Mechanism-Based Inactivation of Cytochrome P450 3A4 by Benzbromarone

Lloyd Wei Tat Tang, Ravi Kumar Verma, Hao Fan, and Eric Chun Yong Chan

Department of Pharmacy, Faculty of Science, National University of Singapore, Singapore (L.W.T.T., E.C.Y.C.) and Bioinformatics Institute (BII), Agency for Science, Technology and Research (A*STAR), Singapore (R.K.V., H.F.)

ABSTRACT

Benzbromarone (BBR), a potent uricosuric agent for the management of gout, is known to cause fatal fulminant hepatitis. Although the mechanism of BBR-induced idiosyncratic hepatotoxicity remains unelucidated, cytochrome P450 enzyme–mediated bioactivation of BBR to electrophilic reactive metabolites is commonly regarded as a key molecular initiating event. However, apart from causing aberrant toxicities, reactive metabolites may result in mechanism-based inactivation (MBI) of cytochrome P450. Here, we investigated and confirmed that BBR inactivated CYP3A4 in a time-, concentration-, and NADPH-dependent manner with $k_i$, $k_{inact}$, and partition ratio of 11.61 $\mu$M, 0.10 minutes $^{-1}$, and 110, respectively. Coincubation with ketoconazole, a competitive inhibitor of CYP3A4, attenuated the MBI of CYP3A4 by BBR, whereas the presence of glutathione and catalase did not confer such protection. The lack of substantial recovery of enzyme activity postdialysis and after oxidation with potassium ferricyanide, combined with the absence of a Soret peak in spectral difference scans, implied that MBI of CYP3A4 by BBR did not occur through the formation of quasi-reversible metabolite–intermediate complexes. Analysis of the reduced CO-difference spectrum revealed an $\sim$44% reduction in ferrous-CO binding and hinted that inactivation is mediated via irreversible covalent addition to both the prosthetic heme moiety and the apoprotein. Finally, our in silico covalent docking analysis further suggested the modulation of substrate binding to CYP3A4 via the covalent addition of epoxide-derived reactive intermediates of BBR to two key cysteine residues (Cys239 and Cys58) vicinal to the entrance of the orthosteric binding site.

SIGNIFICANCE STATEMENT

Although the bioactivation of benzbromarone (BBR) to reactive metabolites has been well characterized, its potential to cause mechanism-based inactivation (MBI) of cytochrome P450 has not been fully investigated. This study reports the MBI of CYP3A4 by BBR via irreversible covalent addition and develops a unique covalent docking methodology to predict the structural molecular determinants underpinning the inactivation for the first time. These findings lay the groundwork for future investigation of clinically relevant drug-drug interactions implicating BBR and mechanisms of BBR-induced idiosyncratic hepatotoxicity.

Introduction

Benzbromarone (BBR) is a benzofuran derivative developed in the 1970s for the therapeutic management of gout. It functions as an uricosuric agent via inhibition of human urate-anion transporter 1, expressed along the apical membrane of the renal proximal tubule epithelial cells (Sorensen and Levinson, 1976). Despite three decades of clinical usage, BBR was abruptly withdrawn from several countries in 2003 amid isolated but fatal case reports of fulminant hepatitis (Lee et al., 2008). Because of the small number of patients affected and the inconsistent temporal relations to drug exposure, BBR-induced hepatotoxicity appears to be idiosyncratic, with its mechanism of toxicity remaining unelucidated.

BBR, benzbromarone; BBR-5,6-BF-Epoxide, benzbromarone-5,6-benzofuran-epoxide; BBR-6,7-BF-Epoxide, benzbromarone-6,7-benzofuran-epoxide; CO, carbon monoxide; DDI, drug–drug interaction; G6P, glucose–6-phosphate; G6PDH, glucose–6-phosphate dehydrogenase; GSH, glutathione; LC/MS/MS, liquid chromatography tandem mass spectrometry; MBI, mechanism-based inactivation; MI complex, metabolite–intermediate complex; OBS, orthosteric binding site; P450, cytochrome P450; rCYP3A4, recombinant CYP3A4; ROS, reactive oxygen species.
Although the specific molecular mechanisms underpinning idiosyncratic drug-induced liver injury often remain nebulous, multiple lines of evidence have demonstrated that its presentation is frequently associated with aberrant P450 enzyme metabolism (Guengerich, 2011). In that regard, the P450s are a ubiquitous class of hemoproteins that play an instrumental role in the oxidative metabolism of endogenous substrates and xenobiotics (Guengerich, 2001; Zanger and Schwab, 2013). It is estimated that the metabolism of >80% of all marketed drugs can be attributed to just six P450 isoforms (Zanger et al., 2008). However, because of the wide substrate diversity and the multiplicity of reactions that P450s can catalyze (Guengerich, 2001), there is increasing recognition that idiosyncratic drug-induced liver injury may arise as an unintended consequence of P450-mediated bioactivation. Bioactivation can generate electrophilic, reactive metabolites that can covalently adduct to nucleophilic centers on biologic macromolecules; generate ROS leading to oxidative stress within cells; or form haptons, which may initiate a cascade of immunologic responses (Stephens et al., 2014). All of these deleterious molecular perturbations can ultimately precipitate as idiosyncratic organ-related toxicities. In fact, it is reported that the most prevalent reason for postmarketing drug withdrawals stems from organ-directed toxicities arising from bioactivation of drugs to electrophilic reactive species (Park et al., 2006).

Although early studies have delineated the roles of CYP3A4 and CYP2C9 in the metabolism of BBR to its two major metabolites (Fig. 1) (De Vries et al., 1989), a surge of recent research efforts has characterized several novel reactive metabolites resulting from P450-mediated bioactivation pathways arising from both the benzofuran and dibrominated phenolic moiety of BBR (McDonald and Rettie, 2007; Kitagawara et al., 2015; Wang et al., 2016). Additionally, the structures of its epoxide metabolites (BBR-5,5-BF-Epoxide and BBR-6,7-BF-Epoxide) arising primarily from CYP3A4-mediated bioactivation of BBR are also shown.

