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The drug-drug effects of rhein on the pharmacokinetics and pharmacodynamics of clozapine in rat brain extracellular fluid by *in vivo* microdialysis

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Abbreviation: DA, dopamine; DOPAC, 3, 4-dihydroxyphenylacetic acid; E, epinephrine; ECF, extracellular fluid; ESI, electrospray ionization; 5-HIAA, 5-hydroxyindole-3-acetic acid; HPLC-ECD, high performance liquid chromatography-electrochemical detection; 5-HT, serotonin; HVA, homovanillic acid; MRM, multiple reaction monitoring; 3-MT, 3-methoxytyramine hydrochloride; NE, norepinephrine; TCM, traditional Chinese medicine; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry.

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## ABSTRACT

Clozapine, an atypical antipsychotic agent, is highly effective in treatment-resistant schizophrenia; however, its major side effect is constipation. Instead of laxatives, rhein is a pharmacologically active component found in *Rheum palmatum* L., a medicinal herbal remedy for constipation. The purpose of this study is to determine whether rhein impacts the pharmacokinetics and pharmacodynamics of clozapine in brain when used to relieve clozapine-induced constipation. Here we have investigated not only pharmacokinetics of clozapine in blood but also the effects of rhein on the pharmacokinetics of clozapine in blood and in brain extracellular fluid (ECF) together with the pharmacodynamics effects on neurotransmitters in ECF. The concentration of clozapine and norclozapine in biological samples were measured by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The drug-drug effects of rhein on extracellular neurotransmitter efflux in the rat mPFC produced by clozapine were assayed by high performance liquid chromatography-electrochemical detection (HPLC-ECD). The results demonstrate that clozapine pharmacokinetics was nonlinear. Pretreatment with rhein for 7 days increased total blood concentration of clozapine, but significantly reduced the unbound clozapine concentrations in the mPFC by approximately 3-fold. Furthermore, 7 days of rhein pretreatment thoroughly abolished the efflux of dopamine and its metabolite (DOPAC) and altered the profile of HVA, another metabolite of dopamine, in the mPFC. In conclusion, rhein was found to substantially decrease clozapine and norclozapine concentrations in the mPFC dialysate, and this is accompanied by lower concentrations in the neurotransmitters in the same biophase. These findings suggest that a detailed clinical study for drug-drug interactions is recommended.

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## Introduction

Antipsychotics are the cornerstone of the management of psychotic disorders and schizophrenia (De Hert et al., 2011) that is a severe mental illness characterized by positive symptoms, negative symptoms, and cognitive impairment. Clozapine is an atypical antipsychotic agent that used for the treatment of schizophrenia. Numerous studies (Murray, 2006; Spina and de Leon, 2007; Fakra and Azorin, 2012) have demonstrated that clozapine, a D<sub>2</sub>-5HT<sub>2</sub> antagonist, is more effective than other antipsychotics against treatment-resistant schizophrenia and is associated with the lowest risk of death such as reducing the risk of suicidal behavior in patients with schizophrenia (Jagodic et al., 2013). Clozapine, second generation antipsychotic, is attributed to some degree to D<sub>2</sub> antagonism, but more to blockade of certain serotonin receptors. The selective blockade of serotonin receptors enhances dopamine function in the mesolimbic pathway, which is relevant in the pathophysiology of schizophrenia (Adams and van den Buuse, 2011). Clozapine is approved for use in patients who are resistant to typical neuroleptics and compliant with strict blood monitoring. Clozapine is primarily metabolized by CYP1A2 into two main metabolites, norclozapine and clozapine-N-oxide. Norclozapine is considered the major metabolite of clozapine because clozapine-N-oxide has a relatively low concentration and little pharmacological activity (Fakra and Azorin, 2012; Wiebelhaus et al., 2012). Clozapine treatment is associated with multiple adverse effects; its most common gastrointestinal side effect is constipation (Fakra and Azorin, 2012).

An estimated one-third of people worldwide suffer from constipation that is a common gastrointestinal problem (Jong et al., 2010; Chey et al., 2011). Laxatives and

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traditional Chinese medicine (TCM) are used to improve symptoms and return the bowel functions to normal physiology (Camilleri and Bharucha, 2010; Candy et al., 2011; Chey et al., 2011). It is reported that the single Chinese herb, rhizomes of *Rheum palmatum* L. (Rhubarb) is used for constipation remedy (Jong et al., 2010). Many active components including aloe-emodin, emodin, and rhein were found in *Rheum palmatum* L. with pharmacologically effects. For example, rhein has moderate anti-inflammatory, analgesic activity and weak laxative effects (Spencer and Wilde, 1997).

