Inactivation of Human Cytochrome P450 3A4 and 3A5 by Dronedarone and N-Desbutyl Dronedarone

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ABSTRACT

Dronedarone is an antiarrhythmic agent approved in 2009 for the treatment of atrial fibrillation. An in-house preliminary study demonstrated that dronedarone inhibits cytochrome P450 (CYP) 3A4 and 3A5 in a time-dependent manner. This study aimed to investigate the inactivation of CYP450 by dronedarone. We demonstrated for the first time that both dronedarone and its main metabolite N-desbutyl dronedarone (NDBD) inactivate CYP3A4 and CYP3A5 in a time-, concentration-, and NADPH-dependent manner. For the inactivation of CYP3A4, the inactivator concentration at the half-maximum rate of inactivation and inactivation rate constant at an infinite inactivator concentration are 35.3 and 36.6. Testosterone protected both CYP3A4 and CYP3A5 from inactivation by dronedarone and NDBD. Although the presence of Soret peak confirmed the formation of a quasi-irreversible metabolite-intermediate complex between dronedarone/NDBD and CYP3A4/CYP3A5, partial recovery of enzyme activity by potassium ferricyanide illuminated an alternative irreversible mechanism-based inactivation (MBI). MBI of CYP3A4 and CYP3A5 was further supported by the discovery of glutathione adducts derived from the quinone oxime intermediates of dronedarone and NDBD. In conclusion, dronedarone and NDBD inactivate CYP3A4 and CYP3A5 via unique dual mechanisms of MBI and formation of the metabolite-intermediate complex. Our novel findings contribute new knowledge for future investigation of the underlying mechanisms associated with dronedarone-induced hepatotoxicity and clinical drug-drug interactions.

Introduction

Atrial fibrillation is the most common sustained arrhythmia in the aging population and is associated with increased cardiovascular morbidity and mortality (Trigo and Fischer, 2012). With the rapid growth of the elderly population, the prevalence of atrial fibrillation is substantially increasing, resulting in a major public health problem. Despite several developments in antithrombotic, antiatherosclerotic, and device-based cardiac therapies, few noteworthy antiarrhythmic drugs have been developed (Pamukcu and Lip, 2011). Dronedarone (Fig. 1A) is the only antiarrhythmic drug approved in 2009 by the US Food and Drug Administration (FDA) since 1999 (De Ferrari and Dusi, 2012). The pharmacological effects of dronedarone are derived from its electrophysiological properties belonging to all four Vaughan-Williams classes (Oyetayo et al., 2010). It is a noniodinated benzofuran derivative of amiodarone (Fig. 1B) and was developed with the intention of improving the safety profile of rhythm-controlling drugs (De Ferrari and Dusi, 2012).

Despite the promises of dronedarone as a safer alternative to amiodarone, postmarketing surveillance revealed that it is not entirely without adverse effects. In January 2011, the FDA released a drug safety alert for dronedarone due to reported cases of severe liver injury, including two cases of acute liver

**ABBRVIATIONS:** ACN, acetonitrile; BEH, ethylene bridged hybrid; CUR, curtain gas; CYP, cytochrome P450 enzyme; DDI, drug-drug interaction; EPI, enhanced product ion; GSH, glutathione; HLM, human liver microsome; IS, internal standard; Kobs, observed inactivation rate constant; LC, liquid chromatography; MBI, mechanism-based inactivation; MI, metabolite intermediate; MS, mass spectrometry; NDBD, N-desbutyl dronedarone; rCYP, human recombinant CYP supernose; RT, retention time; t1/2, half-life; TOF, time of flight; UPLC, ultra performance liquid chromatography.
failure leading to liver transplant (USFDA, 2011). Most recently, there have been a number of reports on significant drug-drug interactions (DDIs) involving dronedarone. The coadministration of dronedarone and simvastatin increased simvastatin levels by 4-fold (Patel et al., 2009). The docetaxel-dronedarone interaction led to grade 4 neutropenia and mucositis, which was probably related to increased systemic exposure of docetaxel (Vodovar et al., 2011). In addition, there were cases of arrhythmic death among patients treated with dronedarone, which might be related to the pharmacokinetic interaction between dronedarone and digoxin (De Ferrari and Dusi, 2012).

Dronedarone undergoes extensive hepatic metabolism by cytochrome P450 enzymes (CYP) (Ferrari and Dusi, 2012). N-desbutyl dronedarone (NDBD) (Fig. 1C), the main metabolite formed primarily by CYP3A is pharmacologically active and has a similar plasma exposure to its parent drug (Klieber et al., 2014). Although the overall metabolic pathway of dronedarone has been reported recently (Klieber et al., 2014), no study investigates the mechanism of inhibition of CYP450 by dronedarone thus far.

As a structural analog of amiodarone, dronedarone retains the tertiary amine group and its associated potential to be oxidized to a nitroso intermediate that may in turn form a metabolite-intermediate (MI) complex with the heme iron of CYP450 (Mansuy et al., 1976; Delaforge et al., 1983). Such quasi-reversible inactivation of CYP450 could lead to the accumulation of the coadministered drug that is a substrate of the same CYP450 and subsequently result in adverse effects arising from DDIs (Grimm et al., 2009). In fact, amiodarone has been shown to cause time-dependent inhibition of CYP3A4 (Ohyama et al., 2000) and CYP2C8 (Polasek et al., 2004), a key feature of irreversible or quasi-reversible inactivation of CYP450. Although dronedarone has been reported to be a moderate inhibitor of CYP3A4 and a weak inhibitor of CYP2D6 (Naccarelli et al., 2011), the potential inactivation of CYP450 by dronedarone and NDBD has not been investigated. In-house preliminary findings demonstrated that dronedarone inhibits CYP3A4 and CYP3A5 in a time-dependent manner (unpublished data). Based on these collective evidences, we hypothesize that dronedarone and its main metabolite NDBD have the propensity to inactivate CYP450.

