Application of Static Modeling in the Prediction of In Vivo Drug–Drug Interactions between Rivaroxaban and Antiarrhythmic Agents Based on In Vitro Inhibition Studies

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ABSTRACT

Rivaroxaban, a direct Factor Xa inhibitor, is indicated for stroke prevention in nonvalvular atrial fibrillation (AF). Studies have revealed that the clearance of rivaroxaban is largely attributed to CYP3A4, CYP2J2 metabolism, and P-glycoprotein (P-gp) efflux pathways. Amiodarone and dronedarone are antiarrhythmic agents employed in AF management. Amiodarone, dronedarone, and their major metabolites, N-desethylamiodarone (NDEA) and N-desbutyl-dronedarone (NDBD), demonstrate inhibitory effects on CYP3A4 and CYP2J2 with U.S. Food and Drug Administration–recommended probe substrates. In addition, both amiodarone and dronedarone are known P-gp inhibitors. Hence, the concomitant administration of these antiarrhythmic agents has the potential to augment the systemic exposure of rivaroxaban through simultaneous impairment of its clearance pathways. Currently, however, clinical data on the extent of these postulated drug–drug interactions are lacking. In this study, in vitro inhibition assays using rivaroxaban as the probe substrate demonstrated that both dronedarone and NDBD produced reversible inhibition as well as irreversible mechanism-based inactivation of CYP3A4– and CYP2J2-mediated metabolism of rivaroxaban. However, amiodarone and NDEA were observed to cause reversible inhibition as well as mechanism-based inactivation of CYP3A4 but not CYP2J2. In addition, amiodarone, NDEA, and dronedarone, but not NDBD, were determined to inhibit P-gp–mediated rivaroxaban transport. The in vitro inhibition parameters were fitted into a mechanistic static model, which predicted a 37% and 31% increase in rivaroxaban exposure due to the inhibition of hepatic and gut metabolism by amiodarone and dronedarone, respectively. A separate model quantifying the inhibition of P-gp–mediated efflux by amiodarone or dronedarone projected a 9% increase in rivaroxaban exposure.

Introduction

Atrial fibrillation (AF) is the most commonly sustained and clinically significant cardiac arrhythmia, affecting approximately 33.5 million people worldwide in 2010 (Chugh et al., 2014). As the prevalence of AF increases with age, the global burden of the disease is projected to increase exponentially, creating a significant public health burden (Rahman et al., 2014). Therapeutic cornerstones of AF management include ventricular rate control, maintenance of sinus rhythm using antiarrhythmic therapy, and prevention of stroke and systemic embolism using anticoagulants (January et al., 2014).

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ABBREVIATIONS: ACN, acetonitrile; AF, atrial fibrillation; AUC, area under the curve; AUCR, area under the curve ratio; DD1, drug–drug interaction; DMEM, Dulbecco's modified Eagle's medium; LC, liquid chromatography; MBI, mechanism-based inactivation; MDCK, Madin-Darby canine kidney; MDR, multidrug resistance protein; MS/MS, tandem mass spectrometry; NDBD, N-desbutyl-dronedarone; NDEA, N-desethyl-amiodarone; P-gp, P-glycoprotein; P450, cytochrome P450; P_app, apparent permeability; PBPK, physiologically based pharmacokinetic; rP450, recombinant cytochrome P450.
the presence of any degree of renal impairment (FDA, 2011b). This attests to the inherent susceptibility of rivaroxaban to complex drug–drug interactions (DDIs) mediated through simultaneous impairment of its multiple clearance pathways. Given the steep relationship between rivaroxaban exposure and major bleeding (FDA, 2011a), it is thus imperative to identify clinically relevant DDI scenarios that could augment rivaroxaban exposure.

A subgroup analysis of the ROCKET AF trial (Rivaroxaban Once Daily Oral Direct Factor Xa Inhibition Compared with Vitamin K Antagonism for Prevention of Stroke and Embolism Trial in Atrial Fibrillation) studying the efficacy and safety of rivaroxaban highlighted the potential combination of antiarrhythmic drug therapy and anticoagulation as mainstays in AF management and concluded that the concomitant use of amiodarone and rivaroxaban warrants further investigation (Steinberg et al., 2014). Dronedarone (Multaq; Sanofi Aventis, Bridgewater, NJ) was approved in 2009 as a structural analog of amiodarone (FDA, 2009a). In a bid to minimize the extracardiac adverse effects associated with amiodarone, iodine substituents were eliminated to avoid amiodarone-linked thyroid toxicities and a methane-sulfonamide group was added to reduce tissue accumulation (Fig. 1, A and B) (FDA, 2009a).

Both amiodarone (Fig. 1A) and dronedarone (Fig. 1B) undergo extensive metabolism by CYP3A4/3A5 to form pharmacologically active metabolites N-desethylamiodarone (NDEA) (Fig. 1C) and N-desbutyldronedarone (NDBD) (Fig. 1D), respectively (Fabre et al., 1993; Klieber et al., 2014). In addition, both drugs are substrates of CYP2J2 (Lee et al., 2010; Karkhanis et al., 2016). The common structural features in these antiarrhythmic agents and their metabolites are the alkylamine and furan that are in turn associated with mechanism-based inactivation (MBI) of P450 (Orr et al., 2012). Indeed, corroborating previous findings, our laboratory established the reversible and irreversible inhibition of CYP3A4- and CYP2J2-mediated metabolism of FDA-recommended probe substrates by amiodarone and NDEA (Ohyama et al., 2000; McDonald et al., 2015; Karkhanis et al., 2016) as well as dronedarone and NDBD (Hong et al., 2016; Karkhanis et al., 2016). Independently, amiodarone and dronedarone have been reported as P-gp inhibitors (FDA, 2009b, 2012).

Taken together, we hypothesized that amiodarone, dronedarone, NDEA, and NDBD could increase systemic exposure of rivaroxaban via their inhibitory effects on CYP3A4, CYP2J2, and P-gp. To date, there remains a paucity of clinical data on the DDIs between rivaroxaban and these antiarrhythmic agents. This study aimed to quantitatively predict the in vivo DDI risk between rivaroxaban and amiodarone or dronedarone via mechanistic static modeling. To characterize the multifaceted DDIs and generate the inhibitory parameters accurately, rivaroxaban was used as the probe substrate in place of the respective FDA-recommended probe substrates of CYP3A4, CYP2J2, and P-gp.

Materials and Methods

In this study, in vitro inhibition data encompassing reversible inhibition, MBI, and inhibition of P-gp–mediated efflux were fitted into mechanistic static models that provided the framework for quantitative predictions of either metabolic or transporter-based DDIs between rivaroxaban and the antiarrhythmic drugs.

