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Title Page

Peony-Glycyrrhiza Decoction, an herbal preparation, inhibits clozapine metabolism via cytochrome P450s, but not flavin-containing monooxygenase in *in vitro* models

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Running Title Page

Running title: Herbal medicine inhibits clozapine metabolism

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ABBREVIATIONS: AICc, akaike information criterion; ANOVA, analysis of variance; BZD, benzydamine; C_{int}, intrinsic clearance; CLZ, clozapine; CLZ-N-oxide, clozapine-N-oxide; CV, coefficients of variation; CYPs, cytochrome P450s; FMOs, flavin-containing monooxygenases; GR, Glycyrrhiza radix (liquorice); HLM, human liver microsomes; HPLC, high-performance liquid chromatography; hyperPRL, hyperprolactinemia; IC_{50}, half maximal inhibitory concentration; K_m, Michaelis-Menten constant; K_i, inhibitory constant; NADPH, nicotinamide adenine dinucleotide phosphate; norCLZ, N-demethyl-clozapine; PGD; Peony-Glycyrrhiza Decoction; PR, Paeonia radix (peony); V_{max}, maximum velocity.

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Abstract

Our previous studies have shown the therapeutic efficacy and the underlying mechanisms of Peony-Glycyrrhiza Decoction (PGD), an herbal preparation, in treating antipsychotic-induced hyperprolactinemia in cultured cells, animal models and human subjects. In the present study, we further evaluated pharmacokinetic interactions of PGD with clozapine (CLZ) in human liver microsomes (HLM), recombinantly expressed cytochrome P450s (CYPs), and flavin-containing monooxygenases (FMOs). CLZ metabolites, N-demethyl-clozapine (norCLZ) and clozapine-N-oxide (CLZ-N-oxide), were measured. PGD, individual peony and glycyrrhiza preparations, and two individual preparations in combination reduced production of CLZ metabolites to different extents in HLM. While the known bioactive constituents of PGD play a relatively minor role in the kinetic effects of PGD on CYP activity, PGD as a whole had a weak to moderate inhibitory potency towards CYPs, in particular CYP1A2 and CYP3A4. FMOs are less actively involved in mediating CLZ metabolism and the PGD inhibition of CLZ. These results suggest that PGD has the capacity to suppress CLZ metabolism in the human liver microsomal system. This suppression is principally associated with the inhibition of related CYP activity but not FMOs. The present study provides *in vitro* evidence of herb-antipsychotic interactions.

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Introduction

Although antipsychotic agents are the mainstay in treating schizophrenia, their clinical use is largely limited due to undesirable side effects that often result in discontinuation and relapse (Kane and Correll, 2010). This has led to a demand for the development of alternative strategies to improve medication compliance and antipsychotic response. Herbal medicines may possess the potential to reduce adverse side effects associated with antipsychotic treatment (Zhang et al., 2010). It has been reported that Peony-Glycyrrhiza Decoction (PGD), a traditional Chinese herbal formula consisting of Paeonia (peony) and Glycyrrhiza (liquorice) radices, can alleviate antipsychotic-induced hyperprolactinemia (hyperPRL) (Costa et al., 2006; Xu, 2003; Yamada et al., 1996, 1997, 1999; Yuan, 2008). In our controlled trial, additional PGD both lowered antipsychotic-induced hyperPRL and improved the related symptoms in women with schizophrenia (Yuan et al., 2008). Studies using cultured cells and animal models have further revealed that the anti-hyperPRL effects of PGD are associated with the modulation of dopamine D_2 receptor and transporters and normalization of sex hormone dysfunction (Wang et al., 2012a). Major bioactive compounds of PGD can modulate multiple anti-apoptotic and pro-apoptotic pathways at subcellular and molecular levels in targeted cells (Wang et al., 2013a,b; 2014a,b). However, it is unclear whether herb-drug kinetic interactions are involved in the therapeutic effects of PGD.

With an increasing use of herbal medicine in managing psychiatric disease, herb-drug interactions have become an important issue in clinical practice (Zhang et al., 2010, 2011). This is particularly apparent in China where herbal medicine has become an important therapeutic approach to psychiatric disorders (Zhang et al., 2010). Our recent epidemiological study has found that approximately 37% of schizophrenic patients sought herbal medicine treatment while they were under antipsychotic medication (Zhang et al., 2011). However, supplementary use of herbal medicine can result in both beneficial and adverse clinical outcomes (Peterson et al., 2008; Przekop and Lee, 2009;

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Zhang et al., 2011). Therefore, identifying potential interactions between herbal and antipsychotic agents is critical for promoting drug safety in clinical psychiatry.

Clozapine (CLZ) is the most commonly prescribed antipsychotic drug for long-term maintenance treatment of schizophrenia in China (Tang et al., 2008). Our epidemiological study has shown that CLZ is often concomitantly used with herbal medicine, which may result in adverse clinical outcomes (Zhang et al., 2011). Clozapine is metabolized to N-demethylated CLZ (norCLZ) and N-oxygenated CLZ (CLZ-N-oxide) via hepatic cytochrome P450 (CYP) enzymes, mainly CYP1A2, CYP3A4, CYP2D6, CYP2C9, and CYP2C19 (Chetty and Murray, 2007). CYPs also play a critical role in the kinetic interaction between liquorice and peony and conventional drugs (Chen et al., 2002; Wang et al., 2013c; Wu et al., 2012; Zhou et al., 2003). Flavin-containing monooxygenases (FMOs) are hepatic microsomal enzymes with importance in catalyzing massive xenobiotics (Krueger et al., 2005). FMOs and CYPs share many similarities in tissue distribution, subcellular location and substrate specificity (Krueger et al., 2005). FMOs, in particular FMO3, may be also involved in the N-oxygenation of CLZ (Tugnait et al., 1997; Fang et al., 1998; Fang, 2000; Zhang et al., 2008).

