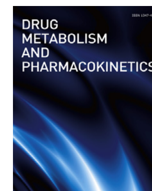
Drug Metabolism and Pharmacokinetics 32 (2017) 46–52

Contents lists available at ScienceDirect

Drug Metabolism and Pharmacokinetics

journal homepage: http://www.journals.elsevier.com/drug-metabolism-and-pharmacokinetics



Regular Article

Identification of novel glutathione adducts of benzbromarone in human liver microsomes

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ARTICLE INFO

Article history:
Received 26 July 2016
Received in revised form
4 October 2016
Accepted 17 October 2016
Available online 27 October 2016

Keywords:
Benzbromarone
Reactive metabolite
Glutathione adduct
Drug-induced liver injury
CYP

ABSTRACT

Benzbromarone (BBR) is a potent uricosuric drug that can cause serious liver injury. Our recent study suggested that 1′-hydroxy BBR, one of major metabolites of BBR, is metabolized to a cytotoxic metabolite that could be detoxified by glutathione (GSH). The aim of this study was to clarify whether GSH adducts are formed from 1′-hydroxy BBR in human liver microsomes (HLM). Incubation of 1′-hydroxy BBR with GSH in HLM did not result in the formation of GSH adducts, but 1′,6-dihydroxy BBR was formed. In addition, incubation of 1′,6-dihydroxy BBR with GSH in HLM resulted in the formation of three novel GSH adducts (M1, M2 and M3). The structures of M1 and M2 were estimated to be GSH adducts in which the 1-hydroxyethyl group at the C-2 position and the hydroxyl group at the C-1′ position of 1′,6-dihydroxy BBR were substituted by GSH, respectively. We also found that the 6-hydroxylation of 1′-hydroxy BBR is mainly catalyzed by CYP2C9 and that several CYPs and/or non-enzymatic reaction are involved in the formation of GSH adducts from 1′,6-dihydroxy BBR. The results indicate that 1′-hydroxy BBR is metabolized to reactive metabolites *via* 1′,6-dihydroxy BBR formation, suggesting that these reactive metabolites are responsible for BBR-induced liver injury.

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1. Introduction

Benzbromarone (BBR) is a uricosuric agent that has been used for the treatment of chronic gout for more than 40 years in many countries, particularly in Europe and Japan. In the early 2000s, BBR was withdrawn from European markets due to cases of fatal fulminant hepatic failure [1–4].

Drug-induced liver injury is often associated with the formation of reactive metabolites mediated by cytochrome P450 (CYP) [5]. Although BBR-induced liver injury has been studied for its association with CYP-mediated metabolic activation, the causal reactive metabolite for liver injury remains unclear. Recently, Thompson et al. [6] reported that CYP3A4 expression increased the cytotoxicity of BBR in SV40 T-antigen-immortalized human liver epithelial cells. It has also been shown that CYP3A4 metabolizes BBR to 1′-hydroxy BBR [7], which is one of the major metabolites detected in human plasma and urine [8–10]. These findings suggest that formation of 1′-hydroxy BBR is involved in BBR-induced liver injury. Moreover, Kobayashi et al. [11] showed that 1′-hydroxy BBR exhibited cytotoxic effects on human hepatocellular carcinoma FLC-4 cells cultured on three-dimensional systems, in which the functional expression of CYPs was higher than that of monolayer culture [12]. In addition, Kobayashi et al. [11] showed that GSH depletion by co-treatment with L-buthionine-sulfoximine (BSO), a well-known GSH synthesis inhibitor, increased the cytotoxicity of 1′-hydroxy BBR in FLC-4 cells cultured on three-dimensional systems. These findings suggest that 1′-hydroxy BBR is metabolized to reactive metabolites, which would be detoxified by GSH. However, it remains unclear whether reactive metabolites are formed from 1′-hydroxy BBR.

The aim of the present study was to clarify whether reactive metabolites are formed from 1′-hydroxy BBR using human liver microsomes (HLM). To accomplish the purpose, 1′-hydroxy BBR was incubated with GSH in HLM and liquid chromatography-

Abbreviations: ABT, 1-aminobenzotriazole; BBR, benzbromarone; BSO, L-buthionine-sulfoximine; CYP, cytochrome P450; GSH, glutathione; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; LC–MS/MS, liquid chromatography–tandem mass spectrometry; TA, tienilic acid.