Chemicals. High-performance liquid chromatography (LC)–grade acetonitrile (ACN) was purchased from Tedia Company Inc. (Fairfield, OH). Amiodarone hydrochloride, dronedarone hydrochloride, NDBD, NDEA, rivaroxaban, and verapamil hydrochloride were acquired from Sigma-Aldrich (St. Louis, MO). Amprenavir was purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). Human recombinant cytochrome P450 P450 (rP450) supermesom and an NADPH regenerating system consisting of NADPH A (NADP+ and glucose-6-phosphate) and B (glucose-6-phosphate dehydrogenase) were obtained from BD Gentest (Woburn, MA). Water was obtained using a Milli-Q water purification system (Millipore, Billerica, MA). For cell culture, Dulbecco’s modified Eagle’s medium (DMEM) with phenol red, Dulbecco’s phosphate buffer solution, fetal bovine serum, and 10,000 IU antibiotic solution (penicillin/streptomycin) were obtained from Gibco Life Technologies (Waltham, MA). Lucifer yellow CH dilitium salt was from Invitrogen Corporation (Carlsbad, CA). t-Glutamine was from HyClone Laboratories (Logan, UT). Sodium bicarbonate powder was from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade.

Fig. 1. Chemical structures of (A) amiodarone, (B) dronedarone, (C) NDEA, and (D) NDBD.
Reversible Inhibition of CYP3A4 and CYP2J2 by Amiodarone, Dronedaron, and Their Metabolites. Amiodarone, dronedaron, and their metabolites (i.e., NDEA and NDBD) were tested as reversible inhibitors using rivaroxaban as the probe substrate. Experiments were performed in 96-well plates and all samples were carried out in triplicates. Rivaroxaban (2.5, 5, 15, 30, and 50 \( \mu M \)) was preincubated at 37°C for 5 minutes with 20 pmol/ml rP450 enzymes, NADPH B, and 100 mM potassium phosphate buffer (pH 7.4) across multiple concentration levels of the inhibitors (amiodarone: 0–5 \( \mu M \); dronedaron, NDEA, and NDBD: 0–10 \( \mu M \)). The reactions were initiated by the addition of 5 \( \mu M \) NADPH A, yielding a final incubation mixture of 100 \( \mu M \) with 1\% ACN (v/v). Incubation was carried out at 37°C for either 2 hours with CYP3A4 or 30 minutes with CYP2J2 before an 80-\( \mu l \) aliquot was removed and quenched with an equal volume of ice-cold ACN containing 0.005 \( \mu M \) verapamil (internal standard). The quenched samples were subjected to centrifugation at 2755 \( g \) at 4°C for 30 minutes. Subsequently, the supernatants were removed to determine the morpholine hydroxylated metabolite (main metabolite) of rivaroxaban by LC–tandem mass spectrometry (MS/MS) analysis (Supplemental Methods; Supplemental Table 1). The data were first fitted to the Michaelis–Menten model. Subsequently, Lineweaver–Burk plots were applied to predict the mode of reversible inhibition. The apparent equilibrium dissociation constant (\( K_i \)) for the enzyme-inhibitor complex was determined by nonlinear least-squares regression based on the best model of reversible inhibition.

**Time- and Concentration-Dependent Inactivation of CYP3A4 and CYP2J2 by Amiodarone, Dronedaron, and Their Metabolites.** Rivaroxaban was used as the probe substrate in this experiment. Incubations (\( n = 3 \)) were conducted in 96-well plates. Primary incubation mixtures comprising various concentration levels of amiodarone (0–5 \( \mu M \)) or NDEA (0–1 \( \mu M \)) were preincubated at 37°C for 5 minutes with rP450 enzymes (20 pmol/ml) and NADPH B in potassium phosphate buffer (100 mM, pH 7.4). To initiate the enzymatic reaction, 5 \( \mu M \) NADPH A was added to the primary incubation. The final primary incubation mixture volume was 100 \( \mu l \) and contained <1\% (v/v) organic solvent. At different preincubation time points (0, 3, 8, 15, 22, 30, and 45 minutes) after the addition of NADPH A, 5-\( \mu l \) aliquots of the primary incubation were transferred to 95 \( \mu l \) of the secondary incubation containing 50 \( \mu M \) rivaroxaban, the NADPH regenerating system, and 100 mM potassium phosphate buffer (pH 7.4) to yield a 20-fold dilution. The secondary incubation mixtures were incubated at 37°C for 2 hours with CYP3A4 or 30 minutes with CYP2J2 before 80-\( \mu l \) aliquots were removed and quenched with an equal volume of ice-cold ACN containing 0.005 \( \mu M \) verapamil (internal standard). The same morpholine hydroxylated metabolite was quantified using LC–MS/MS (Supplemental Methods; Supplemental Table 1). Inactivation of CYP3A4 (40 pmol/ml) and CYP2J2 (20 pmol/ml) by dronedaron (0–2.5 \( \mu M \)) and NDBD (0–5 \( \mu M \)) was investigated using the same two-step incubation protocol except with a 10-fold dilution into the secondary incubation.

**Calculation of Inactivation Kinetic Parameters (\( K_i \) and \( k_{\text{inact}} \)).** The mean of triplicate peak area ratios was normalized to 0 minutes with respect to preincubation time. The percentage of probe substrate activity remaining was computed and the natural logarithmic activity was plotted against inactivation preincubation time for each inactivator concentration. The data were fitted to a linear regression model, and \( k_{\text{inact}} \) values (apparent inactivation rate constants) were calculated as the negative slopes of the lines. Subsequently, a plot of \( k_{\text{inact}} \) values against inactivator concentration ([I]) allowed the fitting of inactivation kinetic parameters (\( K_i \) and \( k_{\text{inact}} \)) to nonlinear least-squares regression based on eq. 1 in GraphPad PRISM software (version 6.01; GraphPad Inc., San Diego, CA).

\[
k_{\text{inact}} = \frac{k_{\text{max}} \times [I]}{K_i + [I]}
\]  

In eq. 1, \( k_{\text{max}} \) represents the maximum inactivation rate constant at infinite inactivator concentration, \( K_i \) is the concentration of inactivator at the half-maximum rate of inactivation, and [I] is the in vitro inactivator concentration. **Inhibition of P-gp Efflux of Rivaroxaban by Amiodarone, Dronedaron, and Their Metabolites,** Madin-Darby canine kidney (MDCK) subclone I cells transfected with multidrug resistance protein (MDR) 1 were maintained in DMEM culture media supplemented with 10% fetal bovine serum, \( l \)-glutamine (862 mg/l), and 1% penicillin/streptomycin. For transport studies, cells were first seeded at a density of 250,000 cells/well and the culture medium was refreshed after 24 hours. At approximately 48 hours postseeding, the culture medium was first removed and each well and insert were rinsed gently with phosphate-buffered saline to ensure no residual metabolic waste. Test inhibitors were dissolved in methanol, whereas rivaroxaban was reconstituted in ACN and dimethyl sulfoxide at lower (2 mM) and higher concentration (20 mM) levels, respectively. Dilutions for all compounds were carried out using ACN. Triplicate sets of wells were used to assess the apparent permeability (\( P_{\text{app}} \)) of rivaroxaban from the apical (A) to basolateral (B) chambers \( P_{\text{app}} \) (A→B) and B to A \( P_{\text{app}} \) (B→A). To initiate transport, the donor solution was added first, followed by the receiver solution. All experiments were carried out at room temperature (24 ± 1°C). Amperanav (10 \( \mu M \)) and propranolol (25 \( \mu M \)) were used as positive controls (substrate and inhibitor, respectively). \( P_{\text{app}} \) (A→B) of lucifer yellow (100 \( \mu M \)) was used as a marker of monolayer integrity. The fluorescence of lucifer yellow of each apical and basolateral solution was measured at an excitation wavelength of 430 nm and an emission wavelength of 540 nm using a Tecan Infinite F500 plate reader (Männedorf, Switzerland). Acceptance criterion for a confluent monolayer was defined as lucifer yellow permeability of <80 nm/s.

