Metabolic Epoxidation Is a Critical Step for the Development of Benzbromarone-Induced Hepatotoxicity^[S]

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ABSTRACT

Benzbromarone (BBR) is effective in the treatment of gout; however, clinical findings have shown it can also cause fatal hepatic failure. Our early studies demonstrated that CYP3A catalyzed the biotransformation of BBR to epoxide intermediate(s) that reacted with sulfur nucleophiles of protein to form protein covalent binding both in vitro and in vivo. The present study attempted to define the correlation between metabolic epoxidation and hepatotoxicity of BBR by manipulating the structure of BBR. We rationally designed and synthesized three halogenated BBR derivatives, fluorinated BBR (6-F-BBR), chlorinated BBR (6-Cl-BBR), and brominated BBR (6-Br-BBR), to decrease the potential for cytochrome P450-mediated metabolic

activation. Both in vitro and in vivo uricosuric activity assays showed that 6-F-BBR achieved favorable uricosuric effect, while 6-Cl-BBR and 6-Br-BBR showed weak uricosuric efficacy. Additionally, 6-F-BBR elicited much lower hepatotoxicity in mice. Fluorination of BBR offered advantage to metabolic stability in liver microsomes, almost completely blocked the formation of epoxide metabolite(s) and protein covalent binding, and attenuated hepatic and plasma glutathione depletion. Moreover, the structural manipulation did not alter the efficacy of BBR. This work provided solid evidence that the formation of the epoxide(s) is a key step in the development of BBR-induced hepatotoxicity.

Introduction

Gout is the most prevalent inflammatory arthritis and occurs in approximately 0.2%–0.35% of the general population (Fernandes et al., 2017). Specifically, gout is an inflammatory arthritis characterized by deposition of urate in articular cartilage or periarticular tissues (Perez-Ruiz et al., 2015; Keenan and Schlesinger, 2016). Overproduction of uric acid and deficiency in renal excretion contribute to elevated serum urate, which is known as hyperuricemia. The increased incidence of gout is considered to partially result from the decline of renal function (Smith et al., 2010). Genetic and dietary factors also influence uric blood levels (Doherty, 2009). Recent studies suggest that hyperuricemia plays essential roles in some important diseases such as cardiovascular disease, diabetes, hypertension, and chronic renal disease (Baker and

Schumacher, 2010; Ruoff and Edwards, 2016). Gout has been a threat to human health, and the need for effective drugs that lower serum urate levels is therapeutically urgent.

Insufficient renal excretion was reported to account for 70% of urate load (Choi et al., 2005; Abhishek et al., 2017). Consequently, uricosurics that act directly on the renal tubule and increase urate renal excretion by inhibiting uric acid reabsorption have emerged. Among commercially available uricosurics, benzbromarone (BBR) (Fig. 1) is extremely potent and well tolerated in most people meeting treatment goals compared with probenecid and sulfapyrazone (Abhishek et al., 2017). As a uricosuric, BBR shows potent inhibition property to human urate transporter 1 (hURAT1), a known transporter responsible for urate reabsorption (Wempe et al., 2011; Ahn et al., 2016). Although effective, BBR is not licensed for use in the United States due to several reported idiosyncratic hepatotoxic events (Arai et al., 2002; Lee et al., 2008; Kydd et al., 2014).

It is widely accepted that cytochrome P450 (P450)-mediated metabolic activation is most frequently responsible for drug-induced idiosyncratic hepatotoxicity and drug withdrawal from the market (Leung et al., 2012; Dekker et al., 2016; Xuan et al., 2016). Our early metabolic studies illustrated that CYP3A metabolized BBR to epoxide intermediate(s) that reacted with sulfur nucleophiles of protein to form

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ABBREVIATIONS: ALT, alanine transaminase; AST, aspartate transaminase; BBR, benzbromarone; 6-Br-BBR, brominated benzbromarone; BSO, buthionine sulfoximine; 6-Cl-BBR, chlorinated benzbromarone; CMC, carboxymethyl cellulose; ESI, electrospray ionization; 6-F-BBR, fluorinated benzbromarone; GSH, glutathione; HLM, human liver microsome; hURAT1, human urate transporter 1; LC-MS/MS, liquid chromatography–tandem mass spectrometry; mBrB, monobromobimane; MDCK, Madin–Darby canine kidney; MLM, mouse liver microsome; NAC, *N*-acetylcysteine; P450, cytochrome P450; PO, potassium oxonate; RLM, rat liver microsome; SUA, serum uric acid; *t*_{1/2}, half-life.

Fig. 1. Structures of BBR and 6-F-BBR, 6-Cl-BBR, and 6-Br-BBR.

protein covalent binding both in vitro and in vivo (Wang et al., 2016a,b). In an attempt to define the association between BBR bioactivation and its hepatotoxicity, we modified the structure of BBR by decreasing the potential for the P450-mediated metabolic activation and then evaluated the toxicological and pharmacological responses to the structural manipulation.

