Inhibition of cytochrome P450 2J2-mediated metabolism of rivaroxaban
and arachidonic acid by ibrutinib and osimertinib

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Running Title

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### Abbreviations

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
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<tr>
<td>ADR</td>
<td>adverse drug reaction</td>
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<tr>
<td>AF</td>
<td>atrial fibrillation</td>
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<tr>
<td>AICc</td>
<td>corrected Akaike Information Criterion</td>
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<td>BTK</td>
<td>Bruton’s tyrosine kinase</td>
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<tr>
<td>CL&lt;sub&gt;int&lt;/sub&gt;</td>
<td>intrinsic clearance</td>
</tr>
<tr>
<td>CYP450</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DDI</td>
<td>drug-drug interaction</td>
</tr>
<tr>
<td>EET</td>
<td>epoxyeicosatrienoic acid</td>
</tr>
<tr>
<td>HLM</td>
<td>human liver microsomes</td>
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<tr>
<td>IS</td>
<td>internal standard</td>
</tr>
<tr>
<td>K&lt;sub&gt;puu&lt;/sub&gt;</td>
<td>unbound distribution coefficient</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>Sy.x</td>
<td>standard error of estimate</td>
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<tr>
<td>TKI</td>
<td>tyrosine kinase inhibitor</td>
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Abstract

Covalent tyrosine kinase inhibitors (TKIs) ibrutinib and osimertinib are associated with cardiac arrhythmia. The interactions between these TKIs with cytochrome P450 2J2 (CYP2J2) that is highly expressed in human heart are unknown. In vitro metabolism experiments were performed to characterize CYP2J2-mediated metabolism of ibrutinib and osimertinib. Unbound distribution coefficient ($K_{puu}$) for both TKIs was determined in AC16 cardiomyocytes. In vitro reversible and time-dependent CYP2J2 inhibition experiments were conducted with exogenous and endogenous substrates, namely rivaroxaban and arachidonic acid (AA), respectively, where kinetic parameters were estimated via one-site and multisite kinetic modeling. Ibrutinib was efficiently metabolized by CYP2J2 to a hydroxylated metabolite, M35, following substrate inhibition kinetics. Osimertinib is not a substrate of CYP2J2. Both TKIs depicted $K_{puu}$ values above 1 and equipotently inhibited CYP2J2-mediated hydroxylation of rivaroxaban in a concentration-dependent manner without time-dependency. The mode of reversible inhibition of CYP2J2-mediated metabolism of rivaroxaban and AA by osimertinib was described by Michaelis-Menten kinetics, while a two-site kinetic model recapitulated the atypical inhibitory kinetics of ibrutinib assuming multiple substrate-binding domains within the CYP2J2 active site. The inhibition of ibrutinib and osimertinib on cardiac AA metabolism could be clinically significant considering the preferable distribution of both TKIs to cardiomyocytes with R cut-off values of 1.160 and 1.026, respectively. The dysregulation of CYP2J2-mediated metabolism of AA to cardioprotective epoxyeicosatrienoic acids by ibrutinib and osimertinib serves as a novel mechanism for TKI-induced cardiac arrhythmia. Mechanistic characterization of
CYP2J2-mediated typical and atypical enzyme kinetics further illuminates the unique catalytic properties of CYP2J2.

**Keywords**

Arachidonic acid; CYP2J2; epoxyeicosatrienoic acids; ibrutinib; multisite kinetics; osimertinib; rivaroxaban.
**Significant statement**

We reported for the first time that ibrutinib is efficiently metabolized by cytochrome P450 2J2 (CYP2J2). By using rivaroxaban and arachidonic acid (AA) as substrates, we characterized the typical and atypical inhibition kinetics of CYP2J2 by ibrutinib and osimertinib. The inhibition of both drugs on cardiac AA metabolism could be clinically significant considering their preferable distribution to cardiomyocytes. Our findings serve as a novel mechanism for drug-induced cardiac arrhythmia and shed insights into the multisite interactions between CYP2J2 and ligands.
Introduction

In pharmacotherapy of cancers, cardiovascular adverse drug reactions (ADRs) have emerged as an important safety concern (Tamargo et al., 2015; Alexandre et al., 2018; Salem et al., 2021). Among the ADRs, cardiac arrhythmia poses a high morbidity burden and is potentially fatal. According to a pharmacovigilance study, tyrosine kinase inhibitors (TKIs) contributed significantly to anticancer drug-associated cardiac arrhythmia, yielding 51.6% of the reported cases within the period of 2014-2018 (Salem et al., 2021). Ibrutinib, a Bruton’s tyrosine kinase (BTK) inhibitor developed for treating B-lymphocyte cell hematologic malignancies, was among the top anticancer drugs that demonstrated the highest disproportional association with ventricular arrhythmia (Salem et al., 2021). Another systemic review also reported a significantly higher incidence of ibrutinib-associated atrial fibrillation (AF) compared with general adult population (Ganatra et al., 2018). Osimertinib, an irreversible inhibitor of both epidermal growth factor receptor (EGFR) and EGFR with T790M resistance mutation, was found to have a significant association with drug-induced long QT (Salem et al., 2021), where such QT prolongation may provoke torsades de pointes (TdP) and ventricular fibrillation.

Cytochrome P450 3A4 (CYP3A4)-mediated metabolism of ibrutinib and osimertinib accounted for 95% and 45% of their total clearance in vivo, respectively (US Food and Drug Administration, 2013a, 2015). CYP2J2, an epoxy oxygenase primarily expressed in cardiomyocytes and coronary arterial endothelial cells (Das et al., 2020), demonstrated similar active site volume as CYP3A4 based on homology model comparison (Lee et al., 2010). Subsequent studies found broad overlap of drugs in terms of metabolism by and inhibition against CYP3A4 and CYP2J2 (Lee et al., 2010; Solanki et al., 2018), while
CYP2J2 has not been included for reaction phenotyping and drug interaction screening during development of both TKIs (US Food and Drug Administration, 2013a, 2015).

As a major cardiac CYP450 enzyme, CYP2J2 is responsible for the metabolism of several endogenous fatty acids and multiple xenobiotics (Solanki et al., 2018), and is implicated in cardiac pathophysiology, which is partially related to its metabolism of arachidonic acid (AA) to cardioprotective regioisomeric epoxyeicosatrienoic acids (EETs), namely, 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET (Karkhanis, Hong, et al., 2017; Das et al., 2020). Cardioprotective and vasoprotective functions of EETs have been extensively reviewed, including vasodilation, anti-inflammation and cardiac ion channel binding (Das et al., 2020).

Multiple molecular mechanisms have been proposed so far in view of the TKI-induced cardiotoxicity, where interruption of several signaling pathways could lead to cardiomyocyte dysfunction and/or death (Alexandre et al., 2018; Salem et al., 2021). However, few studies investigated potential perturbation of CYP2J2-mediated AA-EETs metabolic pathway by ibrutinib and osimertinib, where the association of such interference with cardiac dysfunction has been revealed for several cardiotoxic drugs. For instance, doxorubicin is an anticancer drug with multiple cardiovascular ADRs. Preclinical studies have found the association of doxorubicin treatment with changes in EET formation (Zhang et al., 2009; Zordoky et al., 2010). Dronedarone, an antiarrhythmic drug, potently inhibited CYP2J2-mediated metabolism of AA to 14,15-EET, induced mitochondrial injury in cardiomyocytes, which was rescued by 14,15-EET enrichment in a concentration-dependent manner (Karkhanis, Tram, et al., 2017;
Karkhanis et al., 2018). The findings explained the increased mortality in patients with permanent AF or severe heart failure receiving dronedarone.

