to calculate the range of possible [I\textsubscript{1}]/IC\textsubscript{50} and [I\textsubscript{2}]/IC\textsubscript{50} values. The extent by which this range falls above the decision criteria is a measure of risk associated with the decision, due to IC\textsubscript{50} variability. The risk is expressed as a p value (see below) and describes the probability that the decision criteria would be exceeded and a DDI study indicated, if the IC\textsubscript{50} was measured in any other lab or system.

\textbf{Determination of p-value}
As described above, the p value describes the risk, or probability (p), that for a given IC50 value determined in a single lab, the cutoff criteria may be exceeded based on consideration of the range in possible IC50 values. This risk is equal to the fraction of the confidence interval range of lnIC50 values resulting in (I1 or I2)/IC50 above the cutoff criteria (normal cumulative distribution) and can be calculated using the following formula in R version 2.15.2 (statistical software package):

\[ p \text{ value} = (\text{pnorm}(\ln(I_1 \text{ or } I_2/cutoff), \ln(\text{IC}_{50}),1.08)) \]

where ‘cutoff’ is the (I1 or I2)/(IC50) cutoff derived either by the ROC method or the original FDA value, the I1, I2 and IC50 are the values for the inhibitor of interest and 1.08 is the overall SD for lnIC50 described above. The value returned is the cumulative probability and will be between 0 (low risk) and 1 (high risk).

A value for p can similarly be calculated in Microsoft Excel using the statistical “NORMDIST” function, which returns the normal cumulative distribution (p) for a parameter X, using a specified mean and SD. As an example, consider the I1 of 1.7 µM for maraviroc and the ROC cutoff for I1/IC50 of 0.03, such that the ln(I1/cutoff) for maraviroc equals 4.037. This value equals the lnIC50 at which the I1/IC50 cutoff criterion for maraviroc is reached. This is the parameter X for which to calculate the cumulative probability (normal cumulative distribution) that the I1/IC50 value for maraviroc determined in a single lab falls above the decision criterion, when taking into account the possible range in maraviroc IC50 values due to overall IC50 variability. So the mean in the NORMDIST function is the lnIC50 for maraviroc (5.21) and the SD equals 1.08. Finally, 1 should be entered as the last value in the formula to return the cumulative distribution. The formula should appear as such: ‘=NORMDIST(4.037,5.21,1.08,1)’.
This calculation returns a value of 0.14 for p. In general, a p value less than 0.3 could be considered to indicate low risk and greater than 0.7 to indicate high risk of a transporter-mediated DDI when incorporating variability in a single lab IC\textsubscript{50} measurement.

**RESULTS**

**Clinical Digoxin Data for the Receiver Operating Characteristic Training Set**

Table 2 shows a summary of human digoxin pharmacokinetic DDI data collected from the literature for the fifteen drugs used to investigate \textit{in vitro} P-gp IC\textsubscript{50} variability. The clinical digoxin DDI studies considered were those in which digoxin was dosed orally on multiple days alone and in the presence of the concomitant drug. Studies in which digoxin was administered intravenously and case studies were excluded. For some perpetrators, several clinical digoxin DDI data sets were available as studies were conducted by different investigators. All clinical study results were incorporated in our analysis. For the ROC analysis, the authors chose the PK parameter (AUC or C\textsubscript{max}) that exhibited the greater increase in digoxin exposure in the presence of the perpetrator, this is denoted as the combined PK ratio. Several perpetrator drugs such as carvedilol, diltiazem and felodipine resulted in both positive and negative digoxin drug interactions, \textit{i.e.} changes in digoxin plasma exposure were less than or greater than the clinical threshold value of 1.25.