Incorporation of halogens in drugs is a commonly employed strategy when decrease in P450 metabolic degradation is needed (Wilcken et al., 2013; Ford and Ho, 2016; Obach et al., 2016). In the optimization of ezetimib, a cholesterol inhibitor, benzylic oxidation was blocked by introduction of fluorine at the phenyl ring to mitigate P450-mediated oxidation (Rosenblum et al., 1998; Purser et al., 2008). Bromination or iodination proximal to the phenolic hydroxyl group was carried out in structural optimization of 13 α -estrone to prevent the generation of reactive quinone intermediates that might participate in estrogen-induced carcinogenesis (Patel and Bhat, 2004; Bacsá et al., 2015). It has been shown that by inserting halogens adjacent to metabolically labile sites, or substitution of metabolically labile sites, the potential for metabolic clearance can be attenuated by lowering the metabolic rate or blocking metabolic pathways. Also, halogen substituents are frequently used to improve membrane permeability, and therefore to enhance oral absorption (Lu et al., 2010; Ford and Ho, 2016).

The objective of this study was to define the correlation between BBR bioactivation and its hepatotoxicity by manipulating the structure of BBR. The correlation study was achieved by 1) structural modification of BBR through introducing halogen atoms to the benzofuran ring at the C6 position, which is known to be a site of CYP3A metabolism; 2) evaluation of metabolic activation and toxicity of the test compounds; 3) determination of metabolic stability and pharmacokinetic properties; and 4) assessment of the efficacies of the new compounds in vitro and in vivo.

Materials and Methods

Reagents

BBR (>98%) and potassium oxonate (PO) were purchased from Aladdin Industrial Technology Co., Ltd. (Shanghai, China). Fluorinated BBR (6-F-BBR), chlorinated BBR (6-Cl-BBR), and brominated BBR (6-Br-BBR) (Fig. 1) were synthesized in our laboratory (see the Supplemental Material for synthesis details). Buthionine sulfoximine (BSO), >99%; *N*-acetylcysteine (NAC), >98%; and reduced NADPH were acquired from Sigma-Aldrich (St. Louis, MO). [8-¹⁴C]-Urate was obtained from American Radiolabeled Chemicals (St. Louis, MO). Liquid scintillation was acquired from PerkinElmer (Waltham, MA). All organic solvents and reagents were of either analytical grade obtained from Fisher Scientific (Springfield, NJ) or high-performance liquid chromatography grade from commercial sources.

Assay to Measure the Uricosuric Effect in Madin-Darby Canine Kidney (MDCK) Cells Overexpressing hURAT1

Cell Culture. MDCK cells with overexpressed hURAT1 were preprepared in our laboratory as described in our previously published work. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum,

streptomycin (100 mg/ml), and penicillin (100 U/ml) in an atmosphere of 5% CO₂ and 95% air at 37°C.

[¹⁴C]-Urate Uptake Experiment in MDCK-hURAT1 Cells. Uptake assay was performed as described previously with several modifications (Wu et al., 2017). Briefly, cells were seeded in 24-well plates at a density of 1×10^5 cells/well. After 48 hours, the culture medium was removed, and the resulting cells were washed three times with uptake buffer (125 mM sodium gluconate, 4.8 mM potassium gluconate, 1.3 mM calcium gluconate, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 5.6 mM glucose, and 25 mM HEPES, pH = 7.4). The cells were then incubated in another 0.5 ml of uptake buffer for 10 minutes at 37°C. Then the uptake buffer was removed, followed by addition of 200 μ l of the medium containing [¹⁴C]-Urate (25 μ M) with or without the test compounds to initiate the uptake process. The test compounds were dissolved in dimethylsulfoxide and diluted with the culture medium to the final concentration of dimethylsulfoxide at <1%. The uptake step was set for 2 minutes and terminated by mixing with 0.6 ml of ice-cold Dulbecco's phosphate-buffered saline in wells. The resultant cells were washed with 1 ml of Dulbecco's phosphate-buffered saline two times quickly, and then solubilized in 100 μ l of 0.1 M NaOH. The radioactivity of the cell lysates was determined by a liquid scintillation counter (Micro Beta2; PerkinElmer).

Animals

Male Sprague-Dawley rats (200 $\pm$ 20 g) and male Kunming mice (20 $\pm$ 2 g) were purchased from the Animal Center of Shenyang Pharmaceutical University (Shenyang, China). Animals were housed in a controlled environment (temperature 25°C and 12-hour dark/light cycle) with free access to food and water. Animals were fasted overnight and given tap water ad libitum prior to the experiments. The protocol of the animal experiments was approved by the Ethics Review Committee for Animal Experimentation of Shenyang Pharmaceutical University.

Assay to Determine the Uricosuric Effect in Hyperuricemic Rats

Hyperuricemia was induced in rats using uricase inhibitor PO, according to previous reports (Haidari et al., 2012; Kou et al., 2016; Wang et al., 2016c). Briefly, rats were intraperitoneally treated with PO 1 hour before the final administration of the test compounds. Animals were randomly allocated into six groups ($n = 8$). Groups I and II served as healthy and hyperuricemic controls, respectively. Group III included hyperuricemic rats treated with BBR, which served as the positive control. Groups IV–VI consisted of hyperuricemic rats dosed with 6-F-BBR, 6-Cl-BBR, or 6-Br-BBR. The animals were administered BBR (by mouth) or the other test compounds suspended in 0.9% carboxymethyl cellulose (CMC)-Na at 10 mg/kg once daily for 8 days. On the eighth day, PO suspended in 0.9% saline solution was injected intraperitoneally in the resulting animals at 250 mg/kg 1 hour before the final administration of the test compounds. Blood samples were collected from the retro-orbital sinus 2 hours after PO administration for the assessment of serum uric acid (SUA) levels. The blood samples were allowed to clot for 1 hour at room temperature and then were centrifuged (8000g, 4°C) for 10 minutes to obtain sera. The concentrations of SUA were determined spectrophotometrically using a commercially available standard diagnostic kit, according to manufacturers' instruction (Nanjing Jiancheng Technology Co., Ltd., Nanjing, China).