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http://dx.doi.org/10.1016/j.dmpk.2016.10.412
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tandem mass spectrometry (LC–MS/MS) was used to determine whether GSH adducts are formed from 1′-hydroxy BBR.

2. Materials and methods

2.1. Chemicals and reagents

1′-Hydroxy BBR and 6-hydroxy BBR were provided by Torii Pharmaceutical (Tokyo, Japan). Tienilic acid (TA) was purchased from Cayman (Ann Arbor, MI). 1-Aminobenzotriazole (ABT) and ketoconazole were purchased from Sigma Aldrich (St. Louis, MO). Reduced glutathione (GSH) was purchased from Wako (Tokyo, Japan). HLM (HG3, HG6, HG23, HG42, HG43, HG56, HG66 HG70, HG89, HG93, HG112 and pooled) and microsomes prepared from baculovirus-infected insect cells coexpressing CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 or CYP3A4 with NADPH CYP oxidoreductase were obtained from BD Gentest (Woburn, MA). Recombinant CYP2B6, CYP2C8, CYP2C19, and CYP2E1 were also co-expressed with cytochrome b_5. Microsomes that contain NADPH CYP reductase and cytochrome b_5 were used for controls. Other chemicals and reagents used in this study were purchased from commercial sources.

2.2. Synthesis of 1′,6-dihydroxy BBR

1′,6-Dihydroxy BBR was synthesized from 6-hydroxy BBR *via* allylic hydroxylation as described by Locuson et al. [13]. Briefly, 1.06 mmol of 6-hydroxy BBR was added to 3 equivalents of trimethylamine and 3 equivalents of acetyl chloride in 10 mL of tetrahydrofuran and stirred at room temperature for 1 h to protect the phenol as an acetate. The compound protected as an acetate was added to 1.2 equivalents of *N*-bromosuccinimide and 0.5 equivalents of azobisisobutyronitrile in 20 mL of carbon tetrachloride and heated to reflux for 5 h to form a 1′-bromo compound. Bromine displacement was next carried out in *N*, *N*-dimethylformamide with 2 equivalents of cesium acetate at room temperature for 18 h, after which the solvent was removed under vacuum. The triester compound was then hydrolyzed with 5 equivalents of potassium carbonate in methanol for 5 h at room temperature. Finally, the sample was concentrated and purified *via* SiO_2 chromatography (97:3, chloroform: methanol) to give 1′,6-dihydroxy BBR. Overall yield was 20%. ^{1}H NMR (CD_3OD): δ 7.93 (s, 2H), 7.01 (d, $J = 8.4$ Hz, 1H), 6.93 (d, $J = 1.8$ Hz, 1H), 6.71 (dd, $J = 8.4$, 1.8 Hz, 1H), 4.96 (q, $J = 6.6$ Hz, 1H), 1.56 (d, $J = 6.6$ Hz, 3H). ^{13}C NMR (CD_3OD): δ 189.4, 163.9, 157.6, 157.3, 156.4, 135.0, 133.6, 122.5, 119.7, 116.9, 114.3, 112.2 (br), 98.8, 63.3, 21.5. ESI-MS: *m/z* = 453, 455, 457 (1:2:1) $[M-H]^-$.

2.3. Incubation with pooled HLM

For the formation of GSH adducts, the basic incubation mixture contained pooled HLM (0.4 mg/mL), 100 mM potassium phosphate buffer (pH 7.4), 5 mM GSH, and 100 μM 1′-hydroxy BBR or 100 μM 1′,6-dihydroxy BBR in a final volume of 60 μL. The reaction was initiated by the addition of 1 mM NADPH after 1-min preincubation at 37 °C. After incubation for 60 min at 37 °C, the reaction was stopped by the addition of 25 μL of 10% HCl. The mixture was then centrifuged at 12,000×g for 15 min to remove protein, and the supernatant (30 μL) was analyzed.

