RESEARCH ARTICLE

Searching the Cytochrome P450 Enzymes for the Metabolism of Meranzin Hydrate: A Prospective Antidepressant Originating from Chaihu-Shugan-San

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Abstract

Meranzin hydrate (MH), an absorbed bioactive compound from the Traditional Chinese Medicine (TCM) Chaihu-Shugan-San (CSS), was first isolated in our laboratory and was found to possess anti-depression activity. However, the role of cytochrome P450s (CYPs) in the metabolism of MH was unclear. In this study, we screened the CYPs for the metabolism of MH *in vitro* by human liver microsomes (HLMs) or human recombinant CYPs. MH inhibited the enzyme activities of CYP1A2 and CYP2C19 in a concentration-dependent manner in the HLMs. The K_m and V_{max} values of MH were 10.3 ± 1.3 μM and 99.1 ± 3.3 nmol/mg protein/min, respectively, for the HLMs; 8.0 ± 1.6 μM and 112.4 ± 5.7 nmol/nmol P450/min, respectively, for CYP1A2; and 25.9 ± 6.6 μM and 134.3 ± 12.4 nmol/nmol P450/min, respectively, for CYP2C19. Other human CYP isoforms including CYP2A6, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 showed minimal or no effect on MH metabolism. The results suggested that MH was simultaneously a substrate and an inhibitor of CYP1A2 and CYP2C9, and MH had the potential to perpetrate drug-drug interactions with other CYP1A2 and CYP2C19 substrates.

Introduction

Depression is a severe and recurrent mental disorder that often leads to a significant impairment of daily functions [1–3]. Antidepressants such as selective serotonin reuptake inhibitors (SSRIs) were commonly used to treat depression [4, 5]. However, adverse drug reactions (ADRs) to these drugs were difficult to predict and frequently interfered with the SSRI treatment, leading to treatment failure [6–8]. Antidepressants were facing weak prospects, as several pharmaceutical companies had been forced to withdraw their investment on the research of new antidepressants because of the noncompliance and discontinuation of the present SSRIs owing to ADRs [9, 10].

Common antidepressants were discontinued because they inhibited gastrointestinal kinetics [11, 12]. In contrast, meranzin hydrate (MH), an absorbed bioactive compound originating from the traditional Chinese medicine (TCM) Chaihu-Shugan-San (CSS) [13, 14], was considered a prospective candidate to treat depression because of its gastrointestinal prokinetic properties [15–17]. Xie *et al* reported that MH could increase the amplitude of contractility in the longitudinal and circular jejunum muscles in a dose-dependent manner [15, 16]. Furthermore, Huang *et al* reported that MH significantly accelerated the gastric emptying and intestinal transit in rats [17]. Therefore, MH was considered to be a prospective antidepressant better than other antidepressants.

Although MH showed anti-depression and gastrointestinal prokinetic effects in animal experiments, several questions were unanswered. In addition to the therapeutic effect, the safety of MH was one of the primary concerns. According to various reports, drug interactions could lead to serious adverse events or decreased drug efficacy. These interactions might occur through the inhibition or induction of hepatic and intestinal drug-metabolizing enzymes (e.g., CYPs) and transporters (e.g., p-glycoprotein) [18, 19]. CYP-mediated drug interactions was a major concern because CYP enzymes are involved in the phase metabolism of more than 70% of prescription drugs [20], and to the best of our knowledge, there was little published information about the metabolism of MH. In this study, we examined the CYP enzymes responsible for the metabolism of MH and the potential interactions of MH with typical substrates of the CYP enzymes *in vitro*.

Methods

Enzymes and chemicals

Recombinant human CYP enzymes (Bactosomes), pooled human liver microsomes (HLMs) from ten individual donors (Bactosomes) and NADPH were purchased from Cypex Ltd. (U.K.) and stored at $-80\,^{\circ}\text{C}$ until use. Meranzin hydrate ($C_{15}H_{18}O_5$, MW: 278.30, assay$\geqslant$98%) was obtained from Sigma-Aldrich (Shanghai, China). Furafylline, trans-2-phenylcyclopropylamine hydrochloride, ketoconazole, sulfaphenazole, quinidine, chlormethiazole hydrochloride and ticlopidine hydrochloride were purchased from the National Institutes for Food

and Drug Control (Beijing, China). Phenacetin, coumarin, midazolam, tolbuta-mide, S-Mephenytoin, metoprolol, chlorzoxazone and the standards for their metabolites, including acetaminophen, 7-hydroxyl coumarin, 1-hydroxyl mid-azolam, 4-hydroxyl tolbutamide, 4-hydroxyl mephenytoin, α-hydroxyl metopro-lol, 6-hydroxyl chlorzoxazone and irbesartan (the internal standard) were purchased from Sigma-Aldrich (Shanghai, China). All other chemicals and solvents were of high-performance liquid chromatography (HPLC) grade.