Assessment of Hepatotoxicities of BBR and 6-F-BBR

Male Kunming mice were randomly assigned into six groups ($n = 4$). Two groups of mice were treated (by mouth) with BBR or 6-F-BBR dissolved

in 0.9% CMC-Na at a dose of 50 mg/kg for seven consecutive days. Another two groups of mice were intraperitoneally treated with BSO 1.5 hours before the final administration of BBR or 6-F-BBR. Control groups treated with 0.9% CMC-Na or BSO were included. Blood samples were collected by cardiac puncture 24 hours after the final administration. After clotting at room temperature for 3 hours, the blood samples were centrifuged at 8000g for 10 minutes at 4°C. The resulting sera were collected for the determination

of alanine transaminase (ALT) and aspartate transaminase (AST) activities on a VITROS 5600 Integrated System (Ortho-Clinical Diagnostics, Rochester, NY).

Metabolic Stability Test

Mouse liver microsomes (MLMs) and rat liver microsomes (RLMs) were prepared in our laboratory by following our previously published protocol

Fig. 2. Characterization of 6-F-BBR (A), 6-Cl-BBR (B), and 6-Br-BBR (C) by ^1H NMR analysis.

(Lin et al., 2007). Human liver microsomes (HLMs) were acquired from BD Gentest (Woburn, MA).

BBR or 6-F-BBR at a concentration of 1 or 10 μM was incubated with MLMs, RLMs, or HLMs (1 mg/ml), and MgCl_2 (3.2 mM) in a final volume of 0.5 ml of potassium phosphate buffer (0.1 M, pH 7.4). The mixture was preincubated at 37°C for 3 minutes and then the reaction was commenced by addition of 1.0 mM NADPH. Aliquots of 50 μl were withdrawn at time points of 0, 5, 10, 15, 20, 30, and

60 minutes, and the reaction was immediately quenched by adding an equal volume of ice-cold acetonitrile containing *S*-hexylglutathione (1.28 μM) as an internal standard. The samples were then vortex mixed and spun at 16,000g for 10 minutes at 4°C. The resulting supernatants (5 μl) were subjected to liquid chromatography–tandem mass spectrometry (LC-MS/MS) for analysis of the depletion of the compound. Each stability analysis was performed in triplicate. The natural log of the area ratio was plotted against the incubation time using GraphPad Prism

Fig. 3. Characterization of 6-F-BBR (A), 6-Cl-BBR (B), and 6-Br-BBR (C) by ^{13}C NMR analysis.

5 (GraphPad, San Diego, CA), and the elimination rate constant ($-k$, the slope of the linear regression) was used to determine the in vitro half-life ($t_{1/2}$). For compounds with $t_{1/2} < 60$ minutes, the in vitro intrinsic clearance (CL_{int}) was calculated as

$$CL_{int} = \frac{-k}{[E]} \quad (1)$$

where $[E]$ is the concentration of microsomal protein used in the incubation.

Metabolic Activation Studies of BBR and 6-F-BBR in Liver Microsomes

The incubation mixtures contained 1.0 mg of protein/ml MLMs, 3.2 mM $MgCl_2$, and 75 μM BBR or 6-F-BBR, and 40 mM NAC in a final volume of 0.5 ml of phosphate buffer (pH 7.4). The reactions were initiated by addition of 1.0 mM NADPH and quenched by adding 0.5 ml of ice-cold acetonitrile after 60-minute incubation at 37°C. Control incubations containing no NADPH were included. The reaction mixtures were vortex mixed for 2 minutes, followed by centrifugation at 16,000g for 10 minutes to remove precipitated protein. The resulting supernatants were concentrated by N_2 flushing at 40°C and then redissolved in 100 μl of 50% acetonitrile in water. A 5 μl aliquot of the supernatants was injected into the LC-MS/MS system for analysis.

Determination of In Vivo Protein Adductions Derived from BBR and 6-F-BBR

Mice were treated intraperitoneally with BBR or 6-F-BBR at 50 mg/kg. Liver tissues (0.2 g) harvested 30 minutes postadministration were homogenized in

2.0 ml of phosphate buffer (pH 7.4). The resulting homogenates were denatured by heating in a water bath at 60°C for 30 minutes and then digested overnight with a mixture of chymotrypsin (1.0 mg/ml) and Pronase E (2.0 mg/ml), according to our previous study (Wang et al., 2016b). The supernatants (5 μl) were subjected to the LC-MS/MS system for analysis.

Assessment of Glutathione (GSH) Level

Male Kunming mice were randomly divided into two groups ($n = 4$), and exposed intraperitoneally to 50 mg/kg BBR or 6-F-BBR dissolved in corn oil. Plasma and liver samples were harvested postadministration at times of 0, 10, and 30 minutes and 1, 2, 4, 8, and 12 hours. The liver tissues (0.2 g) were homogenized in 2.0 ml of phosphate buffer (0.1 M, pH 7.4). The assessment of plasma and hepatic GSH was achieved by monobromobimane (mBrB) derivatization as described in our previously published method (Yu et al., 2015), followed by analysis of the mBrB-GSH conjugate by LC-MS/MS.