Moreover, our previous study on AA metabolism based on a CYP2J2 homology model, derived from the crystal structure of CYP2R1, suggested the possibility of two AA substrates binding concomitantly to its orthosteric binding site and alternative binding site (Leow et al., 2021). The observation corroborated the comparable large active site within the binding pocket to that of CYP3A4, which allows for the accommodation of bulky molecules and binding of more than one molecule (Lee et al., 2010). However, the experimentally observed atypical substrate inhibition kinetics of AA could complicate the interpretation of drug-drug interaction (DDI) data with both TKIs. Multisite kinetic models have been proposed to describe CYP3A4-mediated DDI with atypical enzyme kinetics, which consider the simultaneous metabolism, activation and/or inhibition of multiple molecules by CYP3A4 (Segel, 1975; Galetin et al., 2003, 2005; Paragas et al., 2021).

To understand the arcane interactions of ibrutinib and osimertinib vis-à-vis CYP2J2, we firstly investigated the metabolism of ibrutinib and osimertinib by CYP2J2 and their distribution to human cardiomyocytes. We subsequently characterized the in vitro inhibition of CYP2J2-mediated metabolism of two distinct substrates, rivaroxaban and AA, by ibrutinib and osimertinib. By employing one-site and two-site kinetic models to characterize and derive kinetic parameters in the presence of different substrates and inhibitors, our study shed novel insights into the typical and atypical inhibition kinetics of CYP2J2 by ibrutinib and osimertinib.
Materials and Methods

Materials

AA and 14,15-EET were purchased from Cayman Chemical (Ann Arbor, MI). Ibrutinib and osimertinib were purchased from Toronto Research Chemicals (Toronto, ON). M37 (PCI-45227), the major and active metabolite of ibrutinib in vivo, was purchased from MedChemExpress LLC (Monmouth Junction, NJ). Rivaroxaban was purchased from Carbosynth Ltd (Berkshire, UK). Dexamethasone was acquired from Sigma-Aldrich (St. Louis, MO). 4-hydroxybenzophenone (4-HBP) and butylated hydroxytoluene (BHT) were purchased from Thermo Fisher Scientific (Waltham, MA). Pooled human liver microsomes (UltraPool HLM 150, HLM), recombinant human CYP450 enzymes (rCYP3A4, rCYP2J2) and cofactor NADPH (Solution A and Solution B) were commercially purchased from Corning (Woburn, MA). High-performance liquid chromatography (HPLC)-grade acetonitrile (ACN) was purchased from Tedia Company Inc. (Fairfield, OH). Milli-Q water purification system was acquired from EMD Millipore (Billerica, MA). Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12 (DMEM/F-12), Hank’s Balanced Salt Solution (HBSS) and N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) were purchased from Gibco (Grand Island, NY). HyClone fetal bovine serum (FBS) was acquired from Cytiva Life Sciences (Singapore). Pierce BCA Protein Assay Kit was purchased from Thermo Fisher Scientific (Waltham, MA). All other reagents were of analytical or HPLC-grade. Product information for above chemicals and reagents are detailed in Supplemental methods.

Screening of metabolism of ibrutinib and osimertinib by CYP2J2
rCYP2J2 (10 pmol/mL) was mixed with NADPH Solution B, 100 mM potassium phosphate buffer (pH 7.4) and ibrutinib or osimertinib (1 μM) in triplicates. The reaction was initiated after 5 min pre-incubation at 37°C with 5 μL of NADPH Solution A making up the final volume of incubation mixture to 100 μL with < 1% v/v organic solvent. The reaction was kept at 37°C and terminated at 0, 15 and 30 min with ice-cold ACN containing 4 μM dexamethasone as internal standard (IS). The samples were then centrifuged at 2775 g at 4°C for 30 min, and the supernatant was used for the determination of ibrutinib and osimertinib remaining by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

Metabolic stability of ibrutinib was further assessed in rCYP2J2 and compared to that in HLM and rCYP3A4. Similarly, pre-incubation mixture containing HLM, rCYP2J2 or rCYP3A4 was initiated with the addition of 5 μL of NADPH Solution A into the mixture. At 0, 2, 5, 10, 20, 30, 45 and 70 min after initiation, the reaction was terminated. The supernatant after centrifugation was analyzed using LC-MS/MS to monitor the amount of ibrutinib remaining. The peak area ratio of substrate to IS was converted to percentage of substrate remaining and plotted against time. The curve was Ln transformed and fitted with linear regression using Prism 7.05 (GraphPad Software, San Diego, CA). Elimination rate constant, \( k \), was derived from the gradient of the plot and \( \text{in vitro} \) half-life \( (t_{1/2}) \) was calculated by Equation 1.

\[
\text{In vitro } t_{1/2} \text{ (min)} = \frac{\ln 2}{k} \quad \text{Equation 1}
\]

\( \text{In vitro-in vivo} \) extrapolation method was applied for estimation of hepatic clearance of ibrutinib (Choi et al., 2019). \( \text{In vitro} \) intrinsic clearance in HLM \( (\text{in vitro } \text{CL}_{\text{int,HLM}}) \)
(Equation 2) and in vitro intrinsic clearance attributed to CYP3A4 or CYP2J2 (in vitro 
CL_{int_{rCYP3A4}}, in vitro CL_{int_{rCYP2J2}}) (Equation 3) were first calculated.

\[
\text{In vitro } CL_{int_{HLM}} \text{ (mL/min/mg)} = k \times \frac{\text{volume of incubation}}{\text{amount of protein in the incubation}} \quad \text{Equation 2}
\]

\[
\text{In vitro } CL_{int_{CYP}} \text{ (mL/min/pmol)} = k \times \frac{\text{volume of incubation}}{\text{amount of protein in the incubation}} \quad \text{Equation 3}
\]

In vitro CL_{int_{CYP}} for each recombinant enzyme was then extrapolated to in vitro intrinsic 
clearance mediated through individual CYP450 enzyme in HLM (in vitro CL_{int_{CYPj}}) on 
the basis of intersystem extrapolation factor (ISEF) (Equation 4) (Y Chen et al., 2011).

\[
\text{In vitro } CL_{int_{CYP}} \text{ (mL/min/mg)} = \text{In vitro } CL_{int_{CYP}} \times \text{enzyme abundance in HLM} \times ISEF_{rCYP} \quad \text{Equation 4}
\]

where enzyme abundance of CYP3A4 (98 pmol/mg protein) and CYP2J2 (1.2 pmol/mg 
protein) were obtained from product insert of Corning pooled HLM and Simcyp 
Simulator (version 19, Sheffield, UK) built-in database, respectively. ISEF_{rCYP3A4} and 
ISEF_{rCYP2J2} were assigned as 0.157 (Y Chen et al., 2011) and 0.00066 (Lee et al., 2012), 
respectively.