To ensure P-gp assay sensitivity, experiments were first performed to investigate the potential for both concentration- and time-dependent saturation of the transport of rivaroxaban via P-gp (Supplemental Methods). The optimal incubation time was eventually determined to be 90 minutes and a rivaroxaban concentration of 10 \( \mu M \) was also selected (Supplemental Figs. 1 and 2). Subsequently, 25 and 12.5 \( \mu M \) of each inhibitor (i.e., amiodarone, dronedaron, NDEA, and NDBD) was first subjected to a preliminary study to estimate the IC\(_{50}\) values. The inhibitor concentrations were subsequently optimized to encompass the IC\(_{50}\) and ensure inhibition assay sensitivity. The receiver solution was collected and stored at −20°C for further sample processing prior to LC–MS/MS analysis (Supplemental Methods). To determine the mode of P-gp inhibition, three inhibitor concentration levels in proximity of the IC\(_{50}\) value were assayed against two concentration levels of rivaroxaban (5 and 20 \( \mu M \)). A Dixon plot was generated to predict the mode of P-gp inhibition and to determine the \( K_i \) values.

**Estimating the Extent of Metabolic DDIs Using a Mechanistic Static Model.** The kinetic constants accounting for reversible inhibition (i.e., \( K_i \)) and time-dependent inactivation (i.e., \( k_{\text{inact}} \)) of the drug-metabolizing enzymes were incorporated into a mechanistic static model developed previously by Fahimi et al. (2008) and refined by Ishihara et al. (2012) to account for multi-P450 inhibition. The proposed model accounts for the contributions of enzyme inhibition both in the liver and within the gut wall in predicting the extent of DDIs. The area under the curve ratio (AUCR) in the presence of a pharmacokinetic DDI is described by eq. 2:

\[
\text{AUCR} = \frac{1}{1 + \left[I\frac{F_{B}}{K_i} + K_i\right]}
\]

The terms are defined as follows. \( A \) is the term for time-dependent inactivation observed in the liver for each of the P450 enzymes inactivated:

\[
B = \frac{1}{1 + \left[I\frac{F_{B}}{K_i} + K_i\right]}
\]

\( X \) is the term for time-dependent inactivation of CYP3A4 observed in the intestine:

\[
Y = \frac{1}{1 + \left[I\frac{F_{B}}{K_i} + K_i\right]}
\]
where \([I_{\text{H}}]\) and \([I_{\text{G}}]\) represent in vivo concentrations of the inhibitor available to the enzyme in the liver and intestine, respectively (Table 1; Supplementary Table 2). The degradation rates for CYP3A4 in the liver \((k_{\text{deg,H}})\) and intestine \((k_{\text{deg,G}})\) were 0.00032 and 0.00048 min\(^{-1}\) based on a half-life of 36 hours and 24 hours, respectively (Fahmi et al., 2008). For CYP2J2, there are insufficient clinical pharmacokinetic data to perform similar calculations. As a result, the average of the calculated estimates for the various hepatic P450 enzymes (0.00026 min\(^{-1}\)) was used (Yang et al., 2008). The fraction of rivaroxaban metabolized by CYP3A4 (\(f_{\text{m,CYP3A4}}\)) and CYP2J2 \((f_{\text{m,CYP2J2}})\) was reported to be 0.18 and 0.14, respectively (Mueck et al., 2014), whereas the fraction of rivaroxaban escaping intestinal extraction \((F_{\text{G}})\) was calculated to be 0.89 (see the Supplementary Methods).

Estimating the Extent of Transporter-Mediated DDIs Using a Mechanistic Static Model. The kinetic constant describing the inhibition of P-gp-mediated efflux of rivaroxaban was fitted into another mechanistic static model developed to examine the effect of inhibition of renal secretion transporters on plasma exposure of the victim drug (Feng et al., 2013). The AUCR of the victim drug in the presence and absence of the inhibitor is summarized in eq. 3:

\[
\text{AUCR} = \frac{1 + (C_{\text{sec,c}} + C_{\text{I}})}{1 + (C_{\text{sec,c}} + C_{\text{I}})} = \frac{1 - \frac{C_{\text{I}}}{K_{\text{I}}} \left(\frac{1}{1 + \frac{C_{\text{I}}}{K_{\text{I}}}}\right)}{1 - \frac{C_{\text{I}}}{K_{\text{I}}} \left(\frac{1}{1 + \frac{C_{\text{I}}}{K_{\text{I}}}}\right)}
\]

(3)

where \(I\) represents the maximum plasma concentration of the inhibitor (Table 1). The net secretory clearance of rivaroxaban \((C_{\text{sec,c}})\) (55.6 ml/min) was approximated to be five-sixths of the total plasma renal clearance (66.7 ml/min) (Mueck et al., 2014). The total plasma clearance \((CL)\) of rivaroxaban was reported to be 166.7 ml/min and is a composite of the hepatic and renal clearances (Mueck et al., 2014).

**Results**

Reversible Inhibition of CYP3A4 and CYP2J2 by Amiodarone, Dronedarone, and Their Metabolites. The reversible inhibition of CYP3A4 and CYP2J2 by amiodarone, dronedarone, and their metabolites was investigated using the peak area of rivaroxaban metabolite as a proxy for the rate of product formation. Rivaroxaban was evaluated across five concentration levels spanning its \(K_m\) in the presence of varying concentrations of amiodarone, dronedarone, NDEA, and NDBD. In a preliminary study of reversible inhibition of CYP2J2 by amiodarone and NDEA, high concentrations of both amiodarone and NDEA were easily overcome by a small increase in rivaroxaban concentration, thus suggesting that amiodarone and NDEA did not inhibit CYP2J2 with rivaroxaban as the probe substrate (Supplemental Fig. 3A). However, based on the inhibition kinetics plots (Fig. 2, A and B) and their respective Lineweaver–Burk plots (Fig. 2, C and D), dronedarone and NDBD were established to be mixed competitive inhibitors of CYP2J2 when rivaroxaban was used as the probe substrate. On the other hand, amiodarone and NDEA exhibited mixed competitive inhibition of CYP3A4 (Fig. 3, A, B, E, and F), whereas dronedarone and NDBD demonstrated competitive inhibition (Fig. 3, C, D, G, and H). The calculated inhibition constants \(K_i\) of the relevant inhibitors against the respective enzymes are presented in Table 2.