**Receiver Operating Characteristic Analysis**

The purpose of this analysis is to determine the optimal discrimination threshold for [I\textsubscript{1}]/IC\textsubscript{50} or [I\textsubscript{2}]/IC\textsubscript{50}, that corresponds to the lowest classification error, where the classification error is the combined probability of false positive and false negative predictions of the digoxin DDI and greatest accuracy, the highest number of TP and TN. The [I\textsubscript{1}] represents the total maximum
systemic concentration of the inhibitor at steady state and [I₂] is the concentration achieved when the highest approved dose is dissolved in 250 mL. Using the unbound concentration for I₁ did not add any further discriminatory power toward TP, FN, FP and FN’s. The largest and most robust data set of IC₅₀ values were utilized in this analysis, i.e. those values obtained from inhibition of probe substrate transport in the B>A direction and fitted using the logistic equation (values found in Supplemental Table 1). The data set consists of 293 IC₅₀ values generated for fifteen inhibitors by twenty three pharmaceutical laboratories and includes cell-based as well as vesicle-based values. The ROC analysis determined the combined criteria for [I₁]/IC₅₀ and [I₂]/IC₅₀ as 0.03 and 45, respectively, which resulted in the lowest classification error and greatest accuracy. The accuracy was 74%. The percent FPs and FNs were 26 and 27%, respectively.

For the reader’s interest, separate ROC analyses were also performed for each of the individual systems: Caco-2, MDCKII-MDR1, LLC-PK₁, MDR1 and P-gp over-expressing vesicles and several individual labs that obtained 13 or more IC₅₀ values (Supplemental Table 2 and Supplemental Figure 1A-D and Supplementary Figure 2A-C).

**Application of ROC-Derived and FDA Criteria to a Digoxin DDI Test Set**

The digoxin DDI data were collected using the University of Washington Metabolism & Transport Drug Interaction Database and PubMed. The clinical digoxin DDI studies selected were based on oral administration, and included single and multiple dose digoxin regimens and single and multiple dose inhibitor administrations, while intravenous digoxin administration studies and case study reports were excluded. A total of 101 pairs of *in vitro* P-gp IC₅₀ values
and associated *in vivo* digoxin PK changes were created as described in the Materials and Methods section. This set includes forty-seven unique perpetrators, while the fifteen inhibitors from the training set were excluded. The 101 pairs are listed in supplementary Table 3. Digoxin is the main *in vitro* probe substrate with several studies utilizing calcein AM. Cook *et al.*, demonstrated an *in vitro* correlation between digoxin and calcein AM (Cook *et al.*, 2010). These data were then used to generate the TPR, TNR, FPR and FNR using both the ROC derived and the FDA proposed criteria (Table 3). The accuracy for the ROC and FDA criteria was 73% and 69%, respectively. The FPR was 40% and 51% for the ROC and FDA criteria, respectively. The ROC and FDA cut-off values resulted in a FNR of 12% and 10% respectively. The FNR and FPR in the training set are different from the test set since the training set consists of multiple, highly variable *in vitro* IC₅₀ values for each of the 15 perpetrator compounds. For most perpetrator drugs in the test set only one IC₅₀ value could be found in the scientific literature.

As shown in Table 4, the compounds that triggered a FN by both the ROC and FDA criteria are conivaptan, cimetidine, talinolol (2 separate studies). AZD5672 and flecainide were FN by the ROC criteria only and ambrisentan by the FDA criteria only. The ROC and FDA criteria resulted in a common set of twelve compounds triggering a FP result, while the FDA criteria resulted in an additional six FP (sparfloxacin, maraviroc, rofecoxib, levofloxacin, aliskiren (two separate studies) and dabigatran etexilate). For omeprazole and pantoprazole, two clinical digoxin studies for each were reported in which the ROC criteria predicted FP for both cases while the FDA predicted correctly one of the two cases (see supplementary Table 3). For both ritonavir and talinolol, each had one clinical digoxin DDI evaluation indicating no drug interaction while the other studies demonstrated a clinical drug interaction with PK change greater than 25% (see Supplemental Table 3). ROC derived criteria for the individual systems
(Caco-2, MDCKII-MDR1 and P-gp over-expressing vesicles) were also applied to the 101 digoxin test set; however, as expected, the ROC derived criteria when all \textit{in vitro} systems data were incorporated performed better (see Supplemental Table 4).

