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# Mechanism-based inactivation of cytochrome P450 2B6 by isoimperatorin ☆

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ABSTRACT

Isoimperatorin (IIMP), a 6,7-furanocoumarin derivative, occurs in many common medicinal herbs. Human exposure to IIMP mainly results from intake of fruits, foods and medicinal herbs. We examined the irreversible inhibitory effect of IIMP on cytochrome P450 2B6. IIMP was found to cause time-dependent inhibition of CYP2B6. In addition, the loss of CYP2B6 activity occurred in a NAPDH- and concentration-dependent manner. About 60% of activity of CYP2B6 was suppressed after its incubation with IIMP at 25 μM for 9 min. Enzyme kinetic studies were performed, $k_{inact}$ for IIMP was found to be 0.071 $min^{-1}$, and $K_I$ was 17.1 μM, respectively. Glutathione and catalase/superoxide dismutase showed little protective effects on CYP2B6 against the inactivation by IIMP. *S*-Mephenytoin, a substrate of CYP2B6, mildly prevented the enzyme from the inactivation induced by IIMP. The estimated partition ratio of the inactivation was approximately 211. Additionally, a γ-ketoenal intermediate was identified in microsomal incubations with IIMP. CYPs 2B6, 2D6, and 1A2 were the major enzymes responsible for the metabolic activation of IIMP. In conclusion, IIMP is a mechanism-based inactivator of CYP2B6. The formation of γ-ketoenal intermediate may be responsible for the enzyme inactivation.



## 1. Introduction

Isoimperatorin (IIMP), a linear furanocoumarin, occurs in the *Apiaceae* families, which include a variety of medicinal herbs, such as *Angelica Dahurica* and *Radix Glehniae* [1,2]. In addition, many fruits and foods contain IIMP, such as citrus fruits (grapefruit and lemon), umbelliferous vegetables, and culinary herbs (parsley) [3,4]. IIMP is also included in Yuan-hu-zhi-tong (in Chinese) tablets and Huo-xiang-zheng-qi (in Chinese) aqua widely used in China for the treatment of stomachache and headache. As a bioactive component, IIMP exhibited anti-inflammatory, analgesic, anti-spasmodic, and anti-cancer activities [5–8]. It also showed inhibitory effect on β-secretase that has been considered as a valuable target for the treatment of Alzheimer's disease [9,10]. The multiple pharmacological activities of IIMP, along with its natural abundance, have attracted much attention.

Many furanocoumarins have been documented as potent inhibitors of cytochrome P450s (CYPs). Epoxybergamottin and bergapten (5-methoxypsoralen) from grapefruit were proven to inhibit CYP3A4 activity [11,12]. Psoralen and isopsoralen showed inhibitory effects on CYP1A2 [13,14]. 8-Methoxypsoralen 5-methoxypsoralen, 5-hydroxypsoralen, 8-hydroxypsoralen, and psoralen were found to be mechanism-based inactivators of CYP2B1 [15]. Psoralen and 8-methoxypsoralen were reportedly oxidized on the furan ring to form the corresponding furanoepoxides that bind to CYP2B1. We speculated that IIMP might show similar irreversible inhibitory effect on CYP2B subfamily.

*Abbreviations:* CE, collision energy; DMSO, dimethyl sulfoxide; EPI, enhanced product ion; GSH, glutathione; IDA, information-dependent acquisition; IIMP, isoimperatorin; LC, liquid chromatography; LC–MS/MS, liquid chromatography coupled to tandem mass spectrometry; MRM, multiple-reaction monitoring; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NADPH, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt; RLMs, rat liver microsomes; SOD, superoxide dismutase.

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CYP2B6 has been found in liver, brain, kidney, and heart in humans [16,17]. Although CYP2B6 comprises about 0.2% of the total CYPs in human liver microsomes, it is responsible for the metabolism of more than 3% of drugs widely used in clinic [18], such as bupropion, efavirenz, methadone, ifosfamide, and cyclophosphamide, which are preferentially metabolized or stereoselectively metabolized by CYP2B6 [19–22]. To confirm the role of specific CYPs in the clearance of various drugs and to prevent drug–drug interactions induced by the inhibition of CYPs, prediction and identification of compounds that act as mechanism-based inactivators of CYPs have become an important issue in drug discovery process.

The objectives of the study were to examine the irreversible inhibitory effect of IIMP on cytochrome P450 2B6, to characterize the reactive metabolites responsible for the enzyme inactivation, and to identify the P450 enzymes responsible for the metabolic activation of IIMP.

## 2. Materials and methods

### 2.1. Chemicals and materials

Glutathione (GSH), hexyl glutathione, bupropion, and NADPH were purchased from Sigma–Aldrich (St. Louis, MO). Isoimperatorin (IIMP) with purity of 98% was acquired from Shanghai Yuanye Biological Technology Co., Ltd. (Shanghai, China). Recombinant human CYP2B6 was purchased from BD Gentest (Woburn, MA). Rat liver microsomes (RLMs) were prepared by our lab, according to our early work [23]. All organic solvents were from Fisher Scientific (Springfield, NJ). Distilled water was purchased from Wahaha Co. Ltd (Hangzhou, China). All solvents and reagents were either analytical or HPLC grade.