We hypothesize that PGD may affect CLZ metabolism by mediating related CYP and FMO activities in liver microsomes. To test this hypothesis, we evaluated the kinetic effects of PGD, individual peony and liquorice preparations, two individual preparations in combination and the major bioactive constituents of PGD on CLZ metabolism in human liver microsomes (HLM), recombinantly expressed CYP, and FMO enzymes.

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Materials and Methods

Drugs and reagents. Benzydamine (BZD), and BZD N-oxide were purchased from Chemstrong (Shenzhen, China). Clozapine (CLZ), norCLZ, and CLZ-N-oxide were purchased from Selleck Chemicals (TX, USA), Tocris (Bristol, UK), and Enzo (NY, USA), respectively. The five major constituents of PGD, paeoniflorin, liquiritin, liquiritigenin, glycyrrhizic acid, and glycyrrhetinic acid were purchased from Shanghai Yuanye Bio-Technology Co. Ltd. (Shanghai, China). Nicotinamide adenine dinucleotide phosphate (NADPH) tetrasodium salt was obtained from Santa Cruz (TX, USA). High-performance liquid chromatography (HPLC)-grade solvents were supplied by Duksan (South Korea) and other analytical reagents by Sigma Aldrich (St. Louis, USA).

Herbal preparation and determination of quality. Four herbal preparations used in the present study (PGD, individual Paeonia and Glycyrrhiza preparations, and the combination of the two individual herbal preparations) came from the same batches previously reported (Wang et al., 2012a). The raw materials of Paeonia and Glycyrrhiza radices were supplied by the Pharmacy of School of Chinese Medicine at the University of Hong Kong (HKU). For PGD preparation, water extraction was utilized to optimally preserve its bioactive constituents, as recommended by the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010). The extraction was conducted in an automatic boiling machine. Sliced and broiled Paeonia and Glycyrrhiza radices (50 g each) were mixed, immersed, and boiled in a 10-fold volume of distilled water for 2 hours. This process was repeated twice as previously reported (Yuan et al., 2008). The extracted solution was pooled, concentrated, and freeze-dried to yield powder at a 1:5 ratio with raw materials in weight. Individual Paeonia radix (peony) (PR) and Glycyrrhiza radix (liquorice) (GR) were also prepared as done for PGD. The resulting powder was dissolved in water at a concentration of 250 mg/ml to create a stock solution for further use.

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To ensure the quality of the herbal preparation, three batches of PGD used in this study were prepared; inter-batch coefficients of variation (CV) of the contents of the five known constituents (mean ± SEM, mg/g), paeoniflorin (17.76 ± 0.39), liquiritin (1.05 ± 0.11), liquiritigenin (2.07 ± 0.30), glycyrrhizic acid (0.48 ± 0.04), and glycyrrhetinic acid (1.16 ± 0.08), were measured using reverse-phase high-performance liquid chromatography (HPLC). CVs of all constituents measured were <15% across the three batches as reported previously (Wang et al., 2012a).

Experiments in HLM. Pooled HLM from 25 donors (18 males and 7 females) were provided by BD Gentest (Woburn, MA, USA). A stock solution of CLZ was prepared in water with 0.05% acetic acid. Pilot experiments confirmed linear formation of norCLZ and CLZ N-oxide in terms of incubation time and protein concentration.

We first determined the kinetic effects of PGD, individual peony and liquorice preparations, the two individual preparations in combination and major bioactive constituents of PGD on CLZ metabolism in HLM. The transformation rate of norCLZ and CLZ-N-oxide was evaluated at serial concentrations (10, 25, 50, 100, 200, and 400 μM) of CLZ in the absence and presence of PGD (1.25, 2.5, and 5 mg/ml), individual Paeonia and Glycyrrhiza preparations (1.25 mg/ml), the two individual preparations in combination (2.5 mg/ml), paeoniflorin (3 mM), liquiritigenin (200 μM), liquiritin (400 μM), glycyrrhizic acid (400 μM), and glycyrrhetinic acid (100 μM) as inhibitors. Stock solutions of the five constituents were made by dissolving in acetonitrile; the final concentration of acetonitrile was <1% (v/v) in the experimental volume. The concentrations used for the five constituents were 3- to 10-fold higher than those used in *in vitro* pharmacological and therapeutic experiments (Wang et al., 2012a; 2013a,b; 2014a,b). The enzymatic reactions were conducted in polypropylene tubes in triplicate at 37°C in a water bath. CLZ, inhibitor, and HLM (50 μg) were mixed and pre-incubated in potassium phosphate buffer (0.1 M, pH 7.4) for 10 min, initiated by adding the coenzyme NADPH (1 mM), and terminated 15 min later by adding an equivalent volume of ice-cold acetonitrile. The samples were centrifuged at 12,000 × *g*

for 10 min. Supernatants were then separated and stored at -80°C to measure norCLZ and CLZ-N-oxide.