Apparatus and operation conditions

The concentrations of the CYP substrates and their metabolites were quantified using a Waters 2695 separation module HPLC system (Waters Corp., Milford, Massachusetts, USA) coupled to a Quattro micro API triple quadrupole tandem mass spectrometer (Waters Corp., Milford, Massachusetts, USA) with an electrospray ionization source. The samples were separated on a HyPURITY C_{18} column (150 mm × 2.1 mm, 5 μm, Thermo, USA) with a C_{18} security guard column (4.0 mm × 3.0 mm, ID 5 μm). The mobile phases consisted of 20 mM ammonium formate and acetonitrile at a ratio of 60:40. Aliquots of 20 μL were injected at a mobile phase flow rate of 0.3 mL/min. Multiple reaction monitoring was performed in the positive mode. The transitions are listed in table 1. The mass spectra of the metabolites formed in the incubations were identical to those of the corresponding authentic standards.

General incubation conditions

The CYP isoform-specific probe reactions used were phenacetin O-deethylation (for CYP1A2), coumarin 7-hydroxylation (for CYP2A6), tolbutamide 4-hydro-xylation (for CYP2C9), metoprolol α-hydroxylation (for CYP2D6), chlorzox-azone 6-hydroxylation (for CYP2E1), S-Mephenytoin 4-hydroxylation (for CYP2C19) and midazolam 1-hydroxylation (for CYP3A4). The kinetic study of MH was studied with HLMs, an incubation mixture that consisted of MH (as a substrate), the HLMs (0.5 mg/mL) or CYP isoforms (10 pmol) and 0.1 M sodium phosphate buffer (pH 7.4) in a total volume of 0.2 mL was pre-warmed for 5 min at 37°C without (control). The inhibitory effects of MH on the activities of seven different CYP isoforms were studied with the HLMs (and the expressed CYPs, when required), multiple concentrations of MH (as an inhibitor) were included in the above incubation mixture system. The reaction was initiated by the addition of 1 mg/mL triphosphopyridine nucleotide (NADPH). The final incubations were performed in a shaking water bath (37°C) for 30 min. The incubations were performed in triplicates, and the incubation conditions specific to each CYP isoform were within the linear range for the velocity of the reaction (the incubation time as well as the substrate and protein concentrations). All the reagents were dissolved in methanol, and the final solvent concentration in all incubations (including controls) was 1%. The reactions were stopped by adding 0.2 mL ice-cold acetonitrile containing irbesartan (114.9 ng/mL) as the internal

Table 1. Transitions and collision energies used in LC-MS/MS for the detection of meranzin hydrate, the probe substrates, metabolites and the internal standard.

Compound name	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
Meranzin hydrate	279.1	188.9	15
Acetaminophen	152	110	15
7-hydroxyl coumarin	162.9	107.0	20
1-hydroxyl midazolam	342	324	20
4-hydroxyl tolbutamide	287.0	171.0	15
4-hydroxyl mephenytoin	235.0	150.0	10
α-hydroxyl metoprolol	284.3	116.0	20
6-hydroxyl chlorzoxazone	195	138	20
Irbesartan (IS)	429.0	206.9	22

standard. The samples were vortexed for 5 min. After centrifugation ($12000 \times g$ for 10 min), the supernatants were transferred and aliquots of 20 µL were injected into the HPLC-MS/MS system for analysis.

Kinetic analysis of MH

Kinetic analysis was performed for MH, and the data generated were used as a guide for selecting the appropriate concentrations of MH in the subsequent inhibition experiments. Thus, the kinetic parameters for the metabolism of MH was determined by incubating increasing concentrations of MH (0.5–100 µM) (without the inhibitor) at 37℃ with the HLMs and NADPH under the incubation conditions. The equation of MH reaction velocity (V) in the HLMs or CYP isoforms was expressed as $V = (C_0 - C_t)/\text{T}/C_p$, where C_0 and C_t represented the initial and final concentrations of MH in the incubation solution, respectively. T was the incubation time (min) and Cp was the protein concentration (mg/mL or nmol). All values were expressed as the mean ± standard deviation (SD). The mean intrinsic clearance rate (CL_{int}) for the *in vitro* incubation was estimated using V_{max}/K_m.

Specific CYP isoforms screened for the metabolism of MH

To screen the specific CYP isoform responsible for the MH metabolism, we determined the inhibitory effect of specific inhibitors on the metabolism of MH in the HLMs. Inhibitors including furafylline (FUR, inhibitor for CYP1A2), trans-2-phenylcyclopropylamine (TRA, for CYP2A6, 1 µM), sulfaphenazole (SUL, for CYP2C9, 1 µM), quinidine (QUI, for CYP2D6, 1 µM), chlormethiazole (CHL, for CYP2E1, 5 µM), ticlopidine (TIC, for CYP2C19, 1 µM) and ketoconazole (KET, for CYP3A4, 1 µM) were separately incubated with MH (10 µM), the HLMs and NADPH under the same incubation conditions as mentioned above. The concentrations of the inhibitors used were approximately at their respective IC_{50} values from previous reports [21]. The inhibitory effects of the above specific inhibitors on the metabolic clearance rate of MH were evaluated separately to

screen the CYP isoforms responsible for the MH metabolism. The relative activity of the CYP isoforms was calculated by dividing the peak area of MH when incubated with the inhibitor with that of MH from the negative controls.

