Imperatorin Is a Mechanism-Based Inactivator of CYP2B6

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ABSTRACT

Imperatorin (IMP) is the major active ingredient in many common medicinal herbs. We examined the irreversible inhibitory effect of IMP on CYP2B6. IMP produced a time- and concentration-dependent inactivation of CYP2B6. About 70% of activity of CYP2B6 was suppressed after its incubation with 1.5 μM IMP for 9 minutes. K_inact were found to be 0.498 μM and 0.079 min^{-1}, respectively. The loss of CYP2B6 activity required the presence of NADPH. Glutathione and catalase/superoxide dismutase showed little protection against the IMP-induced enzyme inactivation.

Introduction

Imperatorin (IMP), a linear furanocoumarin compound, is the major active ingredient in many common umbelliferous herbs used as traditional Chinese medicines, such as Cnidium monnieri cns and Angelica dahurica (Baek et al., 2000; Lia and Chen, 2004). The IMP-containing herbs are widely used in China for the treatment of osteoporosis and relief of swelling and pain (Zhang et al., 2003). The reported pharmacological activities of IMP include anti-inflammatory properties (Garcia-Argaez et al., 2000), antiosteoporosis (Tang et al., 2008), antibacterial effects (Rosselli et al., 2007), antitumor (Kim et al., 2007), β-secretase inhibition (Marumoto and Miyazawa, 2010), and inhibition of myocardial hypertrophy (Zhang et al., 2010). Recent studies have demonstrated that IMP participates in hypertension by inhibiting voltage-dependent calcium channels and receptor-mediated Ca^{2+} influx and release (He et al., 2007). The studies have demonstrated that IMP may show a similar irreversible inhibitory effect on the CYP2B subfamily.

CYP2B6 has been found in the liver, brain, kidney, and heart in humans (Thum and Borlak, 2000; Miksys and Tyndale, 2004). Hepatic CYP2B6 content ranges from 2 to 10% of the total P450 content (Stresser and Kupfer, 1999). Human CYP2B6 has been proven to be a very important metabolizing enzyme, and the enzyme is responsible for the preferential metabolism of approximately 3–8% of widely used pharmaceuticals, such as bupropion, methadone, ifosfamide, efavirenz, and cyclophosphamide (Faucette et al., 2000; Huang et al., 2000; Ward et al., 2003; Gerber et al., 2004). To determine the role of specific P450s in the clearance of various drugs and to prevent drug-drug interactions induced by the inhibition of P450s, prediction and identification of compounds that act as mechanism-based inactivators of P450s have become an important issue in the drug discovery process. The objectives of this study were to investigate the mechanism-based inactivation of CYP2B6 by IMP, to characterize the reactive metabolites responsible for the enzyme inactivation, and to identify the P450 enzymes responsible for the metabolic activation of IMP.

Materials and Methods

Imperatorin (IMP) with purity of 98% was purchased from Shanghai Yuanye Biologic Technology Co., Ltd. (Shanghai, China). Glutathione (GSH), hexyl glutathione, bupropion, propranolol, ticlopidine, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). Liver microsomes were prepared from male Sprague-Dawley rats by following the published method in Lin et al. (2007). Recombinant human P450 enzymes were acquired from BD Gentest (Woburn, MA). All organic solvents were from Fisher Scientific (Springfield, NJ). Distilled water was purchased from Wahaha Co., Ltd. (Hangzhou, China).

SUPPLEMENTAL MATERIAL

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ABBREVIATIONS: EPI, enhanced product ion; GSH, glutathione; IMP, imperatorin; LC-MS/MS, liquid chromatography–tandem mass spectrometry; P450, cytochrome P450; SOD, superoxide dismutase.
All solvents and reagents were either analytical or high-performance liquid chromatography grade.

Time-, Concentration-, and NADPH-Dependent Inactivation of CYP2B6 by IMP. Recombinant human CYP2B6 was incubated with IMP at concentrations of 0 (●), 0.2 (■), 0.5 (□), 1.0 (▲), 1.5 (△), and 2.5 (○) μM in the presence of NADPH at 30°C for 0, 3, 6, and 9 minutes. Aliquots of incubation mixtures were transferred to the secondary incubation mixtures for the determination of residual enzymatic activity. The residual enzymatic activities at 0 minutes were normalized to 100% at each concentration. (B) Wilson’s plot. The observed inactivation rate constant $K_{\text{obs}}$ was calculated from the slope of the regression lines shown in (A). (C) NADPH-dependent inactivation of CYP2B6 by IMP. CYP2B6 was incubated with vehicle (●) and IMP (1.5 μM) in the presence (▲) or absence (■) of NADPH. Data are mean ± S.D. ($n = 3$).
10 minutes. The supernatants were then collected and analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) as described below.

