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Identification and characterization of reactive metabolites in myristicin-mediated mechanism-based inhibition of CYP1A2

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ABSTRACT

Myristicin belongs to the methylenedioxyphenyl or allyl-benzene family of compounds, which are found widely in plants of the *Umbelliferae* family, such as parsley and carrot. Myristicin is also the major active component in the essential oils of mace and nutmeg. However, this compound can cause adverse reactions, particularly when taken inappropriately or in overdoses. One important source of toxicity of natural products arises from their metabolic biotransformations into reactive metabolites. Myristicin contains a methylenedioxyphenyl substructure, and this specific structural feature may allow compounds to cause a mechanism-based inhibition of cytochrome P450 enzymes and produce reactive metabolites. Therefore, the aim of this work was to identify whether the role of myristicin in CYP enzyme inhibition is mechanism-based inhibition and to gain further information regarding the structure of the resulting reactive metabolites. CYP cocktail assays showed that myristicin most significantly inhibits CYP1A2 among five CYP enzymes (CYP1A2, CYP2D6, CYP2E1, CYP3A4 and CYP2C19) from human liver microsomes. The 3.21-fold IC_{50} shift value of CYP1A2 indicates that myristicin may be a mechanism-based inhibitor of CYP1A2. Next, reduced glutathione was shown to block the inhibition of CYP1A2, indicating that myristicin utilized a mechanism-based inhibition. Phase I metabolism assays identified two metabolites, 5-allyl-1-methoxy-2,3-dihydroxybenzene (M1) and 1'-hydroxymyristicin or 2',3'-epoxy-myristicin (M2). Reduced glutathione capturing assays captured the glutathione-M1 adduct, and the reactive metabolites were identified using UPLC-MS^2 as a quinone and its tautomer. Thus, it was concluded that myristicin is a mechanism-based inhibitor of CYP1A2, and the reactive metabolites are quinone tautomers. Additionally, the cleavage process of the glutathione-M1 adduct was analyzed in further detail. This study provides additional information on the metabolic mechanism of myristicin inhibition and improves risk evaluation for this compound.

1. Introduction

Myristicin, 1-allyl-3,4-methylenedioxy-5-methoxybenzene (Fig. 1), is commonly found in parsley, carrot, black pepper, nutmeg, and many natural oils and flavoring agents [1–3]. For example, in the essential oil of mace, myristicin is the major component of the aromatic ether fraction. Myristicin is also one of main components in nutmeg volatile oils [4]. Some herbs containing myristicin, such as nutmeg, mace and dill preparations, are also used in traditional medicine to treat rheumatism, cholera, psychosis, stomach cramps, nausea, diarrhea, and anxiety [5,6]. Myristicin has been shown to induce glutathione *S*-transferase [7] and inhibit benzo[*a*]pyrene-induced tumorigenesis [8].

However, traditional medicines can also cause adverse reactions, especially when taken inappropriately or in overdoses. Myristicin has weak monoamine oxidase inhibitor properties that are responsible for some cardiovascular symptoms [9]. Myristicin displayed apoptotic activity mediated by the activation of caspases in hamster ovary CHO cells [10], and it also altered mitochondrial membrane function in human leukemia K562 cells, inducing apoptosis and down-regulating DNA damage response genes [11]. Myristicin has also been reported to cause psychotropic effects [12] and even death [5] at high dosages in humans, in which myristicin is metabolized to 3-methoxy-4,5-methylenedioxyamphetamine, the hallucinogenic effect of which was more pronounced than that of mescaline [12,13]. Therefore, as the toxicity of this

Abbreviations: MBI, mechanism-based inhibition; RMs, reactive metabolites; CYPs, cytochrome P450 enzymes; GSH, reduced glutathione; HLMs, human liver microsomes; NADPH, β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt; MI complex, metabolite intermediate complex.

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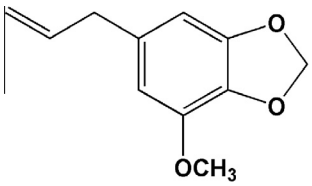

Fig. 1. Structural formula of myristicin.

compound is related to its xenobiotic metabolism, the metabolism of myristicin should be more thoroughly studied to improve risk evaluation.

Tertiary aminopropiophenones were identified as urinary metabolites from rats and guinea pigs treated with myristicin [14], while rat liver converted myristicin into 3-methoxy-4, 5-methylenedioxy amphetamine [15]. In vivo and in vitro studies of rats demonstrated that myristicin could also be transformed into 5-allyl-1-methoxy-2,3-dihydroxybenzene and 1'-hydroxy-myristicin [16]. Through the study of purified recombinant human liver CYPs (cytochrome P450 enzymes) and immunoinhibition assays, Yun et al. determined that the oxidation of myristicin to 5-allyl-1-methoxy-2,3-dihydroxybenzene was catalyzed by CYP3A4 and CYP1A2 in humans [17]. Although these metabolites were characterized in the above reports, the effects of myristicin on the CYPs involved in its metabolism, in particular the identification of RMs (reactive metabolites) in myristicin-mediated MBI (mechanism-based inhibition), remain unknown.