Samples were further analyzed to determine the formation and stability of two specific 
metabolites of ibrutinib, M35 and M37 (described below, Fig. 1). The peak area ratios of 
each metabolite to IS throughout 70 min incubation were compared to characterize the 
stability of metabolites in the presence of HLM, CYP2J2 or CYP3A4.

**Identification of metabolites of ibrutinib generated by CYP2J2**

The incubation experiments were conducted with final substrate concentration at 10 μM 
and 20 pmol/mL of rCYP2J2 or rCYP3A4. The reaction was terminated with ice-cold
ACN at 0 and 30 min after initiation. Samples were then analyzed using LC-MS/MS. Potential metabolites were firstly monitored via Q1 MS scanning experiments based on mass-to-charge ratios (m/z) of the parent ions of in vivo metabolites of ibrutinib (Scheers et al., 2015). Product Ion MS2 scanning experiments were then performed to identify the putative metabolites based on the parent ions and corresponding MS/MS fragmentation patterns.

**Determination of kinetics of CYP2J2-mediated metabolism of ibrutinib**

The CYP2J2 kinetic experiments with ibrutinib as a substrate were performed in triplicates as described previously except with ibrutinib concentrations ranging from 0.1 to 100 μM. Reaction was terminated at 10 min and quenched with equal volume of ice-cold ACN containing 4 μM of IS. Supernatant was withdrawn to quantify the formation of major hydroxylated metabolite (M35, described below, Fig. 1) using LC-MS/MS. The data was fitted to substrate inhibition model using substrate concentration (S) and formation rate of M35 (ν) via nonlinear regression analysis using Prism to obtain relevant kinetic parameters namely, the Michaelis–Menten constant (Kₘ), apparent maximum rate of reaction (Vₘₐₓ) and dissociation constant for the substrate bound to the inhibitory enzymatic site (Kₛᵢ). (Equation 5).

\[
ν = \frac{Vₘₐₓ[S]}{Kₘ + [S] \times \left(1 + \frac{[S]}{Kₛᵢ}\right)}
\]

**Equation 5**

In vitro unbound distribution coefficient (Kₚᵤᵤ) measurement in AC16 cardiomyocytes and estimation of Cₜₐₚₜₑₜₐₚₜₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚئة_xtₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜₚₑₜ₢
AC16 immortalized human ventricular cardiomyocytes were obtained from Dr. Mercy M Davidson of Columbia University, USA (Davidson et al., 2005). The cells were maintained in DMEM/F-12 medium supplemented with 1.2 g/L sodium bicarbonate, 12.5% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere. $K_{puu}$ for ibrutinib and osimertinib in AC16 cells was determined utilizing temperature method (Riccardi et al., 2016) within 6 cell passages. In brief, cells were seeded at a density of 120,000 cells/well in 24-well plates in replicate for steady-state cellular uptake experiments performed at 37°C and 4°C, respectively. Uptake solutions containing 2 µM of ibrutinib or osimertinib were prepared using HBSS with 20 mM HEPES. After cell washing, transport was initiated by the addition of 300 µL of uptake solution, followed by incubation at 37°C or on ice at 4°C. Aliquots of uptake solution were withdrawn after 2 h incubation and mixed with equal volume of ice-cold ACN containing 4 µM of IS. Cells were subsequently lysed by adding 200 µL of ice-cold 0.2 N NaOH containing 6 µM of IS. Protein concentration was determined using the BCA method. Aliquots of cell lysate were neutralized via equal volume of both 0.2 N HCl and ACN. Medium and lysate samples were then centrifuged, and supernatants were analyzed by LC-MS/MS for the quantification of ibrutinib and osimertinib with corresponding standard curves.

Relationship between protein concentration and cell count for AC16 cells was predetermined to be $2.94 \times 10^{-4}$ μg protein/cell, and the volume of each cell was calculated to be $3.59 \times 10^{-6}$ μL based on the assumption of an average cell diameter of 19 μm. Total AC16 cell volume per well ($V_{cell}$) was then estimated by protein concentration and volume of each cell.
In *vitro* $K_{p uu}$ was calculated by **Equation 6**.

$$K_{p uu} = \frac{C_{cell}(37\, ^{\circ}C)/C_{medium}(37\, ^{\circ}C)}{C_{cell}(4\, ^{\circ}C)/C_{medium}(4\, ^{\circ}C)}$$  

**Equation 6**

where $C_{cell}$ is the concentration of drug in each cell and $C_{medium}$ is the concentration of drug in the medium. $C_{cell}$ was calculated by dividing the amount of drug in the cell lysate by $V_{cell}$.

*In vivo* unbound peak drug concentration in heart ($C_{u,\text{max,heart}}$) was then estimated by **Equation 7**.

$$C_{u,\text{max,heart}} = K_{p uu} \times C_{\text{max,ss}} \times f_{up}$$  

**Equation 7**

where $f_{up}$ represents fraction unbound in plasma and $C_{\text{max,ss}}$ represents reported peak plasma concentration of drug at steady state.

**Screening of inhibition of CYP2J2 by TKIs using exogenous probe substrate**

Incubation mixtures in triplicates consisting of 10 pmol/mL rCYP2J2, 10 μM rivaroxaban, NADPH Solution B and varying concentrations of ibrutinib, M37 or osimertinib (0, 0.1, 0.5, 1, 5, 10, 25, 50, 100 μM) in 100 mM potassium phosphate buffer (pH 7.4) were pre-incubated at 37°C for 5 min. The reaction was initiated by the addition of NADPH Solution A, yielding a final incubation mixture of 100 μL with < 1% v/v organic solvent. Aliquot of 80 μL were transferred and quenched with an equal volume of ice-cold ACN containing 4 μM IS. The samples were used for the determination of rivaroxaban morpholinone 2-hydroxylation metabolite by LC-MS/MS analysis. Formation of metabolite was plotted against concentrations of ibrutinib, M37 or
osimertinib to determine half-maximal inhibitory concentration (IC$_{50}$) via nonlinear regression analysis using Prism.

Another experiment was conducted to evaluate potential time-dependent inhibition against CYP2J2. Primary incubation mixtures comprising 40 pmol/mL rCYP2J2, 50 μM ibrutinib or osimertinib, NADPH Solution B and 100 mM potassium phosphate buffer (pH 7.4) were prepared in triplicates. Dronedarone (50 μM) was utilized as a positive time-dependent inhibitor. After pre-incubating at 37°C for 5 min, the reaction was initiated via the addition of NADPH Solution A. The final primary incubation mixture (20 μL) contained <1% v/v organic solvent. Subsequently, at 0 min and 30 min after initiation, a 5 μL aliquot of each primary incubation mixture was withdrawn and diluted 20-fold into a pre-warmed secondary incubation mixture comprising 50 μM rivaroxaban, NADPH Solution A and B and 100 mM potassium phosphate buffer (pH 7.4). The secondary incubation mixtures were further incubated at 37°C for additional 30 min. Samples were then similarly subjected for LC-MS/MS analysis of hydroxylated rivaroxaban.