Inhibition studies for IC_{50} determination

A pilot inhibitory analysis of each CYP isoform was performed to determine the potency of inhibition and to select CYP isoforms for further detailed study of their inhibitions. MH (various concentrations of 0.5–100 µM) and a single CYP isoform-specific substrate (concentration at about the respective K_m value) were used to determine the inhibitory effect of MH on specific CYP isoforms. Substrates including phenacetin, coumarin, tolbutamide, metoprolol, chlorzoxazone, S-Mephenytoin and midazolam were employed at concentrations of 10, 5, 100, 7.5, 40, 100 and 5 µM, respectively [21]. All incubation conditions were the same as mentioned above. The inhibitory effects on the CYP isoforms were investigated individually by incubating the HLMs in the absence or presence of MH. Incubation solution with the solvent that was used to dissolve MH was regarded as the negative control, whereas solutions containing the specific inhibitors mentioned above were regarded as the positive controls. The IC_{50} values of MH were determined and compared with those of the specific inhibitors mentioned above (see Table 2).

Determination of K_i

In pilot experiments (IC_{50} determination), we noted that MH markedly inhibits CYP1A2 and CYP2C19, whereas its effect on the remaining CYPs (CYP2A6, CYP2C9, CYP2D6, CYP2E1 and CYP3A4) was minimal. Therefore, Dixon plots for the inhibition of CYP1A2 and CYP2C19 were determined by incubating the substrate probe at multiple concentrations with or without the test inhibitor at

Table 2. IC_{50} and K_i values of MH against human CYP isoforms compared with that of specific inhibitors reported in literature.

CYP	Activity	IC_{50} (µM)		K_i (µM)	
		MH	Specific inhibitor/reported values[a]	MH	Specific inhibitor/reported values[a]
CYP1A2	Phenacetin O-deethylation	4.47(3.10,6.23)[b]	FUR/1.4 [27]	4.56	FUR/3 [28]
CYP2A6	coumarin 7-hydroxylation	>100	TRA/0.42±0.07 [29]	-	TRA/0.17 [29]
CYP2C9	tolbutamide 4-hydroxylation	>100	SUL/0.3–1.5 [27, 30]	-	SUL/0.3 [28]
CYP2D6	metoprolol α-hydroxylation	>100	QUI/0.02–0.68 [27, 30]	-	SUL/0.027–0.4 [28, 31, 32]
CYP2E1	chlorzoxazone 6-hydroxylation	>100	DIE/21.30 [27]	-	CHL/12 [33]
CYP2C19	S-Mephenytoin 4-hydroxylation	10.91(8.84,13.45)[b]	TCL/0.52–1.6 [30]	42.65	TCL/1.2±0.5 [28]
CYP3A4	midazolam 1-hydroxylation	>100	KET/0.08–0.24 [28]	–	KET/0.015 [28]

[a]IC_{50} and K_i values of specific inhibitors were referred to the reported literatures. [b] represents 95% confidence interval. "–"represents the data that is not calculated. FUR, furafylline; TRA, trans-2-phenylcyclopropylamine hydrochloride; SUL, sulfaphenazole; QUI, quinidine; CHL, chlormethiazole hydrochloride; TIC, ticlopidine hydrochloride; KET, ketoconazole; DIE: diethyldithiocarbamate.

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multiple concentrations with the HLMs and cofactors. The inhibition data obtained from the pilot experiments were used as a guide to generate appropriate probe substrate and test inhibitor concentrations for the determination of the K_i values for each CYP isoform. The isoform-specific probe substrate concentrations used were 3 to 100 μM phenacetin for CYP1A2 and 15 to 120 μM S-mephenytoin for CYP2C19. The MH concentrations used were 0 to 100 μM.

Calculations of enzyme kinetics and statistical method

To determine the major enzymes responsible for MH metabolism in HLMs, the metabolic clearance rate of the incubation solution without any specific inhibitor for MH was considered as 100%. The effects of the specific inhibitors on the metabolic clearance rate of MH were evaluated with SPSS one-way analysis of variance (SPSS Inc., Chicago, IL, USA). $P < 0.05$ denoted significance in all cases. The apparent kinetic parameters of MH (K_m and V_{max}) were determined by fitting the Michaelis-Menten equation using the program GraphPad Prism Enzyme Kinetic 5 Demo (GraphPad Co. Ltd, San Diego, CA, USA). The equation was expressed as $V = V_{max}[S]/(K_m + [S])$. K_m is the substrate concentration at which the reaction velocity is 50% of V_{max}. To determine the inhibition of CYP isoforms, the activity of each CYP isoform was calculated using the metabolic clearance rate of its corresponding probe substrate. The metabolic clearance rate of the probe substrate was considered to be 100% when no specific inhibitor and MH were added in the incubation assay. The $IC_{50}s$ were determined by analyzing the plot of the logarithm of the inhibitor concentration versus the percentage of activity remaining after inhibition, using the SPSS software for Windows (version 11.5, SPSS, Chicago, IL). To calculate the K_i values, the inhibition data were fit to different models of enzyme inhibition (competitive, noncompetitive, and uncompetitive) by nonlinear least-squares regression analysis using the GraphPad Prism 5 software (GraphPad Co. Ltd).

