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Identification of CYP2C19 inhibitors from phytochemicals using the recombinant human enzyme model

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The aim of the present study was to develop the recombinant insect cell-expressed protein as an *in vitro* model for inhibitors screening for human cytochrome P450 2C19 (CYP2C19), and to use the model to investigate the inhibition effect of three phytochemicals on CYP2C19 *in vitro*. Omeprazole was applied as the probe substrate. The estimated inhibitory constant (K_i) of ticlopidine and fluvoxamine were 0.64 ± 0.025 μM and 0.29 ± 0.090 μM, respectively. After co-incubation with ticlopidine or fluvoxamine, the mean omeprazole Michaelis-Menten constant (K_m) increased from 4.99 ± 0.22 μM to 16.25 ± 1.22 μM or 19.20 ± 1.73 μM, respectively, while omeprazole's mean V_{max} did not vary much. Both ticlopidine and fluvoxamine were competitive inhibitors of CYP2C19. The IC_{50} of three phytochemicals, isoalantolactone, curcumol and schisandrin A was determined as 38.91 μM, 121.0 μM and 86.41 μM, and the K_i as 5.02 ± 1.04 μM, 35.84 ± 8.95 μM, and 4.46 ± 0.017 μM, respectively. The *in vitro* model for inhibitor screening established using recombinant CYP2C19 could be used to assess the inhibition potential of drug candidates. Isoalantolactone and schisandrin A are potent inhibitors of CYP2C19, while curcumol is a moderate potent inhibitor of CYP2C19.

1. Introduction

The human cytochrome P450 2C family (CYP2C), mainly includes CYP2C8, CYP2C9, CYP2C18, and CYP2C19, participates in the metabolism of approximately 20 % of marketed drugs. CYP2C19 is one of the most studied CYP enzymes, and phenotyping new chemical entities for CYP2C19-mediated metabolism as well as for their potential to inhibit CYP2C19 is common practice (Foti and Wahlstrom 2008).

As CYP enzymes are responsible for the metabolism of many marketed drugs and xenobiotics, the inhibition of CYPs may be one of the most important factors that cause drug-drug interactions (DDIs) (Zambon et al. 2010). Thus, the evaluation of DDIs has become more important than ever in drug discovery and clinical use. Given that herbal medicines are usually prescribed for long term therapies, which are inevitable from co-administration of other drugs, and an increasing number of people is exposed to herbal preparations, metabolism-based drug-herb interactions should be also paid attention to.

In vitro P450 inhibition studies are of great value for their reliability and convenience. Human liver microsomes (HLM) have been the most widely used *in vitro* model for inhibition studies, but the ethnic rule and individual difference restrict their use. Now, many genetically engineered cells, such as *Escherichia coli*, insect cells, lymphoblastoid cells, HepG2 cells, and yeast have used to express CYPs and provide new tools for investigation of the metabolism and metabolic activation/inhibition of chemicals (Yoshitomi et al. 2001). The Bac-to-Bac™ has been proven to be a significant system expressing mammalian membrane protein in baculovirus-infected insect cells. The coexpressed CYP, NADPH-cytochrome P450 oxidoreductase (CYPOR) and cytochrome b5 (CYPb5) in *Spodoptera frugiperda* (Sf9) cells with recombinant viruses has been established successfully in our laboratory (Wang and Zeng 2009; Qian et al. 2011; Lu et al. 2008).

The objectives of this study were to establish an *in vitro* model for inhibitors screening using the recombinant protein CYP2C19 and apply this model to test the inhibition effect of three phytochemicals extracted from herb medicines, isoalantolactone, curcumol, and schisandrin A (Fig. 1).