Pharmacokinetic Studies

Rats were randomly allocated into two groups ($n = 6$) and administered orally with the test compounds (BBR or 6-F-BBR) suspended in 0.9% CMC-Na by gavage at 10 mg/kg. Blood samples were collected from the retro-orbital sinus at 0 and 30 minutes as well as 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 24 hours after dosing. The collected blood samples were centrifuged (8000g, 4°C) for 10 minutes to obtain the plasma. Aliquots (30 μl) of plasma were mixed with ice-cold acetonitrile (90 μl) containing *S*-hexylglutathione (1.28 nM) as an internal standard. The resulting mixtures were vortex mixed and centrifuged (16,000g,

Fig. 4. Characterization of 6-F-BBR, 6-Cl-BBR, and 6-Br-BBR by high-resolution ESI mass spectroscopy analysis. High-resolution mass spectra of 6-F-BBR (A), 6-Cl-BBR (B), and 6-Br-BBR (C) analyzed by Micro Q-TOF MS (Bruker Corporation, Billerica, MA).

4°C) for 10 minutes to remove the precipitated protein. Aliquots (2 μ l) of the supernatants were subjected to the LC-MS/MS system for quantification of plasma BBR or 6-F-BBR, using validated specific and sensitive LC-MS/MS methods. Both lower limits of quantification of the LC-MS/MS methods for BBR and 6-F-BBR were found to be 0.5 μ g/ml.

LC-MS/MS Method

An AB SCIEX Instruments 5500 triple quadrupole (Applied Biosystems, Foster City, CA) equipped with an Agilent 1260 Series Rapid Resolution LC system (Agilent Technologies, Santa Clara, CA) was employed for the LC-MS/MS analyses. Samples were analyzed by multiple-reaction monitoring scanning in electrospray ionization (ESI) positive ion mode (ESI⁺) using the following conditions: an ion-spray voltage of 5500 V; source temperature, 650°C; curtain gas, 35 psi; ion source gas 1, 50 psi; and ion source gas 2, 50 psi. The parameters of the ion pairs (declustering potential, collision energy, and collision cell exit potential) were m/z 425 $\rightarrow$ 279 (110, 35, and 3) for BBR, m/z 443 $\rightarrow$ 279 (110, 35, and 3) for 6-F-BBR, m/z 392.2 $\rightarrow$ 246.3 (86, 24, and 5) for *S*-hexylglutathione, m/z 586 $\rightarrow$ 279 (110, 35, and 15) for BBR-NAC conjugate derived from epoxide (A1), m/z 544 $\rightarrow$ 279 (110, 25, and 10) for cysteine-based protein adduction by epoxide metabolite(s) of BBR (A2), and m/z 498.2 $\rightarrow$ 192.2 (115, 50, and 3) for mBrB-GSH conjugate. For the analysis of plasma BBR and 6-F-BBR concentrations, A1, A2, and mBrB-GSH conjugate, the mobile phase programs and the columns used were in accordance with our published studies (Wang et al., 2016a,b). Data were processed using Applied Biosystems/SCIEX Analyst software version 1.6.2.

Statistical Analysis

Pharmacokinetic parameters were calculated from the mean plasma concentration by noncompartmental model, using Drug and Statistics 2.1.1 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China). Statistical analyses were performed using a Student's *t* test. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 were considered significant.

Results

Characterization of 6-F-BBR, 6-Cl-BBR, and 6-Br-BBR. The general route for the synthesis of 6-F-BBR, 6-Cl-BBR, or 6-Br-BBR (Fig. 1) is shown in Supplemental Scheme 1. The synthesis started with anhydrous magnesium chloride-catalyzed formylation of the corresponding 3-halogenated phenol with paraformaldehyde to produce halogenated salicylaldehyde **a**. Compound **a** was then cyclized with chloroacetone under the catalysis of to give compound **b**, followed by Wolff-Kishner-Huangminglong reduction, Friedel-Crafts acylation, demethylation, and dibromination to give the final compounds. The C6-fluorinated, -chlorinated, and -brominated BBR, referred to as 6-F-BBR, 6-Cl-BBR, and 6-Br-BBR, respectively, were characterized by ¹H NMR, ¹³C NMR (Figs. 2 and 3), and high-resolution ESI mass spectroscopy (Fig. 4). The ¹H NMR spectra depict the exact integration and the position of each proton present in the individual compounds. The ¹³C NMR spectra show the position of carbons pertaining to each compound. The high-resolution ESI mass spectroscopy spectra display the deprotonated molecular ions [M-H] with the representative isotope abundance patterns.