Results

Kinetic analysis of MH

Fig. 1. A, B and C show the metabolism of MH after incubation with the HLMs, CYP1A2 and CYP2C19. The kinetic plots indicated that the K_m and V_{max} values were 10.3 ± 1.3 μM and 99.1 ± 3.3 nmol/mg protein/min for the HLMs, 8.0 ± 1.6 μM and 112.4 ± 5.7 nmol/nmol P450/min for CYP1A2, 25.9 ± 6.6 μM and 134.3 ± 12.4 nmol/nmol P450/min for CYP2C19, respectively. The *in vitro* CL_{int} values of MH in the HLMs, CYP1A2 and CYP2C19 were 9.7 mL/mg protein$^{-1} \cdot$min^{-1}, 14.1 mL/nmol P450$^{-1} \cdot$min^{-1} and 5.2 mL/nmol P450$^{-1} \cdot$min^{-1}, respectively.
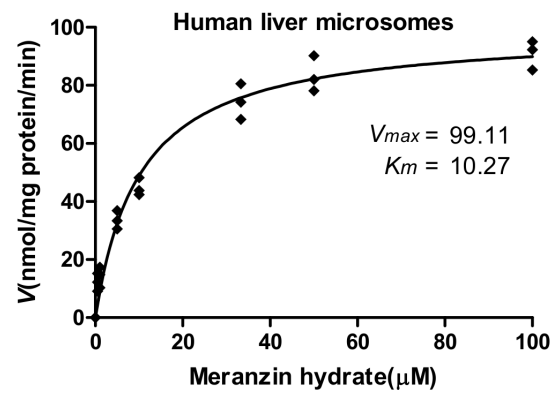
(A)
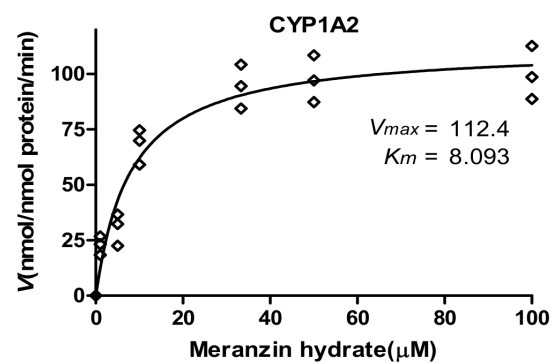
(B)
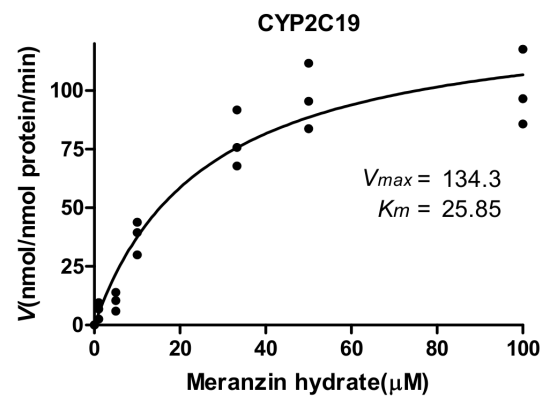
(C)

Figure 1. The concentration-velocity curve for meranzin hydrate metabolism after incubation with HLMs (A), recombinant CYP1A2 (B) and recombinant CYP2C19 (C). Note: The incubation conditions are described in the materials and methods section. The curve was automatically fitted using nonlinear regression and Michaelis-Menten equation, the data were obtained in triplicates.
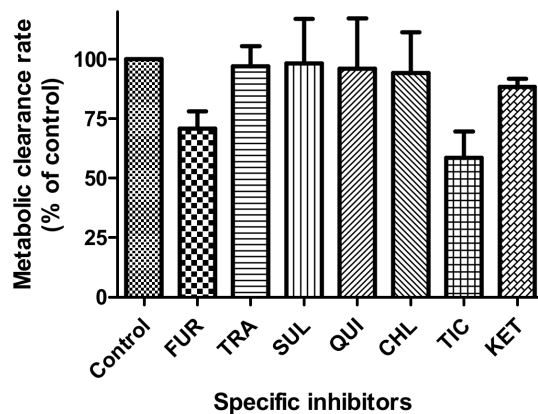
Figure 2. Effect of specific inhibitors on CYP-mediated MH (10 μM) metabolism in HLMs. Note: The concentrations of CHL was 5 μM and that for the other specific inhibitors was 1 μM. The incubation conditions are described in the materials and methods section. Each data point represents the average of triple determinations and error bars (n=3). FUR, furafylline, the specific inhibitor for CYP1A2; TRA, trans-2-phenylcyclopropylamine, the specific inhibitor for CYP2A6; SUL, sulfaphenazole, the specific inhibitor for CYP2C9; QUI, quinidine, the specific inhibitor for CYP2D6; CLM, chlormethiazole, the specific inhibitor for CYP2E1; TIC, ticlopidine, the specific inhibitor for CYP2C19; and KET, ketoconazole, the specific inhibitor for CYP3A4. In the presence of FUR (1 μM) and TIC (1 μM), the metabolic clearance rate of MH decreased to 29.1% and 41.3% of that of the control, respectively, while the other inhibitors had no significant inhibitory effects on the metabolism of MH.