Time- and Concentration-Dependent Inactivation of CYP3A4 and CYP2J2 by Amiodarone, Dronedarone, and Their Metabolites. To investigate the MBI of CYP3A4 and CYP2J2 by amiodarone, dronedarone, and their metabolites, rivaroxaban was used as the probe substrate and the rate of hydroxylation at the morpholinone moiety of rivaroxaban was monitored and used as a surrogate for enzymatic activity. In the presence of NADPH, a time-dependent decrease in CYP2J2 enzymatic activity was not observed when preincubated with amiodarone (5 \(\mu\)M and 50 \(\mu\)M) and NDEA (Supplemental Fig. 3, B and C). However, time-dependent inactivation of CYP2J2 was demonstrated in the presence of dronedarone and NDBD. Preincubation of CYP2J2 with increasing concentration levels of either dronedarone or NDBD resulted in a concentration-dependent increased rate of inactivation of enzymatic activity. The observed first-order rates of inactivation \((k_{\text{obs}})\) calculated from various concentrations of dronedarone (Fig. 4A) and NDBD (Fig. 4B) followed saturation kinetics that approached a maximum rate of inactivation (Fig. 4, C and D). Likewise, as presented in Fig. 5, A–D, time- and concentration-dependent inactivation of CYP3A4 was established for both amiodarone, dronedarone, and their metabolites. Kinetic plots \((k_{\text{obs}} versus inactivator concentration)\) also demonstrated saturation kinetics (Fig. 5, E–H). Calculated inactivation kinetic parameters, \(K_i\) and \(k_{\text{obs,act}}\) are summarized in Table 3. The efficiency of enzyme inactivation \((k_{\text{obs,act}}/K_i\) ratio) is also reported.

**Cell Monolayer Integrity and Compound Recovery.** A-to-B flux determinations of lucifer yellow were used to confirm monolayer integrity when coincubated with the highest concentration levels of the test substrate and inhibitors. These data confirmed that the monolayer was intact under these extreme conditions and validated its suitability for subsequent permeability studies. Using liquid-liquid extraction, a high recovery of rivaroxaban (approximately 100%) and accurate linear calibration (±20% accuracy and \(R^2\approx 0.99\)) were achieved reproducibly from 0.01 to 10 \(\mu\)M. For rivaroxaban concentrations above 10 \(\mu\)M, saturation of the detector was observed and the samples were diluted using DMEM before sample processing to ensure its accurate quantitation. Percentage recovery of rivaroxaban from the apical and basolateral chambers at the end of the assay (mass balance) was >80%, indicating that no significant amount of rivaroxaban was lost during sample transfer and processing nor in MDCK-MDR1, hence demonstrating the reliability of measured \(P_{\text{app}}\) values.

**P-gp Inhibitory IC\(_{50}\) and K\(_i\) of Test Inhibitors.** The concentration-dependent inhibitory effect of amiodarone, dronedarone, and their metabolites on transport of rivaroxaban across MDR1-MDCKII monolayers was tested. IC\(_{50}\) is the inhibitor concentration needed to decrease the efflux ratio by half. Amiodarone (Fig. 6A) and NDEA (Fig. 6B) yielded similar IC\(_{50}\) values of 10.3 \(\mu\)M and 9.2 \(\mu\)M, respectively. Based on an IC\(_{50}\) of 1.83 \(\mu\M) (Fig. 6C), dronedarone was determined to be the most potent P-gp inhibitor, whereas NDBD demonstrated little P-gp inhibition activity, as seen from its high IC\(_{50}\) value of 76.3 \(\mu\M) (Fig. 6D). Subsequently, the respective inhibitory concentrations that produced a linear decrease in efflux ratio were chosen for the Dixon plots (data not shown), in which we confirmed amiodarone’s noncompetitive allosteric inhibition of P-gp efflux of rivaroxaban (5 and 20 \(\mu\M) with a
$K_i$ at 8.94 μM, whereas dronedarone demonstrated competitive inhibition with a $K_i$ at 0.68 μM. Assuming the same mode of non-competitive inhibition of P-gp, the $K_i$ value of NDEA was determined to be 5.36 μM. The $K_i$ value of NDBD was not further elucidated, as its high IC$_{50}$ value was predicted not to produce meaningful inhibitory potential.

Static Modeling of Metabolic and Transport-Based DDIs. In vitro inactivation ($k_{\text{inact}}$ and $K_i$) and inhibition ($K_i$) parameters were subsequently incorporated into a mechanistic static model (eq. 2) that permits comprehensive evaluation of the potential impact of MBI and reversible inhibition of CYP3A4 and CYP2J2 on the systemic exposure of rivaroxaban. In the assessment of metabolic DDI potential using area under the curve (AUC) fold change, inhibition of hepatic metabolism of rivaroxaban by amiodarone, NDEA, dronedarone, and NDBD was predicted to produce AUC fold changes of 1.22, 1.22, 1.17, and 1.26 respectively (Table 4). When the combined effects of altered hepatic and intestinal metabolism were considered, there was an increase in the AUC fold change precipitated by amiodarone and dronedarone to 1.37 and 1.31, respectively (Table 4). Transporter-mediated DDI was quantitatively determined by fitting in vitro parameters describing inhibition of P-gp–mediated efflux of rivaroxaban into eq. 3. As summarized in Table 4, the eventual AUC fold change was 1.09 for both dronedarone and amiodarone, whereas NDEA produced a slightly higher AUC fold change of 1.13.

Fig. 2. Reversible inhibition of recombinant CYP2J2 (rCYP2J2) by dronedarone and NDBD. (A and B) The formation rate of the morpholine hydroxylated metabolite was plotted against inhibitor concentration and fitted in the Michaelis–Menten kinetic model to calculate the inhibition constant $K_i$ for (A) dronedarone and (B) NDBD toward rCYP2J2. (C and D) Lineweaver–Burk plots exhibit mixed competitive inhibition of rCYP2J2 by dronedarone and NDBD. Each point represents the mean ± S.D of triplicate experiments.

Fig. 3. Reversible inhibition of recombinant CYP3A4 (rCYP3A4) by amiodarone, NDEA, dronedarone, and NDBD. (A–D) The formation rate of the morpholine hydroxylated metabolite was plotted against inhibitor concentration and fitted in the Michaelis–Menten kinetic model to calculate the inhibition constant $K_i$ for (A) amiodarone, (B) NDEA, (C) dronedarone, and (D) NDBD toward rCYP3A4. (E–H) Lineweaver–Burk plots exhibit mixed competitive inhibition of rCYP3A4 by (E) amiodarone and (F) NDEA and competitive inhibition of rCYP3A4 by (G) dronedarone and (H) NDBD. Each point represents the mean ± S.D of triplicate experiments.
Discussion

The application of in vitro methodologies to evaluate the inhibitory potential of a drug entity and assess the likelihood of in vivo drug interactions is a critical aspect of the drug development and regulatory review paradigm (Zhang et al., 2009). In this study, we quantified the in vitro inhibition parameters to characterize the DDI between rivaroxaban and the antiarrhythmic agents.

Rivaroxaban was not tested as a substrate in previous in-house studies investigating the CYP3A4 and CYP2J2 inhibitory potencies of amiodarone, dronedarone, and their metabolites (Hong et al., 2016; Karkhanis et al., 2016). In this study, we observed a significantly slower rate of rivaroxaban clearance by CYP3A4 as compared with CYP2J2 during initial assay optimization (data not published). Our optimization culminated in two incubation time periods of 2 hours and 30 minutes for the sensitive detection of inhibitory effects against CYP3A4 and CYP2J2, respectively.