[¹⁴C]-Urate Uptake in MDCK-hURAT1 Cells. hURAT1-transgenic MDCK cells were used to measure the amounts of [¹⁴C]-Urate taken up for BBR, 6-F-BBR, 6-Cl-BBR, and 6-Br-BBR. As shown in Fig. 5, BBR and 6-F-BBR at the concentration of 1 μ M exhibited 59.39% and 63.59% inhibition of the uptake, respectively, compared with that of the control. However, little inhibition was observed at the same concentration for 6-Cl-BBR and 6-Br-BBR. Even at the concentration of 15 μ M, 6-Cl-BBR and 6-Br-BBR only showed weak inhibition of the uptake (21.72% and 13.33%, respectively). Clearly, 6-F-BBR, with similar efficacy as BBR, was a more potent hURAT1 inhibitor than 6-Cl-BBR and 6-Br-BBR.

Fig. 5. [¹⁴C]-Urate uptake in MDCK-hURAT1 transgenic cells in the presence of BBR (1 μ M), 6-F-BBR (1 μ M), 6-Cl-BBR (15 μ M), and 6-Br-BBR (15 μ M). **P* < 0.05; ***P* < 0.01; ****P* < 0.001, compared with the control group.

Uricosuric Efficacy in Hyperuricemic Rats. The effects of the test compounds on SUA levels were evaluated in PO-induced hyperuricemic model rats. As shown in Table 1, after 7 days of treatment with BBR or the test compounds, the SUA levels in each group were significantly reduced (*P* < 0.001, 0.001, 0.01, and 0.01, respectively) compared with the normal control. There was no significant difference between BBR and 6-F-BBR in lowering the SUA levels of healthy rats. As expected, intraperitoneally administration of oxonate significantly (*P* < 0.001) elevated SUA levels compared with the normal control. In addition, the groups treated with BBR, 6-F-BBR, 6-Cl-BBR, or 6-Br-BBR all demonstrated significantly lower levels of SUA (*P* < 0.001, 0.001, 0.01, and 0.01, respectively) compared with the hyperuricemic control. However, the decreased levels of SUA achieved by 6-F-BBR were comparable to that by BBR, which made us select 6-F-BBR as the lead uricosuric benzofuran derivative. As a result, a thorough comparative study on metabolism and toxicities of BBR and 6-F-BBR was carried out as described subsequently.

Hepatotoxicities of BBR and 6-F-BBR. The hepatotoxicities of BBR and 6-F-BBR were evaluated by assessment of serum ALT and AST activities in mice. Administration of BBR by oral gavage at 50 mg/kg for seven consecutive days caused significant elevation of serum AST activity in mice 24 hours after the final administration compared with vehicle-treated animals (*P* < 0.01) and 6-F-BBR-treated animals (*P* < 0.05). A minor increase in serum ALT activity was observed in animals treated with BBR but without statistical difference compared with that of vehicle-treated animals. In a separate study, the

TABLE 1

Effects of BBR and 6-F-BBR, 6-Cl-BBR, and 6-Br-BBR on SUA levels in oxonate-induced hyperuricemic rats (mean $\pm$ S.D., *n* = 8)

Group	Dose	SUA	
		7 Days	8 Days
	mg/kg		
Normal control		76.4 $\pm$ 6.2	75.4 $\pm$ 4.6
Hyperuricemic control		74.7 $\pm$ 4.8	254.1 $\pm$ 21.2 ^a
Hyperuricemic BBR treated	10	54.4 $\pm$ 9.5 ^a	172.2 $\pm$ 20.8 ^d
Hyperuricemic 6-F-BBR treated	10	59.8 $\pm$ 6.2 ^a	186.4 $\pm$ 14.7 ^d
Hyperuricemic 6-Cl-BBR treated	10	63.7 $\pm$ 6.1 ^{b,c}	222.4 $\pm$ 18.4 ^{c,e,f}
Hyperuricemic 6-Br-BBR treated	10	64.3 $\pm$ 5.5 ^{b,c}	215.6 $\pm$ 25.8 ^{c,e,g}

^aCompared with normal control group: *P* < 0.001.

^bCompared with hyperuricemic control group: *P* < 0.001.

^cCompared with normal control group: *P* < 0.01.

^dCompared with BBR-treated group: *P* < 0.05.

^eCompared with hyperuricemic control group: *P* < 0.01.

^fCompared with hyperuricemic 6-F-BBR group: *P* < 0.001.

^gCompared with hyperuricemic 6-F-BBR group: *P* < 0.05.

hepatotoxicities of BBR and 6-F-BBR were examined in mice pretreated with BSO 1.5 hours before the final administration. Pretreatment with BSO was found to potentiate 6-F-BBR- and BBR-induced elevation of serum ALT and AST. However, the elevations of ALT and AST induced by BBR were significantly higher than those induced by 6-F-BBR (Fig. 6). Both studies demonstrated that 6-F-BBR was less hepatotoxic than BBR.

Metabolic Stability. Metabolic stability of BBR and 6-F-BBR was examined in MLMs, RLMs, and HLMs. The substrates all decayed in a monoexponential manner (Supplemental Fig. 1) in the microsomes. As shown in Table 2, 50% of BBR at 1.0 μ M was metabolized in MLMs, RLMs, and HLMs after 5.6, 4.2, and 8.6 minutes, respectively. However, the half-lives of 6-F-BBR in the microsomes were >60, 8.4, and 13.6 minutes, respectively. At the concentration of 10 μ M, similar increased metabolic stability for 6-F-BBR was observed in the microsomes. Taken together, 6-F-BBR was much more stable in the microsomes than BBR, and the introduction of a 6-fluoro substituent on the benzofuran ring (6-F-BBR vs. BBR) offered resistance against the metabolism.