**IC\textsubscript{50} Variability Based Risk Score (p) for \([I_1]/IC\textsubscript{50} and [I_2]/IC\textsubscript{50} Based on Single Lab IC\textsubscript{50} Measurements**

For the inhibitors in the test set, little is known about their lab-to-lab IC\textsubscript{50} variability and this will typically be the case for the IC\textsubscript{50} value of a drug candidate as well. The authors suggest that more confidence in a single IC\textsubscript{50} based risk assessment could be obtained by assigning a risk score to the single laboratory \([I_1]/IC\textsubscript{50} and [I_2]/IC\textsubscript{50} values, which is the probability (p) that for a given IC\textsubscript{50} value determined in a single lab, the cutoff criteria may be exceeded based on consideration of the range in possible IC\textsubscript{50} values. This risk score can be generated based on the variance model utilizing all \textit{in vitro} systems IC\textsubscript{50} values in the training set, generated by the twenty-three laboratories for the fifteen inhibitors (see materials and methods). The p values calculated for several FN and TN compounds identified by ROC-derived criteria are shown in Table 5. Conivaptan (ROC FN based on single IC\textsubscript{50} value) showed a high p value of 0.69 for \([I_1]/IC\textsubscript{50} and a low p value of 0.11 for [I_2]/IC\textsubscript{50} associated with not performing a clinical digoxin DDI, which would indicate a clinical digoxin DDI study is warranted based on IC\textsubscript{50} variability. For sparfloxacin, maraviroc, and aliskiren, both \([I_1]/IC\textsubscript{50} and [I_2]/IC\textsubscript{50} showed low p values associated with not performing a clinical interaction study even when taking into account IC\textsubscript{50} variability, consistent with the clinical observation. For flecainide, the p value was 0.4 and 0.46 for \([I_1]/IC\textsubscript{50} and [I_2]/IC\textsubscript{50} respectively, which is higher than that for sparfloxacin, maraviroc and aliskiren. In reality, flecainide does show a clinically significant PK change of 1.36.

Montelukast showed a high \([I_1]/IC\textsubscript{50} risk of 0.89 which would likely trigger a clinical digoxin
DDI study based on risk associated with IC\textsubscript{50} variability, but in reality no clinical interaction is observed. While not 100\% predictive, the risk score does provide additional information to support decisions on whether or not to run a clinical digoxin study, because the inherent variability in the IC\textsubscript{50} values is taken into account. Moreover, the decision to conduct the clinical digoxin DDI study depends on the level of risk an organization is willing to assume and is one that ultimately the individual company should make. In utilizing the risk score, a value less than 0.3 is considered a low risk and a value greater than 0.7 indicates a high risk of a transporter-mediated DDI.

**Digoxin Decision Tree**

In Figure 2, the digoxin decision tree provides a suggested pathway for the evaluation of the potential for an NCE (new chemical entity) to alter digoxin disposition due to inhibition of drug transporters. The recommended *in vitro* test system is either a polarized cell line such as Caco-2, MDCKII-MDR1, LLC-PK\textsubscript{1}-MDR1, or P-gp over-expressing membrane vesicles. A unidirectional transport assay of digoxin in the B\textgreater{}A direction (polarized cell lines) or uptake of N-methylquinidine or vinblastine (vesicles) is recommended. As noted in Bentz *et al.*, (Bentz *et al.*, under review), no single system out-performs the other based on our IC\textsubscript{50} variability analysis. If no alteration in transport is observed with increasing concentrations of the NCE then the NCE is not likely to interact with digoxin and no further studies are needed. However, if increasing concentrations of the NCE result in the inhibition of probe substrate transport, then the generation of an IC\textsubscript{50} value is recommended. If the [I\textsubscript{1}]/IC\textsubscript{50} or [I\textsubscript{2}]/IC\textsubscript{50} are less than the indicated criteria, a clinical digoxin assessment may not be required, depending on drug class.
and therapeutic area. However, if the \( \frac{I_1}{IC_{50}} \) or \( \frac{I_2}{IC_{50}} \) are greater than the criteria, a clinical evaluation of the potential for the NCE to alter the pharmacokinetics of digoxin is recommended.
DISCUSSION

This manuscript refines the current FDA draft guidance criteria for prediction of digoxin DDIs by applying ROC analysis to IC$_{50}$ data generated for fifteen perpetrators in one of four experimental systems by twenty-three laboratories. The refined ROC derived criteria are $[I_1]/IC_{50} > 0.03$ and $[I_2]/IC_{50} > 45$. These new values were tested against 101 clinical digoxin DDI pairs that were devoid of the fifteen perpetrators utilized to generate the training set. We found that the new criteria resulted in fewer FP and higher accuracy, while the FNR remained at a similar level (12%) when compared to the current FDA criteria (10%). Further, a risk score was developed for single laboratory $[I_1]/IC_{50}$ and $[I_2]/IC_{50}$ values to incorporate IC$_{50}$ variability in the DDI risk assessment.