In the present study, the mechanism of inactivation of CYP3A4 and CYP3A5 by dronedarone and NDBD was investigated. Metabolic stability, enzyme kinetics, and CYP450 reaction phenotyping experiments were performed to identify the major CYP450 responsible for dronedarone metabolism. Metabolite identification was further performed to establish the key metabolites of dronedarone when metabolized by CYP3A4 and CYP3A5. The nature of enzyme inactivation was subsequently characterized based on time-, concentration-, NADPH-dependent inhibition, substrate protection, and spectral difference scanning experiments. Glutathione (GSH)-trapping experiments were further conducted to trace the potential reactive metabolites associated with dronedarone and NDBD.

Materials and Methods

Chemicals. High-performance liquid chromatography-grade acetonitrile (ACN) was purchased from Tedia Company Inc. (Fairfield, OH). Dronedarone hydrochloride, NDBD, verapamil hydrochloride, erythromycin, carbamazepine, ketoconazole, prednisolone, testosterone, and amodiaquin dihydrochloride dihydrate were purchased from Sigma-Aldrich (St. Louis, MO); diclofenac potassium, debrisoquine sulfate, mephenytoin, and phenacetin were obtained from MP Biomedicals (Santa Ana, CA); chlorozoxazone was purchased from Alfa Aesar (Ward Hill, MA); midazolam was purchased from Tocris Bioscience (Bristol, UK); 6β-hydroxytestosterone and 1’-hydroxymidazolam were obtained from Cerilliant Corporation (Round Rock, TX); and potassium ferricyanide was purchased from VWR International (Leuven, Belgium). Pooled human liversomes (HLMs), human recombinant CYP supersomes (rCYP) (enzymes, with the exception of rCYP2C9), and a NADPH-regenerating system consisting of NADPH A (NADP+ and glucose 6-phosphate) and B (glucose-6-phosphate dehydrogenase) were purchased from BD Gentest (Woburn, MA). rCYP2C9 was obtained from Cypex (Dundee, UK). Water was obtained using a Milli-Q water purification system (Millipore, Billerica, MA). All other reagents were of analytical grade.

Metabolic Stability Study. HLMs (0.5 mg/ml) were preincubated with potassium phosphate buffer (100 mM, pH 7.4), NADPH B, and dronedarone (1 μM) at 37°C for 5 minutes. NADPH A was subsequently added to initiate the reaction (i.e., 0 minutes). The final incubation mixture had a total volume of 400 μl and contained <1% v/v organic solvent. The reaction mixtures were incubated at
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37°C with gentle agitation. At the respective time points (0, 5, 15, 30, 45, and 60 minutes), 55 μl of the reaction mixture was quenched using ice-cold ACN with verapamil as the internal standard (IS). The samples were then centrifuged at 15,000g and 4°C for 10 minutes, and 80 μl of each supernatant was transferred to a liquid chromatography (LC)/mass spectrometry (MS) vial. Incubation experiments with verapamil (and dronedarone as the IS) were carried out as the positive control. A third set of incubation mixtures containing Milli-Q water in place of NADPH served as the negative control. The loss of the parent compound (i.e., dronedarone as the test compound or verapamil as the positive control) was determined over time using LC/MS/MS analysis. All samples were analyzed in triplicates.

Enzyme Kinetics Study. For the enzyme kinetics study, incubation experiments were carried out using 96-well plates. Incubation mixtures consisting of 0.5 mg/ml HLM, 100 mM potassium phosphate buffer (pH 7.4), and 100 μM verapamil in ice-cold ACN before centrifugation at 15,000g and 4°C for 10 minutes. The peak area ratio of NDBD (with verapamil as the IS) formed with different concentration levels of dronedarone was determined using LC/MS/MS analysis. The corresponding peak retention times of NDBD formed were correlated with the peak area ratio obtained to the standard curve obtained with NDBD (0.1, 1, 10, 100, and 1000 nM) subjected to the same conditions as the incubation mixtures. All experiments were performed in triplicates.

Cytochrome P450 Reaction Phenotyping Assay. Dronedarone was incubated with rCYP450 to determine the in vitro biotransformation of dronedarone and to elucidate the proportion of hepatic metabolism that is accounted for by various CYP450 enzymes. Each incubation mixture consisted of dronedarone (3 μM) and NADPH B in 100 mM potassium phosphate buffer (pH 7.4). Each of these rCYP450 suprersones (rCYP3A4, rCYP3A5, rCYP2C8, rCYP2C9, rCYP2C19, rCYP2E1, rCYP1A2, or rCYP2D6) was added to obtain a final enzyme concentration of 20 pmol/ml. After prewarming the incubation mixture at 37°C for 5 minutes, NADPH A was added to initiate the metabolic reaction. The final incubation mixture volume was 400 μl with <1% v/v organic phase. At fixed incubation time points (0, 5, 15, 30, 45, and 60 minutes), 55 μl of the reaction mixture was quenched with 0.1 μM verapamil in ice-cold ACN. All quenched samples were centrifuged at 15,000g and 4°C for 10 minutes. Positive and negative controls were tested for each experiment. For the positive control, FDA-recommended CYP substrates were used in place of dronedarone. The specific substrates are testosterone (CYP3A4 and CYP3A5), amodiaquine (CYP2C8), diclofenac (CYP2C9), S-mephenytoin (CYP2C19), chlorozoxone (CYP2E1), phenacetin (CYP1A2), and debromoquine (CYP2D6). The final substrate concentration in the incubation mixture was 1 μM. For the negative control, NADPH was replaced with an equal volume of Milli-Q water. The percentage of substrate remaining at each time point was measured with reference to the 0-minute sample. All experiments were performed in triplicates.

Metabolite Identification. To investigate the metabolites formed by rCYP3A4 and rCYP3A5, dronedarone (20 μM) was incubated with 100 pmol/ml of each supersome, an NADPH regenerating system, and 100 mM potassium phosphate buffer (pH 7.4) at 37°C. After a 0- or 60-minute incubation period, the reaction was terminated and the samples were processed as described above. The 0-minute sample served as the blank control. Metabolite identification was carried out using an ultra performance liquid chromatography (UPLC)/quadrupole time-of-flight (TOF) mass spectrometer system.

