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Metabolic activation of hepatotoxic drug (benzbromarone) induced mitochondrial membrane permeability transition

Maho Shirakawa [a], Shuichi Sekine [a], Ayaka Tanaka [a], Toshiharu Horie [b], Kousei Ito [a,*]

[a] The Laboratory of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan
[b] Faculty of Pharmaceutical Sciences, Teikyo Heisei University, Tokyo, Japan

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ABSTRACT

The risk of drug-induced liver injury (DILI) is of great concern to the pharmaceutical industry. It is well-known that metabolic activation of drugs to form toxic metabolites (TMs) is strongly associated with DILI onset. Drug-induced mitochondrial dysfunction is also strongly associated with increased risk of DILI. However, it is difficult to determine the target of TMs associated with exacerbation of DILI because of difficulties in identifying and purifying TMs. In this study, we propose a sequential in vitro assay system to assess TM formation and their ability to induce mitochondrial permeability transition (MPT) in a one-pot process. In this assay system, freshly-isolated rat liver mitochondria were incubated with reaction solutions of 44 test drugs preincubated with liver microsomes in the presence or absence of NADPH; then, NADPH-dependent MPT pore opening was assessed as mitochondrial swelling. In this assay system, several hepatotoxic drugs, including benzbromarone (BBR), significantly induced MPT in a NADPH-dependent manner. We investigated the rationality of using BBR as a model drug, since it showed the most prominent MPT in our assay system. Both the production of a candidate toxic metabolite of BBR (1′,6-$(OH)_2$ BBR) and NADPH-dependent MPT were inhibited by several cytochrome P450 (CYP) inhibitors (clotrimazole and SKF-525A, 100 μM). In summary, this assay system can be used to evaluate comprehensive metabolite-dependent MPT without identification or purification of metabolites.

Introduction

Drug-induced liver injury (DILI) is the most common major adverse event that causes the failure of candidate compounds during drug testing and the withdrawal of pharmaceuticals from the market. Prediction of DILI in preclinical evaluations of candidates failed to detect 40% of hepatotoxic compounds in humans (McKim, 2010). The liver is one of the most enriched organs in terms of the number and density of mitochondria. Mitochondria have various key functions during energy metabolism and are a master regulator of apoptotic and necrotic cell death. Mitochondrial dysfunction is considered a major mechanism of DILI (Labbe et al., 2008; Pessayre et al., 2010, 2012), and drugs that are potentially toxic to mitochondria are often categorized with a black-box warning by the U.S. Food and Drug Administration (Dykens and Will, 2007). There are several mechanisms of drug-induced mitochondrial toxicity (inhibition of mitochondrial respiration and/or the mitochondrial β-oxidation of fatty acids, depletion of the mitochondrial genome by inhibiting mitochondrial DNA (mtDNA) polymerase γ and/or induction of oxidative damage to mtDNA, or induction of mitochondrial permeability transition (MPT)) (Fromenty and Pessayre, 1995; Begriche et al., 2011; Pessayre et al., 2012). MPT is induced by many anticancer drugs (e.g., tamoxifen) (Mandlekar and Kong, 2001), non-steroidal anti-inflammatory drugs (e.g., diclofenac) (Masubuchi et al., 2002), statins (e.g., simvastatin) (Velho et al., 2006), and some thiazolidinones (e.g., troglitazone) (Masubuchi et al., 2006; Okuda et al., 2010). Loss of membrane impermeability to protons is associated with mitochondrial cell death pathways, and MPT is thought to be a key event in this process (Castilho et al., 1995; Kowaltowski et al., 2001). MPT is characterized by Ca^{2+}-promoted opening of inner membrane pores, sensitive to the immune suppressor cyclosporin A (CsA) (Kowaltowski et al., 2001), and associated with oxidative modifications of inner membrane protein thiol groups (Fagian et al., 1990).

Drug oxidation by microsomal cytochrome P450 (CYP) enzymes is thought to be a major detoxification process in the liver. However, metabolites are sometimes more toxic than the parent drug, the so-called toxic metabolites (TMs). Particularly for metabolites with an unstable nature, they can be classified as reactive metabolites (RMs). Although the molecular targets of TMs are poorly defined, TM formation, including RMs, is considered as one of the factors triggering downstream events culminating in DILI (Lucena et al., 2008; Lammert et al., 2010).