2. Investigations and results

2.1. Validation of the CYP2C19 inhibition model

The results from incubation of omeprazole and ticlopidine or fluvoxamine with S9 of recombinant CYP2C19 confirmed that ticlopidine and fluvoxamine are potent inhibitors of CYP2C19 (Fig. 2 A, B). Then in the Dixon plots assay, the concentrations of ticlopidine were set at 0.5, 1, 2.5, 5, 10, 20 μM, fluvoxamine was set at 0.1, 0.25, 1, 2, 4 μM, respectively, and they were co-incubated with omeprazole at concentrations of 10, 30, 100 μM. From Dixon plots (Fig. 3 A, B), the mechanism of the inhibition by ticlopidine or fluvoxamine appeared to be competitive, and the K_i was 0.64 ± 0.025 μM and 0.29 ± 0.090 μM, respectively. The new enzyme kinetics of omeprazole were then determined after co-incubation with 2.5 μM of ticlopidine or 0.25 μM of

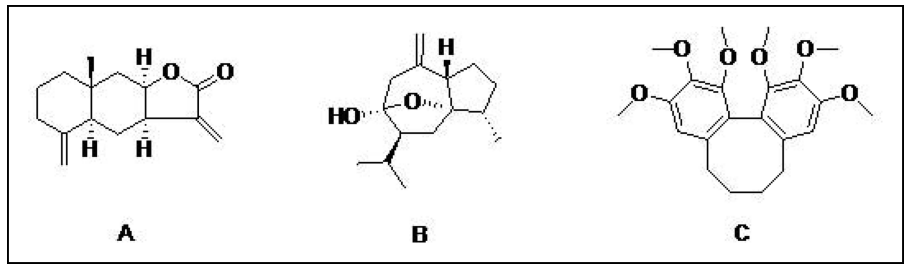

Fig. 1: Structure of isoalantolactone (A), curcumol (B), and schisandrin A (C).

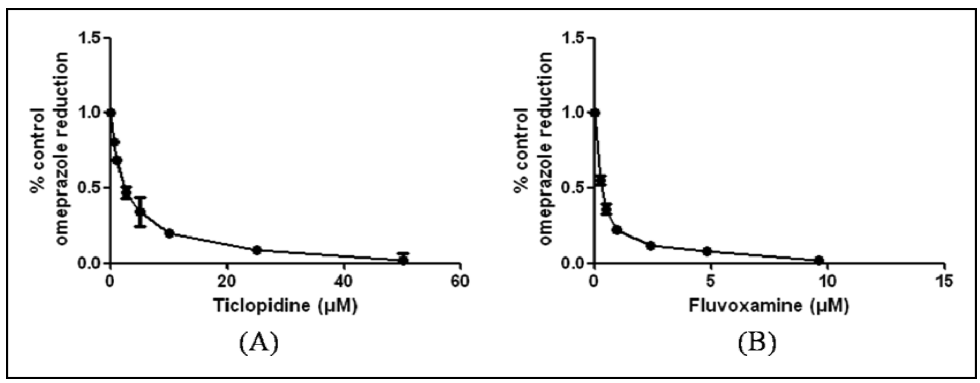

Fig. 2: Inhibition of CYP2C19 by ticlopidine (0 - 50 μM, A) and fluxamine (0 - 9.8 μM, B). Each point represents the mean ± SD, n = 3.

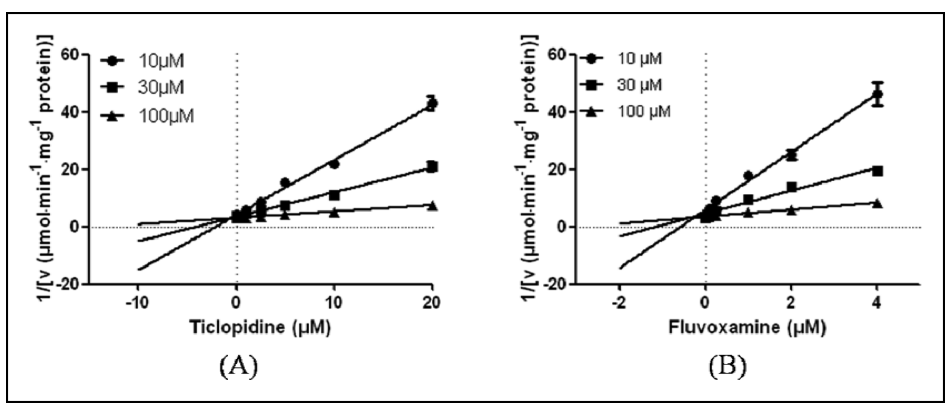

Fig. 3: Dixon plots for the inhibition of CYP2C19 catalyzed omeprazole (10, 30, 100 μM) metabolism by ticlopidine (0 - 20 μM, A) and fluvoxamine (0 - 4 μM, B). Each point represents the mean ± SD, n = 3.