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Specific CYP isoforms for the metabolism of MH

The inhibitory effects of the CYP specific inhibitors on the metabolic clearance rate of MH in the HLMs were shown in Fig. 2. The concentrations of FUR, TRA, SUL, QUI, TIC and KET were 1 μM, except for CHL, which was 5 μM. The concentrations were selected on the basis of previously reported IC_{50} or K_i values for the CYP isoforms to ensure adequate inhibitory selectivity, as well as maximal inhibitory potency. In the presence of FUR (1 μM) and TIC (1 μM), the metabolic clearance rate (MCR) of MH decreased to $29.1 \pm 7.2\%$ and $41.3 \pm 11.1\%$ of that of the control, respectively (Fig. 2). However, other inhibitors had no obvious inhibitory effects on the metabolism of MH. The screened enzymes were further confirmed by human recombinant CYPs by using the specific inhibitors, the MCRs of MH were decreased to 43.5% (MH, 10 μM) and 60.5% (MH, 50 μM) of that of the control for CYP1A2 and to 68.5% (MH, 10 μM) and 80.5% (MH, 50 μM) of that of the control for CYP2C19 (Fig. 3). The results indicated that CYP1A2 and CYP2C19 were possibly the major enzymes responsible for the metabolism of MH *in vitro*.

Estimation of $IC_{50}s$

The inhibitory effects of multiple concentrations of MH (0.5–100 μM) on the activity of each CYP isoform determined by the metabolism of a single concentration of isoform-specific probe were tested with the HLMs (or expressed CYPs, when needed). MH showed potent inhibition of CYP1A2 (phenacetin O-
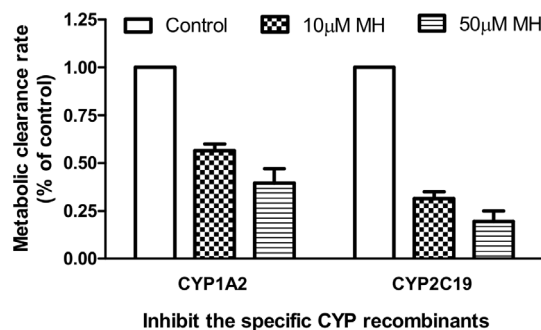
Figure 3. Effect on the metabolic clearance rate (MCR) of meranzin hydrate (MH) under the inhibition of recombinant CYP1A2 and CYP2C19. Note: 10 μM and 50 μM MH were incubated with the CYP recombinants and cofactors in the absence (control) or presence of the inhibitors by furafylline (1 μM) and ticlopidine (1 μM), respectively. Each point represents the average of triplicate incubations. The MCRs of MH were significantly decreased compared with that of the control for CYP1A2 and CYP2C19, for both concentrations of MH.

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deethylation) and CYP2C19 (S-mephenytoin 4-hydroxylation), with IC_{50s} of 4.30 μM and 23.45 μM, respectively. The inhibitory effect of MH on the activity of CYP2A6, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 was negligible (Fig. 4).

Estimation of K_i values

While the $IC_{50}s$ were qualitatively informative and help to address whether inhibition had occurred, their values were of limited use because they could be influenced by the substrate concentration selected, and it might not be accurate to use these parameters for the quantitative prediction of drug interactions *in vivo*.
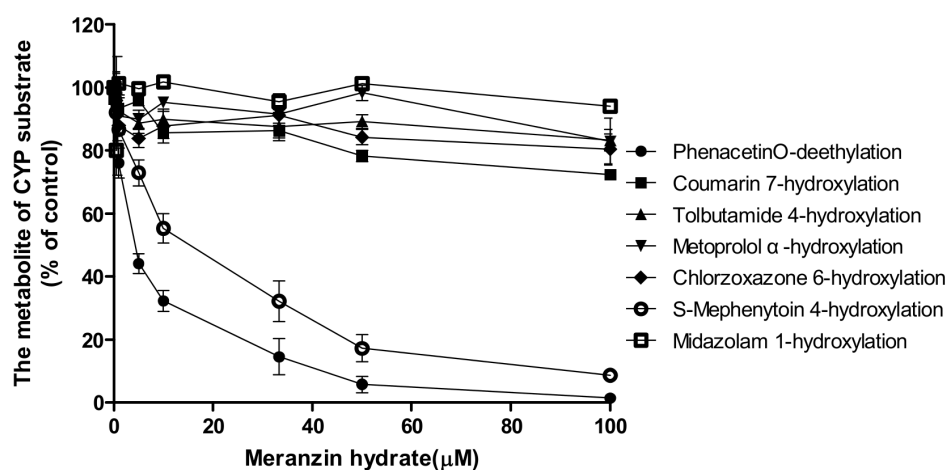
Figure 4. Effect of meranzin hydrate on the metabolic reactions of the seven CYP specific substrates in HLMs. Note: The incubation conditions are described in the materials and methods section. Each data point represents the average of triple determinations and error bars.