Time-, Concentration-, and NADPH-Dependent Inactivation of CYP3A4. Two probe substrates of CYP3A4, testosterone and midazolam, were tested in the experiments. Incubation experiments (n = 3) were performed in 96-well plates. Primary incubation mixtures consisting of 20 pmol/ml rCYP3A4, 100 mM potassium phosphate buffer (pH 7.4), NADPH B, and dronedarone were preincubated at 37°C for 5 minutes. The concentration levels of dronedarone used in the testosterone assay were 0, 0.25, 0.5, 1, 2.5, 5, and 10 μM, whereas a single concentration level of 50 μM was used for the midazolam assay. To initiate the reactions, 5 μl of NADPH A was added to the mixture. The final primary incubation mixture had a total volume of 100 μl and contained <1% v/v organic solvent. At 0, 3, 8, 15, 22, and 30 minutes after the addition of NADPH A, aliquots of the primary incubation mixture were transferred to the secondary incubation mixture containing 200 μM testosterone or 25 μM midazolam, an NADPH regeneration system, and 100 mM potassium phosphate buffer (pH 7.4). For the testosterone assay where 0.25, 0.5, 1, 2.5, 5, and 50 μM of dronedarone was used, 5 μl of the primary incubation mixture was transferred to 95 μl of secondary incubation mixture, resulting in a 20× dilution. For the midazolam and testosterone assays, where 50 μM of dronedarone was used, 10 μl of the primary incubation mixture was transferred to 90 μl of secondary incubation mixture, effecting a 10× dilution. The secondary reaction mixture was incubated for another 10 minutes at 37°C before an 80-μl aliquot was removed and quenched with an equal volume of ice-cold ACN containing 2 μM prednisolone (IS for testosterone assay) or 0.02 μM carbamazepine (IS for midazolam assay). The quenched samples were centrifuged at 9000×g for 20 minutes, and the supernatants were removed for the respective determination of either 6-hydroxytestosterone or 1'-hydroxymidazolam by LC/MS/MS. The experiment was also performed with erythromycin and ketocazole, which are a known mechanism-based inactivator and competitive inhibitor of CYP3A4, respectively. Negative control was performed by replacing 5 μl of NADPH A with 100 mM potassium phosphate buffer (pH 7.4). The incubation assay was repeated by testing NDBD as the inactivator.

To determine the time-, concentration-, and NADPH-dependent activity in the CYP3A4 inactivation assays, the mean of the triplicate analyses was used to calculate the natural log of percentage probe substrate activity remaining that was normalized to 0 minutes against preincubation time. The data were fitted to linear regression, and the observed first-order inactivation rate constant, kobs, was determined. Kinetic parameters, K and k_inact, were determined by using the Kitz-Wilson plot (Kitz and Wilson, 1962) to calculate the potency of inactivation (k, k_inact). The graphs were plotted using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

Time-, Concentration-, and NADPH-Dependent Inactivation of CYP3A5. To investigate the potential of suicide inactivation of CYP3A5 by dronedarone and NDBD, rCYP3A5 (20 pmol/ml) was used in place of rCYP3A4 in the two-step incubation scheme described above. Zero, 0.25, 0.5, 2.5, 5, 10, 15, 25, and 50 μM dronedarone or NDBD were used for the testosterone assay, whereas 50 μM of each compound was used for the midazolam assay. Both the testosterone and midazolam assays were performed using 10× dilution by transferring 10 μl of the primary incubation mixture to 90 μl of the secondary incubation mixture. The kinetics parameters were determined as previously described.

Partition Ratio. The partition ratio, defined as the number of inactivator molecules required to completely inactivate the enzyme, was estimated based on the following experiments. Primary incubations (n = 3) comprising 100 pmol/ml rCYP3A5, NADPH B, dronedarone (0.5, 2.5, 5, 10, 15, 25, and 25 μM), or NDBD (0.25, 0.5, 1, 2.5, 4, and 5 μM) and 100 mM potassium phosphate buffer (pH 7.4) were prepared. After preincubation at 37°C for 5 minutes, the reaction mixtures were initiated by the addition of NADPH A and incubated at 37°C for another 45 minutes, allowing the inactivation to go to completion. Aliquots were then transferred to the secondary incubation mixture with 20× dilution and assayed for residual enzyme activity as described above for rCYP3A4. The experiment was
repeated for dronedarone and NDBD (0, 0.25, 0.5, 2.5, 5, 10, 15, and 25 μM) using rCYP3A4 with 10× dilution. To estimate the partition ratio, the percentage of residual enzymatic activity was plotted against the function of the molar ratios of each test inactivator. The turnover number (partition ratio + 1) was extrapolated from the intercept of the linear regression line plotted at lower ratios and the straight line plotted at the higher ratios to the x-axis. The partition ratio was in turn back calculated from the turnover number by a subtraction of 1.

Substrate Protection. Excess testosterone [in a 1:8 ratio of dronedarone (or NDBD) to testosterone] was added to the primary incubation mixture (n = 3) containing dronedarone or NDBD (5 μM for the rCYP3A4 assay and 25 μM for the rCYP3A5 assay), 20 pmol/ml rCYP3A4 or rCYP3A5, NADPH B, and 100 mM potassium phosphate buffer. The reaction was initiated by the addition of NADPH A after preincubation for 5 minutes at 37°C. Aliquots were then transferred to the secondary incubation mixture and assayed for residual enzyme activity as described in the time-dependent inhibition experiment for CYP3A4 and CYP3A5, respectively. Negative controls were prepared without both testosterone and dronedarone or NDBD or only without testosterone in the primary incubation mixture.

