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Effects of CYP inhibitors on precocene I metabolism and toxicity in rat liver slices

Van T. Ly, Barry Brock *

Bristol-Myers Squibb, Pharmaceutical Candidate Optimization, Department of Biotransformation, P.O. Box 4000, Princeton, NJ 08543, USA

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ABSTRACT

We present a comprehensive *in vitro* approach to assessing metabolism-mediated hepatotoxicity using male Sprague–Dawley rat liver slices incubated with the well characterized hepatotoxicant, precocene I, and inhibitors of cytochrome P450 (CYP) enzymes. This approach combines liquid chromatography mass spectrometry (LC MS) detection methods with multiple toxicity endpoints to enable identification of critical metabolic pathways for hepatotoxicity. The incubations were performed in the absence and presence of the non-specific CYP inhibitor, 1-aminobenzotriazole (ABT) and isoform-specific inhibitors. The metabolite profile of precocene I in rat liver slices shares some features of the *in vivo* profile, but also had a major difference in that epoxide dihydrodiol hydrolysis products were not observed to a measurable extent. As examples of our liver slice metabolite identification procedure, a minor glutathione adduct and previously unreported 7-*O*-desmethyl and glucuronidated metabolites of precocene I are reported. Precocene I induced hepatocellular necrosis in a dose- and time-dependent manner. ABT decreased the toxicity of precocene I, increased exposure to parent compound, and decreased metabolite levels in a dose-dependent manner. Of the isoform-specific CYP inhibitors tested for an effect on the precocene I metabolite profile, only tranylcypromine was noticeably effective, indicating a role of CYPs 2A6, 2C9, 2Cl9, and 2E1. With respect to toxicity, the order of CYP inhibitor effectiveness was ABT > diethyldithiocarbamate∼tranylcypromine > ketoconazole. Furafylline and sulfaphenazole had no effect, while quinidine appeared to augment precocene I toxicity. These results suggest that rat liver slices do not reproduce the reported *in vivo* biotransformation of precocene I and therefore may not be an appropriate model for precocene I metabolism. However, these results provide an example of how small molecule manipulation of CYP activity in an *in vitro* model can be used to confirm metabolism-mediated toxicity.

1. Introduction

Metabolism-mediated toxicity accounts for a substantial proportion of compound attrition in drug discovery and development. The fundamental basis for metabolism-mediated toxicity is the biotransformation of the parent compound to pharmacologically or chemically reactive metabolites [1,2]. However, progression to overt toxicity can involve many factors [3,4] subsequent to biotransformation. The percentage of metabolism-mediated toxicity attrition from one major pharmaceutical company was estimated in 2007 to be 27% of all late stage compounds for an approximately 2 year period [5]. Early identification of metabolism-mediated toxicity would be a valuable contribution towards distinguishing between structure-based and mechanism-based toxicity. With the latter, if the toxicity is relevant to humans, clinical development can only proceed if an adequate safety margin, assurance of reversibility, and the ability to monitor, can be met. Confirmation of metabolism-mediated toxicities could focus synthetic chemistry efforts on alteration of the structure to minimize or eliminate the unwanted metabolism while trying to preserve beneficial attributes such as potency and bioavailability. An ideal tool to assess metabolism-mediated hepatotoxicities would be an *in vitro* model in which biotransformation and toxicity could be measured simultaneously. A more ambitious use of such a model would be the ability to more accurately predict idiosyncratic toxicities that are linked to metabolic transformation. A properly validated model could also aid in addressing selected toxicity issues that arise during the course of late-stage (post-nomination) drug development programs.

A wide variety of *in vivo* and *in vitro* models have shown utility in identifying metabolism-mediated toxicity, in particular, hepatotoxicity. *In vivo* models take advantage of gender-specific polymorphisms in drug metabolizing enzymes [6], transgenic modifications

Abbreviations: CYP, cytochrome P450; LC MS, liquid chromatography mass spectrometry; ABT, 1-aminobenzotriazole; DTNB, dithiobis-nitrobenzoic acid; GSH, reduced glutathione.