The current FDA draft guidance criteria for risk assessment of a clinical digoxin PK interaction through P-gp inhibition are based on single P-gp IC$_{50}$ values, without regard to experimental system or IC$_{50}$ equation. Due to the lack of repetitive data in the literature, it has not yet been possible to consider potential variability in IC$_{50}$ values and the effect of that variability on the decision criteria. The companion manuscript demonstrates that the variability in P-gp IC$_{50}$ values is high, which indicates that a universal decision criteria based on a relatively small set of single IC$_{50}$ values generated by different labs using different experimental systems and transport inhibition equations, may not be ideal for each individual lab. The FDA universal criteria are based on minimizing the FNR to ensure patient safety without consideration of inter-laboratory variability.

This manuscript accounts for variability by applying ROC analysis that incorporates P-gp IC$_{50}$ values generated by twenty-three laboratories for fifteen perpetrator drugs. Because an IC$_{50}$ value is required for the ROC analysis, only drugs that inhibited probe substrate transport \textit{in vitro}
were utilized, which included those associated with positive as well as negative digoxin clinical DDIs. Despite the relatively high rate of FP and FN in the training set (~25%), the ROC-derived criteria performed well when applied to a test set of 101 pairs of $[I_1]/IC_{50}$ and $[I_2]/IC_{50}$ values versus clinical digoxin AUC or $C_{\text{max}}$ ratios.

Agarwal et al., (Agarwal et al., 2012) assembled a data set of eleven compounds approved between 2003 and 2010. The authors observed that the decision criteria in the current FDA guidance was highly accurate in predicting the TP (100%, 5 of 5) and FN (100% 0 of 5), with TN at 67% (4 of 6) and FP at 33% (2 of 6) in this small data set. In the current analysis, the authors have assembled a larger data set of 101 clinical digoxin DDI pairs (the 11 drugs used in Agarwal et al., are included) for a broader, more comprehensive evaluation of the FDA and ROC-derived criteria.

Similar to the training set, the test set contained drugs that inhibited digoxin transport where an IC$_{50}$ value was reported. The literature data set consisted of one or two single laboratory IC$_{50}$ values generated using one of various experimental systems and transport inhibition equations. The predictions based on the FDA and ROC-derived criteria were similar, except the ROC-derived criteria performed slightly better than the FDA criteria with respect to accuracy (73% vs. 69%) and exhibited fewer FP (40% vs. 51%) due to the larger $[I_2]/IC_{50}$ threshold of 45 (compared to 10 for the FDA criteria). The FDA criteria predicted six FP that were classified correctly by the ROC criteria. Both sets of cutoff values performed similarly with respect to generation of FN (12% for ROC and 10% for FDA), in which the FDA and ROC criteria classified talinolol, cimetidine and conivaptan as FN while ambrisentan was FN by FDA criteria and flecainide and AZD5672 were FN by the ROC criteria. The FNR was low and is of greatest concern to both regulatory authorities and sponsors who seek to limit unexpected
digoxin toxicities. The ROC-derived criteria, while a universal criteria, incorporated IC$_{50}$ variability and was derived statistically to identify the best combined [I$_1$]/IC$_{50}$ and [I$_2$]/IC$_{50}$ criteria to achieve the greatest accuracy and minimize FNR and FPR. It is noted that the investigations herein did not compare the ROC derived criteria to the newly endorsed European Medicine Agency (EMA) guidance as it is based only on an [I$_2$]/IC$_{50}$ criterion while the ROC criteria is based on [I$_1$]/IC$_{50}$ and [I$_2$]/IC$_{50}$.