2.4. Incubation with recombinant CYP isozymes

For the formation of 1′,6-dihydroxy BBR from 1′-hydroxy BBR, the basic incubation mixture contained recombinant CYP isozyme (10 pmol P450/mL), 100 mM potassium phosphate buffer (pH 7.4), 0.1 mM EDTA and 100 μM 1′-hydroxy BBR in a final volume of 125 μL. The reaction was initiated by the addition of an NADPH-generating system (0.5 mM $NADP^+$, 2 mM glucose-6-phosphate, 1 U/mL glucose-6-phosphate dehydrogenase and 4 mM $MgCl_2$) after preincubation for 1 min at 37 °C. After incubation for 40 min at 37 °C, the reaction was stopped by the addition of 50 μL acetonitrile followed by the addition of 20 μL of an internal standard (7.5 μM testosterone in methanol). The reaction was performed in a linear range with respect to the protein concentration and the incubation time for each recombinant P450 isozyme. After centrifugation at 12,000×g for 15 min to remove protein, the supernatant (100 μL) was analyzed.

For the formation of GSH adducts from 1′,6-dihydroxy BBR, incubation was performed under the conditions described for incubation with pooled HLM except for the use of recombinant CYP isozyme (50 pmol P450/mL) instead of HLM, and incubation was conducted for 30 min.

2.5. Correlation study

Correlation coefficient of the formation of 1′,6-dihydroxy BBR from 1′-hydroxy BBR with other CYP isozyme-specific activities were calculated in HLM from 11 individual donors. Incubation was performed under the conditions described for incubation with recombinant CYP isozymes, except that HLM (0.1 mg/mL) from each donor were used instead of recombinant CYP isozymes. Data for activities of phenacetin *O*-deethylation for CYP1A2, coumarin 7-hydroxylation for CYP2A6, (*S*)-mephenytoin *N*-demethylation for CYP2B6, paclitaxel 6α-hydroxylation for CYP2C8, diclofenac 4'-hydroxylation for CYP2C9, (*S*)-mephenytoin 4'-hydroxylation for CYP2C19, bufuralol 1′-hydroxylation for CYP2D6, chlorzoxazone 6-hydroxylation for CYP2E1 and testosterone 6β-hydroxylation for CYP3A4 were provided by BD Gentest (Woburn, MA).

2.6. Inhibition study

Effects of tienilic acid on the formation of 1′,6-dihydroxy BBR from 1′-hydroxy BBR were investigated in pooled HLM. Since tienilic acid is a mechanism-based inhibitor of CYP2C9 [14], HLM were incubated with 100 μM 1′-hydroxy BBR for 40 min after preincubation with 10 μM tienilic acid in reaction mixtures containing an NADPH-generating system for 0, 20 or 60 min at 37 °C.

Effects of ABT, a nonselective inhibitor of CYPs [15], and ketoconazole, a selective CYP3A inhibitor [16], on the formation of GSH adducts from 1′,6-dihydroxy BBR were investigated in HLM. Since ABT is a mechanism-based inhibitor of CYPs, pooled HLM were incubated with 100 μM 1′,6-dihydroxy BBR and 5 mM GSH for 30 min after preincubation with 100 or 300 μM ABT in reaction mixtures containing 1 mM NADPH for 30 min at 37 °C. Ketoconazole (2.5 μM) was incubated with 100 μM 1′,6-dihydroxy BBR and 5 mM GSH in HLM in the presence of NADPH for 30 min at 37 °C.

2.7. LC-MS/MS analysis

Structural analyses of metabolites from 1′-hydroxy BBR and GSH adducts generated from 1′,6-dihydroxy BBR and quantitative analysis of GSH adducts generated from 1′,6-dihydroxy BBR were performed by LC–MS/MS. LC–MS/MS analysis was conducted on an amaZon SL ion trap mass spectrometer (Bruker Daltonics, Billerica, MA) connected to a Hitachi HPLC system equipped with two L-2160U pumps and an L-2200 autosampler (Hitachi, Tokyo, Japan). Separation was performed on a TSKgel ODS-100Z column (2.0 mm × 150 mm, 5 μm; Tosoh, Tokyo, Japan). Mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in

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