**Screening of reversible inhibition of CYP2J2 by TKIs using both exogenous and endogenous substrates**

Ibrutinib and osimertinib were further tested as reversible inhibitors using rivaroxaban or AA as probe substrate. Substrate concentration, inhibitor concentration, enzyme concentration and incubation time were modified across each substrate-inhibitor pair (Supplemental Table 1). Incubation mixtures were prepared in triplicates with rCYP2J2, NADPH Solution B as well as varying concentrations of substrate and inhibitor in 100
mM potassium phosphate buffer (pH 7.4). Similarly, the reaction was initiated by the addition of NADPH Solution A after 5 min pre-incubation. The final incubation mixture yielded 100 μL with < 1% v/v organic solvent. After stipulated incubation time, 80 μL of incubation mixture was mixed with an equal volume of ice-cold quenching solution for centrifugation (Supplemental Table 1). The supernatants were analyzed by LC-MS/MS for the quantification of hydroxylated rivaroxaban, 14,15-EET as well as M35. As AA was metabolized by CYP2J2 primarily to 14,15-EET (41%) as the epoxide metabolite (Wu et al., 1996), 14,15-EET was analyzed to measure the surrogate activity of CYP2J2-mediated metabolism of AA to EETs.

Analysis of reversible inhibition kinetics and evaluation of DDI potential

The data of the four substrate-inhibitor pairs (rivaroxaban-ibrutinib, AA-ibrutinib, rivaroxaban-osimertinib, AA-osimertinib) were fitted to several enzyme kinetic models via nonlinear regression analysis using Prism. Model was chosen preferentially according to superior small sample corrected Akaike Information Criterion (AICc) and standard error of estimate (Sy.x) values. Since osimertinib was found not to be metabolized by CYP2J2 (described below), typical reversible inhibition models (competitive, non-competitive, uncompetitive or mixed mode) based on Michaelis-Menten kinetics within Prism were directly adopted to analyze the inhibition of rivaroxaban metabolism by osimertinib. In view of the atypical substrate inhibition kinetics observed for AA (Leow et al., 2021), AA-osimertinib data was fitted using our previously modified reversible inhibition model with substrate inhibition kinetics (Equation 8).
\[ v_A = \frac{V_{\text{max}} [A]}{1 + \frac{[I]}{K_A} + [A] \left( 1 + \frac{|A|h}{K_{si}} \right)} \]  

**Equation 8**

where \( v_A \) = formation rate of 14,15-EET, \( V_{\text{max}} \) = maximal velocity, \([A] = AA\) concentration, \([I] = \) inhibitor (osimertinib) concentration, \( K_A = \) Michaelis-Menten constant for AA, \( K_i = \) inhibition constant of osimertinib, \( K_{si} = \) substrate inhibition constant and \( h = \) Hill’s coefficient. The \( \alpha \) term accounted for mixed \((1 < \alpha < \infty)\), noncompetitive inhibition \((\alpha = 1)\) or competitive inhibition \((\alpha = \infty)\) mode. \( K_i \) was determined based on the best fitted model. Unbound \( K_i (K_{i,u}) \) was corrected by \( f_{u_{\text{mic}}} \) (the fraction of unbound drug in the incubation system) of osimertinib, which were predicted by Simcyp Simulator as 0.46 or 0.30, in the presence 10 or 20 pmol/mL of rCYP2J2 in the incubation system, respectively. \( R \) cut-off value was then calculated to evaluate the reversible inhibition potential (**Equation 9**) (US Food and Drug Administration, 2020).

\[ R = 1 + \frac{C_{u,\text{max}}}{K_{i,u}} \]  

**Equation 9**

where \( C_{u,\text{max}} \) represents maximal unbound concentration of the perpetrator drug, which is corrected by \( f_{u_p} \). An \( R \) value \( \geq 1.02 \) suggests the necessity of further DDI studies (US Food and Drug Administration, 2020).

We then attempted to characterize the formation of metabolites of rivaroxaban or AA by CYP2J2 in the presence of ibrutinib with the previously described models. Due to poor fitting of data based on visual check and CYP2J2-mediated metabolism of ibrutinib (described below), a two-site kinetic model proposed by Galetin et al. to describe the
atypical interactions of CYP3A4 substrates (Galetin et al., 2003) was further utilized to characterize the atypical Michaelis-Menten data. The formation of M35, the major metabolite of ibrutinib, in the presence of rivaroxaban or AA was analyzed as well. 

Supplemental Fig. 1 summarizes the proposed kinetic schemes derived from Galetin et al. (Galetin et al., 2003). Equation 10 and Equation 11 were used to fit rivaroxaban-ibrutinib data, while Equation 12 and Equation 13 were utilized for AA-ibrutinib data. Equation 14 was further incorporated to evaluate the DDI potential in view of the two-site kinetic model (Galetin et al., 2005, p. 4).

\[
\begin{align*}
    v_R &= \frac{V_{\text{max}}[R]\left(1 + \frac{\delta[I]}{aK_B}\right)}{K_A\left(1 + \frac{2[I]}{K_B} + \frac{[I]^2}{\beta K_B}\right) + [R]\left(1 + \frac{[I]}{aK_B}\right)} \\
    v_I &= \frac{V_{\text{max}}[I]\left(1 + \frac{\delta[I]}{aK_A} + \frac{\lambda[I]}{aK_B}\right)}{K_B\left(1 + \frac{[R]}{K_A} + [I]\left(2 + \frac{[I]}{aK_A}\right) + \frac{[I]^2}{\beta K_B}\right)} \\
    v_A &= \frac{V_{\text{max}}[A]\left(1 + \frac{\delta[I]}{aK_B} + \frac{\lambda[I]}{aK_A}\right)}{K_A\left(1 + \frac{2[I]}{K_B} + \frac{[I]^2}{\beta K_B}\right) + [A]\left(2 + \frac{[I]}{aK_A}\right) + \frac{[A]^2}{\beta K_A}} \\
    v_I &= \frac{V_{\text{max}}[I]\left(1 + \frac{\delta[I]}{aK_A} + \frac{\lambda[I]}{aK_B}\right)}{K_B\left(1 + \frac{2[I]}{K_A} + \frac{[I]^2}{\beta K_A}\right) + [I]\left(2 + \frac{[I]}{aK_A}\right) + \frac{[I]^2}{\beta K_B}} \\
    R &= \left(1 + \frac{C_u,\text{max}}{K_{B,u}}\right)^2 \\& 1 + \frac{C_u,\text{max}}{aK_{B,u}}
\end{align*}
\]

where \(v_R\) = formation rate of hydroxylated rivaroxaban, \(v_I\) = formation rate of M35, \(v_A\) = formation rate of 14,15-EET, \(V_{\text{max}}\) = maximal velocity, \([R]\) = rivaroxaban concentration, \([I]\) = ibrutinib concentration, \([A]\) = AA concentration, \(K_A\) and \(K_B\) = respective...
equilibrium dissociation constant in Supplemental Fig. 1, $\alpha$, $\beta$ and $\theta$ = respective factors accounted for the changes in $K_A$ or $K_B$ value, $\delta$, $\lambda$ and $\tau$ = respective factors accounted for the changes in catalytic efficiency. And substrate inhibition was defined by a decrease in product formation from IEI or AEA transitory complex, where I, E and A represent ibrutinib, enzyme and AA, respectively, and $\lambda$ and $\tau$ were defined < 1. Unbound $K_B$ ($K_{B,u}$) was corrected by $f_{\text{uninc}}$ of ibrutinib of 0.57 (Burns et al., 2015).