The authors also propose an additional test for individual laboratories to incorporate variability in their single IC$_{50}$ value by assigning a theoretical variability based on the variance in IC$_{50}$ values observed in the training set. A risk score (p) can be calculated to indicate the probability that [I$_1$]/IC$_{50}$ and [I$_2$]/IC$_{50}$ values will fall above the decision criteria when variability is incorporated into the single lab determined IC$_{50}$ value. This risk score p ranges from 0 to 1. The higher the value of p, the greater the risk of a FN.

Alternatively, a lab may prefer to generate their own ROC derived criteria by choice (Cook et al., 2010) or may observe that their system generates IC$_{50}$ values outside the range reported in our companion manuscript (Bentz et al., under review). Cook et al. generated IC$_{50}$ values for 26 inhibitors in Caco-2 polarized cell mono-layers. As part of the current work, the authors generated ROC-based decision criteria from the Cook 2010 dataset for comparison with the universal ROC-derived criteria (Supplemental Table 1). The decision criteria based on the data in the Cook paper are in line with the universal decision criteria. Thus, if a lab prefers to generate their own decision criteria by ROC analysis, at least 25 inhibitors should be included.

Recently, several laboratories have published in vitro data indicating that, in commonly used cell lines such as MDCKII-MDR1 cells, primary isolated hepatocytes, or HEK293 cells digoxin is recognized and transported by transporters other than P-gp (Bentz et al., 2005; Kimoto
et al., 2011; Taub et al., 2011). It has been demonstrated that in MDCKII-MDR1 cells, digoxin transport involves uptake transporters as well as P-gp and therefore the IC$_{50}$ measured using this cell line is not necessarily specific for P-gp. Since the in vivo disposition of digoxin may involve uptake and efflux transporters, inhibition of such transporters can theoretically contribute to elevated digoxin exposures.

Currently, the only formulation of digoxin commercially available is a tablet (Lanoxin™) which has a bioavailability of 70%. There are several co-medications that increase digoxin exposure greater than 2-fold such as valsparod (Kovarik et al., 1999), dronedarone (Sanofi-Aventis, 2009), quinidine (Rameis, 1985), amiodarone (Robinson et al., 1989) and cyclosporine (Dorian et al., 1988), which is much greater than the theoretical increase in digoxin exposure if only intestinal P-gp was inhibited (~42% increase based on digoxin bioavailability increasing from 70% to 100%). It is likely that inhibition of digoxin uptake in or efflux from the liver or kidney is also a possible contributor to certain DDIs. Also, many of the FP (Table 4) except talinolol were administered orally and on multiple days in which in vivo induction may occur negating the inhibitory effect on digoxin transport. This potential scenario can be anticipated by investigating the induction potential of concomitant medications if decision criteria are exceeded. Interestingly, the EMA (Committee for Human Medicinal Products (CHMP), 2012) has recommended digoxin as a probe substrate for P-gp-mediated renal DDIs only and another probe substrate, dabigatran etexilate, for intestinal P-gp-mediated DDIs.

Since digoxin is not a specific probe substrate for P-gp, in vitro or in vivo, effects on digoxin transport in vitro, or on digoxin exposure in vivo, cannot necessarily be attributed solely to P-gp inhibition. As such, the authors propose a digoxin specific DDI tree to guide digoxin DDI risk assessment. While the authors do not discount the idea that digoxin may serve as a
useful probe substrate to investigate P-gp related DDIs, caution must be exercised in extrapolating observations with digoxin towards other P-gp substrates that may not have similar properties to digoxin. As a note, IC$_{50}$ values obtained using vesicles with NMQ and vinblastine as probe substrates, instead of digoxin, were included in the derivation of the ROC criteria and are an acceptable system to use for prediction of clinical digoxin DDI risk.