Reversibility of Inactivation. The reversibility of enzyme inactivation was investigated by oxidation with potassium ferricyanide based on a method reported previously (Watanabe et al., 2007). Three sequential incubations were performed, including primary 0- or 30-minute incubation with or without dronedarone/NDBD, secondary 10-minute incubation with or without potassium ferricyanide, and tertiary 10-minute incubation with testosterone. The primary incubation solutions consisted of rCYP3A4 or rCYP3A5 (20 pmol/ml), NADPH B, and 100 mM potassium phosphate buffer (pH 7.4), with or without 50 μM dronedarone/NDBD. After adding NADPH A and incubating for 0 or 30 minutes at 37°C, 40 μl of primary incubation was added to 40 μl of secondary incubation containing 100 mM potassium phosphate buffer (pH 7.4) with or without 2 mM potassium ferricyanide. After 10-minute incubation, each secondary reaction mixture was diluted 5-fold with the tertiary incubation, which contained 200 μM testosterone, an NADPH regeneration system, and 100 mM potassium phosphate buffer (pH 7.4). After 10-minute incubation, the reaction mixture was assayed for residual enzyme activity, as described in the time-dependent inhibition experiment. The percentage of metabolic activity [% control0min (% control30min)] was calculated for each sample after 0- or 30-minute preincubation with dronedarone/NDBD and compared with each control sample without dronedarone/NDBD as follows:

\[
\text{% control}_{0\text{min}} = \frac{v \times (0 \text{ min} + \text{inhibitor})}{u \times (0 \text{ min} - \text{inhibitor})} \times 100
\]

\[
\text{% control}_{30\text{min}} = \frac{v \times (30 \text{ min} + \text{inhibitor})}{u \times (30 \text{ min} - \text{inhibitor})} \times 100
\]

v is the residual enzyme activity. Using the above values, the percentage of the enzymatic activity remaining after the 30-minute preincubation relative to the 0-minute preincubation was calculated as follows:

\[
\% \text{remaining} = \frac{\text{% of control (30 min)}}{\text{% of control (0 min)}} \times 100
\]

Spectral Difference Scanning. rCYP3A4 or rCYP3A5 (500 pmol/ml), NADPH B, 50 μM dronedarone, or NDBD and 100 mM potassium phosphate buffer (pH 7.4) were preincubated at 37°C for 5 minutes. The reaction was initiated with the addition of NADPH A. The total volume of the incubation mixture was 500 μl. After incubating for 60 minutes, 500 μl of ice-cold ACN was added to quench the reaction. The mixture was centrifuged at 14,000g for 15 minutes at 4°C. The supernatant was transferred to a clean microtube and dried under a gentle flow of nitrogen gas (TurboVap LV; Caliper Life Science, Hopkinton, MA). The residue was reconstituted with 60 μl of ACN-water mixture (3:7, v:v), vortex mixed, and centrifuged at 14,000g for 15 minutes at 4°C. The supernatant was removed for LC/MS/MS analysis. Negative controls were prepared by the exclusion of test compounds in the incubation mixture. The chemical synthesis of desulfonated dronedarone is described in the Supplemental Methods.

Measurement of Residual CYP450 Activity. Samples were analyzed using the Agilent 1290 Infinity ultra-high pressure liquid chromatography (Agilent Technologies Inc., Santa Clara, CA) interfaced with the AB Sciex QTRAP 5500 MS/MS system (Framingham, MA). The ACQUITY UPLC ethylene bridged hybrid (BEH) C18, 1.7 μM, 2.1 × 50 mm column (Waters, Milford, MA) was used for chromatographic separation. The aqueous mobile phase (A) was 0.1% formic acid in water, whereas the organic mobile phase (B) consisted of 0.1% formic acid in ACN. Mobile phases were delivered at a flow rate of 0.6 ml/min. The column and sample temperatures were maintained at 45 and 4°C, respectively. Gradient elution was first carried out from 20 to 95% of B in 1.40 minutes. This was subsequently followed by isocratic elution at 95% B for a further 0.59 minutes before reducing its percentage from 95 to 20% and maintaining it at 20% for another 0.50 minutes. The source-dependent MS parameters were as follows: IS spray voltage = 5000 V; source temperature = 600°C; curtain gas (CUR) = 20 psi; Ion Source Gas (GS)1 and GS2 = 65 and 45 psi, respectively. The compound-dependent MS parameters are presented in Table 1. Chromatographic peak integration was performed using the Analyst software (AB Sciex). All graphs were plotted using Prism version 5 (GraphPad Software).

**Metabolite Identification.** A Waters ACQUITY UPLC system connected to a quadrupole TOF mass spectrometer (Q-ToF Premier, Waters, Manchester, UK) was operated in a positive and MS/MS mode with electrospray ionization. Dronedarone and its metabolites were separated on a Waters UPLC BEH C18 column (1.7 μM, 2.1 × 100 mm) with 0.2% acetic acid and 5 mM ammonium acetate (solvent A) in water and 0.2% acetic acid in ACN (solvent B). The column heater and sample manager were kept at 45 and 4°C, respectively. The gradient elution comprised a linear gradient of 20–70% B over 0–10 minutes. The optimized MS conditions were as follows: capillary voltage = 3500 V; sampling cone = 40 V; source temperature = 100°C; desolvation gas = 100°C.

**TABLE 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Q1 Mass</th>
<th>Q3 Mass</th>
<th>DP</th>
<th>EP</th>
<th>CE</th>
<th>CXP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dronedarone</td>
<td>557</td>
<td>100</td>
<td>80</td>
<td>10</td>
<td>46</td>
<td>7</td>
</tr>
<tr>
<td>NDBD</td>
<td>501</td>
<td>114</td>
<td>100</td>
<td>11</td>
<td>41</td>
<td>7</td>
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<tr>
<td>Verapamil</td>
<td>455</td>
<td>165</td>
<td>80</td>
<td>10</td>
<td>34</td>
<td>10</td>
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<tr>
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<td>361</td>
<td>147</td>
<td>160</td>
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</tr>
<tr>
<td>6β-Hydroxytestosterone</td>
<td>305</td>
<td>269</td>
<td>200</td>
<td>7</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>Carbamazepine</td>
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<td>194</td>
<td>160</td>
<td>8</td>
<td>26</td>
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</tr>
<tr>
<td>1'-Hydroxymidazolam</td>
<td>342</td>
<td>203</td>
<td>130</td>
<td>13</td>
<td>35</td>
<td>13</td>
</tr>
</tbody>
</table>