Simultaneous monitoring of brain monoamine level changes by the *in vivo* microdialysis sampling technique is an important tool in the discovery of new drug therapies for a large number of neurological disorders such as Parkinson's disease, Alzheimer's disease, epilepsy and neuropsychiatric disorders (Garrison et al., 2002). The advantages of the microdialysis technique include not only simultaneous sampling at multiple sites such as the brain (Gottas et al., 2013) but also no need for sample preparation as the dialysis membrane excludes proteins from the aqueous sample (Tsai, 2003). The use of ion-pair high performance liquid chromatography (HPLC) coupled with electrochemical detection (ECD) is of great interest for the determination of monoamine neurotransmitters norepinephrine (NE), epinephrine (E), 3, 4-dihydroxyphenylacetic acid (DOPAC), dopamine (DA), 5-hydroxyindole-3-acetic acid (5-HIAA), homovanillic acid (HVA), serotonin (5-HT), and 3-methoxytyramine hydrochloride (3-MT) in microdialysis samples (Bicker et al., 2013). Moreover, ultra-performance liquid chromatography (UPLC) coupled with tandem mass spectrometry has been used to quantify the concentrations of drug and its metabolites in microdialysate (Cremers et al., 2012).

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Clozapine may interact with other agents that induce or inhibit CYP1A2 to increase or decrease the metabolism of clozapine (Fakra and Azorin, 2012). For instance, cigarette smoke increases the activity of CYP1A2, thus decreasing the blood concentrations of clozapine. It has been reported that smokers require up to double the dose of clozapine compared with non-smokers to achieve an equivalent plasma concentration due to induced metabolism (Tsuda et al., 2014). Fluoxetine, cimetidine, and a lesser extent valproate inhibit the activities of CYP enzymes will increase levels of clozapine and its metabolites (Watras and Taylor, 2013; Victoroff et al., 2014). Studies on the comparative pharmacokinetics of rhein in normal and constipated rats demonstrate that the loperamide-induced constipation reduced the absorption of rhein (Hou et al., 2014a). Additionally, investigations on gene expression by microarray analysis indicate that five drug-metabolizing genes such as Cyp7a1, Cyp2c6, Ces2e, Atp1b1, and Slc7a2 were significantly altered by the San-Huang-Xie-Xin-Tang (SHXXT) treatment (Hou et al., 2014a). SHXXT, a medicinal herbal product used as a remedy for constipation, consists of rhizomes of *Rheum palmatum* L. (Rhubarb), roots of *Scutellaria baicalensis* Georgi and rhizomes of *Coptis deltoidea* C. Y. Cheng & P. K. Hsiao, with a weight ratio of 2:1:1, respectively. Of the five altered genes, Cyp7a1, Cyp2c6, Atp1b1, and Slc7a2 were up-regulated by approximately 2-fold; however, Ces2e was down-regulated by 19-fold based on the results of microarray analysis. Although no data on the potential for rhein to act as drug-drug interaction perpetrator except the in vitro data regarding inhibition of CYP enzymes by rhein in rat liver microsomes (Tang et al., 2009) were investigated, emodin, a similar compound of rhein, has been reported to have inhibitory properties on P-gp based on in vitro studies (Liu et al., 2011). Therefore, alternations in drug-metabolizing genes

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modulate the functions of drug-metabolizing enzymes, which may potentially impact the therapeutic window of drugs and cause herb-drug interactions. However, there are no reports of the pharmacokinetics of clozapine concerning brain distribution, and the pharmacodynamics of extracellular neurotransmitter changes in medial prefrontal cortex (mPFC) induced by concomitant rhein and clozapine use. Thus, the aims of this study are to investigate the pharmacokinetics of clozapine in freely moving rats by UPLC-MS/MS, to explore whether pretreatment with rhein affects the clozapine and norclozapine levels in blood and in mPFC of rats, and to evaluate whether pretreatment with rhein influences the pharmacodynamics of clozapine on extracellular neurotransmitter efflux in rat mPFC.

