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Metabolism of dictamnine in liver microsomes from mouse, rat, dog, monkey, and human

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ABSTRACT

Dictamnine, a furoquinoline alkaloid isolated from the root bark of *Dictamnus dasycarpus* Turcz. (Rutaceae), is reported to have a wide range of pharmacological activities. In this study, the *in vitro* metabolic profiles of dictamnine in mouse, rat, dog, monkey, and human liver microsomes were investigated and compared. Dictamnine was incubated with liver microsomes in the presence of an NADPH-regenerating system, resulting in the formation of eight metabolites (M1–M8). M1 is an *O*-desmethyl metabolite. M5 and M6 are formed by a mono-hydroxylation of the benzene ring of dictamnine. M8 was tentatively identified as an *N*-oxide metabolite. The predominant metabolic pathway of dictamnine occurs through the epoxidation of the 2,3-olefinic to yield a 2,3-epoxide metabolite (M7), followed by the ring of the epoxide opening to give M4. Likewise, cleavage of the furan ring forms M2 and M3. Slight differences were observed in the *in vitro* metabolic profiles of dictamnine among the five species tested. A chemical inhibition study with a broad and five specific CYP450 inhibitors revealed that most of the dictamnine metabolites in liver microsomes are mediated by CYP450, with CYP3A4 as the predominant enzyme involved in the formation of M7, the major metabolite. These findings provide vital information to better understand the metabolic processes of dictamnine among various species.

1. Introduction

Dictamnine (4-methoxyfuro[2,3-b]quinoline) is the major furoquinoline alkaloid isolated from the root bark of *Dictamnus dasycarpus Turcz.* (Rutaceae). Dictamnine possesses many beneficial biological activities, such as anti-platelet-aggregation, anti-hypertension, antibacterial, anti-mitosis, antifungal, vascular-relaxing, and suppression of Ca^{2+} influx through both voltage-dependent and receptor-operated Ca^{2+} channels [1–7]. Moreover, in cancer-derived cell lines, such as human cervix cells, colon cancer cells, human breast cancer cells, and oral carcinoma cells, dictamnine inhibits cell proliferation and increases cell apoptosis [8,9]. Recently, An et al. reported that dictamnine as an anticancer drug induces human lung adenocarcinoma A549 cell cycle arrest and apoptosis via mitochondria and caspase-independent apoptotic pathway [10]. Furthermore, derivatives from dictamnine received much attention in medicinal research as traditional as well as recent synthetic antitumor agents [11].

During the past two decades, most studies relevant to dictamnine were focused on its biological activity and toxicity, but little attention has been paid to its pharmacokinetics and metabolism. Methods based on ultra-high performance liquid chromatography mass spectrometry (UHPLC-MS/MS) have been developed in our laboratory for the absorption, distribution, and excretion study of dictamnine [12,13]. Dictamnine was found to have considerable oral absorption with absolute bioavailability around 48% (rats, 5 mg/kg). Tissue distribution studies showed that dictamnine can be rapidly and widely distributed into organs and can effectively cross the blood–brain barrier in rats, while an excretion study indicated that very low proportion of dictamnine (<0.3%) was excreted as the unchanged form [13]. All these results support that dictamnine has good membrane permeability, extensive hepatic metabolism, and quick clearance. However, the clearance/metabolic pathway(s) of dictamnine are still unclear.

To date, the metabolism of dictamnine has only been studied by Klier and Schimmer [14]. Metabolites resulting from incubation

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with rat liver microsomes were analyzed by gas chromatography mass spectrometry (GC–MS), suggesting that *O*-demethylation, hydroxylation, and oxidative cleavage of the furan ring are major metabolic pathways in rat microsomal incubation system. They also postulated that the oxidative cleavage of the furan ring may take place via the 2,3-epoxide. It should be noted that the biotransformation of dictamnine reduces its mutagenic activity in *Salmonella typimuriun* TA 98 [14]. On the other hand, significant differences in metabolic pathways among different species may exist, and the various metabolites and their concentrations can potentially affect its in vivo bioactivities and toxicities. All these reasons encouraged us to investigate the metabolic behaviors of dictamnine among different spices.

It is remarkable that there have been no reports about the metabolism of dictamnine in humans. Microsomes from rodent (mice and rats) and nonrodent (dogs and monkeys) animal species are routinely used for the preclinical metabolism studies of drug candidates. Choosing the most relevant animal species on which to conduct studies and extrapolating the results to humans are huge challenges for researchers [15–17]. In this study, the fate of dictamnine in liver microsomes from mouse, rat, dog, monkey, and human were examined by means of LC–MS. The objective of this study were as follows: (1) to gain understanding about the whole metabolic route of dictamnine in liver microsomes from different species including the biotransformation to 2,3-epoxide metabolites and (2) to initially characterize the cytochrome P450 (CYP450) involved in the biotransformation of dictamnine.