**LC-MS/MS method**

Ibrutinib and metabolites were firstly characterized on Agilent 1290 Infinity ultra-high pressure liquid chromatography (UHPLC) system (Agilent Technologies Inc., Santa Clara, CA) coupled with QTRAP 5500 tandem mass spectrometer (MS/MS) (AB SCIEX, Framingham, MA). Quantification of all drugs and metabolites was performed on Agilent 1290 Infinity UHPLC coupled with QTRAP 3500 MS/MS. Analytical method was modified based on our previous studies (Cheong et al., 2019; Leow et al., 2021; Tang, Wu, et al., 2022). In brief, chromatographic separation was achieved on a Luna Omega Polar C$_{18}$ UHPLC column (1.6 μM, 2.1×100 mm, Phenomenex, Torrance, CA). The mobile phase consisted of 0.1 % formic acid in water (A) and 0.1 % formic acid in ACN (B). The gradient elution was delivered at a flow rate of 0.35 mL/min comprising linear gradient from 20 to 80% B (0 - 1.70 min), isocratic at 100% B (1.71 - 2.40 min), linear gradient from 100 to 20% B (2.40 - 2.70 min) and isocratic at 20% B (2.70 - 3.50 min). Rivaroxaban and TKIs were determined in ESI (electrospray ionization) positive mode, where the compound-specific multiple reaction monitoring (MRM) transitions were set for: hydroxylated rivaroxaban, $m/z$ 452.1→406.0; ibrutinib, $m/z$ 441.2→304.3; M37, $m/z$
475.4→304.3; M35, m/z 457.2→319.9; osimertinib, m/z 500.4→385.0; dexamethasone, m/z 393.2→355.1. Quantification for 14,15-EET was carried out in negative ESI mode. Compound-specific transitions were: 14,15-EET, m/z 319→219; 4-HBP, m/z 197→92. Chromatographic peak was integrated using MultiQuant (version 3.0, Applied Biosystems, Foster City, CA).
Results

Ibrutinib is extensively metabolized by CYP2J2 while osimertinib is not a substrate

As shown in Supplemental Fig. 2A, the percentage remaining of ibrutinib in CYP2J2 incubation system declined by more than 50% after 15-min incubation and to below limit of detection at 30 min, while the levels of osimertinib remained almost unchanged. Further studies revealed that ibrutinib was metabolically unstable in HLM, CYP3A4 and CYP2J2 incubation systems (Supplemental Fig. 2B and 2C). The first-order elimination kinetics of ibrutinib by CYP3A4 and CYP2J2 yielded similar trends. Elimination rate constants, $k$, were 0.30 and 0.33 min$^{-1}$, respectively, which were scaled up to yield in vitro $CL_{int,CYP3A4}$ of 0.015 mL/min/pmol and in vitro $CL_{int,CYP2J2}$ of 0.016 mL/min/pmol, respectively. Subsequently, the in vitro $CL_{int,CYP3A4j}$ and $CL_{int,CYP2J2j}$ were calculated as 0.23 and 0.000013 mL/min/mg, respectively, while the in vitro $CL_{int,HLM}$ was determined as 0.27 mL/min/mg.

Ibrutinib forms multiple metabolites via CYP2J2-mediated metabolism

Phase I metabolites of ibrutinib reported by Scheers et al. in a Phase I study were included for screening metabolites generated by CYP2J2-mediated metabolism (Scheers et al., 2015). Fragmentation patterns of parent ibrutinib and putative metabolites are detailed in Supplemental results. The $m/z$ of parent ion, retention time, diagnostic product ions, chromatographic peak area of each putative metabolite derived from CYP2J2 or CYP3A4 incubation systems with ibrutinib are summarized in Supplemental Table 2. The chemical structures of the metabolites, the implicated CYP2J2- and CYP3A4-mediated pathways and additional metabolic pathways derived from Scheers et
al. (Scheers et al., 2015) are summarized in Fig. 1. The extracted ion chromatograms of identified metabolites derived from CYP2J2 and CYP3A4 incubation systems are further illustrated in Supplemental Fig. 3A and B, respectively.

In particular, the peak at 2.24 min yielded the highest intensity in CYP2J2 incubation system, which was approximate 3-fold higher than other metabolites derived from CYP2J2, and 5-fold higher than those derived from CYP3A4 (Supplemental Table 2). Its parent ion of m/z 457 and product ions at m/z 138, 304 and 320 were diagnostic of M35, a phenyl hydroxylated metabolite. Conversely, M37 was not detected in our Q1 MS scan but yielded diagnostic product ions at m/z 304 and 387 in MS2 scan at low intensity for both CYP3A4 and CYP2J2 incubation systems.

**Major hydroxylated metabolite M35 undergoes sequential metabolism rapidly**

The *in vitro* exposures of M35 and M37 derived from HLM, CYP2J2 and CYP3A4 were characterized by the plots of their respective peak area ratios against incubation time (Supplemental Fig. 4). Consistent with metabolite identification results, both CYP2J2 and CYP3A4 generated relatively low *in vitro* exposure of M37, while the abundance of M37 in HLM was relatively higher. Conversely, the exposure of M35 generated by CYP2J2 was remarkably higher than that by HLM or CYP3A4. Notably, the levels of M35 in three enzyme systems declined after 10 min incubation, suggesting rapid sequential metabolism, while the levels of M37 remained relatively stable.

**CYP2J2-mediated metabolism of ibrutinib demonstrates substrate inhibition kinetics**
Based on the high abundance of M35 derived from CYP2J2 metabolism of ibrutinib, it was selected as the surrogate metabolite to determine CYP2J2 kinetics. As shown in Fig. 2A, the metabolism of ibrutinib to M35 adheres to a typical substrate inhibition profile. The formation velocity of M35 declined significantly at concentrations of ibrutinib above 10 μM (Fig. 2A). Corresponding Eadie-Hofstee plot yielded a characteristic ‘hook’ in the upper quadrant indicative of substrate inhibition kinetics (Fig. 2B). The empirical substrate inhibition equation fitted observed values with R² of 0.95. The kinetic parameters describing the metabolism were apparent Vₘₐₓ of 0.4271 peak area ratio/min/pmol CYP2J2, Kₘ of 2.644 μM and Kₛᵢ of 34.77 μM, respectively.

**Ibrutinib and osimertinib show preferable distribution to cardiomyocytes**

Kₚᵤᵤ values of ibrutinib and osimertinib in AC16 cells are summarized in Table 1, where both TKIs achieved Kₚᵤᵤ values above 1, indicating higher unbound intracellular relative to extracellular concentrations. Estimated *in vivo* Cₜₘₐₓ,ss and Cₜₘₐₓ,heart for ibrutinib and osimertinib are illustrated in Table 1 as well.

**Ibrutinib and osimertinib are equipotent in inhibiting CYP2J2-mediated metabolism of rivaroxaban**

As shown in Fig. 3A, ibrutinib, M37 and osimertinib decreased rivaroxaban morpholinone hydroxylation by CYP2J2 in a concentration-dependent manner at concentrations of 0.1-100 μM. The IC₅₀ values for ibrutinib, M37 and osimertinib were 2.43, 66.02 and 7.52 μM, respectively. In view of low inhibitory potency of M37, no further time-dependent inhibition assay against CYP2J2 by M37 is performed. Preincubation of dronedarone with CYP2J2 and NADPH decreased the formation of
hydroxylated rivaroxaban by half as expected of a time-dependent inactivator. Ibrutinib and osimertinib showed no time-dependent inactivation of CYP2J2 (Fig. 3B).