Substrate Protection. Substrate protection from IMP-induced inactivation of CYP2B6 was determined by incorporating ticlopidine (50, 100, and 200 μM) with IMP (1.5 μM) in the primary reaction mixture. The reaction was initiated with NADPH, and aliquots (40 μl) of the mixtures containing 0.1 μM CYP2B6 were transferred at 0, 3, and 9 minutes to the secondary incubation mixture for the determination of bupropion hydroxylase activities of CYP2B6. Control incubations were performed in the absence of IMP or ticlopidine in parallel.

Effects of GSH and Catalase/Superoxide Dismutase on the Enzyme Inactivation. GSH (2.0 mM) was included in the primary reaction mixture containing CYP2B6 (0.1 μM), IMP (1.5 μM), and NADPH (1.0 mM). Aliquots (40 μl) were transferred to the secondary incubation mixture to determine the remaining enzyme activities at various time points. An equal volume of phosphate buffer was added instead of GSH solution as a control sample. In a separate study, CYP2B6 was incubated with IMP and NADPH in the presence or absence of a mixture of catalase and superoxide dismutase (SOD; 800 unit/ml each). After incubation for 0, 3, and 9 minutes, the residual activities were then detected as described below.

Partition Ratio. CYP2B6 (0.1 μM) was mixed with IMP at concentrations of 0 (control), 0.2, 0.5, 1.0, 1.5, 2.5, 5.0, 7.5, and 10 μM. The primary reactions were initiated by adding NADPH at a final concentration of 1.0 mM. Negative control incubations were performed in the absence of NADPH. After incubation for 9 minutes in a water bath shaker at 30°C, aliquots (40 μl) were transferred from the primary reaction mixtures to the secondary reaction mixtures for measurement of CYP2B6 activities as follows:

Irreversibility of Inactivation. Primary incubations containing 2.5 μM IMP and 0.1 μM CYP2B6 were performed in the presence of NADPH at 30°C. The control incubations lacked IMP. Aliquots of the mixtures were withdrawn partly at 0 minutes for enzymatic activity determination (nondialyzed sample). At 9-minute incubation, aliquots of the control and inactivated samples were dialyzed at 4°C against 1-M potassium phosphate buffer (pH 7.4, 3 x 2 hours). The dialyzed samples were brought to room temperature, and the enzyme activities of the resulting samples were assessed as described below.

CYP2B6 Assay. CYP2B6 activity was assessed by measuring the formation of hydroxybupropion analyzed by LC-MS/MS. The LC-MS/MS system consists of AB Sciex Instruments 4000 QTRAP MS (Applied Biosystems, Foster City, CA) interfaced online with an expektor ultraLC 100 system (Applied Biosystems). The chromatographic separations were performed on an AccuBond column (2.1 x 50 mm, 2.6 μm; Thermo Fisher, Pittsburgh, PA). The mobile phase 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 maintained at 0.3 ml/min and the column temperature was operated at 30°C. The gradient elution was set as follows: 0–1.0 minutes, 10% mobile phase A; 1.0–1.5 minutes, 10–30% mobile phase A; 4.0–5.5 minutes, 30–10% mobile phase A; 5.5–8.0 minutes, 10% mobile phase A. Quantification was performed by multiple-reaction monitoring, and ion pairs of 256→246.3 for IMP-GSH conjugates; 260.7→256 for progesterone (internal standard) were acquired in the positive mode.