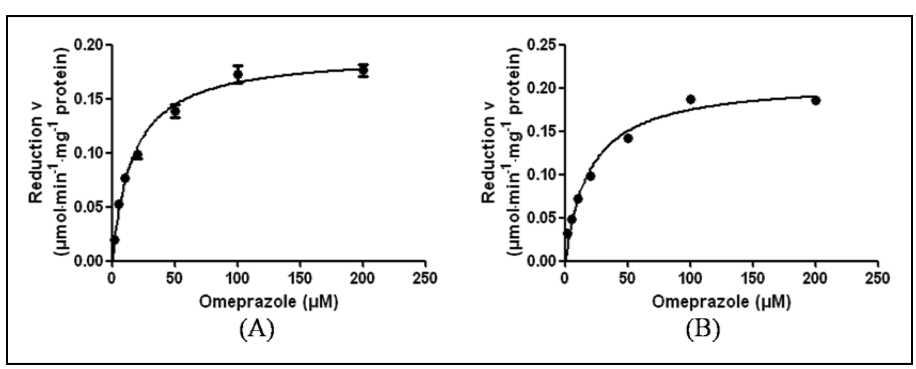

Fig. 4: Enzyme kinetics curve of omeprazole when coincubated with ticlopidine (2.5 μM, A) and fluvoxamine (0.25 μM, B). Each point represents the mean ± SD, n = 3.

fluvoxamine (Fig. 4). The new K_m was increased, while the V_{max} remained almost the same (Table).

2.2. Inhibition studies of three phytochemicals on CYP2C19

Three phytochemicals, isoalantolactone, curcumenol and schizandrin A, were incubated with 10 μM of omeprazole. All of them showed an inhibitory effect on the metabolic capacity of CYP2C19. The log (inhibitor) - response plots indicated that the the IC_{50} of isoalantolactone, curcumenol and schizandrin A were 38.91 μM, 121.0 μM and 86.41 μM, respectively (Fig. 5). Based on their IC_{50}, the incubation concentrations in the Dixon assay of isoalantolactone were set at 0, 10, 25, 50, 75, 100, 150 μM, curcumenol were 0, 50, 75, 100, 125, 150, 200 μM, schizandrin A were 0, 25, 50, 100, 125, 150 μM, respectively. The Dixon plots for the inhibition of three phytochemicals

Table: Enzyme kinetic parameters of omeprazole with or without concurrent inhibitor

	Omeprazole alone (Kong et al. 2012)	Ticlopidine		Fluvoxamine	
		Value	Change ratio	Value	Change ratio
K_m (μM)	4.99 ± 0.22	16.25 ± 1.22	225.7% ↑	19.20 ± 1.73[b]	284.8% ↑
V_{max} (μmol·min-1·mg-1 protein)	0.2539 ± 0.0024	0.1918 ± 0.0040[a]	24.4% ↓	0.2084 ± 0.0055[a]	17.9% ↓
CLint (L·min-1·mg-1 protein)	0.0509	0.0118	76.8% ↓	0.0108	78.8% ↓

[a] $p > 0.05$, [b] $p < 0.05$ vs omeprazole incubated alone by unpaired t-test.

to CYP2C19 (Fig. 6) suggested that the mechanism of the three phytochemicals may be competitive. Both isoalantolactone and schizandrin A showed potent inhibition to CYP2C19 catalyzed omeprazole metabolism (Fig. 6 A, C), with mean K_i of 5.02 ± 1.04 μM and 4.46 ± 0.017 μM, respectively. Curcumenol was a moderately potent inhibitor of CYP2C19, with a mean K_i of 35.84 ± 8.95 μM.

3. Discussion

Induction and inhibition of CYPs are the two major causes for drug interactions. Since the CYP2C19 recombinant protein expressed in insect cell using Bac-to-Bac™ system showed good metabolic activity (Kong et al. 2012), it was designed to establish an *in vitro* model for inhibitors and substrates screening.