As irreversible MBI of P450 persists in vivo even after the perpetrator drug has been systemically eliminated from the body, the extent of endobiotic-xenobiotic interactions and/or drug-drug interaction (DDI) tends to be more profound than with a reversible inhibitor (Björnsson et al., 2003). Moreover, apart from the greater pharmacokinetic DDI liabilities, instances of idiosyncratic immune-mediated hepatotoxicity have also been independently attributed to MBI of P450 (Masubuchi and Horie, 1999).

Because of the implications of MBI of P450 in pharmacology and toxicology, there have been consistent efforts by scientists to screen the MBI of P450 by pharmaceuticals. At this outset, a drug can be characterized as an archetypal MBI if it exhibits the following features: time- and cofactor dependence of inactivation, saturable kinetics of inactivation, protection against inactivation by a competing substrate, lack of protection against inactivation by exogenous nucleophiles or scavengers of ROS, irreversibility of inactivation, and 1:1 binding stoichiometry (Nassar, 2009). Although the inactivation of CYP3A4 by BBR has been hinted at (Masubuchi and Kondo, 2016), the specific nature and mechanism of inactivation remain incompletely characterized.

In this study, the nature of interaction between BBR and several relevant P450 isoforms was investigated, which revealed that it inactivates CYP3A4 in a time-, concentration-, and cofactor-dependent manner. Subsequently, we demonstrated that BBR fulfills the established criteria for an MBI and developed a novel in silico approach to predict the structural determinants underpinning the irreversible covalent inactivation of CYP3A4.

Materials and Methods

Chemicals and Reagents. BBR and sodium dithionite were procured from Tokyo Chemical Industries (Tokyo, Japan). Dexamethasone, diolenc sodium, prednisolone, rivaroxaban, verapamil hydrochloride, glutathione (GSH), catalase, safranin O, and Tergitol-type NP40 were acquired from Sigma-Aldrich (St. Louis, MO). Midazolam was purchased from Tocris BioScience (Bristol, UK). Potassium ferricyanide was obtained from VWR International (Leuven, Belgium). Human recombinant CYP3A4, 2C9, and 2D2
Supersomes coexpressing cytochrome b5 and the NADPH regenerating system comprising NADP+ and glucose-6-phosphate (G6P) (NADPH A) and glucose-6-phosphate dehydrogenase (G6PDH) (NADPH B) were procured from BD Gentest (Woburn, MA). High-performance liquid chromatography–grade acetoneitrile was purchased from Tedia Company, Inc. (Fairfield, OH). Water was obtained using a Milli-Q water purification system (Millipore Corporation, Bedford, MA). All other commercially available chemicals were of analytical or high-performance liquid chromatography grade.

Screening of MBI of P450 by BBR. Potential MBI of CYP3A4, CYP2C9, and CYP2J2 by BBR was investigated by preparing primary incubation mixtures (n = 2) consisting of either 20 pmol/ml rCYP3A4, recombinant CYP2C9 or recombinant CYP2J2, BBR (at either 0, 5, or 25 µM—with the latter two corresponding to low and high concentrations, respectively), G6PDH, and 100 mM potassium phosphate buffer (pH 7.4). The enzymatic reaction was initiated via the addition of NADP+/G6P after prewarming at 37°C for 5 minutes. The final primary incubation mixture (100 µl) contained <1% v/v organic solvent. Subsequently, at various preincubation intervals (0, 3, 8, 15, 22, and 30 minutes), a 5-µl aliquot of each primary incubation mixture was withdrawn and transferred to 95 µl of prewarmed secondary incubation mixture consisting of a P450-specific probe substrate, an NADPH regenerating system, and 100 mM potassium phosphate buffer (pH 7.4). This yielded a 20-fold dilution. The specific probe substrates used in these experiments were 25 µM midazolam (CYP3A4A), 50 µM diclofenac (CYP2C9), and 50 µM rivaroxaban (CYP2J2). The concentration of each probe substrate was higher than the respective Km value with regard to each enzyme. The secondary incubation mixtures were further incubated at 37°C for another 10 (for assays involving midazolam) or 30 minutes (for experiments involving diclofenac or rivaroxaban), after which an 80-µl aliquot was immediately removed and quenched with equal volumes of ice-cold acetoniitrile containing either 1 µM prednisolone (internal standard for midazolam assay) or 4 µM dexamethasone (internal standard for diclofenac or rivaroxaban assay). The quenched samples were centrifuged at 2755g at 4°C for 30 minutes to obtain the supernatant for liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis.

Time-, Concentration-, and NADPH-Dependent Inactivation of CYP3A4. All incubations were performed in 96-well plates. Rivaroxaban was used as the probe substrate in all subsequent CYP3A4 MBI assays. Primary incubation mixtures comprising 40 pmol/ml rCYP3A4, BBR (0, 1, 2.5, 5, 15, 25, and 50 µM), G6PDH, and 100 mM potassium phosphate buffer (pH 7.4) were prepared in triplicate. After preincubating at 37°C for 5 minutes, the reaction was initiated via the addition of NADP+/G6P. The final primary incubation mixture (100 µl) contained <1% v/v organic solvent. Subsequently, at various preincubation intervals (0, 3, 8, 15, 22, and 30 minutes), a 5-µl aliquot of each primary incubation mixture was withdrawn and transferred to a prewarmed secondary incubation mixture consisting of 50 µM rivaroxaban, an NADPH regenerating system, and 100 mM potassium phosphate buffer (pH 7.4). The reaction was initiated by the addition of NADP+/G6P after preincubating at 37°C for 45 minutes to allow inactivation to go into completion. The final primary incubation mixture (50 µl) contained <1% v/v organic solvent. Thereafter, aliquots of the primary incubation mixture were withdrawn and transferred to the secondary incubation mixture (similar to that prepared for the inactivation experiments) and incubated at 37°C for another 2 hours. Samples were then quenched and assayed for residual enzyme activity as described. To estimate the partition ratio, the percentage of residual CYP3A4 activity was plotted against the molar ratio of BBR to CYP3A4 concentration. The turnover number (partition ratio + 1) was obtained by extrapolating the intercept of the linear regression line plotted at lower ratios with the straight line plotted at higher ratios to the abscissa. Finally, the partition ratio was back-calculated by subtracting the turnover number by a numerical value of 1.