In conclusion, decision criteria to predict the potential of a new chemical entity to increase digoxin exposure have been proposed which are based on ROC statistical analysis. The proposed refined criteria incorporate the variability determined for inhibition of digoxin transport in the presence of fifteen inhibitors, generated by twenty-three laboratories. The refined criteria of $[I_1]/IC_{50} = 0.03$ and $[I_2]/IC_{50} = 45$ increased the accuracy and decreased the false positive rate while maintaining a low false negative rate (12%) when tested against a data set of in vitro IC$_{50}$ and clinical digoxin DDI data obtained from the literature. Additionally, a risk score calculation was developed to indicate the probability that $[I_1]/IC_{50}$ and $[I_2]/IC_{50}$ values will fall above the decision criteria when theoretical variability is incorporated into a single lab determined IC$_{50}$ value. This will further facilitate the decision whether or not to conduct a clinical digoxin DDI study. The authors also propose that the decision criteria be specific for digoxin and advocate a digoxin specific decision tree to guide the necessity of a clinical digoxin DDI study.
ACKNOWLEDGEMENTS

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Authorship Contribution:

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Conducted Experiments: Coleman, Chung, Deng, Forsgard, Ragueneau-Majlessi

Contributed new reagents or analytic tools: NA

Performed data analysis: Bentz, Coleman, Deng, Ellens, Lee, O’Connor

Wrote or contributed to the writing of the manuscript: Coleman, Ellens, Lee, Neuhoff, Taub
REFERENCES


Footnotes

DISCLAIMER

The manuscript reflects the views of the authors and should not be construed to represent FDA’s views or policies. Lei Zhang has no conflict of interest to report.

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Figure Legends:

Figure 1. Receiver operating characteristic curve incorporating all company data. The sensitivity on the x axis represents the TPR, while 1-specificity on the y-axis represents the FPR, where the specificity is the TNR

Figure 2. Digoxin DDI decision tree
Table 1. Receiver Operating Characteristic Definition

<table>
<thead>
<tr>
<th>Prediction (([I_1]/IC_{50} \text{ or } [I_2]/IC_{50}))</th>
<th>Truth (clinical AUC or (C_{\text{max}} \text{ ratio}))</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>TP</td>
<td>FP</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>FN</td>
<td>TN</td>
</tr>
<tr>
<td>Total</td>
<td>P</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Inhibitor</td>
<td>Dose (mg/day)</td>
<td>AUC ratio</td>
<td>C&lt;sub&gt;max&lt;/sub&gt; ratio</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------</td>
<td>-----------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Amiodarone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>800</td>
<td>1.68</td>
<td>1.84</td>
</tr>
<tr>
<td>Amiodarone&lt;sup&gt;b&lt;/sup&gt;</td>
<td>400</td>
<td>1.63</td>
<td>1.72</td>
</tr>
<tr>
<td>Captopril</td>
<td>37.5</td>
<td>1.39</td>
<td>1.59</td>
</tr>
<tr>
<td>Carvedilol (female)</td>
<td>12.5</td>
<td>1.24</td>
<td>1</td>
</tr>
<tr>
<td>Carvedilol (male)</td>
<td>12.5</td>
<td>1.56</td>
<td>1.38</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>90</td>
<td>1.4</td>
<td>1.38</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>180</td>
<td>1.24</td>
<td>1.24</td>
</tr>
<tr>
<td>Felodipine</td>
<td>2.5</td>
<td>1.43</td>
<td>1.35</td>
</tr>
<tr>
<td>Felodipine</td>
<td>5</td>
<td>0.76</td>
<td>1.14</td>
</tr>
<tr>
<td>Felodipine</td>
<td>10</td>
<td>1.18</td>
<td>1.35</td>
</tr>
<tr>
<td>Felodipine</td>
<td>10</td>
<td>Not available</td>
<td>1.18</td>
</tr>
<tr>
<td>Isradipine</td>
<td>15</td>
<td>1.11</td>
<td>1.26</td>
</tr>
<tr>
<td>Mibepradil&lt;sup&gt;c&lt;/sup&gt;</td>
<td>150</td>
<td>1.31</td>
<td>1.41</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>20</td>
<td>1.06</td>
<td>1.06</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>15</td>
<td>1.21</td>
<td>1.01</td>
</tr>
<tr>
<td>Drug</td>
<td>Concentration</td>
<td>([I_1] )</td>
<td>([I_2] )</td>
</tr>
<tr>
<td>------------</td>
<td>---------------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>30</td>
<td>1.23</td>
<td>1.06</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>60</td>
<td>1.18</td>
<td>1.08</td>
</tr>
<tr>
<td>Nitrendipine</td>
<td>20</td>
<td>1.15</td>
<td>1.57</td>
</tr>
<tr>
<td>Nitrendipine</td>
<td>10</td>
<td>1.09</td>
<td>1.22</td>
</tr>
<tr>
<td>Quinidine</td>
<td>600</td>
<td>1.76</td>
<td>1.75</td>
</tr>
<tr>
<td>Quinidine</td>
<td>1200</td>
<td>2.65</td>
<td>2.11</td>
</tr>
<tr>
<td>Quinidine</td>
<td>750</td>
<td>Not available</td>
<td>2.65</td>
</tr>
<tr>
<td>Ranolazine</td>
<td>2000</td>
<td>1.6</td>
<td>1.46</td>
</tr>
<tr>
<td>Sertraline</td>
<td>200</td>
<td>1.1</td>
<td>1.05</td>
</tr>
<tr>
<td>Telmisartan</td>
<td>120</td>
<td>1.22</td>
<td>1.58</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>400</td>
<td>1.04</td>
<td>1.05</td>
</tr>
<tr>
<td>Verapamil</td>
<td>240</td>
<td>1.51</td>
<td>1.44</td>
</tr>
<tr>
<td>Verapamil</td>
<td>360</td>
<td>Not available</td>
<td>1.61</td>
</tr>
<tr>
<td>Verapamil</td>
<td>240</td>
<td>Not available</td>
<td>1.77</td>
</tr>
<tr>
<td>Verapamil</td>
<td>240</td>
<td>Not available</td>
<td>1.53</td>
</tr>
</tbody>
</table>