Validation of the CYP2C19 inhibition model was carried out using ticlopidine and fluvoxamine, two classic inhibitors of CYP2C19 recommended by US FDA (US FDA, 2004), and the K_i was estimated to feature the binding affinity. Tateishi et al. (1999) have shown that ticlopidine is a significant inhibitor of CYP2C19 mediated omeprazole 5-hydroxylation in healthy Japanese subjects. An estimated K_i of 3.7 ± 0.2 μM was obtained in HLM (Donahue et al. 1997). Mankowski (1999) has found an essentially similar inhibition profile of CYP2C19 (K_i = 0.64 μM) using recombinant CYP2C19. The K_i we got here was consistent with the reported value and confirmed that ticlopidine is a competitive inhibitor of CYP2C19. It has been long since fluvoxamine was reported to inhibit the metabolism of omeprazole catalyzed by CYP2C19 (Andersson et al. 1993; Christensen et al. 2002; Yasui-Furukori et al. 2004). From the Dixon plots, it could be easily concluded that the inhibition of fluvoxamine was competitive with the K_i (Fig. 3 B), which confirmed previous literature data.

Competitive inhibition is a type of enzyme inhibition where binding of the inhibitor to the active site on the enzyme prevents binding of the substrate and *vice versa*. In competitive inhibition, the V_{max} of the reaction remains unchanged, while the K_m is apparently increased. In the present study, when co-incubated with ticlopidine or fluvoxamine, mean K_m of omeprazole value increased while the changes of V_{max} were not obvious. These also proved the inhibition mechanism of ticlopidine and fluvoxamine were competitive. The data we obtained are consistent with that reported, which means the *in vitro* model we developed is reliable. With this model, we screened 70 phytochemicals from herbal medicines.

Nowadays, more and more people are using herbal medicines to help manage or prevent diseases. Drug-herb interactions were found long before, such as those of grapefruit juice and St John's wort (Sun et al. 2010), and publications showed that herbal medicines were inhibitors and/or inducers of hepatic P450 (Hendersen et al. 1999; Foster et al. 2001; Fan et al, 2007). Therefore

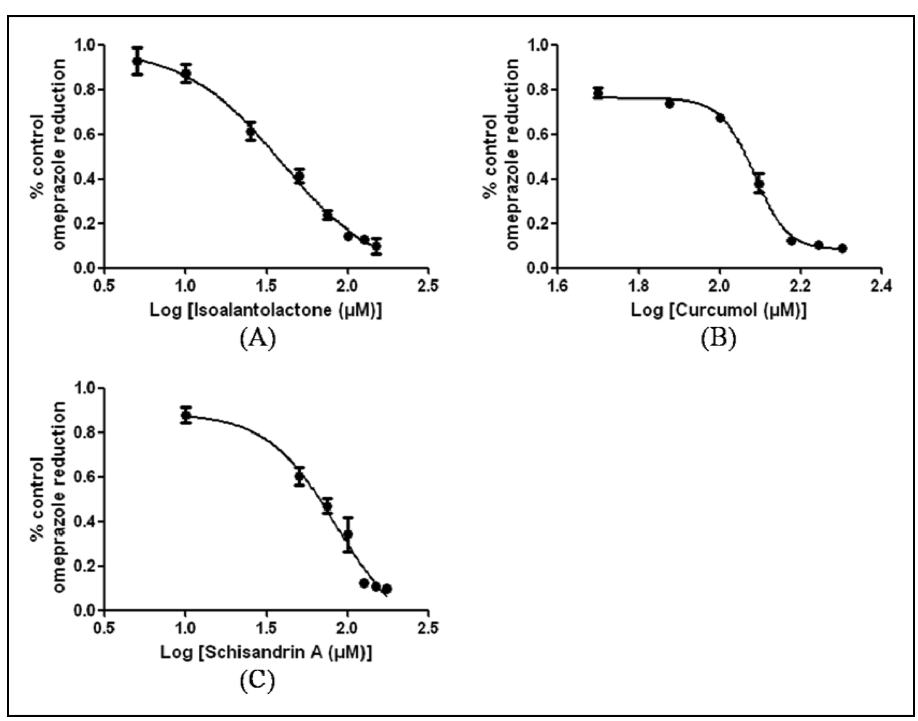

Fig. 5: Log (inhibitor)-response plots of three phytochemicals: isoalantolactone (0 - 150 μM, A); curcumol (0 - 200 μM, B); schisandrin A (0 - 150 μM, C). Each point represents the mean ± SD, n = 3.