Substrate Protection. To investigate whether enzyme inactivation could be protected by a competing substrate, the potent CYP3A4 competitive inhibitor ketoconazole, at a concentration of 0.1 and 1 µM (roughly 1 × 10<sup>-5</sup> and 1 × 10<sup>-4</sup> the Km for CYP3A4), was introduced in triplicate to the primary incubation mixture containing 40 pmol/ml rCYP3A4, 50 µM BBR, G6PDH, and 100 mM potassium phosphate buffer (pH 7.4). The reaction was initiated by the addition of NADP+/G6P after preincubation at 37°C for 5 minutes. Aliquots were then withdrawn at different preincubation time points (0, 3, 8, 15, 22, and 30 minutes) and transferred to the secondary incubation mixture, and the mixtures were subsequently assayed for residual CYP3A4 enzymatic activity as previously described. Primary incubation mixtures that excluded the addition of either ketoconazole or both BBR and ketoconazole served as the negative controls.

Effect of Exogenous Nucleophile and Scavenger of ROS on MBI. The exogenous nucleophilic trapping agent GSH (2 mM) was added to the primary incubation mixture containing 40 pmol/ml rCYP3A4, 50 µM BBR, G6PDH, and 100 mM potassium phosphate buffer (pH 7.4). After preincubating at 37°C for 5 minutes, the enzymatic reaction was initiated via the addition of NADP+/G6P. At specific preincubation time points (0, 3, 8, 15, 22, and 30 minutes), aliquots were transferred to the secondary incubation mixtures and subsequently assayed for residual CYP3A4 enzymatic activity as previously described. Negative controls were prepared without both BBR and GSH or only without GSH in the primary incubation mixture. Parallel experiments involving the incorporation of catalase (800 U/ml) in place of GSH were also performed to investigate the effects of scavenger of ROS on the inactivation of CYP3A4.

from the slope of the linear graphs for each BBR concentration. Subsequently, a plot of k<sub>obs</sub> against BBR concentrations [I] allowed the fitting of inactivation kinetic parameters (K<sub>I</sub> and k<sub>inact</sub>) to nonlinear least-squares regression based on eq. 1 in GraphPad 8.0.2 (San Diego, CA).

\[ k_{\text{obs}} = \frac{k_{\text{inact}} \times [I]}{K_I + [I]} \]  

where k<sub>inact</sub> represents the maximal inactivation rate constant, K<sub>I</sub> is the concentration of the inactivator at half-maximal inactivation rate constant, and [I] is the in vitro concentration of the inactivator. Eq. 1 assumes that there is negligible change of [I] during the incubation period and that the loss of enzyme activity purely commensurate with inactivation by BBR. The ratio of k<sub>inact</sub> to K<sub>I</sub> was determined by dividing the mean values of k<sub>inact</sub> by K<sub>I</sub>. Finally, the time required for half of the enzyme molecules to be inactivated (t<sub>1/2</sub>) was determined by eq. 2.

\[ t_{1/2} = \frac{ln2 \times k_{\text{obs}}}{k_{\text{inact}}} \]
Reversibility of Inactivation. The reversibility of CYP3A4 inactivation was investigated by two distinct approaches that were previously reported (Noseir et al., 2004; Watanabe et al., 2007); namely, equilibrium dialysis and oxidation by potassium ferricyanide. In the dialysis experiments, triplicate primary incubation mixtures comprising 40 pmol/ml rCYP3A4, 50 μM BBR, G6PDH, and 100 mM potassium phosphate buffer (pH 7.4) were preincubated at 37°C for 5 minutes. Enzymatic reaction was initiated by the addition of NADP+/G6P and allowed to proceed for 30 minutes, after which a 5-μl aliquot was transferred to 95 μl of the secondary incubation mixture, yielding a 20-fold dilution. Concurrently, 90 μl of the remaining primary incubation mixture was transferred to a Slide-A-Lyzer mini dialysis device (0.1 ml, molecular weight cutoff of 10,000; Pierce Chemical Co., Rockford, IL) and placed in a beaker filled with 200 ml of ice-cold 100 mM potassium phosphate buffer (pH 7.4). The buffer system was maintained on ice (4°C) with constant stirring and accompanied by one fresh buffer change at the 2nd hour. After 4 hours, 5 μl of the dialyzed mixture was transferred to each prewarmed secondary incubation well. All secondary mixtures were further incubated at 37°C for 2 hours and subsequently assayed for residual CYP3A4 enzymatic activity as previously described. Conversely, in the experiments involving potassium ferricyanide, a series of three incubations were performed sequentially as previously described (Hong et al., 2016). Briefly, the primary incubation comprised 40 pmol/ml rCYP3A4, G6PDH, and 100 mM potassium phosphate buffer (pH 7.4) in the presence or absence of 50 μM BBR. After initiation of the reaction with the addition of NADP+/G6P and incubation at 37°C for either 0 or 30 minutes, 20 μl of the primary incubation mixture was aliquoted into an equal volume of secondary incubation mixture containing 100 mM potassium phosphate buffer (pH 7.4) with or without 2 mM potassium ferricyanide. The secondary mixtures were then allowed to incubate at 37°C for another 10 minutes. Thereafter, 10 μl of the mixture was withdrawn and diluted 10-fold into a tertiary incubation mixture containing 50 μM rivaroxaban (probe substrate), an NADPH regenerating system, and 100 mM potassium phosphate buffer (pH 7.4). The reaction mixture was further incubated at 37°C for another 2 hours and subsequently assayed for residual CYP3A4 activity as previously described. The percentage of CYP3A4 metabolic activity remaining after 0 or 30 minutes of incubation with BBR compared with the corresponding controls in the absence of BBR was calculated using eqs. 3 and 4, respectively.

\[
\frac{\text{activity remaining after } 0 \text{ or } 30 \text{ minutes of incubation with BBR}}{\text{activity as previously described}} \times 100, \quad (3)
\]

\[
\frac{\text{activity remaining after } 30 \text{ minutes of incubation with BBR}}{\text{activity as previously described}} \times 100, \quad (4)
\]

where \(v\) represents the residual metabolic activity of CYP3A4 and its decrease was subtracted from %control\(_{30 min}\) in the presence of potassium ferricyanide with the corresponding values obtained in the absence of potassium ferricyanide.