* Corresponding author. Address: Pharmaceutical Candidate Optimization, Bristol-Myers Squibb, Research and Development, P.O. Box 4000, Princeton, NJ 08543, USA. Tel.: +1 609 252 4460; fax: +1 609 252 3315.
E-mail address: barry.brock@bms.com (B. Brock).

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[7] or small molecule enzyme inhibitors [8]. While these *in vivo* models can be the most definitive, they are resource intensive and the lowest throughput of the models. A variety of cell-based models have been developed such as primary hepatocytes, transformed cell lines, hepatocytes co-cultured with Kupffer cells, primary cells co-cultured with induced S9 fractions, and most recently 3-dimensional hepatocyte monolayers [9–14]. Confirmation of drug metabolizing enzyme involvement, most notably the hepatic cytochrome P450 (CYP) family of enzymes, has been performed by taking advantage of small molecule CYP-specific inhibitors [15] and inducers [16]. Although it is unlikely that a single model will be universally applicable to the study of metabolism-mediated toxicities, and *in vitro* models will always be limited relative to *in vivo* models, there is opportunity for improvement of existing *in vitro* models.

The advantages and disadvantages of tissue slices as *in vitro* models for metabolism and toxicity issues have been addressed extensively [17–25] and only a few features will be described here. For certain types of hepatotoxicities which are linked to an inflammatory response, the non parachymal cells such as Kupffer cells could be critical factors in reproducing some metabolism-mediated hepatotoxicities *in vitro* [26–28]. If a hepatotoxicity were Kupffer cell-mediated, and histological changes were the only reliable endpoint, tissue slices would be considered the first line *in vitro* model. In fact, active Kupffer cells have been demonstrated in liver slices [29]. In support of slices being more similar to the *in vivo* situation than other *in vitro* models, microarray analysis of liver slice gene expression suggests reasonable similarity between slices and gene expression *in vivo* [30–32].

This paper examines in detail the metabolic pathway and toxicity of the well characterized CYP-mediated, hepatotoxicant, precocene I, in precision-cut rat liver slices. In rats *in vivo,* precocene I induces severe centrolobular necrosis with associated decreases in hepatic glutathione, elevations of transaminase activities, and covalent binding to liver proteins and DNA [33,34]. Given the historical link between CYP-dependent metabolic activation and hepatotoxicity for precocene I and the closely related precocene II [34,35], coupled with the extensive evidence of major CYPs being active in liver slices [22,36,37] precocene I was selected as a positive control metabolism-mediated hepatotoxicant. Because of previous success with small molecule inhibitors in liver slices [20,38,39], and because of extensive historical use, we selected ABT as a nonspecific CYP inhibitor. The isoform-specific inhibitors were selected based on published reports of human *in vitro* systems [40–46].

We demonstrate how amenable this model is to small molecule modulation of CYP-mediated biotransformation as a means to confirm the role of metabolism in hepatotoxicity. Using LC MS we report the detailed effects of CYP inhibitors on the precocene I metabolite profile.

2. Materials and methods

2.1. Chemicals and reagents

Precocene I (7-methoxy-2,2-dimethyl-2H-benzo[b]pyran), ABT (1-aminobenzotriazole), Krebs–Henseleit, Insulin (from bovine pancreas), Gentamicin, calcium chloride, glucose, sodium bicarbonate, HEPES were purchased from Sigma–Aldrich (St. Louis, MO). Waymouth MB752/1 was purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum and Amphotericin-B were purchased from MediaTech, Inc. (Manasass, VA). Acetonitrile (HPLC-grade) was obtained from Burdick and Jackson (Muskegeon, MI). Formic acid (analytical-grade) was from J.T. Baker (Phillipsburg, NJ). All other reagents were of analytical grade. Lysing Matrix D Tubes were purchased from MP Biomedical (Solon, OH).