CE, collision energy; CXP, collision cell exit potential; DP, declustering potential; EP, entrance potential; Q1 mass, parent ion; Q3 mass, daughter ion.
temperature = 350°C; cone gas flow = 0 l/h; desolvation gas flow = 300 l/h; collision energy = 25 eV; detector voltage = 1900 V; pusher voltage = 905 V; pusher voltage offset = −0.8 V; and pulser voltage = 610 V. The acquisition rate was set to 1 second, with a 0.1-second interscan delay. The MS was calibrated across the mass range of 100–1500 Da using a solution of sodium formate. All analyses were acquired using the LockSpray to ensure accuracy and reproducibility. Leucine-enkephalin was used as the lock mass (m/z 556.2771) at a concentration of 2 ng/µl and flow rate of 5 μl/min. The LockSpray was operated at a reference scan frequency, reference cone voltage, and collision energy of 10 seconds, 30 V, and 18 V, respectively.

**Detection of GSH Adducts.** GSH adducts of potential electrophilic reactive metabolites were analyzed using the same UPLC/QTRAP/MS system as described above. Chromatographic separation was performed on a Waters ACQUITY UPLC BEH C18 column (1.7 µM, 100 × 2.1 mm). The mobile phases consisted of 0.1% formic acid in water and 0.1% formic acid in ACN and delivered at a flow rate of 0.45 ml/min. The column and sample temperatures were maintained at 45 and 4°C, respectively. The elution gradient was as follows: linear gradient 5–80% (0–6.25 minutes), isocratic at 95% (6.26–7.00 minutes), and isocratic at 5% B (7.01–8.00 minutes). An information-dependent acquisition experiment was performed to detect GSH conjugates, including precursor ion 272 (−) with enhanced product ion (EPI) scan. The compound-dependent MS parameters were declustering potential = 168 V; entrance potential = 10 V; collision energy = 45 V; and collision cell exit potential = 10 V. The source-dependent parameters were as follows: CUR = 20 psi; (collisionally activated dissociation) CAD = high; IS = 5000 V; source temperature = 650°C; GS1 = 45 psi; and GS2 = 60 psi.

For accurate mass measurement of the GSH adducts, the same samples were analyzed using the Ultimate 3000 nanoLC system ( Dionex; Thermo Fisher Scientific, Chelmsford, MA) coupled to AB Sciex 5600 Triple TOF MS (AB Sciex). A 15 cm × 75 µm i.d. packed with Acclaim PepMap RSLC C18 column was used (Dionex; Thermo Fisher Scientific). This column was connected to a spray tip (New Objectives, Woburn, MA), which was directly coupled with the nanospray interface into the triple TOF/MS. Samples were loaded onto a trap column (Acclaim PepMap 100 C18, 2 cm × 75 µm i.d., Dionex; Thermo Fisher Scientific) at a flow rate of 5 µl/min. After a 3-minute wash with loading buffer (2/98 v/v of ACN/water with 0.1% formic acid), the system was switched into the line with the C18 analytical capillary column. A step linear gradient of mobile phase B (2/98 v/v of water/ACN with 0.1% formic acid) from 5 to 7% for 3 minutes, 7 to 60% for 11 minutes, and lastly, 60 to 95% over 1 minute at a flow rate of 300 nl/min was used for the analysis. Other instrumentation settings of triple TOF/MS were as follows: ion spray voltage floating = 2400 V; CUR = 30 psi; GS1 = 12 psi; interface heater temperature = 125°C; declustering potential = 100 V; and nebulizer current = 3 for nitrogen gas. Data were acquired using a product ion MS2 scan and Analyst TF 1.7 software (AB Sciex). The collision energy was set to 44 V, with a collision energy spread (CES) of 5 V.

**Results**

**Metabolic Stability and Enzyme Kinetics.** For the metabolic stability assay, the percentage of dronedarone remaining in the HLM reaction mixture decreased as the incubation time progressed from 0 to 60 minutes. The calculated in vitro metabolism half-life (t1/2) and elimination rate constant of dronedarone were 15.42 minutes and 0.045 minute⁻¹, respectively (Fig. 2A). The metabolism of dronedarone to NDBD was found to be linear from 0 to 30 minutes. Using an incubation time of 15 minutes, our results showed that the metabolism of dronedarone to NDBD follows Michaelis-Menten kinetics. The calculated Km was 8.83 μM, whereas Vmax was 53.4 nmol minute⁻¹ mg⁻¹ proteins (Fig. 2B).

**CYP450 Reaction Phenotyping.** Our results showed that dronedarone was extensively metabolized by CYP3A4 (Fig. 3A) and CYP3A5 (Fig. 3B), with a t1/2 of 5.38 and 13.31 minutes, respectively. On the other hand, CYP2D6 (Fig. 3C) and CYP2C19 (Fig. 3D) metabolize dronedarone at a slower rate, with a t1/2 of 33.43 and >60 minutes, respectively. Finally, our results confirmed that dronedarone was minimally metabolized by CYP2C8, CYP2C9, CYP2E1, and CYP1A2 (data not shown).

**Metabolite Identification.** A total of 14 metabolites were identified from the incubation of dronedarone with rCYP3A4 or rCYP3A5. The detailed information, including the retention time (RT), proposed formula, measured m/z ratio of the MH+ ions, and characteristic product ions, are summarized in Table 2. The most abundant metabolite, M1, with an m/z reduction of 56 (corresponding to a loss of a C6H7 functional group), compared with the parent ion, was confirmed to be NDBD using the standard. Five metabolites (M2-1 to M2-5) were found to have an m/z reduction of 40 and are likely the secondary oxidized metabolites of dronedarone after undergoing N-desbutylation. When compared with the parent drug, seven of the metabolites (M3-1 to M3-7) exhibited an increase in m/z of 16, which is possibly associated with hydroxylation of dronedarone. M4 demonstrated an increase in m/z of 32 and is possibly a result of the dioxidation of dronedarone.