In parallel, the extent of contribution of FMOs in generating CLZ N-oxide was examined in HLM. Benzydamine (BZD), a nonspecific substrate of FMOs, was used as a probe to evaluate FMO activity. The metabolite BZD-N-oxide was measured as an index reaction for FMOs (Tugnait et al., 1997; Zhang et al., 2008). The enzymatic reactions were carried out in triplicate in polypropylene tubes in potassium phosphate buffer (0.1 M, pH 7.4) at a final volume of 100 μl. The reactive system was pre-incubated with 50 μg HLM in the absence and presence of 1 mM NADPH at 37°C in a water bath for 10 min, initiated by adding substrate (100 μM BZD or CLZ), and stopped by adding 100 μl ice-cold acetonitrile at 15 min following the initiation. Supernatants were separated and stored at -80°C to measure BZD-N-oxide, CLZ-N-oxide and norCLZ.

Experiments with recombinantly expressed enzymes. The inhibitory effects of PGD on individual CYP enzymes were further examined in recombinantly expressed CYPs derived from baculovirus-infected insect cells that individually express 1A2, 2C19, 2D6, and 3A4 (BD Gentest, Woburn, MA, USA). The experimental conditions were similar to those described for HLM. The enzymatic reaction system contained individual CYP enzymes at a final concentration of 20 *p*mol/ml, and the substrate CLZ in serial concentrations ranging from 8-100 μM, in the absence and presence of the inhibitor PGD with five serial concentrations of 0.05-4 mg/ml. The reaction lasted 30 min for CYP2C19 and 15 min for other CYPs. Supernatants were separated and stored at -80°C to measure norCLZ and CLZ-N-oxide.

The inhibitory effect of PGD on FMO3 was also assessed in recombinantly expressed FMOs (Corning Gentest, Tewksbury, MA, USA). The enzymatic reactive system consisting of potassium phosphate buffer (0.1 M, pH 7.4), NADPH (1 mM), PGD (5, 10 and 25mg/ml), and FMO3 (0.1 mg/ml) was pre-incubated for 10 min, initiated by

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adding CLZ (50 μM), and terminated 15 min later. After protein precipitation by acetonitrile, the supernatant was obtained to measure norCLZ and CLZ-N-oxide.

Measurement of BZD-N-oxide, norCLZ and CLZ-N-oxide. A Waters 626 series HPLC system (Milford, MA, USA) was used for the measurement of BZD-N-oxide, norCLZ, and CLZ-N-oxide. The separation was achieved on an ACE5 AQ column (5 μm, 4.6 × 250 mm) with mobile phase consisting of solvent A as acetonitrile and solvent B as 0.1 % (v/v) acetic acid in water. The flow rate was 1 ml/min. A gradient program was applied for analysis of CLZ metabolites: 0-18 min, 14% to 20% B; 18-30 min, 20% to 40% B; 30-35 min, 40% to 60% B; 35-40 min, 14%B. The absorbance of norCLZ and CLZ-N-oxide was measured at 240 nm. For BZD-N-oxide quantification, a second gradient program was applied: 0-5 min, 20% to 40% B; 5-14 min, 40% to 60% B; 14-19 min, 20% B. The absorption wavelength was set at 215 nm.

Data Analysis. Data obtained in HLM experiments were analyzed using Prism 5 (GraphPad Software Inc., San Diego, CA, USA). The maximum velocity (V_{max}) and Michaelis-Menten constant (K_m) were obtained and the intrinsic clearance (C_{int}) was calculated from $C_{int} = V_{max}/K_m$. Data obtained from recombinant enzyme experiments were analyzed using SigmaPlot software (Systat Software Inc., Chicago, IL, USA). The inhibition constant (K_i) and the mode of inhibition were determined by both programs with nonlinear regression analysis of the metabolite formation with equations for competitive, noncompetitive, mixed and uncompetitive inhibition. The lowest Akaike information criterion (AICc) value was used as a measure of goodness of fit. The mode of inhibition was further verified by visual inspection of Dixon plots that linearize integrally nonlinear relationships.

One-way analysis of variance (ANOVA) with Dunnett's methods and two-sided Student t-test were applied to compare variables among and between groups, respectively. The half maximal inhibitory concentration (IC_{50}) was determined with plotting log (inhibitor) versus percent activity. Statistical significance was defined as a

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two-sided *P* value less than 0.05.

Results

The effects of PGD and its major constituents on CLZ metabolism in HLM. K_m values of 92.6 μM for norCLZ and 43.9 μM for CLZ N-oxide were obtained from pilot experiments. We then used 100 μM CLZ in dose-response experiment of PGD. In the presence of 100 μM CLZ, IC_{50} of PGD in inhibiting the production of CLZ-N-oxide and norCLZ was 2.41 mg/ml and 2.00 mg/ml, respectively (Fig. 1). We then used 2.5 mg/ml PGD as the reference concentration for further experiments as this concentration is surrounding IC_{50} and has been widely used in our previous studies (Wang *et al*., 2012a; 2013a,b; 2014a,b). Kinetic variables of the four herbal preparations in inhibiting CLZ metabolism are summarized in Table 1 and Fig. 2.

For norCLZ, all four preparations substantially reduced V_{max} values compared to controls ($F = 24.849$, $P < 0.001$). K_m value was significantly increased with Glycyrrhiza treatment alone ($F = 8.283$, $P = 0.003$). PGD and Glycyrrhiza alone and in combination with Paeonia robustly slowed the intrinsic clearance (C_{int}) compared to controls ($F = 28.221$, $P < 0.001$). For CLZ-N-oxide, PGD and Glycyrrhiza alone considerably suppressed V_{max} ($F = 5.241$, $P = 0.015$). Both PGD and the two individual preparations in combination markedly increased K_m values ($F = 12.916$, $P <0.001$) and decreased C_{int} values ($F = 10.813$, $P = 0.001$).