Reactive Intermediate Trapping by GSH. IMP (100 μM) and GSH (1.0 mM) were incubated with rat liver microsomes (1.0 mg protein/ml) or individual human recombinant P450 enzymes, including CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, or CYP3A5 (0.1 μM for each) in the presence or absence of NADPH (1.0 mM) at 37°C. To maximize the quantity of the reactive intermediate to be formed, the enzyme incubations were conducted for 60 minutes. The resulting reactions were quenched by mixing with equal volume ice-cold acetonitrile containing hexyl glutathione as the internal standard, followed by vortexing and centrifuging. The supernatants were harvested and evaporated to dryness. The residue was reconstituted with 50 μl of 50% acetonitrile in water and analyzed by LC-MS/MS. The separation of GSH conjugates was achieved on an Agilent C18 column (4.6 x 150 mm, 5 μm; Thermo Fisher). The solvent system consisted of mobile phase A (acetonitrile with 0.1% formic acid) and mobile phase B (water with 0.1% formic acid), and the flow rate was set at 0.8 ml/min. The gradient elution was set as follows: 0–2.0 minutes, 10% mobile phase A; 2.0–8.0 minutes, 10–90% mobile phase A; 8.0–10 minutes, 90% mobile phase A; 10–13 minutes, 90–10% mobile phase A; and 13–15 minutes, 10% mobile phase A. The GSH conjugates were monitored in positive multiple-reaction monitoring mode (594.0→465.0 for IMP-GSH conjugates; 392.2→246.3 for hexyl glutathione as the internal standard). The information-dependent acquisition standard was followed by triggering the enhanced product ion (EPI) scans. Information-dependent acquisition was used to initiate acquisition of EPI spectra for ions exceeding 500 cps with exclusion of former target ions after three occurrences for 10 seconds. The EPI scan was run in positive mode at a scan range for product ions from m/z 50 to 650. The EPI scanning parameters are listed as follows: scan mode = profile; step size = 0.08 Da; and scan rate = 1000 Da/s (5-millisecond pause between mass ranges).

Synthesis of IMP-GSH Conjugates. IMP (2.5 mg) was dissolved in acetone (200 μl) and mixed with saturated sodium bicarbonate solution (40 μl) and oxone (6.5 mg). The mixture was stirred for 30 minutes at room temperature, followed by addition of GSH (35 mg). The mixture was further stirred for 1 hour at room temperature and centrifuged. The supernatant was divided into two equal portions; one was directly submitted to LC-MS/MS analysis and the other portion of the supernatant was mixed with sodium borohydride (10 mg, NaBH₄). The resulting mixture was gently vortexed for 5 minutes, followed by LC-MS/MS analysis.

Results

Time-, Concentration-, and NADPH-Dependent Inactivation of CYP2B6 by IMP. The remaining CYP2B6 activity was monitored by measuring the amount of hydroxybupropion produced. As shown in Fig. 1A, IMP produced a time- and concentration-dependent inhibition on CYP2B6. The residual enzymatic activities at 0 minutes were normalized to 100% at each concentration. The inactivation of CYP2B6 increased progressively with increasing time and concentrations of IMP applied. About 70% of activity of CYP2B6 was suppressed after its incubation with 1.5 μM IMP for 9 minutes at 30°C. Nevertheless, no loss of enzyme activity was observed in the absence of IMP or NADPH (Fig. 1C). A double-reciprocal plot (Wilson’s plot) of the observed rates of inactivation (kobs) and IMP concentrations was used to calculate the kinetic constants K1 and kinaact (Fig. 1B), where K1 was found to be 0.498 μM, and kinaact was found to be 0.079 min⁻¹.

Substrate Protection. Substrate protection from IMP-dependent inactivation of CYP2B6 was evaluated by including ticlopidine, a substrate of CYP2B6 (Talakad et al., 2011), in the primary incubations that contained CYP2B6 and IMP. The presence of ticlopidine was found to slow down the CYP2B6 inactivation induced by IMP (Fig. 2). In addition, the protective effect of ticlopidine increased with the increase in the concentrations of ticlopidine applied.

Effects of GSH and Catalase/Superoxide Dismutase on the Enzyme Inactivation. To evaluate the protective effect of GSH against the enzymatic inactivation induced by IMP, CYP2B6 inactivation incubation
was conducted in the presence or absence of GSH (2 mM), an electrophile trapping agent. After incubation for 9 minutes, the remaining CYP2B6 activities were 27.4 ± 2.0% (with GSH) and 29.9 ± 2.4% (without GSH). This agent produced little protective effect on the enzyme from inactivation. Additionally, a mixture of catalase and SOD, scavengers of reactive oxygen species, showed little protection against the inactivation of CYP2B6 by IMP, and the remaining enzyme activities were 29.7 ± 1.0% (with catalase/SOD) and 29.9 ± 2.4% (without catalase/SOD) at 9 minutes.

**Partition Ratio.** The partition ratio \( (P) \), a measure of the efficiency of an enzyme inactivator, was estimated by a plot of the percentage remaining activity versus the IMP/CYP2B6 molar ratio (Fig. 3), according to a previously published method (Silverman, 1996). The turnover number \( (P + 1) \) was about 5, and the extrapolated partition ratio of IMP was approximately 4 (Fig. 3).

**Irreversibility of Inactivation.** CYP2B6 was incubated with IMP (2.5 μM) in the presence of NADPH, followed by 6-hour dialysis (3 × 2 hours). The remaining activities of the control samples and those treated with IMP were determined before and after dialysis. Only 10% of CYP2B6 activity was recovered after dialysis.