Abbreviations: AOC, area over the curve; BBR, benzbromarone; CsA, cyclosporin A; CYP, cytochrome P450; DRLM, dexamethasone-treated Sprague–Dawley rat liver microsome; DILI, Drug-induced liver injury; G6P, glucose 6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; mtDNA, mitochondrial DNA; MPT, mitochondrial permeable transition; NAPQI, *N*-acetyl-*p*-benzoquinone imine; RMs, reactive metabolites; TMs, toxic metabolites.

* Corresponding author at: Inohana 1-8-1, Chuo-ku, Chiba 260-8675, Japan.
E-mail address: itokousei@chiba-u.jp (K. Ito).

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Indeed, the FDA noted in a draft guidance document that radiochemical in vitro assays should be considered to detect and quantify covalent binding of the drug or its metabolites to liver proteins to assess the risk of idiosyncratic drug-induced toxicity in the early stages of drug development. However, it is difficult to determine the target of TMs associated with exacerbation of DILI because of the difficulty in the identification and purification of TMs. Moreover, during the drug development process, it is not practical to predict the potential hepatotoxicity induced by each metabolite, including minor metabolites (less than 10% of the total amount). In this study, we hypothesized that TM formation might cause direct mitochondrial perturbation and constructed a prototype in vitro assay system that enabled us to evaluate TM-dependent MPT in a single assay system without any purification process. To confirm the proof of concept, a detailed analysis was performed with a representative drug (benzbromarone: BBR), because 1) BBR showed the most significant NADPH-dependent mitochondrial swelling in this system (Supplemental Fig. 1) and 2) CYP3A4 and/or CYP2C9 dependent production of several metabolites of dihydroxylated BBR (e.g., 1′,6-$(OH)_2$ BBR and 5,6-$(OH)_2$ BBR; Fig. 1) are reported to cause hepatotoxicity (Kobayashi et al., 2012; McDonald and Rettie, 2007).

Materials and methods

Chemicals. BBR, clotrimazole (CLO) and sulfaphenazole (SPZ) were purchased from the Sigma Chemical Co. (St. Louis, MO); dexamethasone (DEX), CsA, and flutamide were from Wako Pure Chemical Industries (Osaka, Japan); SKF-525A was from Research Biochemicals International (Natick, MA); β-$NADP^+$, glucose 6-phosphate (G6P), and glucose-6-phosphate dehydrogenase (G6PDH) were from Oriental Yeast Co., Ltd. (Tokyo, Japan); and human liver microsomes (HLMs) pooled from 53 individual donors were purchased from BD Gentest (Woburn, MA). Three metabolites of BBR (1′-OH BBR; 6-OH BBR; and 5,6-$(OH)_2$ BBR) were chemically synthesized by Torii Pharmaceutical Co. Ltd. (Chiba, Japan). All other chemicals and solvents were of analytical grade.

Animals. Sprague–Dawley male rats (SLC Japan Inc., Tokyo, Japan), 6–7 weeks old, were used throughout the experiments. Animals were treated humanely in accordance with guidelines issued by the National Institutes of Health (Bethesda, MD). In addition, all procedures were approved by the Animal Care Committee of Chiba University.

Preparation of rat liver microsomes. Sprague–Dawley rats (6–7 weeks old) were administered 100 mg/kg of DEX dissolved in olive oil for 3 days via intraperitoneal injection. Twenty-four hours after the last administration of DEX, rat livers were removed and perfused with ice-cold 1.15% KCl. Liver homogenates were centrifuged at 9000 ×*g* for 30 min, and then the supernatant fraction was ultracentrifuged twice at 105,000 ×*g* for 1 h. The resulting microsomal pellet was resuspended in 0.25 M sucrose. The protein concentration of the microsomal fraction was determined by the method of Lowry et al. (1951).