In Vitro Metabolism (Epoxidation) of BBR and 6-F-BBR. Our previous study demonstrated that epoxide metabolite(s) was a primary reactive metabolite of BBR, which may be related to BBR hepatotoxicity (Wang et al., 2016a). The potentials of metabolic activation (epoxidation) of BBR and 6-F-BBR were evaluated in MLMs supplemented with NAC as a trapping agent. The representative chromatograms of NAC conjugate derived from epoxide metabolite(s) of BBR and 6-F-BBR are shown in Fig. 7. As expected, only minor epoxide-derived NAC conjugate (A1) (Supplemental Scheme 1) was detected in 6-F-BBR-supplemented microsomal incubations. Apparently, approximately 300-fold more A1 was produced in microsomal incubation of BBR. In addition, we failed to detect the corresponding NAC conjugate derived from 6-F-BBR, indicating that no epoxide metabolites were formed in the metabolism of 6-F-BBR and that the introduction of the fluorine atom to BBR deactivated the aromatic ring toward epoxidation.

Epoxide-Derived Hepatic Protein Modification by BBR and 6-F-BBR. The detection of epoxide-derived liver protein covalent binding

in 6-F-BBR- and BBR-treated mice was achieved by proteolytic digestion of the protein adducts to the corresponding cysteine conjugates. The representative chromatograms of the cysteine adducts are shown in Fig. 8. Epoxide-derived cysteine adduct A2 (Supplemental Scheme 1) was detected in BBR-treated mice, while no such adduct was observed in mice given 6-F-BBR.

Hepatic and Plasma GSH Depletion Induced by BBR and 6-F-BBR in Mice. Depletion of cellular GSH has been considered as a biomarker for the production of reactive metabolites and early stage of cell injury. Time courses of hepatic and plasma GSH levels were monitored in 6-F-BBR- and BBR-treated mice (Fig. 9). Hepatic and plasma GSH were dramatically depleted by as much as 87% and 92%, respectively, in mice after a hepatotoxic dose (50 mg/kg) of BBR, while 6-F-BBR demonstrated much less potential to deplete hepatic and plasma GSH than BBR at the same dose.

Pharmacokinetic Behaviors. Plasma BBR and 6-F-BBR were monitored in rats given a single dose of BBR or 6-F-BBR at 10 mg/kg by oral gavage. The concentration-time (0–24 hours) curves of the compounds are shown in Fig. 10, and the corresponding pharmacokinetic parameters are presented in Table 3. Both BBR and 6-F-BBR revealed a double-peak property in their pharmacokinetic profiles. 6-F-BBR achieved C_{max} (54.59 μ g/ml) and area under the curve (256.20 μ g/ml·h) values over twice as high as those of BBR-treated animals (26.80 μ g/ml and 123.66 μ g/ml·h, respectively). In addition, with the observed good metabolic stability in RLMs, 6-F-BBR displayed much lower in vivo clearance (0.04 l/h per kilogram) than BBR (0.09 l/h per kilogram). There was no significant difference in the half-life between BBR (3.86 hours) and 6-F-BBR (2.59 hours).

Discussion

Although BBR shows great potential in the treatment of gout, it has not been licensed for use in Europe due to several reports of fatal idiosyncratic hepatic failure. Idiosyncratic toxicities are considered to be associated with metabolic activation of protoxicants. Our previous studies demonstrated that CYP3A-mediated epoxidation of BBR at the benzofuran ring was related to BBR-induced protein adduction and hepatotoxicity (Wang et al., 2016a,b). To avoid metabolic

Fig. 6. Hepatotoxicities of BBR and 6-F-BBR. Mice were pretreated (intraperitoneally) with BSO (666 mg/kg) 1.5 hours before the final administration (by mouth) of BBR or 6-F-BBR (50 mg/kg) for seven consecutive days. The groups of BBR or 6-F-BBR alone without BSO pretreatment as well as control groups treated with 0.9% CMC-Na or BSO were all included. Serum ALT and AST activities were examined 24 hours after the final administration ($n = 4$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ were considered significantly different from the vehicle-treated group; # $P < 0.05$; ### $P < 0.001$ were considered significantly different from the 6-F-BBR-treated group.

TABLE 2

Metabolic stability of BBR and 6-F-BBR in liver microsomes

The units of $t_{1/2}$ and CL_{int} are in minutes and ml/min per milligram protein, respectively; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with BBR.

Compound	Concentration μM	MLM		RLM		HLM	
		$t_{1/2}$	CL_{int}	$t_{1/2}$	CL_{int}	$t_{1/2}$	CL_{int}
BBR	1	5.6	0.124	4.2	0.163	8.6	0.081
	10	10.9	0.064	11.3	0.061	>60	—
6-F-BBR	1	>60	—	8.4**	0.082***	13.6*	0.051*
	10	>60	—	15.1	0.046	>60	—

CL_{int} , intrinsic clearance.

—, CL_{int} was not calculated

activation-induced liver injury by BBR, we replaced the hydrogen at the metabolically labile site with a halogen atom, which is a commonly used strategy to attenuate the potential of P450-mediated metabolism (metabolic activation). Currently, 50% of the leading drugs on the pharmaceutical market are halogenated (Xu et al., 2014), and there is great potential to exploit halogens into drug design in medicinal chemistry.