\(^a[I_1] = \) maximum inhibitor concentration at steady state and \([I_2] = \) nominal intestinal concentration assumed gut volume is 250 mL.

\(^b\) Amiodarone: Doses are from the same study. 800 mg was dosed for one week (maintenance dose) and concentration represents the one week dosing and the 400 mg was the next 5 weeks.

\(^c\) No digoxin clinical DDI study so selected highest mibebradil dose.
Table 3. Application of *In Vitro* Criteria to a Test Set of *In Vitro* IC$_{50}$ Values Paired with Clinical DDI Data$^a$

<table>
<thead>
<tr>
<th></th>
<th>[I$<em>1$/IC$</em>{50}$]</th>
<th>[I$<em>2$/IC$</em>{50}$]</th>
<th>True Positive$^b$ (Percentage)</th>
<th>True Negative$^c$ (Percentage)</th>
<th>False Positive$^c$ (Percentage)</th>
<th>False Negative$^b$ (Percentage)</th>
<th>Accuracy (Percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDA</td>
<td>0.1</td>
<td>10</td>
<td>45 (90)</td>
<td>25 (49)</td>
<td>26 (51)</td>
<td>5 (10)</td>
<td>70 (69)</td>
</tr>
<tr>
<td>ROC</td>
<td>0.03</td>
<td>45</td>
<td>44 (88)</td>
<td>30 (58)</td>
<td>21 (40)</td>
<td>6 (12)</td>
<td>74 (73)</td>
</tr>
</tbody>
</table>

$^a$Digoxin DDI data set contains 101 pairs of *in vitro* IC$_{50}$ data and clinical DDI data.

Value represents total hits out of $^b$50 and $^c$51 pairs of *in vitro* IC$_{50}$ data and clinical DDI data.
Table 4. False Negative and False Positive Compounds Utilizing FDA and ROC Criteria Values