Microsomal incubation of test drugs. The concentration of 44 test drugs (including BBR) was set at 1, 10, and 100 times higher than that of the C_{max} value in clinical use. Among these 3 concentrations, the tested concentration of 44 drugs was set at the maximal concentration in which the parent drugs do not induce significant mitochondrial swelling as determined by a rapid decrease in absorbance at 540 nm (e.g. BBR, 54 μM at 10 × C_{max} (5.4 μM)). Microsomes (1 mg of protein/ml) were preincubated for 4 min at 37 °C in reaction buffer containing 100 mM potassium phosphate (pH 7.4), 51.1 mM sucrose, 10 mM HEPES–KOH, 2.5 μM rotenone, 5 mM sodium succinate, 10 mM $MgCl_2$, and test drugs at the set concentration (54 μM BBR). Reactions were initiated by the addition of a NADPH regenerating system (0.1 mM $NADP^+$, 1 mM G6P, and 1 U/ml G6PDH) and were performed at 37 °C.

Measurement of BBR metabolites. BBR metabolites were measured using a liquid chromatography–tandem mass spectrometry (LC–MS/MS) system (amaZon SL, Bruker Daltonics Inc., MA). Samples were prepared and assayed according to the method previously described by Wu et al. with modifications (Wu et al., 2012). After microsomal incubation, the reaction was terminated by the addition of 500 μl of ice-cold 100 mM Na_2CO_3. Each sample was mixed with a 300 μl of 1 M HCl and 5 ml of ethyl acetate. Warfarin was added as the internal standard. The resultant solution was shaken for 20 min and centrifuged at 1,700 ×*g* for 20 min. The upper organic layer was transferred and evaporated. The residue was reconstituted with 150 μl of the initial mobile phase, and filtered using 0.45 μm polytetrafluoroethylene filters. Samples (5 μl) were injected into an LC–MS/MS system consisting of an ion-trap amaZon SL device equipped with a high-performance liquid chromatography system (Chromaster; Hitachi High-Tech, Tokyo, Japan). A Wakosil-II C18 column (150 mm × 2.1 mm internal diameter, 3 μm particle size; Wako Pure Chemical Industries, Ltd, Osaka, Japan) was employed at 40 °C. The mobile phase consisted of acetonitrile with 0.1% formic acid (A) and water with 0.1% formic acid (B). A constant flow rate elution at 0.2 ml/min was performed in gradient mode (20% A for 20 min, 60% A for 10 min, 90% A for 5 min, and 20% A for 10 min.). All tested drugs were analyzed using Quant Analysis 2.2 (Bruker Daltonics Inc., MA) with a chromatogram of specific retention time and product ions.

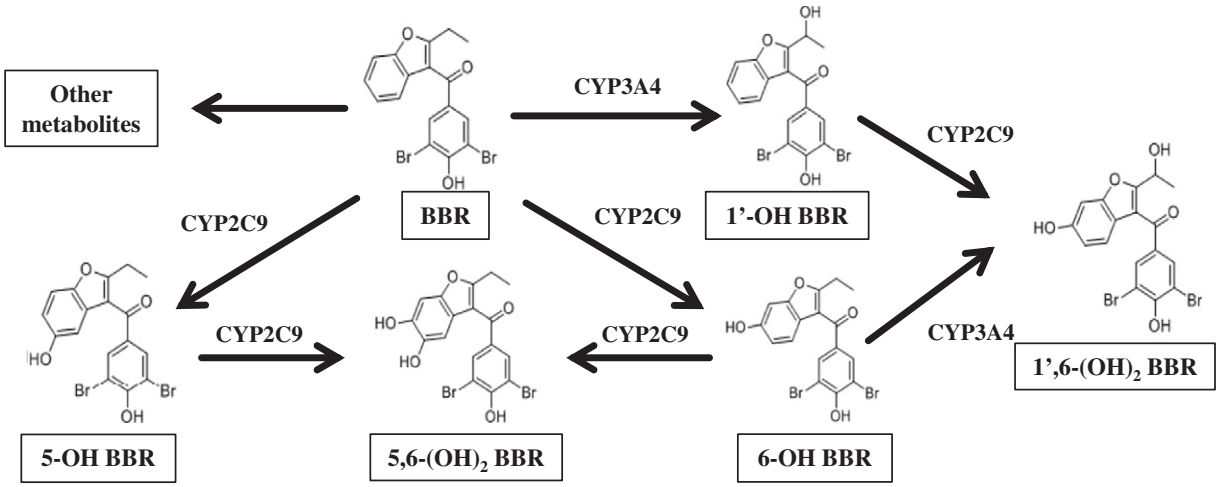

Fig. 1. Major metabolic pathways of BBR.