Spectral Difference Scanning. Incubation mixtures (200 μl) containing 200 pmol/ml rCYP3A4, 50 μM BBR, G6PDH, and 100 mM potassium phosphate buffer (pH 7.4) were prepared and preincubated at 37°C for 5 minutes. Thereafter, the enzymatic reaction was initiated via the addition of NADP+/G6P and immediately scanned from 430 to 490 nm at 5-minute intervals over a 45-minute duration using an Infinite M200 Tecan microplate reader (Tecan Group, Männedorf, Switzerland) maintained at a constant 37°C. The spectral differences were obtained by comparing the UV absorbances between the sample and reference wells, which comprised vehicle in place of BBR. Positive control wells were prepared using 10 μM verapamil, a known MBI of CYP3A4, through the formation of quasi-reversible metabolite-intermediate complex (MI complex). Finally, the extent of MI complex formation was also quantitatively assessed by measuring the absorbance difference between 454 and 490 nm.

Reduced Carbon Monoxide–Difference Spectroscopy. Triplicate incubation mixtures (50 μl) comprising 640 pmol/ml rCYP3A4, 50 μM BBR, G6PDH, and 100 mM potassium phosphate buffer (pH 7.4) were prepared and preincubated at 37°C for 5 minutes, after which NADP+/G6P was added to initiate the reaction, and the mixture was incubated for another 30 minutes at 37°C. Subsequently, the reaction was terminated via the addition of 450 μl ice-cold quenching buffer comprising 1 mM EDTA, 20% glycerol, 1% Tergitol-type NP40, 2 μM safranin O, and 100 mM potassium phosphate buffer (pH 7.4). The quenched samples were then mixed by inversion and evenly divided into two 250-μl tubes (sample and reference tubes). Carbon monoxide (CO) was slowly bubbled into one of the tubes (sample tube) and stopped after roughly 60 bubbles had been passed into the mixture. Thereafter, approximately 1 mg of sodium dithionite was added to both tubes, and 200 μl of the mixture was transferred out from each tube into a 96-well plate. The reduced CO-difference spectra for sample and reference wells were acquired by scanning from 400 to 500 nm using an Infinite M200 Tecan microplate reader (Tecan Group). Negative controls were prepared by omitting NADPH from the incubation mixture. The extent of reduction in the peak at 450 nm was determined by calculating the P450 concentration according to eq. 5 (Guenegerich et al., 2009).

\[
\text{CYP450 concentration (nmol of CYP450 per mL)} = \frac{(\Delta A_{091} - \Delta A_{091})}{0.091}
\]

Measurement of Residual P450 Activity. All samples were analyzed using the LC/MS/MS system consisting of an Agilent Infinity ultra-high-pressure liquid chromatography (Agilent Technologies Inc., Santa Clara, CA) interfaced with AB SCIEX TRAP 3500 tandem mass spectrometer (AB SCIEX, Framingham, MA). Chromatographic separation was achieved with an ACQUITY ultra-performance liquid chromatography ethylene bridged hybrid C18, 1.7 μm, 2.1 × 50 mm column (Waters, Milford, MA). The aqueous mobile phase (A) was 0.1% formic acid in water, whereas the organic mobile phase (B) was 0.1% formic acid in acetonitrile. Mobile phases were delivered at a flow rate of 0.5 ml/min. The column and sample temperatures were set at 45 and 4°C, respectively. The gradient elution conditions were as follows: linear gradient from 20% to 80% B (0–1.20 minutes), isocratic at 100% B (1.21–2.00 minutes), and isocratic at 20% B (2.01–2.50 minutes). All analytes were detected in positive electrospray ionization (ESI) mode. The source-dependent mass spectrometry parameters are as follows: ion spray voltage = 5500 V; source temperature = 500°C; curtain gas = 25 psi; ion source gas 1 (sheath gas) = 30 psi; ion source gas 2 (drying gas) = 30 psi. The multiple reaction monitoring transitions and compound-dependent mass spectrometry parameters of the analytes are summarized in Supplemental Table 1. Chromatographic peak integration was performed using Analyst software version 1.6.2 (Applied Biosystems). For all LC/MS/MS analyses, the peak area of the analyte was expressed as a ratio to the peak area of the internal standard.

In Silico Protein Structure Preparation. To glean possible mechanistic insights on the structural molecular determinants of CYP3A4 inactivation arising from covalent adduction by BBR, we used 49 high-resolution crystal structures of human CYP3A4 (<3 Å; comprising 3 apo- and 46 holo-forms) from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (Supplemental Table 2) (Rose et al., 2015). For each protein structure retrieved, only chain A was considered. All cocrystallized ligands except for the heme cofactor were removed from the protein structures. Additionally, mutated amino acids in each crystal structure were reverted back to their corresponding wild-type counterparts. Thereafter, protein structures were processed with the protein preparation wizard in Maestro (Schrodinger, NY), and the missing residues and loop segments were added to reconstruct the protein. Finally, hydrogen atoms were added, and the ionization states of titratable groups were determined.

In Silico Ligand Preparation. Previously, it was reported that BBR undergoes P450-mediated bioactivation to electrostatic reactive epoxide intermediates (Fig. 1) (Wang et al., 2016). To investigate whether these reactive epoxides could be implicated in the covalent
adduction of CYP3A4, we obtained three-dimensional coordinates of BBR from the PubChem data base (Kim et al., 2019), from which two epoxide metabolites—namely, benzbromarone-6,7-benzofuran-epoxide (BBR-6,7-BF-Epoxide) and benzbromarone-5,6-benzofuran-epoxide (BBR-5,6-BF-Epoxide)—were derived using the sketcher module in the Schrödinger suite. Thereafter, the lowest energy conformer was generated for both epoxide metabolites using the LigPrep (Schrödinger). A similar approach was employed to prepare the noncovalent substrate rivaroxaban without considering possible metabolite derivatives.