**Reactive Metabolite Trapping by GSH.** GSH was included in the IMP incubation systems containing rat liver microsomes or recombinant P450 enzymes to trap the reactive metabolites. The samples obtained from the incubations in the presence or absence of NADPH were analyzed by LC-MS/MS. A peak with protonated molecule ion...
[M + H]+ at m/z 594 at retention time of 6.8 minutes was observed (Fig. 4D); however, no such peak was found in the control sample (absence of NADPH). The MS/MS spectrum of the conjugate was obtained by multiple-reaction monitoring/EPI scanning (ion transition m/z 594/465). The spectrum showed the indicative characteristic fragment ions associated with the cleavage of the GSH moiety (Fig. 4B). The product ions at m/z 465 and 519 were derived from the neutral loss of γ-glutamyl portion (−129 Da) and glycine portion (−75 Da) from parent ion m/z 594, respectively.

To verify the characterization, IMP was chemically oxidized by oxone in acetone, followed by reaction with GSH. As expected, the product responsible for the IMP-derived GSH conjugate showed the same chromatographic behaviors and identical mass spectrum (Fig. 4C) as that of the conjugate generated in the microsomal reactions (Fig. 4B). Unfortunately, we were unable to obtain enough of the product for NMR characterization due to poor reaction yield. Furthermore, the other portion of the reaction solution was mixed with sodium borohydride for reduction and then analyzed by LC-MS/MS. A new peak at retention time of 6.7 minutes was observed (Fig. 5A), and the [M + H]+ was m/z 596.2, two Daltons higher than that of the conjugate observed before the reduction by sodium borohydride (Fig. 5B).

**P450 Enzymes Responsible for IMP Bioactivation.** To determine which P450 enzymes preferentially catalyze the oxidation of IMP, IMP was incubated with individual recombinant human P450 enzymes, including CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, or CYP3A5 (0.1 μM for each) in the presence of GSH as the trapping agent. The IMP-GSH conjugate was assessed in the incubation with CYP1A2, CYP2A6, CYP2B6, CYP2D6, CYP2E1, CYP3A4, or CYP3A5. Apparently, little IMP-

![Fig. 5.](https://example.com/fig5.png) (A) Extracted ion (m/z 596 → 467) chromatogram obtained from LC QTRAP MS analysis of the chemical oxidation reaction followed by reaction with NaBH₄. (B) MS/MS spectrum of the reduced product.
GSH adduct was found in the incubations with CYP2C9 or CYP2C19 (Fig. 6). The experiments demonstrated that multiple P450 enzymes are involved in bioactivation of IMP, especially for CYP1A2.

Discussion

Our kinetic study clearly demonstrated that IMP produced a time-dependent inhibition of CYP2B6. The observed enzyme inactivation was also found to be concentration dependent, and it reached about 70% inactivation at the concentration of 2.5 μM. CYP2B6 inactivation induced by IMP was not observed unless NADPH was present in the primary incubation mixtures. This indicates that IMP itself is not an inactivator of CYP2B6 and that the enzyme inactivation by IMP needs biotransformation with assistance from P450 cofactor NADPH. Taken together, these results provided strong evidence that IMP is a mechanism-based inactivator of CYP2B6.

Little protection by catalase and SOD against enzyme inactivation by IMP was observed. Catalase and SOD are known as enzymes quenching hydrogen peroxide, superoxide anion, and other reactive oxygen species that are possible agents to inactivate enzymes (Klaassen et al., 1986). The observed lacking of protection effect by catalase and SOD indicates that reactive oxygen species may not play a significant role in CYP2B6 inactivation induced by IMP. Additionally, GSH, a nucleophilic agent, did not show protective effect on CYP2B6 inactivation by IMP. This implies in part that CYP2B6 is covalently modified by electrophilic metabolites of IMP before escaping from the active site. The protective effect of ticlopidine on CYP2B6 inactivation observed in the competition experiment indicates the competition between ticlopidine and IMP to reach the active site of the enzyme, which slows down the generation of the reactive metabolites of IMP. This critical finding provided evidence that bioactivation of IMP took place in the active site of CYP2B6.

The P value is defined as the number of product molecules produced per inactivation event and reflects the efficiency of the inactivator. The P values reported in the literature for mechanism-based inactivators of P450 enzymes vary from 2 (very highly efficient inactivators) to >1000 (inefficient) (Kent et al., 2001). Bergamottin, a furanocoumarin compound, was an efficient mechanism-based inactivator of CYP2B6 and CYP3A5 with partition ratios of approximately 2 for CYP2B6 and 20 for CYP3A5 (Lin et al., 2005). Therefore, IMP (P = 4) may be ranked as an efficient inactivator of CYP2B6.