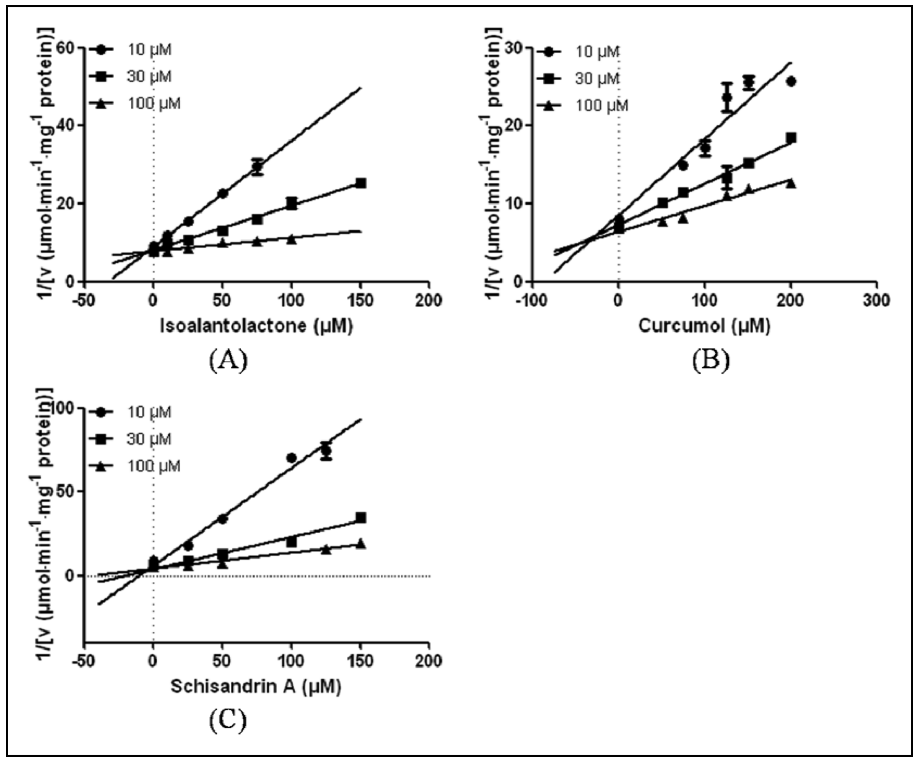

Fig. 6: Dixon plots for the inhibition of CYP2C19 catalyzed omeprazole (10, 30, 100 μM) metabolism by isoalantolactone (0 - 150 μM, A), curcumol (0 - 200 μM, B) and schisandrin A (0 - 150 μM, C). Each point represents the mean ± SD, n = 3.

it is imperative to evaluate phytochemicals' potential to induce DDIs.

Curcumenol, a sesquiterpenoid which has been found to possess anticancer and antivirus activities, was considered to be the main biological active ingredient of Ezhu essential oil (Sun et al. 2010). It was demonstrated to competitively inhibit the activity of CYP3A4 with other CYP isoforms (CYP1A2, CYP2C9, CYP2A6, CYP2C8, CYP2D6 and CYP2E1) negligibly affected (Sun et al. 2010). Here, curcumenol presented potent inhibition to CYP2C19 with low IC_{50} and K_i, and the mechanism of inhibition was competitive according to the Dixon plots. Isoalantolactone, a major constituent of *Inula racemosa* (Compositae), caused Nrf2-mediated induction of detoxifying enzymes (Seo et al. 2009). Our study showed that it exhibited moderate potent inhibition to the CYP2C19 mediated omeprazole metabolism. Schisandrin A is the abundant active dibenzocyclooctadiene derivatives isolated from *F. schisandrae*. Schisandrin A could significantly reduce the CYP3A activity either *in vitro* in a concentration-dependent manner (LI et al., 2010) or *in vivo* in male SD rats (Li et al. 2012). We also proved that schisandrin A was a competitive potent inhibitor of CYP2C19.