The five constituents of PGD, paeoniflorin (3 mM), liquiritigenin (200 μM), liquiritin (400 μM), glycyrrhizic acid (400 μM) and glycyrrhetinic acid (100 μM), were also tested in HLM. Only liquiritigenin exhibited a significant effect in reducing the V_{max} of CLZ-N-oxide formation compared to controls without liquiritigenin ($t = 6.279$, $P = 0.003$) (Table 2). The other four constituents had no effect on either norCLZ or CLZ-N-oxide (data not shown).

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Role of FMOs in CLZ metabolism in HLM. The amount of BZD-N-oxide produced in the presence of the coenzyme NADPH was approximately one-fold higher than in the absence of the coenzyme (t = 10.885, P < 0.001). Under the same reaction conditions, the amount produced was similar to those of CLZ-N-oxide (t = 1.168, P = 0.308) and norCLZ (t = 0.573, P = 0.597) in the presence and absence of NADPH (Table 3).

The effects of PGD on CYP activity in CLZ metabolism. The inhibitory potency of PGD towards the four CYPs (1A2, 2C19, 2D6 and 3A4) involved in the metabolism of CLZ was further evaluated in recombinant CYPs. The results are summarized in Table 4 and Fig. 3. PGD weakly to moderately inhibited the four CYPs in the formation of norCLZ with K_i values of 0.3-2.4 mg/ml, V_{max} of 15-53 pmol/min/pmol enzyme, and K_m of 12-51 μM. PGD also moderately suppressed CYP3A4 activity in the N-oxide formation of CLZ with K_i values of 0.5 mg/ml, V_{max} of approximately 28 pmol/min/pmol enzyme, and K_m of 16 μM.

The effects of PGD on FMO3 activity in CLZ metabolism. Values for V_{max} of 2.9 nmol/mg protein/min and K_m of 51 μM of for CLZ-N-oxide were obtained in recombinantly expressed FMO3 without PGD. Concentration-response experiments of PGD (2.5-25 mg/ml) were then carried out in the presence of 50 μM CLZ. Plotting log showed an IC_{50} of 44.3 mg/ml of PGD in inhibiting the production of CLZ-N-oxide (Fig. 4). The decrease in the production of CLZ-N-oxide in the presence of 2.5 and 5 mg/ml of PGD was not significantly different from that in the absence of PGD (96.8% and 88.3%, respectively). Even at 10 and 25 mg/ml of PGD, 74.9% and 59.0% CLZ-N-oxide was still produced, respectively (Fig. 4).

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Discussion

The present study represents an attempt to characterize metabolism-based interactions between herbal and antipsychotic agents. PGD often serves as adjunctive therapy to treat antipsychotic-induced hyperPRL (Zhang et al., 2010). Our previous studies have demonstrated the anti-hyperPRL effects of PGD in cultured cells, animal models and patients with schizophrenia and the underlying mechanisms (Wang et al., 2012a; 2013a,b; 2014a,b; Yuan et al., 2008). In the present study, a series of *in vitro* experiments were employed to further evaluate the kinetic properties of PGD, the individual herbal preparations, and its major constituents on CLZ metabolism.

Clozapine (CLZ) is metabolized to norCLZ and CLZ-N-oxide mainly via hepatic cytochrome P450 (CYP) enzymes (Chetty and Murray, 2007). The present study first examined the kinetic effects of PGD in HLM. We found that PGD at 2.5 mg/ml, an effective dose inducing pharmacological and therapeutic response, exerted significant effects in reducing V_{max} and C_{int} and increasing K_m values of the two CLZ metabolites, norCLZ and CLZ-N-oxide, indicating that this herbal preparation possesses the capacity to suppress CLZ metabolism *in vitro*. Of the CYP enzyme systems, CYP1A2 and CYP3A4 play a major role in the metabolism of CLZ, with a minor contribution from other CYP isoforms (Chetty and Murray, 2007). We therefore further examined the effects of PGD on individual CYP activity using recombinantly expressed CYPs. The current study showed that PGD had a moderate inhibitory potency toward CYP1A2, CYP2C19, and CYP3A4 and a weak potency toward CYP2D6 in the demethylation of CLZ. PGD also had a moderate potency in inhibiting the oxidation of CLZ by CYP3A4. These results suggest that PGD suppression of CLZ metabolism appears to be at least partly derived from the inhibition of related CYP enzymes, in particular CYP1A2 and CYP3A4.

In addition to CYPs, HLM also express abundant FMOs (Krueger et al., 2005). Previous studies have suggested that FMOs, in particular FMO3 may be involved in