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(A)

(B)
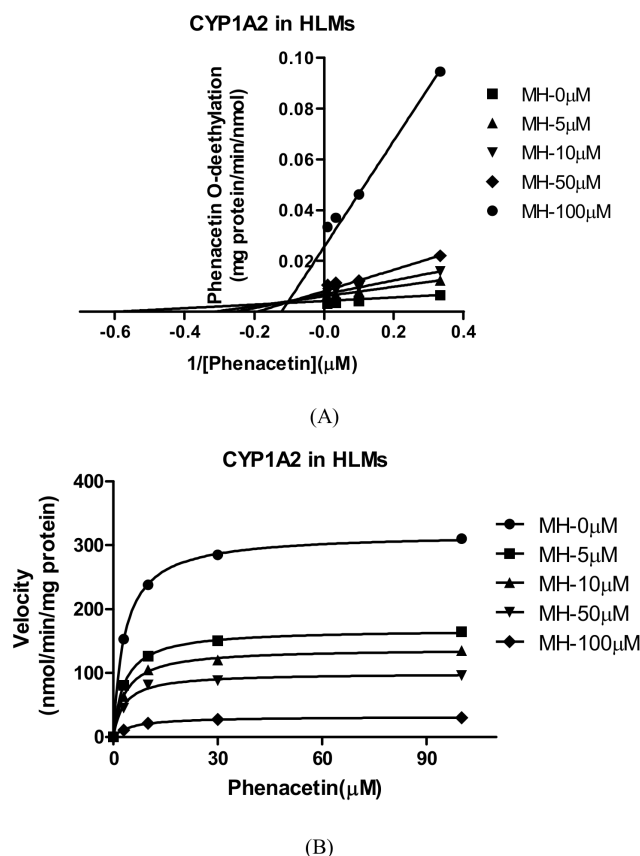
Figure 5. Double reciprocal (Lineweaver-Burk) plots for direct inhibition of phenacetin O-deethylation (A) and K_i values. Note: The inhibition of CYP1A2 activity by MH can be best described as a mixed full inhibition mechanism by different concentrations of MH (0–100 µM) in HLM incubations (0.5 mg·mL^{-1} protein). The data points are mean values of triplicate incubations. Non-linear regression analysis of the phenacetin O-deethylation versus substrate concentration was performed to obtain K_i values.

Therefore, we performed additional experiments designed to estimate the K_i values. The preliminary inhibition data generated from a single probe substrate reaction were used to simulate the appropriate range of substrate and inhibitor concentrations for use in the construction of Dixon plots for the inhibition of the CYP isoforms by MH in the HLMs, from which precise K_i values were estimated.

For CYP1A2, the K_i values were determined using phenacetin as the probe substrate. Of all CYPs tested, CYP1A2 was the most sensitive to the inhibition by MH (Table 2). The representative Dixon plots for the inhibition of CYP1A2 in the HLMs were shown in Fig. 5. Visual inspection of the Dixon plots and further analysis of the parameters of the enzyme inhibition models suggested that the inhibition data fit well to a competitive type of inhibition. The K_i values estimated by using a nonlinear regression model for the competitive enzyme inhibition of CYP1A2-catalyzed phenacetin O-deethylation in the HLMs were less than 5 µM (Table 2, Fig. 6). Fig. 7. Shows the Dixon plots for the inhibition of CYP2C19 by
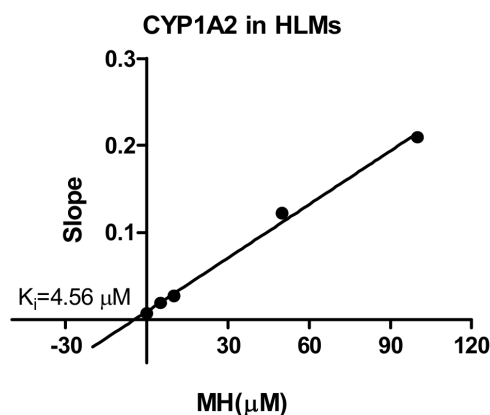
Figure 6. Secondary plot of CYP1A2 activity using the slopes of the primary Lineweaver-Burk plots versus concentrations of MH. Note: The effects of MH on the metabolism of phenacetin O-deethylation in human liver microsomes. Each point represents the mean of triplicate determinations.

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MH in the HLMs. MH inhibited CYP2C19 competitively, with estimated K_i values of 42.65 μM (Table 2, Fig. 8).