**Osimertinib inhibits metabolism of rivaroxaban and AA by CYP2J2 competitively**

In the presence of varying concentrations of test inhibitor osimertinib, CYP2J2-mediated metabolism of rivaroxaban displayed typical Michaelis-Menten kinetics (Fig. 4A and 4B) while CYP2J2-mediated AA metabolism yielded atypical substrate inhibition kinetics (Fig. 4C). Eadie-Hofstee plot demonstrated the persistence of AA-mediated substrate inhibition of CYP2J2 despite the increasing concentration levels of osimertinib (Fig. 4D). Comparison of goodness-of-fit parameters for the fitting of various inhibition modes based on nonlinear regression analysis is summarized in Supplemental Table 3.

Osimertinib exhibited competitive inhibition when either rivaroxaban or AA was the probe substrate, with comparable $K_i$ values of 10.73 and 6.60 μM, and $K_{u,i}$ values of 4.94 and 1.98 μM, respectively (Table 2). R value for rivaroxaban was then calculated as 1.001, where $C_{u,max,ss}$ of 0.0072 μM of osimertinib was applied. And R value for AA was 1.004 or 1.026, when either $C_{u,max,ss}$ or $C_{u,max,heart}$ was utilized.

**Ibrutinib inhibits metabolism of rivaroxaban and AA by CYP2J2 via two-site kinetic model**

Metabolite formation profiles derived from both pairs of substrate-inhibitor data (rivaroxaban-ibrutinib and AA-ibrutinib) with fitted two-site kinetic models are illustrated in Fig. 5 and Fig. 6, respectively. Formation of hydroxylated rivaroxaban fitted with competitive inhibition model is presented in Supplemental Fig. 5. Formation of
14,15-EET and M35 fitted with modified competitive inhibition model coupled to substrate inhibition is shown in Supplemental Fig. 6. Goodness-of-fit parameters for the fitting of various inhibition modes are summarized in Supplemental Table 3. By comparing AICc and Sy.x values as well as visual inspection, the two-site kinetic model best characterized the formation of hydroxylated rivaroxaban at various ibrutinib concentrations (Fig. 5A), which in turn accounted for M35 formation in the presence of rivaroxaban (Fig. 5B). Similarly, the two-site kinetic model yielded better fitting than the modified reversible inhibition models with substrate inhibition for AA-ibrutinib data (Fig. 6).

The estimated kinetic parameters based on the two-site kinetic model are summarized in Table 2. Notably, $K_A$ and $K_B$ values obtained with either substrate were comparable within the same corresponding dataset, except for $K_A$ value for AA-ibrutinib pair, representing the binding kinetics of AA to CYP2J2, where one value was estimated to be unstable and another relatively high, although the two-site kinetic model showed superior modeling parameters among other models (Table S2). It could be attributed to the formation of multiple primary metabolites of AA besides 14,15-EET (Wu et al., 1996).

Similarly, the DDI between ibrutinib and rivaroxaban could be marginal in view of the R value of 1.050 and the fraction of metabolism of rivaroxaban attributed to CYP2J2, while the inhibition of CYP2J2-mediated AA metabolism by ibrutinib resulted in a much higher R of 1.160, when considering the $K_{puu}$ of ibrutinib in cardiomyocytes (Table 2).

In general, reduced metabolite formation at high substrate concentrations and low inhibitor concentrations was associated with substrate inhibition of CYP2J2 by both AA
and ibrutinib (Fig. 5B, Fig. 6A and Fig. 6C). Notably, at the highest inhibitor concentration (rivaroxaban for Fig. 5B; ibrutinib for Fig. 6A; AA for Fig. 6C), the shape of each implicated metabolite profile was restored to a hyperbolic type, and the corresponding Eadie-Hofstee plots demonstrated the gradual disappearance of the characteristic ‘hook’ profile (Fig. 5B inset, Fig. 6B and Fig. 6D).
Discussion

Ibrutinib and osimertinib, two favorable substrates of CYP3A4, are associated with cardiac arrhythmia (Salem et al., 2021). CYP2J2 is a highly expressed CYP450 in human heart, and was demonstrated to share several common substrates of CYP3A4 (Lee et al., 2010). Here, our results revealed that ibrutinib, but not osimertinib, is a substrate of CYP2J2. Moreover, ibrutinib was rapidly metabolized by CYP2J2 and CYP3A4, yielding comparable \textit{in vitro} \( \text{CL}_{\text{int, CYP}} \) values. However, the \textit{in vitro} \( \text{CL}_{\text{int, CYP3A4}} \) of ibrutinib was 0.23 mL/min/mg, which was comparable to \textit{in vitro} \( \text{CL}_{\text{int, HLM}} \) of 0.27 mL/min/mg and substantially higher than its \textit{in vitro} \( \text{CL}_{\text{int, CYP2J2}} \) of 0.000013 mL/min/mg. Taken together, our extrapolation results confirmed the major contribution of CYP3A4 towards the metabolism of ibrutinib (US Food and Drug Administration, 2013a), consistent with higher protein expression of hepatic CYP3A4 than CYP2J2 (Lee et al., 2012).

M35 was identified as the major hydroxylated metabolite of ibrutinib derived from CYP2J2. The sulfate conjugate of M35, M21 (Fig. 1), was the predominant metabolite in human plasma at 1 h post oral administration of ibrutinib, and was rapidly eliminated through 4 h post oral administration of ibrutinib, while M37, the main circulating metabolite of ibrutinib, was still detectable at 24 h (US Food and Drug Administration, 2013a). Consistently, M37 was found to be more stable than M35 throughout our \textit{in vitro} incubation studies. M35 was subsequently selected as the major metabolite \textit{in vitro} to monitor metabolism of ibrutinib by CYP2J2. Here, we established for the first time that ibrutinib exhibited substrate inhibition of CYP2J2, where the same phenomenon was previously reported for several other substrates of CYP2J2 (Karkhanis, Hong, et al., 2017).
The next question arose whether the TKIs are inhibitors of CYP2J2? Rivaroxaban has been widely prescribed for stroke prevention in patients with non-valvular AF and is a substrate of CYP2J2 (Cheong et al., 2019), which was then selected as the exogenous probe substrate. We demonstrated that ibrutinib and osimertinib are equipotent inhibitors of CYP2J2-mediated metabolism of rivaroxaban to its hydroxylated metabolite, while the major metabolite of ibrutinib in vivo, M37, was notably less potent in inhibiting CYP2J2. Covalent inhibitors exert their pharmacology via covalent binding to therapeutic targets while their electrophilic warheads may also yield off-target covalent adduction to non-therapeutic proteins (Dahal et al., 2013). For instance, osimertinib elicited irreversible binding to human and rat hepatocytes (Dickinson et al., 2016). Our laboratory has further reported the metabolic activation of the acrylamide Michael acceptor warhead in futibatinib to an epoxide intermediate, culminating in the covalent inactivation of CYP3A4 (Tang, Fu, et al., 2022). Nevertheless, it has been reported that there is no anticipated risk of time-dependent CYP450 inhibition by both ibrutinib and osimertinib (US Food and Drug Administration, 2013a, 2015). In corroboration, our study confirmed that the inhibition of CYP2J2 by both TKIs is not time-dependent.