The metabolism of drugs by CYPs to form RMs that bind covalently to a catalytic site or sites of the enzyme itself leads to irreversible inhibition of the enzymes. This phenomenon is referred as MBI. MBI inhibitors have very specific features that make them recognizable by in vitro testing. Fontana et al. [18] summarized the most common substructures causing the MBI of CYPs, including methylenedioxyphenyl-containing compounds such as myristicin and safrole. The drug-induced MBI of CYPs is initiated by conversion of the drug into highly RMs, and this group of drugs may be involved in metabolism-dependent drug toxicities.

In this work, we demonstrated that myristicin is an MBI inhibitor of CYP1A2 and determined that its reactive metabolites are quinone tautomers using the following assays: (i) Using a CYP cocktail assay, the effects of myristicin on specific marker reactions of five CYP isoforms were measured in HLMs (human liver microsomes). Myristicin inhibited CYP1A2 more strongly than it inhibited the other CYPs. (ii) It could be estimated that myristicin may be the MBI inhibitor of CYP1A2 according to IC_{50} shift value. (iii) In phase I metabolic reactions, NADPH-dependent inhibition assays were used to identify the generation of two metabolites (M1 and M2). (iv) In GSH capturing assays, GSH acted as a trapping agent to identify the structure of the RMs. It was concluded that myristicin could inhibit CYP1A2 through an MBI mechanism and produce electrophilic reactive metabolites with tautomeric structures (quinone and its tautomer). Such information concerning the MBI by this compound and its RMs can be helpful for thoroughly understanding the drug metabolism of myristicin.

2. Materials and methods

2.1. Materials

Myristicin was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Phenacetin, midazolam, dextromethorphan, (*s*)-mephenytoin, chlorzoxazone and NADPH (*β*-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). GSH was obtained from Sigma-Aldrich (Milwaukee, WI, USA). Pooled HLMs, which were purchased from the Research Institute for Liver Diseases Co., Ltd. (Shanghai, China), were prepared from human liver tissues under Chinese organ donation regulations with the full consent of the patients. All other reagents were of high-performance liquid chromatography grade.

2.2. CYP cocktail assays—screening for significant myristicin-mediated inhibition of CYPs

CYP cocktail assays were employed to analyze and screen the myristicin-mediated inhibition of five CYP isoforms. Phenacetin (CYP1A2), dextromethorphan (CYP2D6), chlorzoxazone (CYP2E1), midazolam (CYP3A4), and (*s*)-mephenytoin (CYP2C19) were chosen as probe substrates for each corresponding CYP. The assays consisted of two steps: (i) A mixture of 20 μL myristicin (stock concentration: 200 μM or 2 mM) and 20 μL HLMs (stock concentration: 8 mg protein/mL) were pre-incubated in the presence or absence of 40 μL NADPH (denoted as +/− NADPH; stock concentration: 2 mM) for 30 min at 37 °C. For the (−)NADPH reaction, 40 μL of 0.1-M potassium phosphate buffer, pH 7.4, was used instead of NADPH. (ii) Next, 80 μL of the probe substrates and 100 μL NADPH were added to the 20 μL pre-incubation mixtures and incubated for 30 min under the same conditions. The reactions were stopped by adding a 2-fold volume (400 μL) of ice-cold methanol containing 50-ng/ml carbamazepine. Control reactions were performed without myristicin. The final concentrations of the components were as follows: myristicin (5 μM or 50 μM), HLMs (0.2 mg protein/mL), and NADPH (1 mM). The final incubation concentrations of the probe substrates were fixed around their reported K_m values: phenacetin (10 μM), dextromethorphan (2.5 μM), chlorzoxazone (20 μM), midazolam (5 μM), and (*s*)-mephenytoin (20 μM). The assays were performed in triplicate for all test specimens.

2.3. IC_{50} shift assays and the blocking of CYP1A2 inhibition by GSH—an evaluation of myristicin MBI potential

The same experimental conditions described above in *2.2. Cocktail assays* were used in the IC_{50} shift assays except that six different concentrations of myristicin were used: 0.5, 1.6, 4, 8, 20 and 50 μM. The assays were performed in triplicate for all test specimens. The IC_{50} values were calculated using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

Based on the IC_{50} shift value, we predicted that GSH would be able to block the inhibition of CYP1A2 by myristicin in the presence of NADPH and designed an assay to test this prediction. The same experimental conditions described above were used except for the following two exceptions: (1) Phenacetin was chosen as a probe substrate for CYP1A2, and (2) the final incubation concentration of myristicin was 2 μM, which was close to its IC_{50} value of 1.796 μM. The assays were performed in triplicate. The relative activity (%) of CYP1A2 was monitored to determine whether GSH was able to block inhibition.

2.4. Phase I metabolism—identification of the metabolites

Mixtures of 50 μL myristicin (stock concentration: 200 μM; final concentration: 50 μM) and 50 μL HLMs (stock concentration: 8 mg protein/mL; final concentration: 2 mg protein/mL) were pre-incubated 5 min at 37 °C. Subsequently, 100 μL NADPH (stock concentration: 2 mM; final concentration: 1 mM) were added to the above reaction mixture. After incubation at 37 °C for 2 h, the reaction was stopped by adding 400 μL ice-cold methanol. After centrifugation at 10,000 rpm for 10 min, the supernatant was