In conclusion, an *in vitro* model for inhibitors screening was established here using recombinant insect cell-expressed CYP2C19, and was validated to be reliable using two classic inhibitors. This model can be applied to screen substrates and inhibitors of CYP2C19, and predict potential DDIs. *In vitro* CYP2C19 inhibition data of isoalantolactone, curcumol and schisandrin A were obtained for the first time, which can provide useful information for DDI prediction.

4. Experimental

4.1. Chemicals

Omeprazole was granted by CONBA Pharmaceutical Co., LTD (Zhejiang, China). Ticlopidine HCl, fluvoxamine, nifedipine, schisandrin A were purchased from National Institutes for Food and Drug Control (Beijing, China). Curcumol was purchased from Aladdin (Aladdin Reagent, Shanghai, China). Isoalantolactone was of HPLC grade. NADP and NADPH were purchased from Sigma-Aldrich Chem Co. (St Louis, MO, USA). All other chemicals were purchased from the common commercial sources. Plasmid and bacmid containing human CYP2C19 gene were constructed by our laboratory (Kong et al., 2012).

4.2. Cell culture

Sf9 insect cells were maintained in Grace's medium supplemented with 10 % fetal bovine serum (Gibco), at 28 °C in a humidified incubator.

4.3. Preparation of recombinant enzyme S9 expressed in Sf9 cells

Recombinant baculoviruses were produced with the Bac-To-Bac™ System and co-expression of the CYP2C19 with the CYPOR and CYPb5 was performed as described previously (Wang and Zeng 2009; Qian et al. 2011; Lu et al. 2008). Briefly, 72 h after infected with recombinant baculovirus, Sf9 cells were harvested by centrifugation (800 x g) for 15 min at 4 °C, and washed two times in 1 × PBS. The cell bolus was re-suspended in cell lysate buffer (100 mM sodium phosphate buffer (pH 7.4), 1 mM dithiothreitol, 1 mM EDTA, 20 % glycerol, and 100 mM PMSF), and sonicated on ice for 4 min (5 s bursts, allowing at least 15 s between bursts). The mixture was then centrifuged 9000 x g for 20 min at 4 °C. The supernatant used as the S9 of recombinant protein. The concentration of S9 was measured using BSA method.

4.4. Incubation condition and HPLC analysis

The incubation time and protein concentration used here were within the linear range of substrate metabolism according to our previous research (Kong et al. 2012). The typical 100 μL incubation mixture contained 0.5 g L^{-1} S9 of recombinant CYP2C19 protein in 300 mM potassium phosphate, pH 7.4, 3.3 mM $MgCl_2$ with an NADPH generating system (0.3 mM NADP, 3.3 mM glucose 6-phosphate, and 0.4 U·mL^{-1} of glucose 6-phosphate dehydrogenase), and 10 μM of omeprazole was added as the substrate. After 5 min pre-incubation at 37 °C, the reaction was initiated by adding 2.5 μL of NADPH-regenerating system (10 mg NADP and 5 mg NADPH in 100 μL of 1 % $NaHCO_3$). Reaction was stopped 20 min later by adding 400 μL of ethyl acetate containing 0.02 g L^{-1} nifedipine as the internal standard. The mixtures were vortexed for 90 s and centrifuged at 6000 rpm for 10 min at room temperature. Then the ethyl acetate (upper layer) of 380 μL was transferred to a new centrifuge tube and evaporated under vacuum to dryness. The sample residues were reconstituted in 100 μL of mobile phase (methanol: 10 mM phosphate buffer (pH 7.0) = 1: 1) by vortex mixing for

90 s. After centrifugation at 13,000 rpm for 20 min, 20 μL of the supernatant was sampled into HPLC system. All reactions were run in triple.
The Agilent 1200series HPLC system was comprised of a G1322A degasser, a G1311A quaternary pump, G1316A column compartment maintained at 40 °C and a G1314B VWD detector which was set at a wave length of 305 nm for measurement. Separation was performed on a Diamonsil C18 column (5 μm particle size, 200 × 4.6 mm, Dikma Technologies Inc.). The mobile phase consisted of 10 mM KH_2PO_4 buffer (contained 0.1 % triethylamine, pH 7.0) (solution A) and methanol (solution B), using a gradient elution. The proportion of solvent B was 50 % at 0 min to 13 min, increased from 50 % to 60 % linearly from 13 min to 20 min, then decreased from 60 % to 50 % linearly from 20 min to 23 min, and the system was re-equilibrated for 2 min. The flow rate was set at 1 $mL \cdot min^{-1}$.