2.2. Liver slice culture and sample preparation

Male Sprague–Dawley rats (325–450 g, 10–15 weeks old), obtained from Charles River Laboratories, Raleigh, NC were individually housed in a temperature- and light-controlled room. They were fed standard diet and water *ad libitum*. On the morning of each experiment rats were euthanized with CO_2 and exsanguinated prior to removing the liver. Intact liver lobes were placed in ice-cold Krebs–Henseleit buffer (pH 7.4). Slices were prepared the day of each experiment using a protocol closely following previously published procedures [29,47]. Tissue cores were prepared with an 8 mm diameter motor-driven tissue coring tool (Alabama Research and Development Corporation, Munford, AL). Tissues slices, approximately 250 μm thick, were prepared in ice-cold Kreb–Henseleit buffer using a Krumdieck tissue slicer (Alabama Research and Development Corporation, Munford, AL). Liver slice thickness was determined by macroscopic comparison to a 250 μm thickness gauge.

The slices were placed onto Vitron (Vitron Inc., Tucson, AZ) type C titanium roller inserts and cultured in glass scintillation vials containing 1.75 mL culture medium and continously exposed to Carbogen (95% O_2/5% CO_2). The culture medium consisted of Waymouth's media containing 25 mM HEPES, 10% fetal bovine serum, 5 μg/mL insulin, 50 μg/mL gentamicin, 2.5 μg/mL amphotericin B, 25 mM glucose, 2.4 g/L sodium bicarbonate with the final pH adjusted to 7.4. Prior to treatment, samples were equilibrated for 1 h in a Vitron dynamic organ culture incubator at 37 °C. After equilibration, the culture media was replaced with fresh media containing the test compounds and incubated overnight (ca. 15–24 h). Stock solutions of precocene I, ABT, and isoform-specific inhibitors were made in dimethyl sulfoxide (DMSO). The final DMSO concentration never exceeded 1.3% (v/v). Liver slices were incubated with precocene I (100 or 200 μM) in the absence or presence of 10, 30, 90 and 270 μM ABT or 200 μM isoform-specific inhibitors for 20–24 h. The isoform-specific inhibitors and the reported target CYPs [46] were furafylline (1A2), diethyldithiocarbamate (2A6, 2C9, 2E1), sulfaphenazole (2C9), quinidine (2D6), ketoconazole (3A) and tranylcypromine (2A6, 2C19, 2E1). All individual slice treatments were run in triplicate.

Following incubations, slices and media were separated to facilitate processing for biochemical and bioanalytical analysis. Entire slices were homogenized in 1 mL of 0.1 M phosphate buffer, pH 7.4 using Lysing Matrix D Tubes in the Savant FastPrep 120 (MP Biomedical, Solon, OH) Shaker for 20 s. Liver slice supernatants were then obtained by centrifugation at approximately 5000 *g*, for 5 min. Aliquots of the supernatant were used for GSH and ATP measurement (see below). For bioanalytical assessment, 100 μL slice supernatants were combined with 100 μL of the respective media. These 200 μL samples were then combined with 200 μL of acetonitrile (ACN) then mixed by vortexing for 20 s, followed by centrifugation at 1000 *g* for 10 min.

2.3. Bioanalytical

2.3.1. Semi quantitative analysis

ACN-extracted samples were injected onto a Phenomenex Synergi Hydro-RP, 4 μm, 2.1 × 150 mm column (Torrance, CA) for the analysis of precocene I and its metabolites. The mobile phases A (0.1% formic acid in water) and B (acetonitrile) were used for all assays. Mobile phase B increased from 0% to 100% in 5 min and then back to 0% in 3 min, held for 2 min for re-equilibration. Total run time was 10 min with a flow rate of 0.3 mL/min. The HPLC system used was a HP1100 series with autosampler (Agilent Technologies, Santa Clara, CA). The mass spectrometer was an Applied Biosystems MDS Sciex (Toronto, Canada) API 4000 Q Trap equipped with a turbo spray ionization source. The instrument was used in the