### 2.2. Time-, concentration-, and NADPH-dependent inactivation of CYP2B6 by IIMP

The composition of the primary incubation mixtures was CYP2B6 (0.1 μM), $MgCl_2$ (3.2 mM), and IIMP at a series of concentrations (0, 2.5, 5.0, 7.5, 10, and 25 μM) in potassium phosphate buffer (pH 7.4). The total volume was 0.2 mL. To determine the requirement of metabolism for the enzyme inactivation, IIMP (25 μM) and CYP2B6 were incubated in the absence of NADPH as negative control. The primary mixture was preincubated at 30 °C for 3 min. The reactions were initiated by addition of NADPH (1.0 mM) and were incubated at 30 °C. At various time points, aliquots (40 μL) of the primary mixtures were transferred into the secondary incubation mixtures containing bupropion (100 μM) and NADPH (0.45 mM) in 0.1 M potassium phosphate buffer (pH 7.4). The secondary incubation mixtures were further incubated at 30 °C for 30 min, followed by the addition of 0.12 mL ice-cold acetonitrile containing propranolol as internal standard. After vortexed for 3 min, the mixture was centrifuged at 16,000 rpm for 10 min. The supernatant was subjected to LC–MS/MS analysis as described below.

### 2.3. Substrate protection

Substrate protection from IIMP-induced inactivation of CYP2B6 was determined by including IIMP and *S*-mephenytoin (1:4) in the primary reaction mixture. The primary mixture was preincubated at 30 °C for 3 min. The reactions were initiated by the addition of NADPH (1.0 mM), and aliquots (40 μL) of the primary mixtures were transferred at 0, 3, and 9 min to the secondary incubation mixtures for the determination of bupropion hydroxylase activity of CYP2B6. Control incubations were performed in the absence of IIMP or *S*-mephenytoin in parallel.

### 2.4. Effects of GSH and catalase/superoxide dismutase on the enzyme inactivation

The primary mixtures including CYP2B6 (0.1 μM), IIMP (10 μM)), and GSH (2 mM) were preincubated at 30 °C for 3 min. The reactions were initiated by the addition of NADPH (1.0 mM) and were incubated at 30 °C for 0, 3 and 9 min. Aliquots (40 μL) were taken to the secondary incubation mixture for determining the remaining enzyme activity. In control samples, GSH was replaced by an equal volume of phosphate buffer. In a separate study, CYP2B6 was incubated with IIMP and NADPH in the presence or absence of a mixture of catalase and superoxide dismutase. The concentrations of superoxide dismutase and catalase were both 800 unit/mL. After incubation for 0, 3, and 9 min, the residual CYP2B6 activities were examined as described below.

### 2.5. Partition coefficient

To estimate the partition coefficient, IIMP was added to the primary reaction mixtures (final concentrations: 0, 1.5, 2.5, 5, 7.5, 10, 25, 50, and 150 μM) containing CYP2B6 (0.1 μM). The reactions were initiated by the addition of NADPH (1.0 mM) and incubated at 30 °C for 9 min to assure that the inactivation had proceeded to completion. Negative control incubations lacked NADPH. Aliquots (40 μL) were withdrawn from the primary reaction mixtures and transferred to the secondary incubation. The samples were analyzed as follows.

### 2.6. Irreversibility of enzyme inhibition

The primary reaction mixture was incubated with 25 μM IIMP (inactivated sample) or without of IIMP (control sample) at 30 °C. Aliquots of the control and inactivated samples were withdrawn at 9 min and dialyzed using Slide-A-lyzer membranes (molecular mass cut off, 3,500 Da, Pierce, Rockford, IL) against 0.1 M potassium phosphate buffer (pH 7.4, 3 × 2 h) at 4 °C. The dialyzed samples were brought to room temperature and were added to the secondary incubation mixture for the determination of the residual enzyme activities as below.

### 2.7. CYP2B6 Assay

CYP2B6 activity was monitored by measuring the formation of hydroxybupropion analyzed by LC–MS/MS, using an Accuore $C_{18}$ column (2.1 × 50 mm, 2.6 μM, ThermoFisher, Pittsburgh, PA). The LC–MS/MS system consisted of AB Sciex Instruments 4000 Q-Trap mass spectrometry (Applied Biosystems, Foster City, CA) interfaced online with an ekspert ultra LC 100 system (Applied Biosystems, Foster City, CA). The mobile phases consisted of 0.1% formic acid in acetonitrile (mobile phase A) and 0.1% formic acid in water (mobile phase B). The flow rate was 0.5 mL/min, and the column temperature was maintained at 30 °C. The gradient elution was set as follow: 0–1.0 min, 10% A; 1.0–1.5 min, 10–30% A; 1.5–4.0 min, 30% A; 4.0–5.5 min; 30–10% A; 5.5–8.0 min, 10% A. Injection volume was 5.0 μL. Quantification was performed by multiple reaction monitoring (MRM), and ion pairs of $m/z$ 256.2 → 238.3 for hydroxybupropion and $m/z$ 260.7 → 116.3 for propranolol (internal standard) were acquired in positive mode.

### 2.8. Reactive intermediate trapping by GSH

IIMP (120 μM), rat liver microsomes (1.0 mg protein/mL), and GSH (1.0 mM) were incubated in the presence or absence of