2. Materials and methods

2.1. Reagents

Microsomes and NADPH-regenerating systems were purchased from BD Biosciences (Bedford, MA, USA). Dictamnine, robustine, and skimmianine (purity >98%) were isolated from the root bark of *Dictamnus dasycarpus* Turcz. (Rutaceae) in our laboratory. 1-Aminobenzotriazole (ABT), ketoconazole (Ket), α-Naphthoflavone (α-NF), methoxsalen (Met), quercetin (Quer), and quinidine (Qui) were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC and LC–MS grade solvents were obtained from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Microsomal incubations of dictamnine

2.2.1. Experiment 1

Human liver microsomes (HLM) (final concentration at 0.1 or 0.5 mg/mL in the incubation system) were incubated with dictamnine (20 μM) for several time points. The experimental incubation mixture consisted of 100 mM potassium phosphate buffer (pH 7.4), a prepared NADPH-regenerating system, and HLM. Stock solution of dictamnine was prepared in dimethylsulfoxide (DMSO). The final concentration of DMSO in the incubation did not exceed 1% (v/v). After 15 min pre-incubated at 37 °C in water bath, the reactions were initiated by the addition of dictamnine and were further incubated at 37 °C. The reaction was terminated at 0, 30, 45, 60, and 90 min by the addition of an equal volume of ice-cold acetonitrile (ACN) containing of 2% acetic acid. The mixture was vortexed and centrifuged at 16,100 × *g* for 5 min. Aliquots of supernatant were stored at −20 °C until analysis. Incubations with different substrate concentrations (5, 10, 20, 50 μM) incubated with fixed HLM concentration (0.5 mg/mL) were also performed to investigate the effect of substrate concentration and facilitate the MS detection and avoid potential saturation and inhibition of drug-metabolizing enzymes at a high substrate concentration. Control incubations without NADPH-regenerating system, without substrate, or without microsomes were performed to ensure that metabolite formation was microsome- and NADPH- dependent.

2.2.2. Experiment 2

Based on the optimized conditions in experimental 1, mouse liver microsomes (MLM), Spragur-Dawley rat liver microsomes (RLM), beagle dog liver microsomes (DLM), cynomolgus monkey liver microsomes (CyLM), and HLM (each 0.5 mg/mL, respectively), were mixed with the aforementioned incubation mixture and were held in 37 °C water bath for 15 min before initiating by the addition of dictamnine (20 μM). Reactions were quenched by the addition of an equal volume of ice-cold ACN containing of 2% acetic acid. The mixture was vortexed and centrifuged at 16,100 × *g* for 5 min. Aliquots of supernatant were stored at −20 °C until analysis.

2.2.3. Chemical inhibition study

The metabolism of dictamnine in liver microsomes (MLM, RLM, DLM, CyLM, and HLM, 0.5 mg protein/mL) in the absence or presences of CYP450 inhibitors were measured to explore the enzyme(s) involved in the *in vitro* metabolism of dictamnine. In brief, dictamine (20 μM) was incubated in liver microsomes (0.5 mg protein/mL) in the absence (control) and presence of CYP450 inhibitors/substrate. The selective inhibitors and their concentrations were as follows [18,19]: 10 μM for α-NF (CYP1A2), 10 μM for methoxsalen (CYP2A6), 30 μM for quercetin (CYP2C8), 10 μM for quinidine (CYP2D6) and 5 μM for ketoconazole (CYP3A4), and ABT 500 μM for broad-spectrum CYP450s [20]. After pre-incubated with liver microsomes in experimental incubation mixture at 37 °C for 15 min, the substrate was added to initiate the reaction. After 60 min incubation, reactions were quenched with equal volumes of ice-cold ACN containing 2% acetic acid. For the chemical inhibition study, incubations without inhibitor were regarded as controls. The yield of M7 was estimated using the ratio of the areas of M7 and skimmianine (internal standard). The relative metabolic rate (%) was calculated as the yield of M7 divided by the mean of the yield of M7 in the control group. The relative metabolic rate (%) in the control group was examined using the same method, the mean obtained as 100%. All incubations in each experiment were performed in triplicate.

2.3. LC-ESI-MS analysis

Samples were first screened for metabolites using an Agilent 6520 quadrupole time-of-flight (Q-TOF) mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) coupled with an Agilent 1260 Series LC system (Agilent Technologies, Wilmington, DE, USA), and exact mass data were obtained for all detected compounds. The (potential) precursor ($[M+H]^+$) ion was selected and further fragmented on a Thermo-Finnigan Spectra System consisting of an LTQ Velos Pro mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Dionex Ultimate 3000HPLC (Dionex, sunnyvale, CA, USA). For both MS experiments, mass spectrometric detection was accomplished using electrospray ionization (ESI) interface in positive ion mode.

Using the LTQ Velos Pro system, data dependent scanning was performed to obtain the MS/MS spectra up to MS^3 with the following settings: (1) scan event one to collect the full MS spectrum of all the ions in the sample; (2) scan event two to collect the MS^2 spectra of the most intense ion at each time point form the MS spectra obtained from the scan event one; and (3) scan events three, four, and five to collect the MS^3 spectra of the most intense, second most intense, and third most intense ions from the MS^2 spectra obtained from scan event two, respectively. The collision-induced dissociation (CID) was conducted with an isolation width of 2 Da and normalized collision energy of 35 for both MS^2 and MS^3. Default automated gain control target ion values were used for MS, MS^2,