CYP2J2 metabolizes endogenous AA to EETs which have pleiotropic cardioprotective effects (Karkhanis, Hong, et al., 2017; Das et al., 2020). We previously reported that dronedarone and its active metabolite, \textit{N}-desbutyldronedarone, inactivated CYP2J2 potently and irreversibly and disrupted AA-EETs pathway, that was in turn predicted to decrease cardiac 14,15-EET level by 15% and 25%, respectively (Karkhanis, Tram, et al., 2017). Subsequently, we demonstrated that dronedarone caused mitochondrial injury in rat cardiomyocytes which was mitigated via exogenous 11,12-EET and 14,15-EET.
enrichment (Karkhanis et al., 2018). Recently, we demonstrated that dronedarone treatment or CYP2J2 knockdown in human cardiomyocytes resulted in proarrhythmia (Karkhanis et al., 2022). Our collective findings provided compelling evidences that the dysregulation of CYP2J2-mediated AA-EETs metabolism affects both the viability and normal beating function of cardiomyocytes. As inhibition of CYP2J2 is substrate-dependent (Leow et al., 2021), AA was additionally adopted as the physiologically relevant substrate.

Our results suggested that osimertinib competitively inhibited CYP2J2 at equipotency with rivaroxaban and AA as probe substrates. R value of 1.001 implied low risk of DDI between osimertinib and rivaroxaban. It is important to mention the extensive distribution of osimertinib in multiple tissues as reported previously (Dickinson et al., 2016). Specifically, the brain exposure of $^{11}$C-osimertinib was 3.8-times higher than its systemic level clinically (Varrone et al., 2020). Our $K_{puu}$ results demonstrated extensive distribution of osimertinib to cardiomyocytes as well. The R value of osimertinib using AA as the probe substrate was 1.026 when calculated based on unbound concentration of osimertinib in heart tissue. The inhibition of CYP2J2-mediated AA-EETs metabolism by osimertinib could therefore be clinically significant considering its favourable distribution to human heart tissue.

In many instances, CYP3A4 exhibits atypical enzyme kinetics due to its large active site that can accommodate the binding of multiple ligands. Multisite kinetic models have been employed to fit the atypical Michaelis-Menten experimental data of CYP3A4 (Segel, 1975; Korzekwa et al., 1998; Galetin et al., 2003; Paragas et al., 2021). The multisite kinetic theory assumes multiple substrate-binding domains within the CYP3A4...
active site with access to the heme ion. Such atypical kinetics was also observed for other CYP450 (Hutzler et al., 2001). Interestingly, homology models depicted similar active site volumes of CYP2J2 and CYP3A4 (1420 Å³ versus 1585 Å³) (Lee et al., 2010). Our previous molecular dynamic simulations also revealed two substrate-binding sites of AA within the binding pocket of CYP2J2 (Leow et al., 2021), underscoring the relevance of multisite kinetic theory for the analysis of atypical CYP2J2 kinetics.

In view of the poor fitting of data with inhibition models based on Michaelis-Menten kinetics, the two-site kinetic model for CYP3A4 was adopted, which successfully characterized the metabolite profiles of hydroxylated rivaroxaban or 14.15-EET as well as the experimentally observed kinetics of M35 in the presence of rivaroxaban or AA. At higher substrate and inhibitor concentrations, the formation of M35 and 14,15-EET transitioned to hyperbolic saturation kinetics with declined substrate inhibition effects (Fig. 5B, Fig. 6A and Fig. 6C), due to the existence of nonproductive rivaroxaban-CYP2J2-ibrutinib or AA-CYP2J2-ibrutinib complex, which was in accordance with the observation of CYP3A4 substrates (Galetin et al., 2003). However, this phenomenon contrasted with the inhibition of CYP2J2-mediated metabolism of AA by osimertinib (Fig. 4) and several other perpetrator drugs (Leow et al., 2021), where substrate inhibition of CYP2J2 by AA was persistently observed. We posit this is possibly due to the competitive binding of osimertinib to the active site of CYP2J2 without the formation of AA-CYP2J2-osimertinib complex.

Notably, the $K_A$ for AA was unable to be accurately estimated during ibrutinib-AA data fitting (Table 2). 14,15-EET was generally considered as the dominant metabolite of CYP2J2-metadiaed AA metabolism (41%), while 8,9-EET and 11,12-EET accounted for
28% and 27% of total EET products, respectively (Wu et al., 1996). Paragas et al. found that metabolism of midazolam via CYP3A4 to its primary metabolites 1’-OH-midazolam and 4-OH-midazolam showed distinct atypical kinetics, which could be successfully reproduced via a two-site kinetic model (Paragas et al., 2021). In view of the multiple primary metabolites of AA, further investigation monitoring all the 4 EETs of AA with the application of a more robust multisite kinetic model is warranted to better describe the interaction between AA and ibrutinib.

The $C_{u,\text{max,ss}}$ of ibrutinib was 0.0081 µM (US Food and Drug Administration, 2013a), consequently its substrate inhibition against CYP2J2 and inhibition on CYP2J2-mediated metabolism of rivaroxaban might not be remarkable, where CYP2J2 contributes less than 50% to total clearance of rivaroxaban (Cheong et al., 2019). Preclinical studies found that tissue:plasma concentration ratios of ibrutinib were less than 1 for most tissues through 24 h postdose in rats (US Food and Drug Administration, 2013b). Nevertheless, the $K_{pui}$ value of ibrutinib is greater than 1 for human cardiomyocytes, indicating its favorable exposure in human heart. Consequently, the $\text{R}$ value of ibrutinib was calculated as 1.160, suggesting the potential of clinically significant inhibition of cardiac CYP2J2. Future studies are warranted to further explore the association between ibrutinib-induced dysregulation of AA metabolism and proarrhythmia in cardiomyocytes.

Apart from human heart, abundant CYP2J2 expression was observed in leukemia cells derived from patients with hematologic malignant diseases (C Chen et al., 2011; Karkhanis, Hong, et al., 2017). Overexpression of CYP2J2 and addition of exogenous EETs markedly promoted viability and accelerated proliferation of human leukemia cells in vitro. Such proliferation could be significantly reversed by a selective CYP2J2
inhibitor with enhanced cell apoptosis (C Chen et al., 2011). Our observation of inhibition of CYP2J2-mediated AA metabolism by ibrutinib suggested a novel but currently arcane anticancer pharmacology of ibrutinib for the treatment of human hematologic malignant diseases.

To our knowledge, this is the first study to establish the metabolism of ibrutinib by CYP2J2, substrate inhibition of CYP2J2 by ibrutinib, distribution of ibrutinib and osimertinib to cardiomyocytes, and their inhibition of CYP2J2-mediated metabolism of rivaroxaban and AA. Our findings revealed the unique mechanism of dysregulation of CYP2J2-mediated AA-EETs metabolism in defining the cardiotoxicity associated with both TKIs. By using rivaroxaban and AA as substrates and employing different enzyme kinetic models, we characterized the typical and atypical inhibition kinetics of CYP2J2 by ibrutinib and osimertinib. Our findings shed novel insights into the multisite interactions between CYP2J2 and ligands, which are important in guiding future CYP2J2-related in vitro experiments.
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Authorship contributions

Participated in research design: Wang, Chan

Conducted experiments and performed data analysis: Wang

Wrote or contributed to the writing of the manuscript: Wang, Chan
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US Food and Drug Administration (2013b) *Pharmacology Reviews Ibrutinib*.