4.5. Inhibition studies of ticlopidine and fluvoxamine

Omeprazole of 10.0 μM was incubated in triplicate with S9 of recombinant CYP2C19 and NADPH-generating system in the absence (control) or presence of varying concentrations of ticlopidine (1, 2, 5, 10, 25, 50 μM) or fluvoxamine (0.24, 0.48, 0.96, 2.4, 4.8, 9.6 μM). The approximate value obtained was used to generate appropriate inhibitor concentrations for the determination of K_i for each inhibitor. In Dixon plot analysis, omeprazole was set at three different concentrations (10, 30, 100 μM), and were co-incubated with a range of appropriate concentrations of ticlopidine or fluvoxamine. The extraction methods and the HPLC conditions were the same as those described above.
Then the enzyme kinetics of omeprazole co-incubated with inhibitors was determined. Incubation experiments were carried out using a range of omeprazole concentrations (2, 5, 10, 20, 50, 100, 200 μM) and a single concentration of ticlopidine or fluvoxamine around their K_i. The new kinetic parameters were calculated and compared with the data obtained without inhibitors.

4.6. Inhibition studies of three phytochemicals on CYP2C19

Three phytochemicals, isoalantolactone, curcumenol and schizandrin A were employed to examine the potency as inhibitors of CYP2C19 based on our previous study by screening of 70 phytochemicals (Kong et al. 2012).
Omeprazole of 10.0 μM was co-incubated with the three phytochemicals. The concentration of isoalantolactone was set at 5, 10, 25, 50, 75, 100, 125, 150 μM, curcumenol at 50, 75, 100, 125, 150, 175, 200 μM, and schizandrin A at 5, 10, 50, 75, 100, 125, 150, 175 μM, respectively. The IC_{50} values of the phytochemicals were determined for setting the inhibitor concentrations used to measure their K_i. Then Dixon plots assay for the inhibition by phytochemicals were performed using the same procedure. Omeprazole was set at three different concentrations (10, 30, 100 μM), and incubated with varying concentrations of three phytochemicals as the section "Inhibition studies of ticlopidine and fluvoxamine" mentioned above.

4.7. Data analysis

All the data are presented as the mean ± SD, and were analyzed using GraphPad Prism version 5.01 software (GraphPad Software, Inc.). K_m and V_{max} were calculated using the model of Michaelis-Menten equation. The K_i was calculated with Dixon plots with using the equation assuming competitive inhibition.
Correlations were estimated by regression analysis. Analysis of significant differences was performed by the unpaired Student's t-test. A p-value of < 0.05 was considered to represent a statistically significant difference.

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References

Andersson T, Miners JO, Veronese ME, Tassaneeyakul W, Tassaneeyakul W, Meyer UA, Birkett DJ (1993) Identification of human liver cytochrome P450 isoforms mediating omeprazole metabolism. Br J Clin Pharmacol 36: 521–530.

Christensen M, Tybring G, Mihara K, Yasui-Furokori N, Carrillo JA, Ramos SI, Andersson K, Dahl ML, Bertilsson L (2002) Low daily 10-mg and 20-mg doses of fluvoxamine inhibit the metabolism of both caffeine (cytochrome P4501A2) and omeprazole (cytochrome P4502C19). Clin Pharmacol Ther 71: 141–152.

Donahue SR, Flockhart DA, Abernethy DR, Ko JW (1997) Ticlopidine inhibition of phenytoin metabolism mediated by potent inhibition of CYP2C19. Clin Pharmacol Ther 62: 572–577.

Fan L, Wang G, Wang LS, Chen Y, Zhang W, Huang YF, Huang RX, Hu DL, Wang D, Zhou HH (2007) Herbal medicine yin zhi huang induces CYP3A4-mediated sulfoxidation and CYP2C19-dependent hydroxylation of omeprazole. Acta Pharmacol Sin 28: 1685–1692.