<table>
<thead>
<tr>
<th>FDA</th>
<th>ROC</th>
</tr>
</thead>
<tbody>
<tr>
<td>False Positives</td>
<td>False Negatives</td>
</tr>
<tr>
<td>Propafenone</td>
<td>Talinolol</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Cimetidine</td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>Conivaptan</td>
</tr>
<tr>
<td>Quinine</td>
<td>Ambrisentan</td>
</tr>
<tr>
<td>Etravirine</td>
<td></td>
</tr>
<tr>
<td>Montelukast</td>
<td></td>
</tr>
<tr>
<td>Disopyramide</td>
<td></td>
</tr>
<tr>
<td>Ritonavir</td>
<td></td>
</tr>
<tr>
<td>Fidaxomicin</td>
<td></td>
</tr>
<tr>
<td>Eplerenone</td>
<td></td>
</tr>
<tr>
<td>Omeprazole</td>
<td></td>
</tr>
<tr>
<td>Pantoprazole</td>
<td></td>
</tr>
<tr>
<td>Sparfloxacin</td>
<td></td>
</tr>
<tr>
<td>Maraviroc</td>
<td></td>
</tr>
<tr>
<td>Rofecoxib</td>
<td></td>
</tr>
<tr>
<td>Levofloxacin</td>
<td></td>
</tr>
<tr>
<td>Aliskiren</td>
<td></td>
</tr>
<tr>
<td>Dabigatran etexilate</td>
<td></td>
</tr>
</tbody>
</table>
Table 5. Risk Score p for $[I_1]/IC_{50}$ and $[I_2]/IC_{50}$

<table>
<thead>
<tr>
<th>Compound</th>
<th>PK Change*</th>
<th>Lower Bound $[I_1]/IC_{50}$</th>
<th>Upper Bound $[I_1]/IC_{50}$</th>
<th>p</th>
<th>Lower Bound $[I_2]/IC_{50}$</th>
<th>Upper Bound $[I_2]/IC_{50}$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conivaptan</td>
<td>1.80</td>
<td>0.006</td>
<td>0.43</td>
<td>0.69</td>
<td>1.4</td>
<td>99</td>
<td>0.11</td>
</tr>
<tr>
<td>Flecaainide</td>
<td>1.36</td>
<td>0.003</td>
<td>0.19</td>
<td>0.40</td>
<td>4.8</td>
<td>334</td>
<td>0.46</td>
</tr>
<tr>
<td>Sparfloxacin</td>
<td>1.18</td>
<td>0.001</td>
<td>0.07</td>
<td>0.12</td>
<td>1.6</td>
<td>113</td>
<td>0.13</td>
</tr>
<tr>
<td>Montelukast</td>
<td>1.11</td>
<td>0.013</td>
<td>0.92</td>
<td>0.89</td>
<td>1.0</td>
<td>71</td>
<td>0.06</td>
</tr>
<tr>
<td>Maraviroc</td>
<td>1.0</td>
<td>0.001</td>
<td>0.08</td>
<td>0.14</td>
<td>1.5</td>
<td>106</td>
<td>0.12</td>
</tr>
<tr>
<td>Citalopram</td>
<td>1.08</td>
<td>0.0005</td>
<td>0.03</td>
<td>0.03</td>
<td>1.0</td>
<td>71</td>
<td>0.06</td>
</tr>
<tr>
<td>Aliskiren</td>
<td>1.15</td>
<td>0.001</td>
<td>0.08</td>
<td>0.14</td>
<td>4.4</td>
<td>301</td>
<td>0.42</td>
</tr>
</tbody>
</table>

*PK change represents either AUC or $C_{\text{max}}$ ratio of digoxin in the absence and presence of inhibitor, value is the higher of the two PK parameters.
Figure 1

![ROC Curve](image)

- **Sensitivity**
- **1 - Specificity**

- **AUC' = 0.78**
Figure 2.

Cellular and Vesicle Assays (digoxin, NMQ or vinblastine)  
(Caco-2, MDR1-MDCK, MDR1-LLCPK1, MDR1-Vesicles)

Probe substrate Papp (B>A or into vesicles) decreasing with increased concentrations of the investigational drug

- Probably a digoxin transporter inhibitor, determine IC\(_{50}\)
  - I/IC\(_{50}\) > 0.03 or I\(_2/IC_{50}\) > 45 (based on ROC analysis)
    - An \textit{in vivo} drug interaction study with digoxin is recommended
  - I/IC\(_{50}\) < 0.03 and I\(_2/IC_{50}\) < 45 (based on ROC analysis)
    - An \textit{in vivo} drug interaction study with digoxin may not be needed

Probe substrate Papp (B>A or into vesicles) not changing with increased concentrations of the investigational drug

- Poor or not inhibitor of probe substrate disposition