**Fig. 2.** In vitro (A) metabolic stability of dronedarone and (B) enzymatic kinetics of metabolism of dronedarone to NDBD.
Time-, Concentration-, and NADPH-Dependent Inactivation of CYP3A4 and CYP3A5. Dronedarone inactivated CYP3A4 in a time- and NADPH-dependent manner with testosterone as the probe substrate (Supplemental Fig. 1A). As shown in Fig. 4, A and B, the inactivation of CYP3A4 by dronedarone and NDBD was also concentration dependent. The observed rate of inactivation ($k_{obs}$) calculated from various concentration levels of dronedarone and NDBD followed saturation kinetics that approached a maximum rate of inactivation (Fig. 4, C and D). The $K_I$ and $k_{Inact}$ values for the inactivation of CYP3A4 by dronedarone were determined to be 0.873 $\mu M$ and 0.039 minute$^{-1}$, respectively, which in turn yielded a $k_{Inact}/K_I$ ratio of 0.0445 minute$^{-1} \cdot \mu M^{-1}$ (Fig. 4C). The time required for half of the enzyme molecules to be inactivated ($t_{1/2}$) was 17.8 minutes. In a similar fashion, as shown in Fig. 4D, NDBD inactivated CYP3A4, with $K_I$ and $k_{Inact}$ values of 6.242 $\mu M$ and 0.099 minute$^{-1}$, respectively, and a $k_{Inact}/K_I$ ratio of 0.0158 minute$^{-1} \cdot \mu M^{-1}$. The inactivation $t_{1/2}$ was 7.0 minutes. To further assess the inactivation of CYP3A4 by dronedarone and NDBD, another well characterized CYP3A4 probe substrate, midazolam, was also tested. Time- and NADPH-dependent inactivation of CYP3A4 was also observed when midazolam was used as the probe substrate for both dronedarone (Supplemental Fig. 1C) and NDBD (Supplemental Fig. 1D). However, a lower potency of inactivation of CYP3A4 was observed as compared with when

### TABLE 2

<table>
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<tr>
<th>Description</th>
<th>Assignment</th>
<th>Retention Time (min)</th>
<th>Measured MH(^+) (m/z)</th>
<th>Theoretical MH(^+) (m/z)</th>
<th>Proposed Formula</th>
<th>Product Ions (m/z)</th>
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<td>M0</td>
<td>6.11</td>
<td>557.3036</td>
<td>557.3049</td>
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<td>N-desbutylation</td>
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<td>4.75</td>
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<td>501.2423</td>
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<td>517.2372</td>
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<td>517.2372</td>
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<td>M3-3</td>
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<td>573.2996</td>
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<td>589.2984</td>
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</table>

Fig. 3. Metabolism of dronedarone by selected recombinant CYP isoforms, namely, (A) rCYP3A4, (B) rCYP3A5, (C) rCYP2D6, and (D) rCYP2C19.
testosterone was used as the probe substrate. The positive control, erythromycin (a known mechanism-based inactivator of CYP3A4), demonstrated time-, concentration-, and NADPH-dependent inactivation of CYP3A4 (Supplemental Fig. 1E). On the other hand, the negative control, ketoconazole (a competitive inhibitor of CYP3A4), did not yield time dependency of CYP3A4 inhibition (Supplemental Fig. 1F).

Inhibition of CYP3A5 by dronedarone (Supplemental Fig. 2A) and NDBD (Supplemental Fig. 2B) demonstrated time, concentration, and NADPH dependence with testosterone as the probe substrate. Observed inactivation rates ($k_{obs}$) were used to calculate the inactivation kinetic constants $K_I$ and $k_{inact}$ using nonlinear regression. The $K_I$ and $k_{inact}$ values were (C) 0.873 μM and 0.039 minute$^{-1}$ for dronedarone and (D) 6.242 μM and 0.099 minute$^{-1}$ for NDBD, respectively. Each point in (A and B) represents the mean and S.D. of triplicate experiments.

**Fig. 4.** Time- and concentration-dependent inactivation of CYP3A4 by (A) dronedarone and (B) NDBD using testosterone as the probe substrate. Observed inactivation rates ($k_{obs}$) were used to calculate the inactivation kinetic constants $K_I$ and $k_{inact}$ using nonlinear regression. The $K_I$ and $k_{inact}$ values were (C) 0.873 μM and 0.039 minute$^{-1}$ for dronedarone and (D) 6.242 μM and 0.099 minute$^{-1}$ for NDBD, respectively. Each point in (A and B) represents the mean and S.D. of triplicate experiments.

**Fig. 5.** Determination of the partition ratios for inactivation of CYP3A4 by (A) dronedarone and (B) NDBD. Substrate protection of CYP3A4 inactivation by (C) dronedarone and (D) NDBD. rCYP3A4 was incubated with dronedarone (■), 1:8 dronedarone/testosterone (▲), and neither dronedarone nor testosterone (○). Each point represents the mean and S.D. of triplicate experiments.
the substrate. As shown in Supplemental Fig. 2C, the $K_I$, $k_{\text{Inact}}$, and $t_{1/2}$ values for dronedarone were determined to be 2.186 µM, 0.0056 minute$^{-1}$, and 123.1 minutes, respectively. On the other hand, the $K_I$, $k_{\text{Inact}}$, and $t_{1/2}$ values for NDBD were 5.445 µM, 0.0563 minute$^{-1}$, and 12.3 minutes, respectively (Supplemental Fig. 2D). Time- and NADPH-dependent CYP3A5 inactivation was also observed when midazolam was used as the probe substrate. Similarly, the potency of inactivation was lower (data not shown).

**Partition Ratio.** The partition ratios of dronedarone and NDBD with regards to the inactivation of CYP3A4 were 51.1 and 35.3, respectively (Fig. 5, A and B). As for the inactivation of CYP3A5, the respective partition ratios were 32.2 and 36.6 (Supplemental Fig. 3, A and B).