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the oxygenation of CLZ (Fang et al., 1998; Fang, 2000; Tugnait et al., 1997; Zhang et al., 2008). In the present study, we revealed that, under physiological conditions, the production of CLZ metabolites, CLZ-N-oxide and norCLZ, was not different between partial and full activation of FMOs in the presence and absence of the coenzyme NADPH, respectively. In contrast, the amount of BZD-N-oxide, an index of FMO reactions and produced in the presence of NADPH, was almost doubled compared to that produced in the absence of NADPH. It is likely then that the human hepatic FMO-mediated metabolic pathway plays a relatively minor role in the biotransformation of CLZ. Similar results were also observed in a previous study (Zhang et al., 2008), demonstrating that N-oxide formation of CLZ in HLM was not affected when FMO activity was selectively inhibited. On the other hand, FMO3 is the prominent functional form in the human hepatic FMO system (Tugnait et al., 1997). We thus evaluated the potential of PGD in mediating FMO3 activity using recombinantly expressed FMO3. The current study showed that PGD in effective pharmacological concentration (2.5 and 5 mg/ml) presented a slight effect (<12%) in inhibiting FMO3 from catalyzing the N-oxide formation of CLZ. Even at 10-25 mg/ml, 4- to 10-fold higher than the reference concentration (2.5 mg/ml), the inhibitory magnitude still could not reach the IC_{50} level (44.3 mg/ml) at which 50% of the production of CLZ-N-oxide should be suppressed. Taken together, PGD suppression of CLZ metabolism in HLM is principally associated with the inhibition of CYPs. FMOs are less actively involved in the kinetic interactions of PGD with conventional drugs in human liver microsomal systems.

We further examined the kinetic effects of the five pharmacologically active constituents in PGD, paeoniflorin, liquiritigenin, liquiritin, glycyrrhizic acid, and glycyrrhetinic acid on CLZ metabolism in HLM. Only liquiritigenin exhibited a weak effect in inhibiting the formation of CLZ-N-oxide while the other four constituents had no effects on the production of either norCLZ or CLZ-N-oxide. Previous studies have shown a weak interaction potential between glycyrrhizic acid or glycyrrhetinic acid with CYP enzymes (Pandit et al., 2011; Zhao et al., 2012). Therefore, the known

bioactive constituents of PGD may play a relatively minor role in the inhibitory effects of PGD on human liver CYP enzymes. Given that the anti-hyperPRL effects of PGD are closely associated with the constituents tested (Wang et al., 2012a; Yuan et al., 2008), it is unlikely that the anti-hyperPRL effects of PGD observed previously (Wang et al., 2012a; Yuan et al., 2008) are directly related to the kinetic interactions of the major constituents of PGD with antipsychotic drugs. Nevertheless, many naturally occurring compounds, such as triterpenoids and flavonoids, have been shown to have kinetic effects on conventional drugs (Zhou et al., 2003). The kinetic properties of other ingredients contained in PGD requires further evaluation.

The present study also compared the kinetic properties of PGD with individual peony and liquorice preparations as well as the mixture of the two individual preparations in HLM. While all four preparations inhibited CYP activity on the formation of both norCLZ and CLZ-N-oxide to different extents, PGD exhibited the highest potency toward most variables, not only was the potency of PGD greater than that of the two individual preparations, but it was also greater than the mixture of the two individual preparations at equivalent concentration, suggesting a potential synergistic herb-herb interaction. In clinical practice of Chinese medicine, most patients are prescribed polyherbal formulae rather than singular herbal preparations. This is based on the empirical view that combining multiple herbs should enhance their positive effects and reduce adverse side effects, leading to greater therapeutic response (Pei et al., 2013). Here, we provide pharmacokinetic evidence supporting such synergistic herb-herb interaction. In further support of our hypothesis, it has been found that the interaction potential on CYP3A4 of liquorice extract was greater than for the pure compound (Tsukamoto et al., 2005). Combinations of peony and liquorice in different ratios resulted in significant differences in the bioavailability of the major bioactive constituents (Xu et al., 2013). There were also marked differences in gastrointestinal absorption rate and blood concentration of 15 ingredients between PGD and the mixture of the two individual preparations (Shen et al., 2012). Therefore, it appears that different herbal preparation procedures yielded bioactive components of varying

quantity and quality with differing pharmacokinetic profiles among each preparation.

In summary, this study suggests that PGD has the capacity to suppress CLZ metabolism in the human liver microsomal system. This suppression is mainly associated with the inhibition of related CYP activity, in particular CYP1A2 and CYP3A4 but not FMOs. The known pharmacologically active constituents contained in PGD may play relatively minor role in kinetic interactions of PGD with CLZ. Further, it suggests that further investigation into herb-drug interactions may be of interest.

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Authorship Contributions

Participated in research design: W Wang, D Wang, Tan, CY Wang, Zhang.

Conducted experiments: W Wang, Zheng, Tian.

Performed data analysis: W Wang, Zhang.

Wrote or contributed to the writing of the manuscript: W Wang, CY Wang, Zhang.

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Footnotes

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Legends for figures

Fig. 1. Concentration response of PGD (0.5-5 mg/ml, log) in inhibiting the metabolism of clozapine (CLZ, 100 μM) was examined in the human liver microsomes. The metabolites of CLZ, N-demethylated CLZ (norCLZ) and N-oxygenated CLZ (CLZ-N-oxide), were measured. IC_{50} of PGD in inhibiting the production of CLZ-N-oxide and norCLZ was 2.41 mg/ml and 2.00 mg/ml, respectively. Experiments were conducted in triplicate (n = 3).

Fig. 2. Michaelis-Menten plots illustrate the effects of PGD, individual Paeonia radix (peony) (Pr) and Glycyrrhiza radix (liquorice) (Gr) preparations and the two individual preparations in combination on the formation of N-demethyl-clozapine (norCLZ, A) and clozapine-N-oxide (CLZ-N-oxide, B) in the human liver microsomes. Serial concentration of clozapine (CLZ) was co-incubated in the presence of herbal preparations. The absence of herbal preparations serves as control (CON).