In Silico Binding Site Prediction and Covalent Docking. All 49 preprocessed CYP3A4 crystal structures were first superimposed, after which potential ligand-binding pockets for each of the included protein structures were identified using the site recognition software Sitemap (Schrödinger). We then computed a list of accessible cysteine residues using an in-house analytical algorithm for each identified pocket. The docking of BBR-6,7-BF-Epoxide and BBR-5,6-BF-Epoxide was performed in two sequential stages. We first noncovalently docked the metabolites in the binding pockets containing the accessible cysteines using GLIDE (Friesner et al., 2004). If noncovalent docking was successful, we then performed covalent docking for accessible cysteine residues within the pocket using CovDock (Zhu et al., 2014) in the Schrödinger suite. In particular, we covalently docked each BBR epoxide metabolite at Cys58 and Cys239 residues in 18 and 25 CYP3A4 crystal structures, respectively (Supplemental Table 2), where these corresponding cysteine residues are accessible. The covalent docking scores obtained were then combined, sorted, and ranked. To further investigate whether BBR adducts hinder the approach and/or binding of rivaroxaban by restricting the flexibility of the F-F’ loop, noncovalent substrate rivaroxaban was docked inside the orthosteric binding site (OBS) in the absence of BBR adducts using GLIDE. The minimum distance between the heavy atoms of docked rivaroxaban and BBR adducts were subsequently computed for comparison. Furthermore, to interrogate whether BBR epoxide metabolites could form covalent adducts with the porphyrin ring of the heme moiety, we also simultaneously docked BBR-6,7-BF-Epoxide and BBR-5,6-BF-Epoxide to the heme residue of CYP3A4 and calculated their corresponding covalent docking scores.

Results

Screening of MBI of P450 by BBR. To elucidate the nature of interaction between BBR and CYP3A4, CYP2C9, and CYP2J2, we performed an initial screening experiment involving BBR at three different concentrations (0, 5, and 25 μM). Preliminary results revealed that BBR resulted in a time-dependent decrease in CYP3A4 enzymatic activity (Fig. 2A). The loss of enzyme activity was also more pronounced at the higher concentration of BBR—with residual CYP3A4 activity reaching a nadir when preincubated with 25 μM BBR for 30 minutes. Time-dependent reduction in CYP2C9 and CYP2J2 activities by BBR was not observed (Fig. 2, B and C).

Time-, Concentration-, and NADPH-Dependent Inactivation of CYP3A4. After demonstrating that BBR potentially inactivates CYP3A4 from our screening experiments, we proceeded to characterize the inactivation kinetics of CYP3A4 using rivaroxaban as the clinically relevant probe substrate. Previous in-house studies have demonstrated the feasibility of utilizing rivaroxaban as a probe substrate—in place of the prototypical US Food and Drug Administration—recommended substrates—to investigate the inhibition and inactivation potencies of CYP3A4 and CYP2J2 by amiodarone, dronedarone, and their respective metabolites for subsequent static modeling of DDI (Cheong et al., 2017). Our findings revealed that BBR inactivated CYP3A4 in both a time- and concentration-dependent manner (Fig. 3A). Moreover, the omission of NADPH abrogated the loss of CYP3A4 activity when preincubated with BBR for up to 30 minutes (Fig. 3B). This observed NADPH dependence implied that prior metabolic activation of BBR was a crucial molecular initiating event leading to the inactivation of CYP3A4. Additionally, the observed first-order rate of inactivation (k_inact) derived from the various concentrations of BBR approached a maximum inactivation rate constant (k_inact) (Fig. 3C), hence denoting that the loss in CYP3A4 catalytic activity elicited by BBR followed pseudo-first-order kinetics. Specifically, the inactivation kinetic parameters (K1 and k_inact) derived from the Kitz-Wilson plot (Kitz and Wilson, 1962) were 11.61 ± 2.40 μM and 0.10 ± 0.008 minutes⁻¹ respectively, which in turn yielded a k_inact/K1 ratio of 8.82 minutes⁻¹ mM⁻¹. The half-life was calculated to be 6.8 minutes. Similarly, time-, concentration-, and NADPH-dependent inactivation of CYP3A4 by BBR was also observed when midazolam was used as an alternative probe substrate (Supplemental Fig. 1, A and B).

Partition Ratio. A titration method previously reported (Silverman, 1995) was adopted, which determined the partition ratio of BBR to be 110 (Fig. 4A).

Substrate Protection. Enzyme inactivation was protected in the presence of a competitive inhibitor of CYP3A4. Coincubation with ketoconazole attenuated the rate of CYP3A4 inactivation by BBR as evident by the diminished rate of enzyme inactivation with time (Fig. 4B). Moreover, the protective effects conferred by ketoconazole appeared to be concentration-dependent, with inactivation being completely nullified when 1 μM of ketoconazole was coincubated with BBR and CYP3A4 in the primary incubation mix.

Effect of Exogenous Nucleophile and Scavenger of ROS on MBI. The addition of exogenous nucleophiles (GSH) or scavengers of ROS (catalase) had negligible effects on the
rate of enzyme inactivation. As illustrated in Fig. 4, C and D, CYP3A4 was inactivated to a similar extent in incubation mixtures containing BBR alone.

Reversibility of Inactivation. To determine whether inactivation of CYP3A4 by BBR is quasi-irreversible or irreversible, the nature of inactivation was investigated via dialysis and oxidation with potassium ferricyanide. The magnitude of CYP3A4 activity did not increase after extensive dialysis at 4°C for 4 hours (Fig. 5A). It should be noted that the marginal decrease in residual enzyme activity observed for both vehicle and BBR containing reaction mixture postdialysis was due to enzymatic degradation that occurred during the course of dialysis. Oxidation with potassium ferricyanide restored metabolic activity of CYP3A4 by 0.9% ± 0.25% after inactivation by BBR (Fig. 5B). Conversely, in control experiments involving verapamil (a known quasi-irreversible inactivator of CYP3A4), the metabolic activity of CYP3A4 was restored by 24.5% in the presence of potassium ferricyanide (Supplemental Fig. 2).