At time 0 minutes, a decrease in enzyme activity was observed with the increase of applied IMP concentrations. The observed enzyme inhibition may result from reversible inhibition at the beginning of the incubation.

Generation of reactive metabolites is a key step for mechanism-based enzyme inactivation. We hypothesized that IMP is metabolized to furanooxepoxide intermediate 2 (Scheme 1) and/or γ-ketoenal intermediate 3, and that γ-ketoenal is also suggested to be generated by rearrangement of the furanooxepoxide. To probe the hypothesis, we searched for the electrophilic intermediates using GSH as a trapping agent. An IMP-derived GSH conjugate in microsomal incubations

![Diagram](image-url)
with IMP was detected by LC-MS/MS (Fig. 4D). The detected protonated molecular ion [M + H]^+ matched the molecular weight of GSH conjugates 4–6 (Scheme 1). The MS/MS spectrum of the metabolite showed the indicative characteristic fragments of GSH and some other fragments valuable for metabolite identification (Fig. 4B). A fragment at m/z 162 responsible for the molecular formula of C9H6O3 was detected, indicating that the five-member ring fused with the coumarin ring was opened (Scheme 1). This allowed us to exclude the formation of GSH conjugate 4, since the ring opening of conjugate 4 would produce a thio-ester. Only GSH conjugate 6 had the ring opening structure, and conjugate 5 could be spontaneously converted to conjugate 6. To further characterize conjugate 6, we chemically reduced the conjugate with NaBH4. The reduction reaction afforded a new product with retention time at 6.7 minutes and [M + H]^+ at m/z 596.2 (Fig. 5A), 2.0 Da higher than that of the one detected before the reduction reaction. The MS/MS spectrum of the reduced GSH conjugate demonstrated that the fragment at m/z 162 was retained, indicating that no reduction took place in the coumarin ring. In addition, the original IMP-GSH conjugate showed a fragment at m/z 177 that resulted from the elemental composition of C9H6O3 of the conjugate losing the side chain, GSH, and CO2. In other words, the fragment of m/z 177 contained the aldehyde group (Fig. 4B). However, no such fragment was observed in the MS/MS spectrum of the reduced IMP-GSH conjugate (Fig. 5B). Instead, the reduced IMP-GSH conjugate showed a fragment of m/z 179, 2 Da higher than that (m/z 177) observed in the MS/MS spectrum of the original IMP-GSH conjugate (Fig. 5B). This provided evidence that the reduction took place on the aldehyde not on the lactone group. An additional experiment showed that IMP was resistant to reduction by NaNBH4 (data not shown). We anticipate that the NaNBH4-based reduction of conjugate 6 gave conjugate 7. The observed conjugate 7 indicates that conjugate 6 was the primary GSH conjugate resulting from metabolic activation of IMP. However, we are unable to tell whether conjugate 6 came from intermediate 2 or 3, since conjugate 5 may be spontaneously converted to conjugate 6, and vice versa. In other words, whether intermediate 2 or 3 was the primary metabolic intermediate remains unknown. We speculate that IMP was oxidized to the corresponding γ-ketoenal/epoxide (on the furan rings), which chemically attacked the host enzyme to form covalent binding, and the enzyme modification initiated the process of the enzyme inactivation.

The bioactivation studies with individual recombinant enzymes demonstrated that multiple P450 enzymes catalyzed the metabolism of IMP to the reactive intermediate (Fig. 6). CYP1A2 was found to be the major enzyme responsible for the bioactivation of IMP. Whether IMP is a mechanism-based inactivator of CYP1A2 is under investigation. In summary, we have shown that IMP is characterized as a mechanism-based inactivator of CYP2B6. A GSH conjugate derived from a γ-ketoenal intermediate was identified in microsomal incubations with IMP trapped with GSH.

Authorship Contributions

References


Correction to: “Imperatorin Is a Mechanism-Based Inactivator of CYP2B6”

In the above article [Zheng L, Cao J, Lu D, Ji L, Peng Y, and Zheng J (2015) Drug Metab Dispos 43:82–88] the correct value of $k_{\text{inact}}$ is 0.18 min$^{-1}$ ($0.079 \times 2.3$), not 0.079 min$^{-1}$, after conversion from log$_{10}$ to the natural logarithm of the data.

The Full Text article is corrected with the issue of this erratum.

The authors apologize for any inconvenience this may have caused.