Fig. 3. The inhibitory effects of PGD on individual CYP enzymes were examined in recombinantly expressed CYPs, including CYP 1A2 (A), CYP2C19 (B), CYP 2D6 (C), CYP 3A4 (D), and CYP 3A4 (E). The enzymatic reaction system contained individual CYP enzyme at the final concentration of 20 *p*mol/ml and the substrate clozapine (CLZ) with three different concentrations (μM) indicated on the end of each linear regression line, in the absence and presence of the inhibitor PGD with five serial concentrations of 0.05□4 mg/ml. The inhibition constant (K_i) and the mode of inhibition were determined with nonlinear regression analysis of the metabolite formation and further verified by visual inspection of Dixon plots that linearize integrally nonlinear relationships.

Fig. 4. Concentration response of PGD (2.5-25 mg/ml, log) in inhibiting the metabolism of clozapine (CLZ, 50 μM) was examined in recombinantly expressed FMO3. The metabolite of CLZ, N-oxygenated CLZ (CLZ-N-oxide) was measured. IC_{50} of PGD in inhibiting the production of CLZ-N-oxide was 44.3 mg/ml. Experiments were conducted in triplicate (n = 3).

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Tables

TABLE 1
Kinetic effects of various herbal preparations on clozapine metabolites in HLM [a,b]

	Control	PGD (2.5 mg/ml)	PR (1.25 mg/ml)	GR (1.25 mg/ml)	PR (1.25 mg/ml) + GR (1.25 mg/ml)
NorCLZ					
V_{max} (*p*mol/min/mg protein)	774.3 ± 63.2	348.4 ± 23.7*	488.0 ± 6.0*	634.7 ± 10.4*	442.6 ± 31.3*
K_m (μM)	91.3 ± 6.0	115.6 ± 10.1	72.3 ± 6.2	138.6 ± 13.9*	107.9 ± 2.9
C_{int} (μl/min/mg protein)	8.5 ± 0.2	3.0 ± 0.2*	6.8 ± 0.5	4.7 ± 0.6*	4.2 ± 0.4*
CLZ-N-oxide					
V_{max} (*p*mol/min/mg protein)	959.5 ± 1.5	549.0 ± 90.3*	773.9 ± 44.8	656.2 ± 49.6*	792.0 ± 100.5
K_m (μM)	45.0 ± 5.1	88.2 ± 3.2*	49.7 ± 1.9	35.2 ± 9.4	102.5 ± 14.4*
C_{int} (μl/min/mg protein)	21.9 ± 2.6	6.3 ± 1.2*	15.6 ± 0.3	20.5 ± 3.5	8.1 ± 1.6*

[a] The data are expressed as mean ± SEM (n = 3) and analyzed using one-way ANOVA, followed by Dunnett's methods for multiple pair comparisons. * $P < 0.05$ vs. control.

[b] PGD, Peony-Glycyrrhiza Decoction; PR, Paeonia radix (peony) preparation; GR, Glycyrrhiza radix (liquorice) preparation; CLZ, clozapine; norCLZ, N-demethyl-clozapine; CLZ-N-oxide, clozapine-N-oxide.

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TABLE 2
Kinetic effects of liquritigenin on clozapine metabolites in HLM [a]

	Control	Liquritigenin (0.2 mM)
NorCLZ		
V_{max} (*p*mol/min/mg protein)	584.9 ± 49.4	481.9 ± 62.8
K_m (μM)	43.7 ± 14.3	57.2 ± 34.7
C_{int} (μl/min/mg protein)	16.0 ± 4.2	15.2 ± 6.1
CLZ-N-oxide		
V_{max} (*p*mol/min/mg protein)	920.7 ± 48.1	605.9 ± 14.2*
K_m (μM)	46.3 ± 10.7	18.1 ± 3.7
C_{int} (μl/min/mg protein)	21.5 ± 3.5	36.7 ± 7.7

[a] The data are expressed as mean ± SEM (n = 3) and analyzed using two-sided Student t-test. * $P < 0.05$ vs. control.

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TABLE 3
Effects of full and partial activation of FMOs with and without the coenzyme NADPH on BZD and CLZ metabolism in HLM

Substrate (100 μM)	NADPH (1 mM)	N-oxidation (*p*mol/mg protein/min)[a]	N-demethylation (*p*mol/mg protein/min)[a]
BZD	+	498.4 ± 15.2	
BZD	-	247.5 ± 17.4*	
CLZ	+	682.6 ± 7.8	393.0 ± 7.7
CLZ	-	664.4 ± 13.5	400.1 ± 9.7

[a] The data are expressed as mean ± SEM (n = 3) and analyzed using two-sided Student t-test. * $P < 0.05$ vs. NADPH (+) with the same substrate. BZD, benzydamine; CLZ, clozapine; NADPH, nicotinamide adenine dinucleotide phosphate.

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TABLE 4
The inhibitory potency of PGD towards CYPs in CLZ metabolism in recombinantly expressed CYPs

CYPs	Metabolites of CLZ	V_{max} (*p*mol/min/mg protein)	K_m (μM)	K_i (mg/ml)	Inhibition model
1A2	norCLZ	28.5 ± 4.7	50.8 ± 0.5	0.9 ± 0.1	noncompetitive
2C19	norCLZ	23.9 ± 1.3	30.9 ± 1.4	0.3 ± 0.0	noncompetitive
2D6	norCLZ	53.1 ± 4.3	12.0 ± 4.0	2.4 ± 0.5	mixed
3A4	norCLZ	15.2 ± 0.2	40.2 ± 10.5	0.6 ± 0.1	competitive
3A4	CLZ-N-oxide	27.7 ± 3.8	16.1 ± 2.9	0.5 ± 0.1	mixed

[a] The data are expressed as mean ± SEM (n = 3).