Spectral Difference Scanning. Spectral differences obtained by scanning from 430 to 490 nm at 5-minute intervals for 45 minutes highlighted a lack of an observable peak in the Soret region (448–458 nm) when CYP3A4 was incubated with BBR (Fig. 6A). Furthermore, tracking the increase in absorbance at 454 nm and the isosbestic point at 490 nm further confirmed the lack of MI complex formation with BBR (Fig. 6B), whereas control experiments involving verapamil (which is known to form MI complex with CYP3A4) demonstrated the presence of a Soret peak and a time-dependent increase in absorbance at 454 nm relative to 490 nm that correlates with the extent of MI complex formation (Supplemental Fig. 3, A and B).

Reduced CO-Difference Spectroscopy. The reaction between CO and the ferrous (reduced) form of P450s gives rise to a complex that produces a spectrum with a maximum absorbance at ∼450 nm (Omura and Sato, 1964). Incubating CYP3A4 with 50 μM BBR in the presence of NADPH for

Fig. 3. (A) Time- and concentration-dependent inactivation of CYP3A4 by BBR using rivaroxaban as a probe substrate. (B) Cofactor NADPH-dependent inactivation of CYP3A4 by BBR using rivaroxaban as a probe substrate. (C) Nonlinear regression of observed first-order inactivation rate constants (k_{obs}) vs. concentration levels of BBR. The calculated K_I and k_{inact} based on nonlinear regression are 11.61 ± 2.40 μM and 0.10 ± 0.008 minutes^{-1}, respectively. Each point in (A and B) represents the mean and S.D. of triplicate experiments.

Fig. 4. (A) Partition ratio for the inactivation of CYP3A4 by BBR estimated to be 110 was determined by extrapolating the intercept of the linear regression line at lower ratios and the straight line for the high ratios to the x-axis. (B) Inactivation of CYP3A4 was attenuated in the presence of the reversible inhibitor ketoconazole (KTC). Conversely, the presence of either (C) GSH or (D) catalase did not protect against enzymatic inactivation. Each point in (A–D) represents the mean and S.D. of triplicate experiments.
30 minutes resulted in a mean reduction of \(~\sim\~\) 44% in the spectrally detectable peak at 450 nm as compared with negative control incubations lacking NADPH (Fig. 6C). The measured concentrations of CYP3A4 in the presence and absence of NADPH were 33 ± 6 and 58 ± 24 pmol/ml, respectively.

**CYP3A4 Crystal Structure Analysis and Covalent Docking.** Inspection of the spatial orientations of nucleophilic cysteine residues revealed that none of the seven cysteine residues in CYP3A4 (Cys58, Cys64, Cys98, Cys239, Cys377, Cys442, and Cys468) is present within the OBS where the cognate ligand binds close to the heme group. Our in silico ligand-binding pocket prediction identified four cysteine residues (Cys58, Cys64, Cys239, and Cys468) on the CYP3A4 apoprotein that are accessible for covalent ligand binding (Supplemental Table 2). Interestingly, two of the four accessible cysteine residues, Cys58 and Cys239, are located in the vicinity of the F-F’ loop (residues 211–220), an overhanging segment of the protein that forms the roof of the active site cavity, with a minimum distance between heavy atoms of 8.05 ± 2.07 and 6.09 ± 0.77 Å, respectively. Subsequently, superimposition of all 49 preprocessed CYP3A4 crystal structures revealed a large degree of conformational flexibility of the F-F’ loop (Fig. 7A). CYP3A4 crystal structures that yielded the best covalent docking scores for BBR-6,7-BF-Epoxide and BBR-5,6-BF-Epoxide at Cys58 and Cys239 residues are summarized in Table 1. Results from our covalent docking experiments demonstrated that BBR adducts at Cys239 could adopt a binding pose adjacent to (minimum distance < 4 Å) the F-F’ loop (Fig. 7B). In contrast, the BBR adducts at Cys58 are oriented further away from the F-F’ loop. However, it was observed that the F-F’ loop and Cys58 are flanked by a common C-terminal loop (residue 469–491) (Fig. 7C).

The minimum distances between the heavy atoms of docked rivaroxaban and BBR adducts were computed, which revealed that BBR adducts did not occupy the same space as rivaroxaban (minimum distance between the docked rivaroxaban and BBR adducts is > 10 Å) (Table 2). Notably, our results demonstrated that rivaroxaban forms extensive interactions with both F-F’ and the C-terminal loop (Fig. 7D). Specifically, the minimum distances between rivaroxaban and the F-F’ and C-terminal loop are always determined to be < 4 and < 8 Å, respectively, which suggests that the F-F’ loop not only functions as a lid gating the OBS but also has to undergo a conformational change to facilitate productive rivaroxaban binding.

Finally, our covalent docking analyses revealed that the epoxide metabolites of BBR could also directly alkylate the carboxyl groups on the porphyrin ring of the prosthetic heme (Supplemental Fig. 4, A and B). Covalent docking scores for BBR-6,7-BF-Epoxide and BBR-5,6-BF-Epoxide at the heme residue in selected crystal structures are tabulated in Supplemental Table 3.

**Discussion**

BBR is a potent uricosuric agent that was withdrawn from several global markets because of its propensity to cause idiosyncratic hepatotoxicity. Although the exact molecular mechanisms underpinning idiosyncratic toxicities often remain unelucidated, covalent addition by reactive metabolites generated via P450-mediated bioactivation is generally regarded as the underpinning molecular initiating event. However, beyond merely causing aberrant toxicities, these electrophilic reactive species may also cause MBI of P450s. In that regard, our study confirmed time-, concentration-, and NADPH-dependent

---

**Fig. 5.** (A) Percent activity of CYP3A4 remaining did not increase after extensive dialysis at 4°C for 4 hours. (B) Potassium ferricyanide (KFC) only restored the metabolic activity of CYP3A4 by a modest 0.9% ± 0.25% after a 30-minute incubation with 50 μM BBR. Results from both graphs show the mean and S.D. of two independent experiments conducted in triplicate.