In this study, three BBR derivatives substituted with a halogen (F, Cl, or Br) at C6 of BBR, the site of epoxidation catalyzed by CYP3A, were designed and synthesized to block the metabolic activation of BBR. As the initial step, we evaluated the uricosuric efficacy of the resulting compounds (6-F-BBR, 6-Cl-BBR, and 6-Br-BBR) to identify the most effective compound for further toxicity and metabolism investigation. The efficacy evaluation started with assessment of the inhibitory effect on urate transport in hURAT1-transgenic cells since uricosuric BBR is known to be a potent inhibitor of hURAT1. 6-F-BBR was found to inhibit hURAT1 the most, followed by BBR, 6-Cl-BBR, and 6-Br-BBR

in that order. In addition, we evaluated their suppressive effects on SUA in rats with hyperuricemia (Table 1). No statistical significance was observed in SUA levels in healthy rats treated with 6-F-BBR, 6-Cl-BBR, or 6-Br-BBR, presumably due to the protective compensation reaction of healthy rats. However, 6-F-BBR elicited more potency to decrease the SUA level than 6-Cl-BBR ($P < 0.001$) and 6-Br-BBR ($P < 0.05$) in hyperuricemic rats. Both in vitro and in vivo studies demonstrated that 6-F-BBR was as effective as BBR and more effective than 6-Cl-BBR and 6-Br-BBR. The observed favorable efficacy of 6-F-BBR was possibly attributed to the highly electronegative nature of fluorine, the similarity in size of hydrogen (BBR) to that of fluorine (fluorinated BBR), along with the low polarizability of fluorine (Hernandes et al., 2010). These chemical characteristics of fluorine can productively influence the physicochemical and conformational properties of 6-F-BBR, which may contribute to the improvement in oral absorption in rats (Table 3). As a result, this makes 6-F-BBR present pharmacological properties.

As the core of the present study, the hepatotoxicities of BBR and 6-F-BBR were evaluated in vivo. We succeeded in the development of a mouse model for the assessment of the hepatotoxicities of BBR and 6-F-BBR in mice pretreated with BSO. BSO is known to have the ability to deplete GSH contents by inhibiting γ -glutamylcysteine synthetase, a rate-limiting enzyme in the biosynthesis of GSH. Significant elevations of serum ALT and AST were observed in BSO-pretreated mice given BBR, while a minor or slight increase in serum ALT and AST was found in animals treated with BBR alone (Fig. 6). This made it possible to evaluate the hepatotoxicity of BBR and hopefully its idiosyncratic liver injury, although the mechanisms of the development of idiosyncratic toxicity remain unknown.

6-F-BBR was selected for further hepatotoxicity and metabolic activation evaluation since the fluorinated BBR was found to be the most effective uricosurics among the compounds tested and to be as effective as BBR (Fig. 5; Table 1). The toxicity study was performed in

Fig. 7. Extracted ion chromatograms of multiple-reaction monitoring scan of m/z 586 $\rightarrow$ 279 obtained from the analysis of mouse liver microsomal incubations containing NAC and 6-F-BBR (A) or BBR (B).

Fig. 8. Extracted ion chromatograms of multiple-reaction monitoring scan of m/z 544 $\rightarrow$ 279 obtained from analysis of cysteine-based protein adduction of mice after 30-minute administration (intraperitoneally) of 6-F-BBR (A) and BBR (B) at 50 mg/kg.

Fig. 9. Assessment of GSH contents in mouse liver (A) and plasma (B). Mice were treated (intraperitoneally) with BBR or 6-F-BBR at 50 mg/kg. The assessment of GSH was achieved by mBrB derivatization and LC-MS/MS analysis. GSH levels were measured postadministration at 0, 10, and 30 minutes and 1, 2, 4, 8, and 12 hours. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with BBR.

mice pretreated with BSO. 6-F-BBR was found to be much less hepatotoxic than BBR (Fig. 6). The metabolism study demonstrated that the metabolic activation (epoxidation) of 6-F-BBR was almost abolished. Microsomal incubations of 6-F-BBR fortified with NAC revealed a significant decrease in the formation of epoxide-derived NAC conjugate by nearly 99.7% compared with BBR, and no epoxide-derived protein adduction was detected in mice given 6-F-BBR (Figs. 7 and 8). It is likely that the introduced fluorine blocked the site from metabolic epoxidation. Additionally, the electron-withdrawing effect of the fluorine decreased the electron density of the phenyl ring, which disfavors the oxidation reaction. The observation of a high volume of protein adduction derived from BBR, combined with the finding of more potent hepatotoxicity induced by BBR, led us to propose that epoxidation at C6 of BBR is a key step for hepatotoxicity of BBR.

Our previous study demonstrated that the reaction of the epoxide metabolite(s) of BBR with GSH contributes to the depletion of GSH (Wang et al., 2016b). The present study found that treatment of BBR caused significant elevation in serum AST activity in opposition to a decrease in hepatic and plasma GSH content. On the contrary, slight hepatotoxicity, along with attenuated GSH depletion, was observed in mice given 6-F-BBR, indicating that the formation of the epoxide metabolite(s) was slowed down by fluorine substitution. It should be noted that 6-F-BBR did not protect mice completely from GSH depletion. A decrease in GSH level was also observed during the first 30 minutes postadministration of 6-F-BBR. This might result from the formation of catechol (Supplemental Scheme 1) and hydroquinone through *ipso*-substitution of the dibromophenol ring (Kitagawara et al., 2015). We cannot underestimate the possible contribution of their oxidized forms (quinone metabolites, unpublished data), which are known to be able to deplete GSH. The observed attenuated GSH depletion at least partially resulted from the fluorine substitution. The supporting evidence was that little metabolites derived from the epoxidation of 6-F-BBR were observed.