We report for the first time the competitive inhibition as well as MBI of CYP3A4 and CYP2J2 by dronedarone and NDBD with rivaroxaban as the probe substrate. On the other hand, amiodarone and NDEA demonstrated mixed competitive inhibition of CYP3A4 but not CYP2J2. Similarly, MBI was established for amiodarone and NDEA with respect to CYP3A4 but not CYP2J2. On the basis of our reported $K_i$ values (Table 2), both amiodarone and NDEA exhibited similar potencies for mixed competitive inhibition against CYP3A4, whereas dronedarone was a relatively more potent inhibitor of both CYP3A4 and CYP2J2 as compared with NDBD. For MBI, our results confirmed that the inactivation efficiency of NDEA was approximately 3-fold higher than that of amiodarone against CYP3A4 (Table 3). Between dronedarone and NDBD, dronedarone exhibited a stronger inactivation efficiency against CYP3A4 compared with NDBD, whereas both dronedarone and NDBD demonstrated comparable CYP2J2 inactivation efficiency (Table 3).

Using two concentrations of rivaroxaban as the test substrate, our monolayer efflux studies also illustrated the noncompetitive allosteric inhibition of P-gp–mediated rivaroxaban efflux by amiodarone. This finding corroborated previous in silico studies, which also predicted amiodarone to inhibit P-gp by noncompetitive allosteric inhibition (Seelig and Landwojtowicz, 2000). Based on the structural similarities of NDEA to amiodarone, NDEA was assumed to follow a similar mode of P-gp inhibition. Intriguingly, although dronedarone was determined to be a competitive inhibitor of P-gp efflux, NDBD demonstrated a...
The systemic exposures of NDEA and NDBD are comparable to that of their parent compounds, and regulatory guidance proposes that metabolites present at ≤25% of the AUC of the parent drug warrant further consideration with regard to their potential in precipitating in vivo DDIs (FDA, 2012). Taken together, our results indeed underscore the potential contributions of NDEA and NDBA as inhibitory metabolites. NDEA ($k_{\text{inact}}/K_i = 387 \text{ min}^{-1}\text{mM}^{-1}$) being a more potent time-dependent inactivator of CYP3A4 compared with amiodarone ($k_{\text{inact}}/K_i = 130 \text{ min}^{-1}\text{mM}^{-1}$) was consistent with previous in vitro studies using midazolam as a probe substrate (McDonald et al., 2015). Likewise, NDEA ($K_i = 5.36 \mu\text{M}$) being more potent than amiodarone ($K_i = 8.94 \mu\text{M}$) in the inhibition of P-gp-mediated rivaroxaban transport was also well aligned with previous transport assays using digoxin as a probe substrate (Katoh et al., 2001). In summary, it is evident that a holistic prediction of in vivo clinical DDIs would require consideration of the combined inhibitory effects of both the parent compound and its metabolite.

Comparison of inactivation parameters obtained in our study with previous in-house data generated using FDA-validated probe substrates established a probe substrate differential response (Supplemental Table 3). In the case of CYP3A4, the inactivation efficiency ($k_{\text{inact}}/K_i$) of dronedarone and NDBD was greater when rivaroxaban was used as the probe substrate (i.e., 185 and 53.7 min$^{-1}$mM$^{-1}$) as compared with testosterone (i.e., 44.8 and 15.9 min$^{-1}$mM$^{-1}$). This phenomenon of probe substrate-dependent inhibition profiles has been emphasized mainly for CYP3A4, which possesses multiple probe substrate binding regions within its active site (Kenworthy et al., 1999). Consequently, the interactions observed with one CYP3A4 probe may not accurately reflect those observed with another probe substrate (Galetin et al., 2005; Foti et al., 2010). Although CYP2J2 metabolism is generally restricted to a single active site, our findings suggested differential binding and metabolism among different substrates. Previous studies utilizing FDA-recommended astemizole demonstrated both reversible and irreversible inhibition of CYP2J2 metabolism by amiodarone (Lee et al., 2012). However, our investigation revealed that amiodarone did not inhibit CYP2J2 reversibly and irreversibly with rivaroxaban as the probe substrate (Supplemental Figs. 3, A and B), suggesting the possibility of independent access of the active site of CYP2J2 by amiodarone and rivaroxaban (Shou et al., 1994).

Taken together, our study 1) reiterates that the variability of in vitro inhibitory potencies against CYP3A4 and CYP2J2 is highly dependent on the choice of probe substrates and 2) confirms the importance of using a specific victim drug as the probe substrate in enzyme and transporter interaction studies.

In our study, the static prediction of DDIs between amiodarone, dronedarone, and rivaroxaban yielded an AUC fold change of 1.22 and 1.17, respectively when inhibition of hepatic metabolism was considered (Table 4). On the basis of FDA (2012) guidelines (Table 5), this would imply that no significant inhibition was present. However, upon inclusion of gut metabolism, amiodarone and dronedarone were predicted to precipitate an AUC fold change in rivaroxaban exposure by 1.37 and 1.31, respectively. This attests to the significance of intestinal wall metabolism (Galetin et al., 2007), which when ignored can underestimate the extent of DDIs involving CYP3A4. In addition, our results underscore the potential contribution of NDEA and NDBD to the eventual DDI magnitude, where AUC fold changes of 1.22 and 1.26 were observed, respectively. Furthermore, the inhibition of P-gp–mediated rivaroxaban efflux by amiodarone, NDEA, and dronedarone was also predicted to independently produce AUCR increases of 1.09, 1.13, and 1.09, respectively. However, the simultaneous influences of both parent drug and metabolite as well as the cumulative impact of enzyme-transporter interplay cannot be incorporated in the current static modeling. Moreover, there is also no consensus

<table>
<thead>
<tr>
<th>TABLE 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4 and CYP2J2 inactivation kinetic parameters for amiodarone, NDEA, dronedarone, and NDBD using morpholinone hydroxylation of rivaroxaban as a surrogate measurement of residual enzymatic activity</td>
</tr>
<tr>
<td>Data represent means ± S.D.</td>
</tr>
<tr>
<td>P450</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>CYP3A4</td>
</tr>
<tr>
<td>NDEA</td>
</tr>
<tr>
<td>Dronedarone</td>
</tr>
<tr>
<td>NDBD</td>
</tr>
<tr>
<td>CYP2J2</td>
</tr>
<tr>
<td>NDEA</td>
</tr>
<tr>
<td>Dronedarone</td>
</tr>
<tr>
<td>NDBD</td>
</tr>
</tbody>
</table>

NA, not applicable.
on an appropriate surrogate concentration of the inactivator/inhibitor [I]_{in vivo} available to the enzyme. Typically, measures of [I]_{in vivo} are selected based on the estimates that provide the best correlation between predicted and observed DDIs reported in literature (Fahmi et al., 2008). Yet, in our case, our model cannot be cross-validated, since clinical interaction data between rivaroxaban and amiodarone or dronedarone are not available. Nevertheless, it has been shown that model predictability was optimal when free portal steady-state $C_{\text{max}}$ was used for the reversible inhibition portion of the expression (term B) and free systemic steady-state $C_{\text{max}}$ was used for the time-dependent inactivation portion (term A) (Fahmi et al., 2009). Hence, these validated estimates of [I]_{in vivo} were adopted in our study to enhance DDI predictability.