Foster BC, Foster MS, Vandenhoek S, Krantis A, Budzinski JW, Arnason JT, Gallicano KD, Choudri S (2001) An *in vitro* evaluation of human cytochrome P450 3A4 and P-glycoprotein inhibition by garlic. J Pharm Pharm Sci 4: 176–184.

Foti RS, Wahlstrom JL (2008) CYP2C19 inhibition: the impact of substrate probe selection on *in vitro* inhibition profiles. Drug Metab Dispos 36: 523–528.

Henderson GL, Harkey MR, Gershwin ME, Hackman RM, Stern JS, Stresser DM (1999) Effects of ginseng components on c-DNA-expressed cytochrome P450 enzyme catalytic activity. Life Sci 65: 209–214.

Kong LM, Hu HH, Zeng S (2012) Coexpression model and inhibitors screening of the human CYP2C19 with the CYPOR and CYPb5. Chin J Pharm Anal 32: 2099–2107.

Li WL, Xin HW, Su MW (2012) Inhibitory effects of continuous ingestion of schisandrin A on CYP3A in the rat. Basic Clin Pharmacol Toxicol 110: 187–192.

Li WL, Xin HW, Su MW, Xiong L (2010) Inhibitory effects of schisandrin A and schisandrin B on CYP3A activity. Methods Find Exp Clin Pharmacol 32: 163–169.

Lu K, Zeng S, Yao TW (2008) Expression of human CYP2E1 in insect cells using bac-to-bac expression system. J Zhejiang Univ (Med Sci) 37: 118–125.

Mankowski DC (1999) The role of CYP2C19 in the metabolism of (+/-) bufuralol, the prototypic substrate of CYP2D6. Drug Metab Dispos 27: 1024–1028.

Qian MR, Chen J, Liu Y, Yu LS, Chen SQ, Zeng S (2011) CYP2D6*1, CYP2D6*10 co-expressed with CYPOR in Bac-to-Bac expression system and activity determination. Acta Pharm Sin 46: 207–212.

Seo JY, Park J, Kim HJ, Lee IA, Lim JS, Lim SS, Choi SJ, Park JH, Kang HJ, Kim JS (2009) Isoalantolactone from Inula helenium caused Nrf2-mediated induction of detoxifying enzymes. J Med Food 12: 1038–1045.

Sun DX, Fang ZZ, Zhang YY, Cao YF, Yang L, Yin J (2010) Inhibitory effects of curcumenol on human liver cytochrome P450 enzymes. Phytother Res 24: 1213–1216.

Tateishi T, Kumai T, Watanabe M, Nakura H, Tanaka M, Kobayashi S (1999) Ticlopidine decreases the *in vivo* activity of CYP2C19 as measured by omeprazole metabolism. Br J Clin Pharmacol 47: 454–457.

US FDA (2004) Drug interaction studies-study design, data analysis, and implications for dosing and labeling. Available at: http://www.fda.gov/ohrms/dockets/ac/04/briefing/2004–4079B1_04_Topic2-TabA.pdf. Accessed on 28 Dec 2012.

Wang XW, Zeng S (2009) Heterogenous expression and activity analysis of human cytochrome P450 3A4 mutants CYP3A4.3, CYP3A4.4, CYP3A4.5 and CYP3A4.19. Chin J Pharmacol Toxicol 23: 456–463.

Yasui-Furukori N, Takahata T, Nakagami T, Yoshiya G, Inoue Y, Kaneko S, Tateishi T (2004) Different inhibitory effect of fluvoxamine on omeprazole metabolism between CYP2C19 genotypes. Br J Clin Pharmacol 57: 487–494.

Yoshitomi S, Ikemoto K, Takahashi J, Miki H, Namba M, Asahi S (2001) Establishment of the transformants expressing human cytochrome P450 subtypes in HepG2, and their applications on drug metabolism and toxicology. Toxicol *In Vitro* 15: 245–256.

Zambon S, Fontana S, Kajbaf M (2010) Evaluation of cytochrome P450 inhibition assays using human liver microsomes by a cassette analysis /LC-MS/MS. Drug Metab Lett 4: 120–128.