**Fig. 6.** (A) Spectral difference measured at over 45 minutes failed to elucidate a Soret peak in the absorbance ranges of 448–458 nm for CYP3A4 incubated with 50 μM BBR. (B) Similarly, a comparison of the absorbance at the reference of 454 nm against the isosbestic point at 490 nm failed to demonstrate an increase in the extent of MI complex formation over time. (C) Reduced CO-difference spectrum of CYP3A4 in the reconstituted system incubated with 50 μM BBR for 30 minutes in the absence and presence of NADPH.
inactivation of CYP3A4 by BBR. Interestingly, comparison of its $k_{\text{inact}}/K_I$ ratio with those reported for other known MBI of CYP3A4 (Table 3) revealed that although the inactivation potency of BBR is eclipsed by other potent inactivators, such as amprenavir (Ernest et al., 2005), its $k_{\text{inact}}/K_I$ ratio was in the same order of magnitude as other clinically relevant MBIs of CYP3A4, such as clarithromycin and lapatinib (Polasek and Miners, 2008; Teng et al., 2010). With regard to the latter, it is intriguing to note that, like BBR, multiple reports of idiosyncratic hepatotoxicity have also been described with lapatinib (Teo et al., 2012). Notably, our group previously characterized the unique dichotomous MBI of CYP3A4 and CYP3A5 by lapatinib and proposed that the presence of MBI can be considered as a premonitory biomarker of reactive metabolite-induced hepatotoxicity (Chan et al., 2012; Ho et al., 2015). Although it is tempting to speculate that BBR, with similar inactivation potency as lapatinib, could be capable of inducing hepatotoxicity in the same manner, such direct comparison of $k_{\text{inact}}/K_I$ ratios is highly reductionistic and does not embody all the clinical complexities involved in the inactivation of P450s (Fowler and Zhang, 2008). Consequently, additional in vivo and clinical data are warranted to elucidate the exact molecular mechanisms of BBR-induced idiosyncratic hepatotoxicity.

Seminal work in the field of X-ray crystallography has helped shed light on the ligand promiscuity of CYP3A4. These studies revealed the existence of a large degree of conformational flexibility of the F-F loop. (B) Superimposition of top scored binding poses for BBR adducts at Cys239 in CYP3A4 (PDB ID: 4I4G). (C) Superimposition of top scored binding poses for BBR adducts at Cys58 in CYP3A4 (PDB ID: 4D78). (D) Rivaroxaban forms extensive interactions with the F-F loop and the C-terminal loop of CYP3A4. The relative positions of Cys58 and Cys239 are labeled in (B and C).

The efficiency of an MBI is assessed by its partition ratio, which is defined as the number of parent drug molecules required to completely inactivate the enzyme. Partition ratios ranging from near zero to several thousands have been reported—with lower values ascribed to more efficient inactivators (Kent et al., 2001). The partition ratio of 110 suggests that BBR is a moderately efficient inactivator of CYP3A4 and implies that metabolism of BBR to the putative reactive metabolite(s) responsible for MBI of CYP3A4 is subjected to intrinsic competition by other CYP3A4-mediated metabolism pathways of BBR. Coincubation with ketoconazole conferred protection against inactivation, further lending support to the notion that enzymatic inactivation by BBR occurred within the active site. Although reactive metabolites and ROS produced as by-products of xenobiotic metabolism can have...
deleterious effects on enzyme function, the lack of protection conferred by GSH or catalase confirmed that the putative reactive metabolite of BBR inactivates the enzyme before it is liberated from the active site and ROS is not involved in enzyme inactivation.

Although our aforementioned findings collectively substantiated MBI of CYP3A4 by BBR, it remains unclear whether the observed inactivation is mediated via the formation of quasi-irreversible MI complex or through irreversible covalent addition to either the P450 heme moiety and/or apoprotein (Polasek and Miners, 2007). MI complexes, which arise from the formation of coordinate bonds between the inactivator and the prosthetic heme iron, are pseudoirreversible and can be reversed in vitro through equilibrium dialysis or with a strong oxidizing agent. The dissociation of these complexes revives enzymatic catalysis and restores metabolism of the probe substrate. This contrasts starkly with enzymatic inactivation that proceeds via irreversible covalent adduction where there is an irrevocable loss of enzymatic activity. Collectively, our experiments demonstrated that the metabolic activity of CYP3A4 could not be recovered after extensive dialysis and that the addition of potassium ferricyanide only restored activity by 0.9%, which when interpreted under the context of a published criterion (Watanabe et al., 2007), confirms the MBI of CYP3A4 by BBR via covalent addition. Furthermore, it is also well established that a reactive intermediate that forms quasi-irreversible MI complex tends to exhibit a characteristic Soret peak (Polasek and Miners, 2008). The absence of this peak in our spectral analysis further augmented our findings that BBR inactivates CYP3A4 via irreversible covalent addition. Reduced CO-difference spectroscopy further revealed that although a considerable fraction of the reactive intermediate may covalently modify the prosthetic heme, it did not represent the sole pathway by which CYP3A4 is inactivated, as the loss of ferrous-CO binding (∼44%) was not commensurate with the reduction in CYP3A4-catalyzed hydroxylation of rivaroxaban (∼95%).