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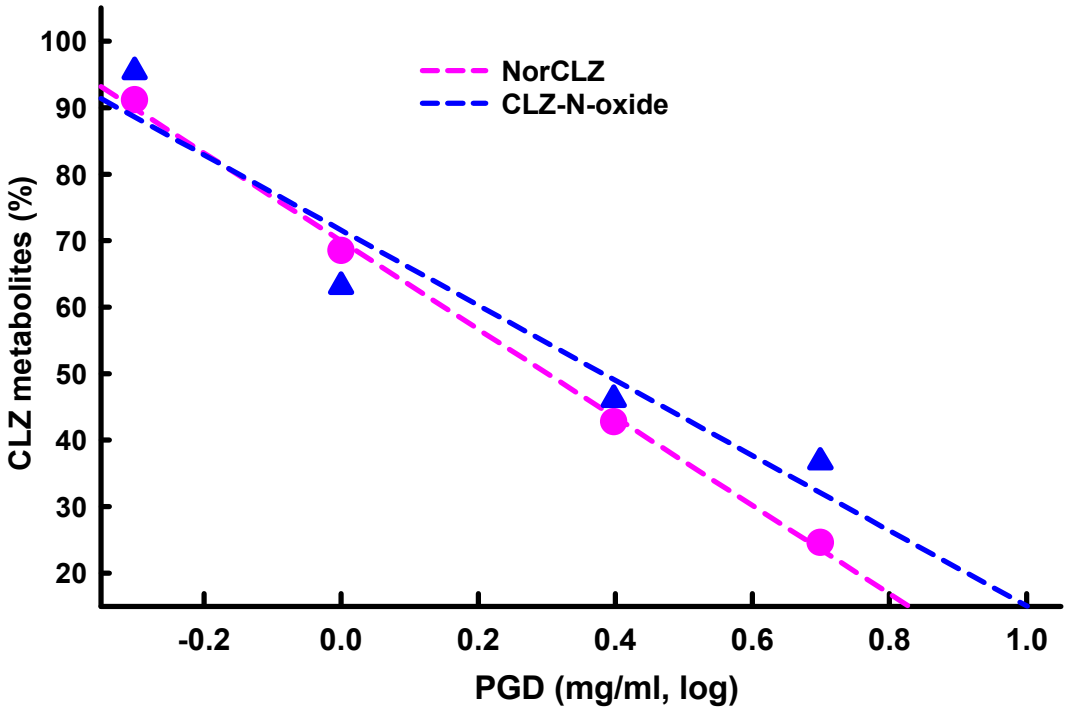

Figure 1

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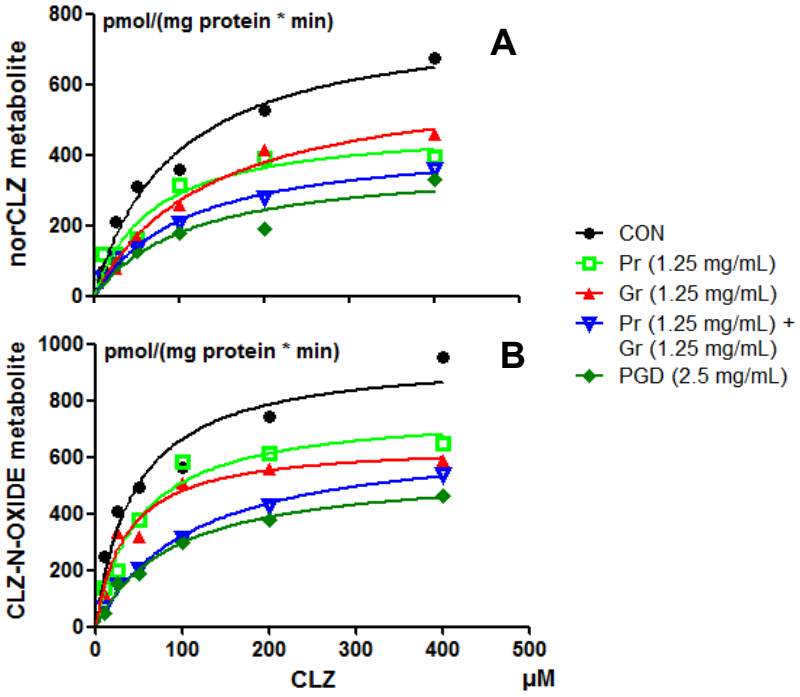