Given that the in vivo interactions might culminate in a more significant DDI than that estimated using mechanistic static modeling, the use of PBPK models presents several theoretical advantages. First, PBPK modeling utilizes a dynamic approach that allows consideration of changes in concentrations of enzyme, substrate, and inactivator/inhibitor with time, instead of relying on static point estimates. Second, PBPK modeling allows the evaluation of both intrinsic (e.g., organ dysfunction, age, genetics) and extrinsic (e.g., DDIs) factors, alone or in combination, on drug exposure. Third, DDIs across multiple pathways and the variability of these DDIs in different populations can be examined.

Another factor that could potentially affect the in vitro to in vivo correlation is the presence of nonspecific protein binding. The $K_i$, $K_I$, and $k_{\text{inact}}$ values were computed without accounting for protein binding in the recombinant system. However, Ishigam et al. (2001) reported how the conversion of inhibition constants to their unbound values could eventually lead to more accurate predictions of the AUC fold change upon coadministration of drugs. Furthermore, considering the high protein binding of amiodarone, dronedarone, and their metabolites, the derived in vitro inhibition and inactivation parameters might be relatively higher when unbound concentrations are considered. Hence, to increase confidence in the DDI prediction, the fraction unbound in each incubation should be determined either computationally (Austin et al., 2002) or through equilibrium dialysis experiments (Banker et al., 2003).

Table 4
Prediction of metabolic and transporter-mediated DDI upon concomitant administration of rivaroxaban with amiodarone, dronedarone, and their metabolites using mechanistic static modeling

<table>
<thead>
<tr>
<th>Precipitant</th>
<th>Predicted AUC Fold Change</th>
<th>Inhibition of Hepatic Metabolism</th>
<th>Inhibition of Hepatic and Gut Metabolism</th>
<th>Inhibition of P-gp–Mediated Efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amiodarone</td>
<td>1.22</td>
<td>1.37</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>NDEA</td>
<td>1.22</td>
<td>NA</td>
<td>1.13</td>
<td></td>
</tr>
<tr>
<td>Dronedarone</td>
<td>1.17</td>
<td>1.31</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>NDBD</td>
<td>1.26</td>
<td>NA</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

NA, not applicable; ND, not determined.
In conclusion, amiodarone, dronedarone, NDEA, and NDBD cause reversible inhibition and irreversible MBI of CYP3A4 with rivaroxaban as the probe substrate. Amiodarone and NDEA, unlike dronedarone and NDBD, do not inhibit CYP2J2. Amiodarone, dronedarone, and NDEA, but not NDBD, inhibit P-gp-mediated efflux of rivaroxaban. Static modeling predicted a weak DDI risk between rivaroxaban and amiodarone or dronedarone. Fundamental limitations of the static model implied that molecular interactions between rivaroxaban and the antiarrhythmic agents and their metabolites via CYP3A4, CYP2J2, and P-gp were not considered in entirety. Future work would involve the assimilation of these in vitro inhibition parameters into a dynamic PBPK model, from which a more accurate quantitation of DDI magnitude can be derived.

Authorship Contributions

Participants in research design: Cheong, Goh, Hong, Kojodjojo, Chan.

Conducted experiments: Cheong, Goh, Hong, Venkatasen, Liu.

Contributed new reagents or analytic tools: Chiu.

Performed data analysis: Cheong, Goh, Hong, Chan.

Wrote or contributed to the writing of the manuscript: Cheong, Goh, Hong, Chan.

References


FDA guidelines for relative risk of DDIs based on observed increases in AUC for the victim drug

<table>
<thead>
<tr>
<th>Category of Relative Risk</th>
<th>Observed Changes in AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong DDI</td>
<td>$\geq 5$-fold increase in AUC</td>
</tr>
<tr>
<td>Moderate DDI</td>
<td>$\geq 2$ but $&lt;5$-fold increase in AUC</td>
</tr>
<tr>
<td>Weak DDI</td>
<td>$\geq 1.25$ but $&lt;2$-fold increase in AUC</td>
</tr>
</tbody>
</table>


FDA (2009a) Multaq (Dronedarone) Briefing Document, U.S. Food and Drug Administration, Silver Spring, MD.

FDA (2009b) Multaq (Dronedarone) Clinical Pharmacology and Biopharmaceutics Review, U.S. Food and Drug Administration, Silver Spring, MD.

FDA (2011a) Kaxirola (Rivaroxaban) Clinical Pharmacology and Biopharmaceutics Review, U.S. Food and Drug Administration, Silver Spring, MD.

FDA (2011b) Kaxirola (Rivaroxaban) Product Information, U.S. Food and Drug Administration, Silver Spring, MD.


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Supplemental Data:

Application of static modeling in the prediction of *in vivo* drug-drug interactions between rivaroxaban and anti-arrhythmic agents based on *in vitro* inhibition studies

Eleanor Jing Yi Cheong, Janice Jia Ni Goh, Yanjun Hong, Gopalakrishnan Venkatesan, Yuanjie Liu, Gigi Ngar Chee Chiu, Pipin Kojodjojo and Eric Chun Yong Chan

*Drug Metabolism and Disposition*

Supplemental Methods:

**Measurement of Residual Enzyme Activity and Rivaroxaban Metabolite Formation via LC/MS/MS.** All samples were analyzed using an Agilent 1290 Infinity ultra-high pressure liquid chromatography (UHPLC) (Agilent Technologies Inc., Santa Clara, CA, USA) interfaced with the AB SCIEX QTRAP 5500 tandem mass spectrometry (MS/MS) system (AB SCIEX, Framingham, MA, USA). Separation was performed on an ACQUITY UPLC BEH C₁₈, 1.7 µM, 2.1 × 50 mm column (Waters, Mildord, MA, USA). The column and sample temperatures were maintained at 45°C and 4°C respectively. Samples were delivered using an injection volume of 5 µL. The aqueous mobile phase (A) was 0.1% [v/v] formic acid in milli-Q water whereas the organic mobile phase (B) consisted of 0.1% formic acid [v/v] in ACN. Mobile phases were delivered at a flow rate of 0.6 mL/min. The gradient program was as follows: linear gradient from 25% to 95% B (0-1.21 min), isocratic at 95% B (1.21-2 min), linear gradient from 95% to 25% B (2-2.01 min) and isocratic at 25% B (2.01-2.5 min). All analyses were performed in ESI positive mode. The MS source conditions were as follows: source temperature 650°C, curtain gas 20 psi, ion source gas 1 (sheath gas) 45 psi, ion source gas 2 (drying gas) 60 psi, ion spray voltage +5000V, collision gas (nitrogen) medium. The compound-dependent MS parameters are summarized in **Supplemental Table 1.** Chromatographic peak integrations were performed with Analyst software ver. 1.6.2 (Applied Biosystems).
**Kinetics and linearity of transport of rivaroxaban by P-gp.** To determine the optimal incubation time for the transwell assay, 10 µM of rivaroxaban was tested for both A→B and B→A transport by sampling 200 µL of receiver solution at 0, 60, 90, 120 and 180 min. The samples were stored at -20°C for further processing prior to LC/MS/MS analysis. The P_{app} in each direction was determined and plotted against time. The optimal incubation time was selected when bidirectional P_{app} (A→B, B→A) of rivaroxaban demonstrated a linear correlation with time. To determine the optimal concentration of rivaroxaban, P_{app} (A→B) and P_{app} (B→A) were measured at increasing rivaroxaban concentrations (10, 20, 30, 50 and 100 µM). An optimal concentration of rivaroxaban was defined as a concentration where P_{app} (B→A) was consistent, indicating non-saturated P-gp transporters. After a 90 min incubation, 200 µL of receiver solution was aliquoted into a 2 mL Eppendorf tube and stored at -20°C for further processing prior to LC/MS/MS analysis.