**Substrate Protection.** Testosterone, an alternate substrate of CYP3A4 and CYP3A5, was included in the primary incubations along with dronedarone or NDBD. As shown in Fig. 5, C and D, the inactivation of CYP3A4 by both dronedarone and NDBD was protected in the presence of 8-fold excess testosterone. Similar observations were made for the substrate protection of CYP3A5 inactivation (Supplemental Fig. 3, C and D).

**Reversibility of Inactivation.** To investigate whether the inactivation of CYP3A4 and CYP3A5 by dronedarone and NDBD is quasi-irreversible or irreversible, oxidation with potassium ferricyanide was performed based on a method reported previously (Watanabe et al., 2007). Upon oxidation using potassium ferricyanide, the metabolic activity of CYP3A4 and CYP3A5 was restored by 10.9 and 19.4% postinactivation by dronedarone and 22.6 and 32% postinactivation by NDBD (Table 3).

**Spectral Difference Scanning.** Spectral difference scanning experiments were performed to investigate whether the inactivation of CYP3A4 and CYP3A5 by dronedarone and NDBD occurred via the formation of the MI complex. A clear peak at 448–458 nm was observed when verapamil (forms an MI complex with CYP3A4) (Ma et al., 2000) was incubated with rCYP3A4 (Supplemental Fig. 4A) and rCYP3A5 (data not shown) based on the precursor ion scan experiments performed at $m/z$ 272 in the (Electrospray Ionization) ESI negative mode. The EPI spectrum yielded a product ion at $m/z$ 479, corresponding to the desulfonated metabolite of dronedarone. This was further confirmed by the detection of the same GSH adduct in the incubation of chemically synthesized desulfonated dronedarone with rCYP3A4/5 and GSH. For NDBD, a potential GSH adduct with $m/z$ 744 (RT: 4.42 minutes) was found in the incubation with rCYP3A4 (Supplemental Fig. 5B) and rCYP3A5 (data not shown). Similarly, a product ion at $m/z$ 423, corresponding to the desulfonated metabolite of NDBD, was detected (Supplemental Fig. 5B). To further confirm the identity of the potential GSH adduct, an accurate mass measurement was performed using triple TOF/MS. The proposed chemical formulae, experimental accurate $m/z$, theoretical exact $m/z$, and mass accuracy (ppm) associated with the parent and product ions of the dronedarone-GSH adduct are summarized in Table 4. Our data suggested the GSH adducts were formed via covalent binding with the oxime metabolites derived from both desulfonated dronedarone and desulfonated NDBD. The MS/MS spectrum (Fig. 7) confirmed the accurate mass fragmentation patterns of the oxime metabolite–GSH adduct derived from dronedarone.

**Discussion**

Dronedarone causes DDIs and liver toxicity, but the mechanism is currently unknown. The present study provides evidence for the first time that demonstrates the inactivation of CYP3A4 and CYP3A5 by dronedarone and its main metabolite NDBD.

Our in vitro assay using rCYP450 confirmed that dronedarone is extensively metabolized by CYP3A4 and CYP3A5, in agreement with a previous report (Klieber et al., 2014). Additionally, we found that although CYP2D6 and CYP2C19 contribute to the metabolism of dronedarone,
CYP2C8, CYP2C9, CYP1A2, and CYP2E1 do not metabolize dronedarone. Therefore, subsequent metabolite identification and the CYP450 inactivation studies focused mainly on CYP3A4 and CYP3A5.

Dronedarone was found to be extensively metabolized to a number of metabolites by CYP3A4 and CYP3A5, mainly through N-desbutylation and hydroxylation. Our in vitro observation that NDBD is the main metabolite of dronedarone is consistent with clinical data that the plasma systemic exposure of NDBD is approximately half that of its parent drug following oral administration (USFDA, 2008). Notably, NDBD is pharmacologically active in vivo (USFDA, 2008) and is further oxidized by CYP3A4 (Klieber et al., 2014).

As hypothesized, our results demonstrated that both dronedarone and NDBD inactivate CYP3A4 and CYP3A5 in a time-, concentration-, and NADPH-dependent manner. To estimate and appreciate the inactivation potency of dronedarone, $k_{\text{Inact}}/K_I$ ratios were calculated and compared with those of known inactivators, such as clarithromycin, erythromycin, and amprenavir, which inactivate CYP3A4 via the formation of the MI complex formation over time for both dronedarone and NDBD incubated with (E) CYP3A4 and (F) CYP3A5.

TABLE 4

<table>
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<tr>
<th>Proposed Chemical Formula</th>
<th>Experimental m/z</th>
<th>Theoretical m/z</th>
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complex (Table 5). Based on the inactivation of CYP3A4-mediated testosterone–6β-hydroxylation, dronedarone and NDBD were found to have lower potencies as compared with a potent inactivator, such as amprenavir (Ernest et al., 2005), but comparable inactivation potencies as compared with moderate inactivators, such as clarithromycin and erythromycin (Polasek and Miners, 2006). In other words, the inactivation of CYP3A4 by dronedarone and NDBD is clinically important. Notably, both dronedarone and NDBD demonstrated greater inactivation potencies against CYP3A4 as compared with CYP3A5. This underscores the important difference between the two CYP3A isoforms in terms of inactivation characteristics.

The efficiency of CYP450 inactivation is estimated by its partition ratio, which is the number of inactivator molecules required to completely inactivate the enzyme. In theory, a lower partition ratio implies a more efficient CP450 inactivation. The partition ratio for the inactivation of CYP3A4 by dronedarone is higher than that of NDBD, suggesting the parent drug is less efficient in inactivating the enzyme as compared with its main metabolite. This finding supports our hypothesis as NDBD is possibly the intermediate metabolite leading to the formation of the reactive intermediates. On the other hand, dronedarone and NDBD displayed similar partition ratios against CYP3A5, suggesting equal efficiency in its inactivation. The less distinctive partition ratios in the case of CYP3A5 might be due to the overall lower potency of dronedarone and NDBD in inactivating this CYP3A isoform (Table 5). Coincubation with another specific CYP3A substrate, testosterone, protected both CYP3A4 and CYP3A5 from inactivation by dronedarone and NDBD, further confirming that inactivation occurred within the active site of the enzymes.