Discussion

Depression is an incapacitating psychiatric ailment and widely recognized as one of the three major diseases of the new century, besides cancer and AIDS [22]. Antidepressants are commonly used to treat depression; however, most of them were withdrawn from treatment due to serious side effects [6]. Therefore, new drugs with high efficiency and low toxicity are urgently required for treating depression. MH, an absorbed bioactive compound from Chaihu-Shugan-San (CSS), displayed potent preclinical anti-depression and gastrointestinal prokinetic functions [13, 14], rendering it a prospective candidate to treat depression. However, several questions need to be answered in order for MH to be introduced as a new antidepressant.

The drug interactions of MH were one of the critical questions to be resolved. These interactions might occur through the inhibition or induction of drug-metabolizing enzymes and lead to serious adverse events or decreased drug efficacy [12]. The cytochrome P450 (CYP) enzymes are a large family of drug-metabolizing enzymes that play a critical role in Phase I drug metabolism, and most of the endogenous and exogenous substances are the substrates of CYPs [13]. CYP-mediated drug interactions is a major concern; the inhibition of a CYP enzyme can cause an increase in drug plasma levels via decreased drug metabolism, which could result in significant adverse reactions or toxicities. Therefore, inhibition-based drug interactions are a primary cause of clinically significant drug interactions [14]. The major CYPs involved in the hepatic metabolism of most drugs are CYP1A2, CYP2A6, CYP2C9, CYP2D6, CYP2E1,
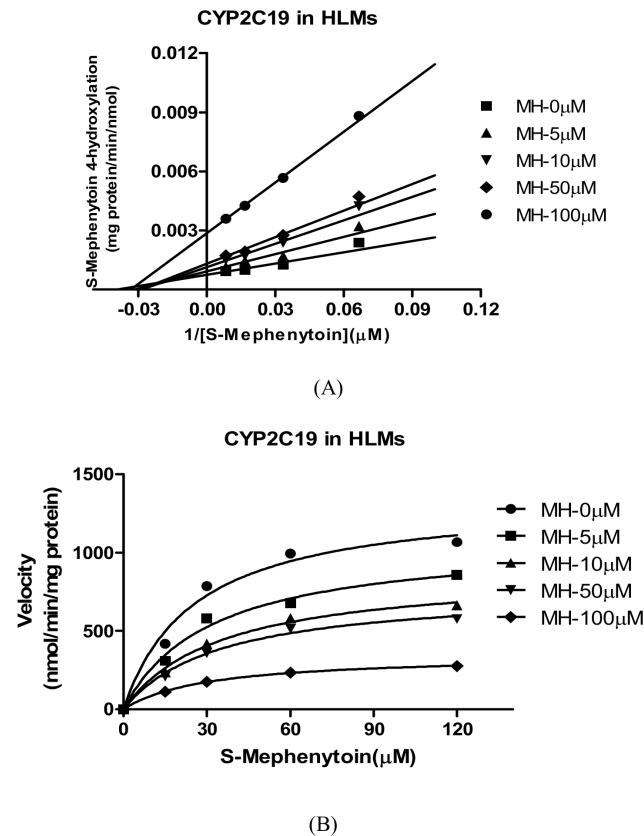
Figure 7. Double reciprocal (Lineweaver-Burk) plots for direct inhibition of S-Mephenytoin 4-hydroxylation (A) and K_i values (B). Note: The inhibition of CYP1A2 activity by MH can be best described as a full competitive inhibition by different concentrations of MH (0–100 μM) in HLM incubations (0.5 mg·mL^{-1} protein). The data points are mean values of triplicate incubations. Non-linear regression analysis of the S-Mephenytoin 4-hydroxylation versus substrate concentration was performed to obtain K_i values.

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CYP2C19 and CYP3A4 [14]; therefore, screening the CYPs for the metabolism of MH could predict its drug interactions.

In the present study, we have shown that MH is a substrate of CYP1A2 and CYP2C19, especially CYP1A2. Our data and findings suggested that MH was unlikely to alter the pharmacokinetics of drugs metabolized by CYP2A6, CYP2C9, CYP2D6, CYP2E1 and CYP3A4. Of the seven CYPs studied, CYP1A2 and CYP2C19 were screened out as participating in the metabolism of MH, by using specific inhibitors on CYP in the HLMs. The MCRs of MH were decreased to 29.1% and 41.3% of that of the control, respectively ([Fig. 2](#)). The results were further confirmed by the recombinant CYP1A2 and CYP2C19 experiment; after inhibiting the activities of the enzymes, the MCRs of MH were significantly decreased to 43.5% (MH, 10 μM) and 60.5% (MH, 50 μM) of that of the control for CYP1A2 and to 68.5% (MH, 10 μM) and 80.5% (MH, 50 μM) of that of the control for CYP2C19 ([Fig. 3](#)). The kinetics study indicated that the K_m and V_{max} values of MH were 10.3 ± 1.3 μM and 99.1 ± 3.3 nmol/mg protein/min for the