6-F-BBR was more stable than BBR in liver microsomes, particularly in MLMs (as much as 10-fold more stable) (Table 2). This indicates that the incorporation of fluorine kept the molecule from P450-mediated metabolism. It is believed that the electron-withdrawing property of the fluorine substituent deactivated the aromatic system toward metabolic degradation by P450 enzymes. Besides epoxidation of BBR at the benzofuran ring, BBR was suggested to be oxidized to a quinone metabolite via sequential oxidation of 6-OH-BBR (McDonald and Rettie, 2007). Additionally, Cho et al., 2017 demonstrated that BBR was metabolized to a quinone methide derived from 1',6-dihydroxy BBR. We failed to detect the formation of such reactive metabolites directly from BBR in liver microsomal systems, indicating that the two metabolic pathways are minor in comparison with the epoxidation. The observed improvement of 6-F-BBR metabolic stability probably resulted mainly from retarding of the epoxidation of the benzofuran ring, and unlikely from that of the two minor pathways.

The metabolic stability studies demonstrated that the $t_{1/2}$ of 6-F-BBR increased twice as much as that of BBR in RLMs, and over 2-fold increases in C_{max} and area under the curve values were observed in 6-F-BBR-treated rats relative to animals treated with BBR at the same dose. This might result from the improvement in oral absorption, presumably due to the increase in membrane permeability by fluorine substitution. Permeability is one of the major considerations in the optimization of oral drugs. Many drugs with aromatic fluorination are prone to penetrate cell membranes, thereby increasing the intracellular concentration (Smart, 2001; Murphy and Sandford, 2015). Furthermore, 6-F-BBR was much more stable than BBR in MLMs with $t_{1/2} > 60$ minutes. It might be reasonable to assume that exposure to 6-F-BBR would be greater than that to BBR in mice. Surprisingly, 6-F-BBR displayed much lower hepatotoxicity than BBR (Fig. 6). This suggests that the observed toxicity of BBR was induced by its metabolite but not the parent compound itself. Interestingly, with the observed good metabolic stability in RLMs, 6-F-BBR displayed a slightly increased elimination rate than that of BBR in vivo (Fig. 10). An explanation for the observed discrepancy could arise from the elevated phase II metabolism, such as glucuronidation or sulfation, involved in the biotransformation of 6-F-BBR. The resulting polar conjugates could be eliminated quickly.

Conclusively, replacement of the hydrogen in the metabolically labile site by a fluorine atom significantly attenuated the hepatotoxicity of BBR but did not alter its uricosuric efficacy. The present work provided solid evidence that the formation of the epoxide(s) is a key step in the development of BBR-induced hepatotoxicity. Additionally, the fluorinated BBR was identified to be as effective as BBR, and this study is an excellent example of drug metabolism-guided rational drug design.

Fig. 10. Time course of plasma BBR and 6-F-BBR in rats after administration (by mouth) of BBR or 6-F-BBR at 10 mg/kg. Plasma BBR and 6-F-BBR were measured at time intervals of 0 and 30 minutes and 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 24 hours after administration. Data represent the mean $\pm$ S.D., $n = 6$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with BBR.

TABLE 3

Summary of pharmacokinetic parameters obtained in rats after administration (by mouth) of BBR and 6-F-BBR^a

Parameter	BBR	6-F-BBR
$C_{\max}$ ($\mu\text{g/ml}$)	26.80 $\pm$ 11.19	54.59 $\pm$ 10.17 ^b
$T_{\max}$ (h)	1.25 $\pm$ 0.27	1.75 $\pm$ 0.27 ^c
$AUC_{(0-1)}$ ($\mu\text{g/ml}\cdot\text{h}$)	121.62 $\pm$ 39.20	245.35 $\pm$ 28.80 ^d
$AUC_{(0-\infty)}$ ($\mu\text{g/ml}\cdot\text{h}$)	123.66 $\pm$ 38.41	256.20 $\pm$ 43.88 ^d
CL/F (l/h/kg)	0.09 $\pm$ 0.03	0.04 $\pm$ 0.01 ^b
$t_{1/2}$ (h)	3.86 $\pm$ 1.27	2.59 $\pm$ 0.55
V_z/F (l/kg)	0.52 $\pm$ 0.28	0.15 $\pm$ 0.02 ^c

AUC, area under the curve; $T_{\max}$, time required to reach maximum concentration; CL/F, clearance rate; V_z/F , apparent volume of distribution.

^a10 mg/kg, $n = 6$.

^bSignificantly different from BBR-treated mice ($P < 0.01$).

^cSignificantly different from BBR-treated mice ($P < 0.05$).

^dSignificantly different from BBR-treated mice ($P < 0.001$).

Authorship Contributions

Participated in research design: Zheng, S. Wang, Pang.

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