The substrate specificity of CYP3A is relatively low due to its large active site (Fowler and Zhang, 2008). Research has shown that there are multiple binding modes of substrates within CYP3A (Ekins et al., 2003). In the present study, the inactivation of CYP3A4 and CYP3A5 by dronedarone and NDBD using midazolam as a probe substrate was also investigated. Compared with the inactivation of testosterone 6β-hydroxylation, inactivation of midazolam 1β-hydroxylation by dronedarone and NDBD was less significant. This demonstrates the inactivation of the two CYP3A isoforms by dronedarone and NDBD is substrate specific. As reported before, lapatinib also yields the site-specific MBI of CYP3A5 (Chan et al., 2012). As the potential clinical DDIs arising from the inactivation of CYP3A4 and CYP3A5 by dronedarone and NDBD are substrate specific, prudent clinical interpretation needs to be exercised.

The MI complex forms a Soret peak at approximately 448–455 nm due to the presence of a coordinate bond formed between the reactive intermediate and ferrous iron of the CYP450 heme group (Franklin, 1972; Buening and Franklin,

![Fig. 7. Accurate mass spectrum and proposed fragmentation pattern of oxime metabolite–GSH adduct derived from dronedarone.](molpharm.aspetjournals.org at ASPET Journals on June 21, 2017)
inactivated CYP3A4 and CYP3A5 via the formation of MI complexes.

The reaction sequence to MI complex formation from the tertiary alkylamine has been proposed by Hanson et al. (2010). N-dealkylation occurs to yield a secondary amine, followed by...
two potential pathways, namely: 1) hydroxylation of the secondary amine leading to the nitroso intermediate; or 2) a second N-dealkylation forms a primary amine, ultimately leading to the same nitroso intermediate. By comparing the inactivation rate constant \(k_{\text{inact}}\), the MI complex formation by NDBD is more efficient than dronedarone against both CYP3A4 and CYP3A5. This implies that N-desbutylation of dronedarone to NDBD is possibly the first bioactivation step toward the formation of the MI complex. Interestingly, five metabolites (M2-1 to M2-5) were identified to be hydroxylated NDBD as confirmed using accurate mass spectrometry (Table 2). Based on the evidence, we propose the formation of the MI complex between dronedarone and CYP3A is mediated through hydroxylation of the amino group of the NDBD followed by subsequent formation of a nitroso intermediate (Fig. 8).

The quasi-irreversible MI complex can be dissociated by oxidation with potassium ferricyanide (Buening and Franklin, 1976). This is followed by the recovery of CYP450 enzymatic activity. In contrast, for MBI, the inactivated CYP450 enzymatic activity is irreversible and cannot be restored. Based on a published criterion (Watanabe et al., 2007), CYP3A enzymatic activity inactivated after a 30-minute preincubation with quasi-irreversible inactivators can be restored by more than 20% with the addition of potassium ferricyanide. For irreversible inactivation, the recovery of enzyme activity is less than 20%. The metabolic activity of inactivated CYP3A4 and CYP3A5 was restored by less than 20% for dronedarone and more than 20% for NDBD after the addition of potassium ferricyanide (Table 3). Our findings hinted of the possibility of an electrophilic reactive metabolite of dronedarone that inactivates CYP3A via an additional irreversible MBI pathway.

Indeed, reactive metabolite-GSH adducts were found for the first time when dronedarone and NDBD were incubated with CYP3A4 and CYP3A5. The EPI spectrum suggested that the GSH adduct was formed via the formation of a covalent bond between the cysteine thiol of GSH and the electrophilic oxime metabolite derived from desulfonated dronedarone (Fig. 7; Table 4). Similar observations were made for GSH adducts derived from NDBD and synthesized desulfonated dronedarone. Collectively, our findings confirmed that desulfonation of both dronedarone and NDBD precedes the formation of the oxime metabolites and supports the latter as the species that inactivate CYP3A4 and CYP3A5 via covalent and irreversible MBI. Although the recovery of enzymatic activity was more than 20% after adding potassium ferricyanide to NDBD incubations, one has to consider two key factors before ruling out the MBI of CYP3A by NDBD. First, the stipulated criterion (Watanabe et al., 2007) may not be applicable to a mixed mode inactivator, such as NDBD, where it inactivates CYP3A via both MBI and the formation of a MI complex. Second, the greater recovery of enzymatic activity observed for NDBD (>20%) is supportive of our earlier finding that MI complex formation by NDBD is more efficient than dronedarone against both CYP3A4 and CYP3A5.

In conclusion, we demonstrated for the first time that dronedarone and NDBD inactivate CYP3A4 and CYP3A5 site specifically via the formation of both quasi-irreversible MI complexes and quinone oxide-mediated covalent adducts (Fig. 8). With a deeper elucidation of the mechanisms of CYP3A inactivation, our findings fuel new knowledge in understanding clinical DDIs and hepatotoxicity associated with dronedarone. Nevertheless, further clinical or physiologic-based pharmacokinetics studies are necessary to confirm the clinical significance of CYP3A inactivation by dronedarone in DDIs. In addition, although our study discovers the potential of dronedarone and NDBD in covalent binding to cellular nucleophiles, further studies need to be performed to elucidate the molecular mechanisms associated with dronedarone-induced hepatotoxicity.

Authorship Contributions

Participated in research design: Hong, Kojodjojo, Chan.
Conducted experiments: Hong, Chia, Yeo, Venkatesan, Koh.
Contributed new reagents or analytic tools: Chai, Zhou.

Performed data analysis: Hong, Chia, Yeo, Venkatesan, Koh.
Wrote or contributed to the writing of the manuscript: Hong, Chia, Yeo, Venkatesan, Chan.

References


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