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Identification of the rat liver cytochrome P450 enzymes involved in the metabolism of the calcium channel blocker dipfluzine hydrochloride

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ABSTRACT

This study aimed to identify the specific cytochrome P450 (CYP450) enzymes involved in the metabolism of dipfluzine hydrochloride using the combination of a chemical inhibition study, a correlation analysis and a panel of recombinant rat CYP450 enzymes. The incubation of Dip with rat liver microsomes yielded four metabolites, which were identified by liquid chromatography-coupled tandem mass spectrometry (LC/MS/MS). The results from the assays involving eight selective inhibitors indicated that CYP3A and CYP2A1 contributed most to the metabolism of Dip, followed by CYP2C11, CYP2E1 and CYP1A2; however, CYP2B1, CYP2C6 and CYP2D1 did not contribute to the formation of the metabolites. The results of the correlation analysis and the assays involving the recombinant CYP450 enzymes further confirmed the above results and concluded that CYP3A2 contributed more than CYP3A1. The results will be valuable in understanding drug–drug interactions when Dip is coadministered with other drugs.

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1. Introduction

CYP450 is a superfamily of heme-containing monooxygenases, many of which are expressed in the liver, and they are significant phase-I enzymes in drug metabolism and detoxification. There are three subfamilies (CYP1, CYP2 and CYP3) that are mainly involved in the metabolism of drugs in both humans and rats (Nedelcheva and Gut, 1994). It has been proposed that the expression levels and activities of CYP450 enzymes directly affect the bioavailability of many drugs. Moreover, CYP450 enzymes can be inhibited, activated or induced by concomitant drug treatments, which have been recognized by regulatory authorities as an important cause of drug–drug interactions. For example, terfenadine, astemizole and cisapride are all extensively metabolized by CYP3A4 and can cause Torsades de Pointes (ventricular tachycardia) when coadministered with other drugs that inhibit CYP3A4, including ketoconazole and erythromycin (Nassar et al., 2009). Thus, the CYP450-mediated metabolism of drugs is one of the major kinetic profile determinants, and the prediction of this metabolism is highly relevant during the drug discovery and development process (Rydberg et al., 2009).

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Dipfluzine hydrochloride (1-diphenylmethyl-4-(3-(4-fluorobenzoyl))-piperazine hydrochloride, Dip), a diphenylpiperazine calcium channel blocker, was synthesized according to the chemical structure of cinnarizine (CZ). Previous studies have demonstrated that Dip is a highly selective cerebral vasodilator that exerts protective effects against focal or global cerebral ischemic injury *via* multiple mechanisms (Wang and He, 1993; Bai and Wang, 2002; Zhang et al., 2005a,b). In addition, studies have shown that Dip also inhibits platelet aggregation *in vitro* and prevents thrombus formation *in vivo* (Wang and He, 1994a,b). Evidence from *in vivo* and *in vitro* experiments has revealed that the pharmacological effects of Dip are more potent than its analogs, CZ or flunarizine (FZ) (Wang and He, 1993, 1994; Zhu et al., 1996). Therefore, Dip is a promising drug candidate for the treatment of cerebral vascular diseases.

A previous study showed that Dip was transformed to five metabolites *via* multiple pathways, mainly by N-dealkylation at the 1- and 4-positions of the piperazine ring (Liu et al., 2005). These five metabolites found in rat urine were identified by LC/DAD/MS methods as 1-(4-fluoro-benzene)-4-piperazine-butanone (M1), 4-hydroxy-benzophenone (M2), 4-fluoro-γ-hydroxy-benzenebutanoic acid (M3), diphenylmethanol (M4) and benzophenone (M5) (Fig. 1). In recent studies, we identified Dip and its metabolites using LC/MS/MS analysis in rat liver microsomes (Guo et al., 2012). The results demonstrated that only four of the five Dip metabolites found in rat urine were detected (all except M3). We presumed that the sample processing method (plasma had not been hydrolyzed) applied in our study might lead to the concentration of M3 being too low to be detected by LC/MS/MS. However, there is no evidence that the CYP450 enzymes are involved in Dip metabolism or of their relative contributions to the formation of the metabolites. Additional knowledge regarding the influence of the CYP450 enzymes on the metabolism of Dip will facilitate the development of better therapeutic strategies to enhance the efficacy and minimize the toxicity in patients. Thus, in the present study, we characterized the CYP450 enzymes involved in the metabolism of Dip using the combination of selective chemical inhibitor studies, a correlation analysis with a bank of rat liver microsomes and a panel of recombinant rat CYP450 enzymes. These results would provide useful information for future pharmacological, toxicological and clinical evaluations of Dip.