**Time- and concentration- dependent optimization of MDCK-MDR1 Transport Assay.** From 90 to 180 min, the gradient for A→B rivaroxaban transport started to plateau (Supplemental Figure 1A). On the other hand, B→A transport of rivaroxaban was linear from 0 to 180 min (Supplemental Figure 1B). Hence, 90 minutes was chosen as the optimal incubation time due to the linearity of rivaroxaban transport bidirectionally, indicating that dynamic equilibrium has not been attained. Over the concentration range of 10 to 100 µM rivaroxaban (Supplemental Figure 2), the efflux ratio of P_{app} (B→A) to P_{app} (A→B) was relatively consistent, indicating that rivaroxaban efflux was not saturable within the concentration range tested. A low concentration of 10 µM rivaroxaban was subsequently selected as it could be sensitively detected.
Supplemental Figure 1. Time-dependent (A) A→B and (B) B→A transport of rivaroxaban (10 µM). The gradients of the graphs represent dQ/dt, the flux of rivaroxaban across MDCK-MDR1. Both graphs were fitted with nonlinear regression Michaelis Menten models. Each point in (A and B) represents the mean and S.D. of triplicate experiments.

Supplemental Figure 2. Concentration dependent A→B and B→A transport of rivaroxaban (10 to 100 µM). Each point represents the mean and S.D. of triplicate experiments.

Sample processing for P-gp transport studies. For amprenavir and rivaroxaban samples, calibration standards and controls, the samples were spiked with 2 µL of 50 µM verapamil and subjected to a 2-step liquid-liquid extraction (LLE). In the first extraction, 1 ml of methyl tert butyl ether (MTBE) was aliquoted into each tube and vortexed at high speed for 5 min. Samples were then centrifuged using a microfuge at 10 000 g for 5 min at 4 °C to ensure complete separation of the media from the extraction solvent. 800 µL of MTBE was carefully aliquoted from each tube to a corresponding 2 mL
Eppendorf tube. For the second extraction, another 500 µL of MTBE was added, vortexed and spun down in a similar manner before 600 µL of MTBE was aliquoted and added to the same corresponding tube. The extraction solvent was then dried down in a turbovap using nitrogen gas at 3-5 psi before reconstituting in 200 µL of 0.1% formic acid in 50% ACN/methanol for rivaroxaban and 0.1% formic acid in methanol for amprenavir. The sample processing for propranolol followed a similar protocol except the extraction solvent used was 30% ethyl acetate in MTBE. The samples were then transferred to vials for LC/MS/MS analysis.

**LC/MS/MS quantitation.** A calibration curve was built using 0.01, 0.1, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 10 µM of rivaroxaban reconstituted in 50% ACN/methanol in DMEM. To measure accuracy, quality control (QC) samples were prepared in triplicates using a similar method where low, medium and high QCs were 0.125, 2.5 and 7.5 µM respectively. Each standard and QC was also spiked with 2µl of 50µM verapamil as internal standard. To account for chemical degradation that might have occurred during the cell assay, the calibrants were exposed at room temperature for 90 min similar to test samples. Both calibrants and QCs were subjected to the same sample processing method.

The LC/MS/MS system was similar to that described before. The sample injection volume was 1 µL for rivaroxaban, amprenavir and propranolol. Solvent A composed of 0.1% [v/v] formic acid in MiliQ water while solvent B composed of 0.1% [v/v] formic acid in acetonitrile. The solvents were pumped into the column at a flow rate of 0.6 mL/min. The gradient program for rivaroxaban was as follows: linear gradient from 25% to 95% B (0-1.21 min), isocratic at 95% B (1.21-2 min), linear gradient from 95% to 25% B (2-2.01 min) and isocratic at 25% B (2.01-2.5 min). To prevent compound accumulation on the needle, 50 % methanol in ACN was used as needle wash for 30 s per sample. For amprenavir, the gradient program was: linear gradient from 20% to 25% B (0-1.00 min), 25% to 40% B (1.00-1.5 min), 40% to 50% B (1.5 – 2 min), isocratic at 50% B (2-2.5 min), linear gradient from 50% to 95% B (2.5 – 2.51 min), isocratic at 95% B (2.51 – 2.80 min), linear gradient 95% to 20% B (2.80 – 2.81 min) and isocratic at 20 % B (2.81 – 3 min). 100 % methanol was used as the needle wash for amprenavir. For propranolol, the gradient program was linear gradient from 25% to 30% B
(0-1 min), 30% to 35% B (1 – 1.2 min), 35% to 40% B (1.2 – 1.5 min), 40% to 50% B (1.5 – 1.75 min), 50% to 65% B (1.75 -2 min), 65% to 95% B (2 – 2.01 min), 95% to 25% B (2.01 to 2.3 min) and isocratic at 25% B (2.3 – 2.5 min). The MRM transition and compound dependent MS parameters of the analytes are summarized in Supplemental Table 1.

**Calculation of apparent permeability, percentage recovery and efflux ratio.** $P_{app} (A \rightarrow B, B \rightarrow A)$ was calculated based on Equation 1, where $Q$ is the amount of substrate (unit), $C_0$ is the concentration at $t = 0$ s, SA is the surface area (cm$^2$) and $t$ is the incubation time (s).

$$P_{app} of substrate (\frac{cm}{s}) = \frac{dQ/dt}{C_0 \times SA} \quad (1)$$

The percentage recovery of a drug from one chamber to the other chamber was calculated based on Equation 2, where $D_{90}$ is the amount of substrate at donor chamber at $t = 90$ min (unit), $R_{90}$ is the amount of substrate at receiver chamber at $t = 90$ min (unit) and $D_0$ is the amount of substrate at donor chamber at $t = 0$ min (unit).

$$Percentage\ recovery\ (%) = \frac{D_{90} + R_{90}}{D_0} \quad (2)$$

Efflux ratio, defined as the ratio of $P_{app} (B \rightarrow A)$ to $P_{app} (A \rightarrow B)$, was calculated using Equation 3 where $P_{app} (B \rightarrow A)$ is the apparent permeability of substrate from B to A (cm/s) and $P_{app} (A \rightarrow B)$ is the apparent permeability of substrate from A to B (cm/s).

$$Efflux\ ratio = \frac{P_{app} (B \rightarrow A)}{P_{app} (A \rightarrow B)} \quad (3)$$