Footnotes

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The authors declare that they have no conflicts of interest with the contents of this article.
Figure legends

Fig. 1 Chemical structures of ibrutinib, its metabolites and respective metabolic pathways, which are denoted by the black arrow representing pathways confirmed by our study and grey arrow representing the proposed pathways by Scheers et al. (Scheers et al., 2015).

Fig. 2 (A) Substrate inhibition kinetics of metabolism of ibrutinib to M35 by CYP2J2 and (B) its corresponding Eadie-Hofstee plot in rCYP2J2 incubation system. Data are expressed as mean±SD (n = 3). Line in (A) represents nonlinear regression.

Fig. 3 (A) Concentration-response relationship on the inhibition of CYP2J2-catalyzed hydroxylation of rivaroxaban by ibrutinib, M37 and osimertinib in rCYP2J2 incubation system. Data are normalized to control samples in the absence of inhibitors and expressed as mean±SD (n = 3). Line represents nonlinear regression. (B) Time-dependent inactivation of CYP2J2 by dronedarone, ibrutinib and osimertinib in rCYP2J2 incubation system. Data are normalized to control samples with 0 min primary incubation in the absence of inhibitors and expressed as mean±SD (n = 3).

Fig. 4 (A) Competitive inhibition of CYP2J2-mediated hydroxylation of rivaroxaban by osimertinib shown as formation rate of hydroxylated rivaroxaban against osimertinib concentrations and (B) corresponding Lineweaver-Burk plot. (C) Competitive inhibition with substrate inhibition of CYP2J2-mediated metabolism of AA to 14,15-EET by osimertinib shown as formation rate of 14,15-EET against osimertinib concentrations and (D) corresponding Eadie-Hofstee plot. Data are expressed as mean±SD (n = 3).
**Fig. 5** (A) Kinetic plots of formation of hydroxylated rivaroxaban at various concentrations of ibrutinib and (B) formation of M35 at various concentrations of rivaroxaban by CYP2J2. Inset represents Eadie-Hofstee plot for M35. Data are expressed as mean±SD (n = 3). Line represents nonlinear regression of two-site kinetic model.

**Fig. 6** (A) Kinetic plots of formation of 14,15-EET at various concentrations of ibrutinib by CYP2J2 and (B) corresponding Eadie-Hofstee plots. (C) Kinetic plots of formation of M35 at various concentrations of AA by CYP2J2 and (D) corresponding Eadie-Hofstee plots. Data are expressed as mean±SD (n = 3). Line in (A) and (C) represents nonlinear regression of two-site kinetic model.
Table 1  Reported and estimated peak total and unbound concentrations of ibrutinib and osimertinib in plasma and heart at steady state, calculated based on unbound distribution coefficient ($K_{puu}$).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Reported value</th>
<th>In house and estimated data</th>
<th>Reference</th>
<th>$K_{puu}$</th>
<th>$C_{u,max,ss}$ (µM)</th>
<th>$C_{u,max,heart}$ (µM)</th>
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<td>$f_{up}$</td>
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<tr>
<td>Osimertinib</td>
<td>0.55</td>
<td>0.013</td>
<td>(Pilla Reddy et al., 2018)</td>
<td>7.1</td>
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<tr>
<td>Ibrutinib</td>
<td>0.30</td>
<td>0.027</td>
<td>(US Food and Drug Administration, 2013a)</td>
<td>2.1</td>
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</table>

$C_{max,ss}$, in vivo total peak plasma concentration; $C_{u,max,ss}$, in vivo unbound peak plasma concentration; $C_{u,max,heart}$, in vivo unbound peak drug concentration in heart; $f_{up}$, fraction unbound in plasma; $K_{puu}$, unbound distribution coefficient.

$C_{max,ss}$ for osimertinib was obtained from patients receiving 80 mg of osimertinib orally once daily.

$C_{max,ss}$ for ibrutinib was obtained from patients receiving 420 mg of ibrutinib orally once daily dosing (132 ng/mL).
<table>
<thead>
<tr>
<th>Dataset</th>
<th>Substrate for estimation</th>
<th>Kinetic parameter</th>
<th>R cut-off value</th>
<th>Estimation method by</th>
<th>( C_{u,\text{max},\text{ss}} )</th>
<th>( C_{u,\text{max},\text{heart}} )</th>
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<td>Rivaroxaban-osimertinib</td>
<td>Rivaroxaban</td>
<td>( V_{\text{max}} ) (area ratio/min/pmol CYP2J2)</td>
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<td>Competitive inhibition mode</td>
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<td>( K_{i,u} ) (µM)</td>
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<td></td>
<td>( K_{A} ) (µM)</td>
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<td>( K_{B} ) (µM)</td>
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<td>K&lt;sub&gt;A&lt;/sub&gt;</td>
<td>K&lt;sub&gt;B&lt;/sub&gt;</td>
<td>Cu&lt;sub&gt;max,ss&lt;/sub&gt;</td>
<td>Cu&lt;sub&gt;max,heart&lt;/sub&gt;</td>
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</tbody>
</table>

V<sub>max</sub>, maximal metabolite formation velocity of respective substrate; K<sub>A</sub> and K<sub>B</sub>, equilibrium dissociation constants illustrated in Supplemental Fig. 1; Cu<sub>max,ss</sub>, in vivo unbound peak plasma concentration; Cu<sub>max,heart</sub>, in vivo unbound peak drug concentration in heart.

* unstable parameter estimated by Prism 7.05.
Fig 1

M37 (m/z 475)

CYP2J2
Epoxide
hydrolyase

CYP2J2

CYP3A4

Epoxidation

CYP2J2

CYP3A4

M35 (m/z 457)

Ibrutinib (m/z 441)

CYP3A4

M39/M40 (m/z 457)

M34 (m/z 459)

CYP2J2

M20 (hydroxylation
metabolite)

M25 (m/z 473)

M11, M17, M38
(sequential metabolism)
Fig 3

A

Formed of hydroxylated rivaroxaban compared to control (%)

Concentration (μM)

Ibrutinib

Osimertinib

M37

B

Formed of hydroxylated rivaroxaban compared to control (%)

0 min

30 min

Droneje

Ibrutinib

Osimertinib
Fig 6

A

B

C

D

Formation rate of 14,15-EE1 (area ratio/min/pmol CYP2J2)
Formation rate of 14,15-EE1/[AA] (area ratio/min/[AA]/μM)
Formation rate of M35 (area ratio/min/pmol CYP2J2)
Formation rate of M35/[Ibrutinib] (area ratio/min/[Ibrutinib]/μM)

Ibrutinib (μM)
- 0
- 1
- 5
- 10
- 40

AA (μM)
- 0
- 10
- 20
- 40
- 60
- 80

[AA] (μM)
[1brutinib] (μM)