2. Materials and methods

2.1. Chemicals

Dip and its metabolite reference standards were provided by the Department of Medicinal Chemistry, Hebei Medical University. Their purities were >99%. α-Naphthoflavone, pilocarpine, orphenadrine, cimetidine, diethyldithiocarbamate, ketoconazole, β-nicotinamide adenine dinucleotide phosphate, *O*-demethylation dextromethorphan, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, tolbutamide, dextromethorphan, chlorzoxazone and 6-hydroxy chlorzoxazone were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetaminophen, diclofenac, phenacetin, testosterone, sulfaphenazole and quinidine were purchased from Wako Pure Chemicals (Osaka, Japan). 7α-Hydroxytestosterone, 16α-hydroxytestosterone and 16β-hydroxytestosterone were purchased from Ultrafine Chemicals (Manchester, UK). Midazolam was obtained from F. Hoffman-La Roche (Nutley, NJ). 1′-Hydroxymidazolam was obtained from Ultrafine Chemicals (Manchester, England). 4-Hydroxydiclofenac and nine different rat recombinant CYP450 isoforms (CYP1A2, CYP2A1, CYP2B1, CYP2C6, CYP2C11, CYP2D1, CYP2E1, CYP3A1 and CYP3A2) were purchased from BD Gentest (Woburn, MA). All of the recombinant CYP450s were co-expressed with NADPH-P450 oxidoreductase. The solvents were high-performance liquid chromatography grade (Fisher, USA) and all other chemicals were of the highest quality available.

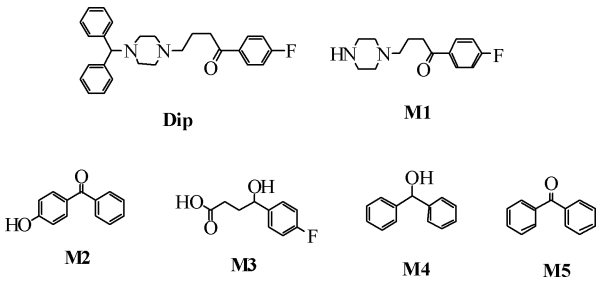

Fig. 1 – The structures of Dip and its five metabolites M1, M2, M3, M4 and M5.

2.2. Animals

Male Sprague-Dawley (SD) rats weighing 210–230 g were purchased from the Department of Experimental Animals, Hebei Medical University (Shijiazhuang, China). The rats were acclimated to temperature (22 ± 2 °C)- and humidity (55 ± 5%)-controlled rooms with a 12-h light–dark cycle for 1 week before use. The regular laboratory rat diet and tap water were given to the rats *ad libitum*. All animal care and experiments were conducted in accordance with the guidelines provided by the Department of Experimental Animals, Hebei Medical University. Before each experiment, the rats were starved for 24 h but were given drinking water freely. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital and sacrificed by decapitation. The livers were rapidly removed, weighed and stored at −80 °C for further microsomal study *in vitro*.

2.3. The preparation of rat liver microsomes

Whole liver was homogenized in an ice-cold 0.25 M sucrose buffer (consisting of 10 mM Tris and 1 mM EDTA, pH 7.4). The homogenate was centrifuged at 20,000 × *g* for 20 min (Optima LE-80K high-speed centrifuge, Beckman, USA), and the supernatant was further ultracentrifuged at 100,000 × *g* for 60 min (Allegra 64 R Ultrarapid Centrifuge, Beckman, USA). The microsomal pellet was suspended in 0.1 M potassium pyrophosphate buffer (containing 1 mM EDTA, pH 7.4) and ultracentrifuged again at 100,000 × *g* for 60 min. Liver microsomes were prepared and suspended in 10 mM Tris–HCl buffer

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