CYP2C19 in HLMs
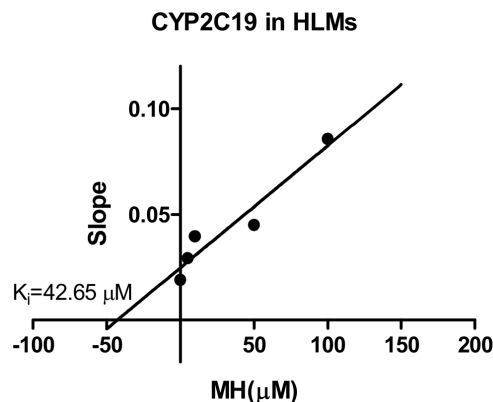
Figure 8. Secondary plot of CYP2C9 activity using the slopes of the primary Lineweaver-Burk plots versus concentrations of MH. Note: illustrating the effects of MH on the metabolism of S-Mephenytoin via 4-hydroxylation in human liver microsomes. Each point represents the mean of triplicate determinations.

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HLMs, 8.0 ± 1.6 μM and 112.4 ± 5.7 nmol/nmol P450/min for CYP1A2 and 25.9 ± 6.6 μM and 134.3 ± 12.4 nmol/nmol P450/min for CYP2C19, respectively (Fig. 1). CYP1A2 and CYP2C19 might be the major enzymes involved in the metabolism of MH *in vitro*.

Our study showed that CYP1A2 was most sensitive to the inhibition by MH ($IC_{50}=4.47$ μM), and CYP2C19 was less sensitive to the inhibition by MH ($IC_{50}=10.91$ μM). CYP2A6, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 were hardly sensitive to the inhibition of MH (IC_{50s} were all more than 100 μM) (Table 2). The IC_{50} value of CYP1A2 for the O-deethylation of phenacetin was 3.2-fold higher than that of FUR in the HLMs. The effect of MH on the O-deethylation of phenacetin metabolism was further assayed using CYP1A2 (10 pmol), by the co-incubation of phenacetin (final concentration of 3, 10, 30 or 100 μM) with MH (final concentrations of 0, 5, 10, 50 and 100 μM) for 30 min. The K_i values were evaluated using the Dixon plot method (Fig. 5). The results showed that MH, at concentrations lower than 100 μM, significantly inhibited the CYP1A2-catalysed O-deethylation of phenacetin with $K_i=4.56$ μM, which was approximately 1.5-fold higher than that of FUR in the HLMs. The type of competition appears to be competitive inhibition. Fig. 7. Showed the Dixon plots for the inhibition of CYP2C19 by MH in the HLMs. The IC_{50} value of CYP2C19 for 4-hydroxyl of S-Mephenytoin was 6.8–21.8-fold higher than that of TCL in the HLMs. MH was a moderate inhibitor for CYP2C19, with an estimated K_i value of 42.65 μM (Table 2). According to Kong *et al* [23], the potency of a test compound could be classified according to its IC_{50} values, as potent, if $IC_{50} \leq 20$ μg/mL or ≤ 10 μM; moderate, if IC_{50} 20–100 μg/mL or 10–50 μM; or weak, if $IC_{50} \geq 100$ μg/mL or ≥ 50 μM. Thus, MH is a potent inhibitor for CYP1A2, a moderate inhibitor for CYP2C19 and a weak inhibitor for the other five CYPs tested in this study.

Drug interactions associated with the induction or inhibition of CYP enzymes was among the most important causes of side effects in humans, and the inhibition effect was considered as the most common mechanism involved in CYP-associated drug-drug interactions. The results of the present work indicated that MH had the potential to interact with a wide range of xenobiotics or endogenous chemicals that were CYP1A2 or CYP2C19 substrates during clinical treatment. Because these two enzymes are involved in the clearance of 6–7% of currently marketed drugs, there is a strong possibility that MH would be co-prescribed with drugs that interact and thus elicit frequent and severe adverse drug interactions in the population. However, it is important to note that the extent of drug interactions with MH might highly vary among individuals due to genetic polymorphisms of CYP1A2 and CYP2C19. In previous studies, allelic variants in the genes encoding CYP1A2 and CYP2C19 had been shown to affect enzyme activities[24–26]. The alleles were classified as belonging to three major phenotypes: extensive metabolism, intermediate metabolism and poor metabolism. Thus, the effect of the gene polymorphisms of these two enzymes should be considered during clinical trials for MH in the future.

In conclusion, our results show that MH was simultaneously a substrate and an inhibitor of CYP1A2 and CYP2C9, and MH had the potential to perpetrate drug-drug interactions with other CYP1A2 and CYP2C19 substrates. Because CYP1A2 and CYP2C19 are polymorphic enzymes, the drug interactions between MH and the substrates of these enzymes should be considered during future experiments.

Author Contributions

Conceived and designed the experiments: YC XH. Performed the experiments: YC YG WZ ZRT JBP YCW. Analyzed the data: YC DSO. Contributed reagents/materials/analysis tools: DLH ML YW JX WHH. Wrote the paper: YC XH WHH.

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