Figure 2

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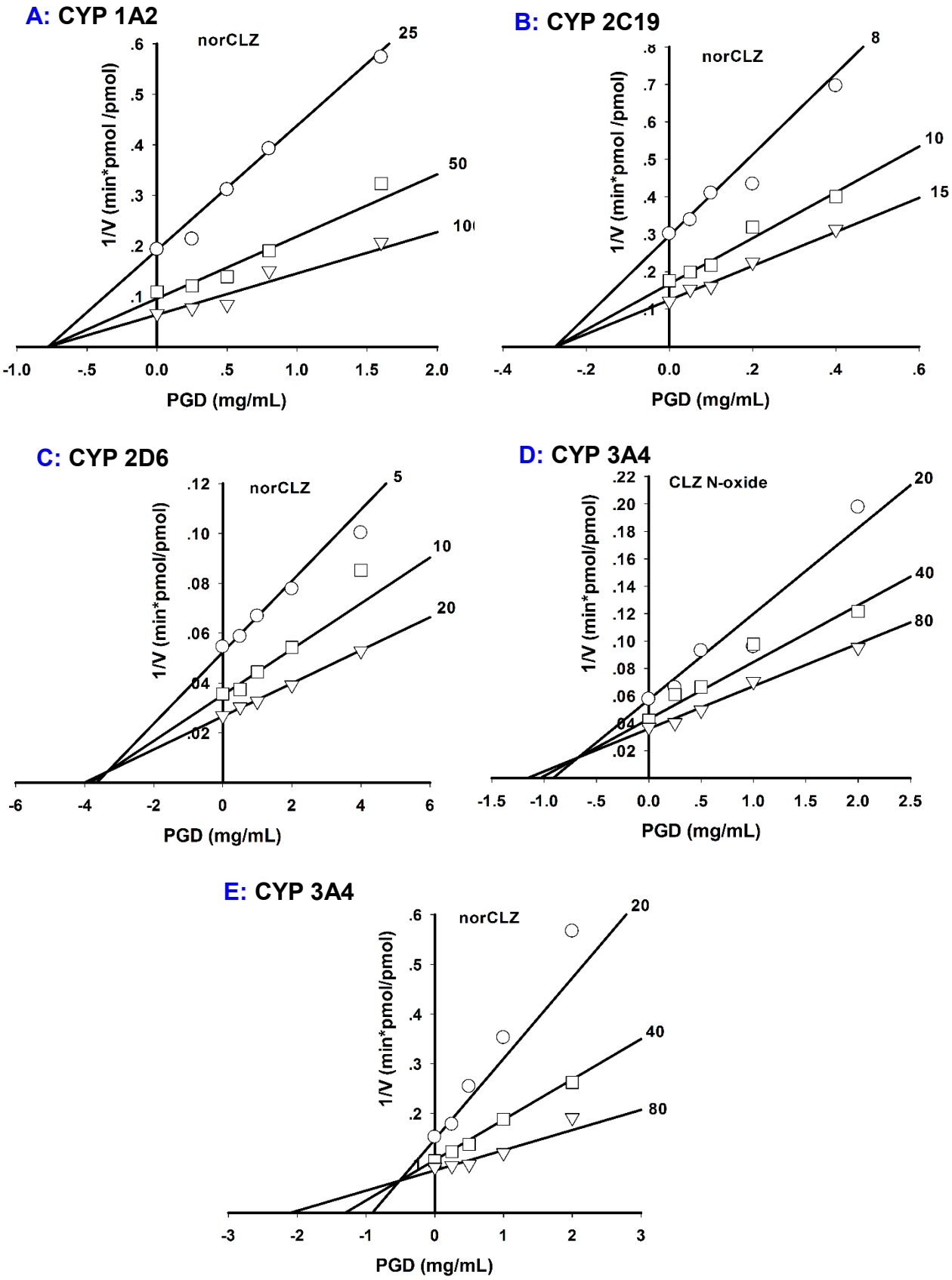

Figure 3

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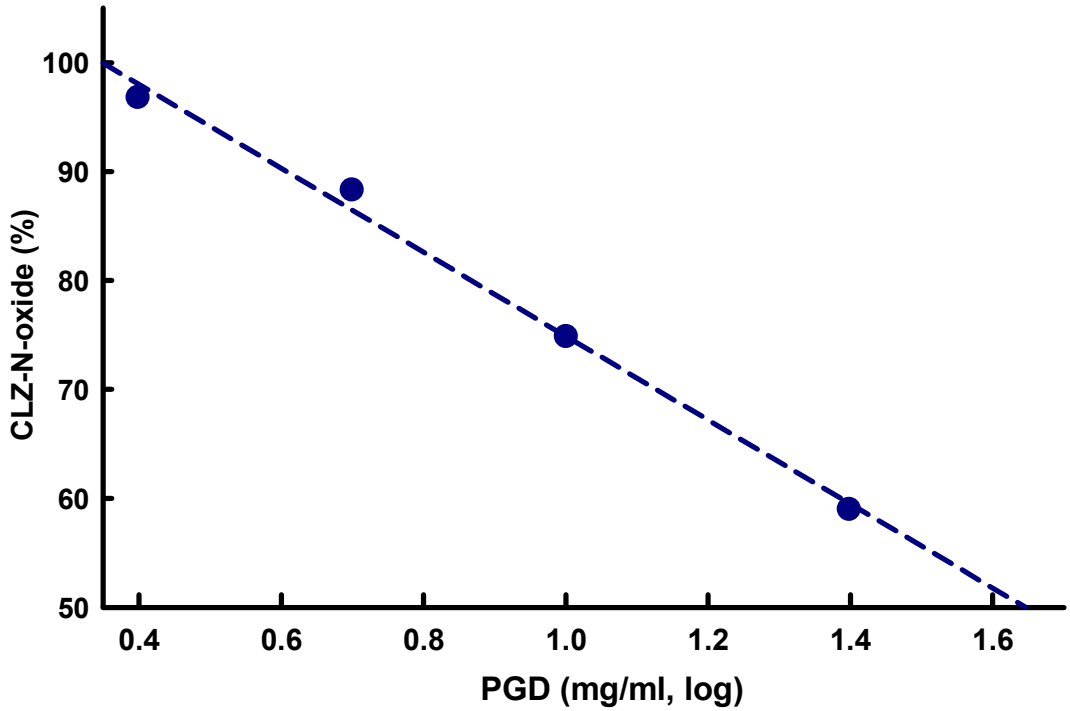

Figure 4