Finally, our docking analysis provided novel insights on the possible structural determinants that define the inactivation of CYP3A4 by BBR. We chose to interrogate the previously reported epoxide-derived intermediates, as metabolic epoxidation of BBR is predominantly mediated by CYP3A4 (Wang et al., 2016). Furthermore, BBR was more liable to be bioactivated to the epoxide metabolite in vivo as compared with other known reactive intermediates stemming from P450-mediated bioactivation pathways (Wang et al., 2019). The highly malleable nature of the F-F loop in CYP3A4 has been previously characterized and is proposed to play an instrumental role in productive substrate-enzyme binding by functioning as a gating mechanism involved in substrate access and egress (Sevrioukova and Poulos, 2013; Benkaidali et al., 2019). The successful recapitulation of this phenomenon in our structural analyses gave us confidence that our methodology could account for active site plasticity to predict covalent adduction sites. Using multiple crystal structures of CYP3A4, we identified two cysteine residues (Cys239 and Cys58) vicinal to the F-F loop as potential sites of covalent addition. Consequently, based on our docking assays and current understanding on the structural biology of CYP3A4, we posit that covalent addition of Cys239 and/or Cys58 by the epoxide metabolites of BBR could induce a conformational change in the protein structure that could directly or indirectly restrict F-F loop dynamics and adversely modulate OBS-rivaroxaban binding. Our postulations are also in concordance with our in vitro findings, which hinted that MBI also occurs via the formation of irreversible covalent adducts to the CYP3A4 apoprotein. Although covalent modification of Cys239 has been reported to be the underpinning mechanism of MBI of CYP3A4 by several drugs (Baer, Wienkers, and Rock, 2007; Kang et al., 2008; Henne et al., 2012), the indirect perturbation of the F-F loop via the interaction of Cys58 adducts and the C-terminal loop has to be further investigated using site-directed mutagenesis and molecular dynamics simulations. Additionally, the structural insights gleaned from docking BBR epoxide metabolites to heme also align with our experimental data. Such a bimodal adduction profile has recently been reported for 17α-ethynylestradiol against several P450 isoforms (Lin, Zhang, and Hollenberg, 2018). However, it should be noted that our covalent docking experiments do not provide direct evidence on the formation of adducts by BBR with one or more of these cysteine residues or the propionates of the heme of CYP3A4. Further high-mass-accuracy proteomic experiments need to be performed to confirm the adduction sites via peptide mapping.

### Table 3

Comparison of CYP3A4 enzyme inactivation kinetic parameters

<table>
<thead>
<tr>
<th>Compound</th>
<th>( K_I )</th>
<th>( k_{inact} )</th>
<th>( k_{inact}/K_I )</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBR</td>
<td>11.61</td>
<td>0.1024</td>
<td>8.82</td>
<td>Teng et al., 2010</td>
</tr>
<tr>
<td>Lapatinib</td>
<td>1.71</td>
<td>0.0202</td>
<td>11.8</td>
<td>(Polasek and Miners, 2008)</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>2.25</td>
<td>0.84</td>
<td>17.78</td>
<td>(Polasek and Miners, 2008)</td>
</tr>
<tr>
<td>Amprenavir</td>
<td>0.26</td>
<td>0.73</td>
<td>2808</td>
<td>Ernest et al., 2005</td>
</tr>
</tbody>
</table>

### Table 2

Minimum distances between docked rivaroxaban and functionally important loops in CYP3A4 and BBR adduct docking poses

<table>
<thead>
<tr>
<th>Residue</th>
<th>PDB ID</th>
<th>Minimum Distance with Docked Rivaroxaban (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F-F' Loop</td>
<td>C-Terminal Loop</td>
</tr>
<tr>
<td>Cys58</td>
<td>4D78</td>
<td>3.01</td>
</tr>
<tr>
<td></td>
<td>6BD5</td>
<td>3.78</td>
</tr>
<tr>
<td>Cys239</td>
<td>4HG</td>
<td>3.41</td>
</tr>
</tbody>
</table>

*aMinimum distance computed from superimposed rivaroxaban and BBR adduct docking structures.*
Although the clinical ramifications of irreversible P450 MBI in pharmacokinetic DDI are well established, the haptenized P450 may also be implicated in idiosyncratic immunemediated hepatotoxicity. Tienilic acid, an MBI of CYP2C9, is a frequently cited paradigm of this phenomenon (Lopez-Garcia et al., 1994). Autoimmune hepatitis associated with tienilic acid was attributed to de novo anti-liver-kidney microsomal type 2 antibodies toward the covalently alkylated CYP2C9 (Homburg et al., 1984). Although the exact cascade of molecular events leading to the hepatotoxic sequelae of BBR remains unknown, the covalent adduction of BBR to CYP3A4 might similarly engender the production of such autoantibodies.

In conclusion, our results demonstrated that BBR fulfills the established criteria as an MBI of CYP3A4. Moreover, the nature of inactivation was also elucidated to be via irreversible covalent modification of its prosthetic heme and possibly to either Cys239 and/or Cys58 of CYP3A4 apoprotein. Future investigations aimed at clarifying the exact molecular mechanisms of hepatotoxicity by BBR are currently underway.

**Authorship Contributions**

**Participated in research design:** Tang, Verma, Fan, Chan.
**Conducted experiments:** Tang, Verma.
**Contributed new reagents or analytic tools:** Verma, Fan.
**Performed data analysis:** Tang, Verma.
**Wrote or contributed to the writing of the manuscript:** Tang, Verma, Fan, Chan.

**References**


Benkaidali L, André F, Moroy G, Tangour B, Maurel F, and Petitjean M (2019) Four investigations aimed at clarifying the exact molecular mechanism of BBR to CYP3A4 might similarly engender the production of such autoantibodies.

In conclusion, our results demonstrated that BBR fulfills the established criteria as an MBI of CYP3A4. Moreover, the nature of inactivation was also elucidated to be via irreversible covalent modification of its prosthetic heme and possibly to either Cys239 and/or Cys58 of CYP3A4 apoprotein. Future investigations aimed at clarifying the exact molecular mechanisms of hepatotoxicity by BBR are currently underway.

**Authorship Contributions**

**Participated in research design:** Tang, Verma, Fan, Chan.
**Conducted experiments:** Tang, Verma.
**Contributed new reagents or analytic tools:** Verma, Fan.
**Performed data analysis:** Tang, Verma.
**Wrote or contributed to the writing of the manuscript:** Tang, Verma, Fan, Chan.

**References**


Address correspondence to: Eric Chun Yong Chan, Department of Pharmacy, National University of Singapore, 18 Science Drive 4, Singapore 117543. E-mail: phaccye@nus.edu.sg