The efflux ratio data was subsequently processed by non-linear regression analysis using GraphPad PRISM® software version 6.01 (San Diego, CA, USA).
**Supplemental Table 1**: MRM transition and compound-dependent MS parameters of various analytes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MRM Transition (m/z)</th>
<th>MRM Transition</th>
<th>Collision Energy (CE) (V)</th>
<th>Declustering Potential (DP) (V)</th>
<th>Entrance Potential (EP) (V)</th>
<th>Collision Exit Potential (CXP) (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxylated Rivaroxaban</td>
<td>452.000 → 147.100</td>
<td>45</td>
<td>150</td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Amprenavir</td>
<td>506.220 → 245.200</td>
<td>20.160</td>
<td>169</td>
<td>7.7</td>
<td>10.510</td>
<td></td>
</tr>
<tr>
<td>Rivaroxaban</td>
<td>436.000 → 145.000</td>
<td>32</td>
<td>136</td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Propranolol</td>
<td>260.300 → 56.100</td>
<td>9.48</td>
<td>98.8</td>
<td>13.77</td>
<td>45.060</td>
<td></td>
</tr>
<tr>
<td>Verapamil</td>
<td>455.000 → 165.000</td>
<td>34</td>
<td>80</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>
**Supplemental Table 2.** Drug-dependent parameters for determination of relevant *in vivo* concentrations of amiodarone, dronedarone and their metabolites.

<table>
<thead>
<tr>
<th></th>
<th>Amiodarone</th>
<th>NDEA</th>
<th>Dronedarone</th>
<th>NDBD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D, mg</strong></td>
<td>400(^1)</td>
<td>NA</td>
<td>800</td>
<td>NA</td>
</tr>
<tr>
<td><strong>MW, g/mol</strong></td>
<td>645.32</td>
<td>617.25</td>
<td>556.76</td>
<td>500.66</td>
</tr>
<tr>
<td><strong>C(_{ss,max}), (µg/L)</strong></td>
<td>1930(^1)</td>
<td>1790(^1)</td>
<td>85-150(^1)</td>
<td>107(^5)</td>
</tr>
</tbody>
</table>

(Ave: 117.5)
B/P, blood to plasma partition ratio; D, total daily oral dose of the inhibitor; EH, hepatic extraction ratio; Foral, oral bioavailability, fa, fraction of dose absorbed into the gut wall; Fa, product of the fractions absorbed and escaping intestinal metabolism; ka, first order absorption rate constant

\[
fa = \frac{FG}{1 - f_{nl}}
\]

where \( f_a \) is the fraction of the dose entering the cellular space of the enterocytes, \( FG \) is the fraction of the drug entering the enterocytes that escapes first pass intestinal metabolism and \( FH \) is the fraction of the drug entering the liver that escapes first pass hepatic metabolism and/or biliary secretion

B/P, blood to plasma partition ratio; D, total daily oral dose of the inhibitor; EH, hepatic extraction ratio; Foral, oral bioavailability, fa, fraction of dose absorbed into the gut wall; Fa, product of the fractions absorbed and escaping intestinal metabolism; ka, first order absorption rate constant

<table>
<thead>
<tr>
<th></th>
<th>a Calculated using the equation</th>
<th>b Reported by PubChem</th>
<th>c Predicted using physicochemical properties of the drug</th>
<th>d Predicted with in vitro to in vivo extrapolation using Simcyp’s in vitro analysis toolkit</th>
</tr>
</thead>
</table>

**Derivation of \( F_G \) of rivaroxaban.**

Plasma clearance after intravenous administration: 10 L/h

Fraction excreted unchanged (\( f_c \)): 0.36

Hepatic clearance = \((1 - 0.36) \times 10\) L/h = 6.4 L/h

Human plasma to blood partition coefficient is 1.40 averaged

\[
CL_{H,B} = 6.4 \times 1.40 = 8.96 \text{ L/h}
\]

\[
EH = \frac{CL_{H,B}}{Q_{H,B}}
\]
\[ E_H = \frac{8.96}{87} = 0.103 \]

Where \( CL_{\text{Hib}} \) is the hepatic blood clearance obtained by correcting plasma clearance after iv administration with blood:plasma ratio, \( Q_{\text{Hib}} \) is the hepatic blood flow.

\( F_a \) and \( F_G \) was corrected for \( F_a \) when data was available, otherwise complete absorption was assumed, as permeability is known to be high.

Oral bioavailability \( \sim 80-100\% \) with the administration of a 10 mg tablet.

\[
F = F_a \times F_G \times (1 - E_H) \\
0.8 = 1 \times F_G \times (1 - 0.103) \\
F_G = 0.89
\]
Results:

Absence of inhibitory effect of amiodarone and NDEA on CYP2J2 with rivaroxaban as probe substrate. In the preliminary study of reversible inhibition for CYP2J2, the plot of the amount of metabolite against rivaroxaban concentration was fitted using a competitive inhibition model as CYP2J2 was shown to have a single large active site where most of its enzymatic activity occurred. When tested for competitive inhibition, (Supplementary Figure 3A) high concentrations of both amiodarone and NDEA were easily overcome with a small increase in rivaroxaban concentration. Amiodarone demonstrated a lack of mechanism based inactivation (MBI) with CYP2J2. MBI can be characterized based on the presence of time-dependent inactivation, where plotting the natural logarithm of the percentage of enzyme activity remaining versus the pre-incubation time would yield a negative slope ($k_{obs}$). Time-dependent inactivation was not demonstrated in preliminary studies of MBI of CYP2J2 with both amiodarone and NDEA (Supplemental Figure 3B). In order to ascertain that lack of inhibition was not confounded by weak enzymatic activity, an assay using a high concentration of amiodarone 50µM along with dronedarone 3µM was used. As MBI of dronedarone was reproduced successfully, it was confirmed that amiodarone demonstrated no time-dependent inactivation (Supplemental Figure 3C).

It was thus concluded that both amiodarone and NDEA did not inhibit CYP2J2 when rivaroxaban was used as the probe substrate.
Supplemental Figure 3. (A) Competitive inhibition of CYP2J2 using 5 µM amiodarone and 5 µM NDEA; (B) Mechanism based inactivation of CYP2J2 by 5 µM amiodarone and 5µM NDEA and (C) Mechanism based inactivation of CYP2J2 by 50 µM amiodarone and 3 µM dronedarone (positive control). Each point represents the mean of triplicates.
Supplemental Table 3. Inactivation efficiency of dronedarone and NDBD against CYP3A4 and CYP2J2 in the presence of different probe substrates.

<table>
<thead>
<tr>
<th></th>
<th>Testosterone</th>
<th>Rivaroxaban</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dronedarone</td>
<td>44.8</td>
<td>185</td>
</tr>
<tr>
<td>NDBD</td>
<td>15.9</td>
<td>53.7</td>
</tr>
</tbody>
</table>

$k_{inact}/K_I (\text{min}^{-1}/\text{mM}^{-1})$
Supplemental References:


5. US FDA (2009b) MULTAQ (dronedarone) clinical pharmacology and biopharmaceutics review.