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## Materials and methods

### Chemicals and Reagents

The chemicals rhein, clozapine, norclozapine, carbamazepine, sodium 1-octanesulfonate monohydrate, sodium metabisulfite, 3-hydroxytyramine hydrochloride (dopamine, DA), 3, 4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine hydrochloride (3-MT), homovanillic acid (HVA), (-)-norepinephrine (NE), (-)-epinephrine (E), serotonin hydrochloride (5-HT), and 5-hydroxyindole-3-acetic acid (5-HIAA) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). LC/MS grade solvents were obtained from J.T. Baker, Inc. (Phillipsburg, NJ, USA) and chromatographic reagents were obtained from Tedia Co., Inc. (Fairfield, OH, USA). Sodium chloride, sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$ ), orthophosphoric acid ( $\text{H}_3\text{PO}_4$ , 85%), hydrochloric acid, disodium edetate, potassium chloride, and sodium hydroxide were purchased from E. Merck (Darmstadt, Germany). Triple deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

### Clozapine and norclozapine assay

All the experiments were carried out on a Waters Acquity UPLC-MS/MS system (Waters, Manchester, UK) equipped with an Acquity UPLC type BEH C18 column, maintained at 40 °C in a column oven. The UPLC system was coupled with a Waters Xevo<sup>TM</sup> tandem quadrupole mass spectrometer in electrospray ionization (ESI) mode. The multiple reaction monitoring (MRM) mode was used for quantification. All ion transitions and collision energies were determined and optimized by using the



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MassLynx 4.1 software data platform. The MS conditions were set as follows: ESI, positive mode; source temperature, 150 °C; collision gas, argon; desolvation temperature, 400 °C; desolvation gas flow, 800 L/h. The optimized cone voltages (CV) were 34 V for clozapine, 36 V for norclozapine, and 32 V for carbamazepine. The ion transitions monitored were m/z 327.2, 192.1 for clozapine, m/z 313.3, 192.1 for norclozapine, and m/z 237.1, 165.1 for carbamazepine. Carbamazepine was used as the internal standard (IS) for positive ion mode analytes. Chromatographic separation was achieved using a C18 column (100 x 2.1 mm, 1.7 µm). Mobile phase A consisted of 5 mM ammonium formate, pH 6.1, and mobile phase B consisted of acetonitrile:methanol 3:2, v/v. A gradient elution of 95% (v/v) A at 0-2 min, 60% A at 2.1-7 min, 52% A at 7.1-11 min, 20% A at 11.1-14 min, and 95% A at 14.1-17 min was used. The flow rate was set at 0.25 mL/min, and the injection volume was 5 µL. A MassLynx 4.1 software data platform was used spectral acquisition, spectral presentation and peak quantification.

The method validation assays for quantification of clozapine and norclozapine in rat plasma and in rat mPFC dialysates were conducted based on the current US Food and Drug Administration (FDA) bioanalytical method validation guidance (Zimmer, 2014). The specificity, matrix effects and recovery were evaluated. Matrix effects can be described as the difference between the mass spectrometric response for an analyte in standard solution and the response for the same analyte in a biological matrix, such as plasma. Matrix effects result from co-eluting matrix components that affect the ionization of the target analyte, resulting either in ion suppression, or in ion enhancement (Van Eeckhaut et al., 2009). To evaluate matrix effect (ME) and recovery (RE), six different lots of blank plasma were extracted and then spiked with

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clozapine or norclozapine at three concentrations. The corresponding peak areas of clozapine or norclozapine in the spiked biological samples post-extraction (A) were compared to those of the aqueous standards in mobile phase (B) at equivalent concentrations. The ratio  $(A/B \times 100)$  is defined as the ME. The corresponding peak areas of standards in the spiked biological samples before extraction (C) were compared to those of standards in the spiked biological samples post-extraction (A) at equivalent concentrations. The ratio  $(C/A \times 100)$  is defined as the RE. All linear calibration curves were required to have a coefficient of estimation of at least  $>0.995$ . The intra- and inter-day variability, accuracy (bias %), and the relative standard deviation (RSD) were calculated.

### **Animal experiments**

All animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC number: 1011202) of National Yang-Ming University. Total fifty-six male specific pathogen-free Sprague-Dawley rats weighing  $220 \pm 20$  g were used in this study. For the pharmacokinetic study, six rats per group were anesthetized with pentobarbital (50 mg/kg, i.p.) for cannulation. The detailed procedures of cannulation were performed as the previous study (Hou et al., 2014a). Additionally, ten rats per group were used for *in vivo* microdialysis study.

The dose of clozapine for animals was derived from a human dose by following a conversion equation recommended by the US Food and Drug Administration guidelines: Human equivalent dose (HED, mg/kg) = animal dose (mg/kg)  $\times$  (animal  $K_m$ /human  $K_m$ ) (Reagan-Shaw et al., 2008). The  $K_m$  factor, body weight (kg) divided

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by body surface area (BSA,  $m^2$ ), is used to convert the mg/kg dose in a study to an  $mg/m^2$  dose. The  $K_m$  factors are 6 and 37 for a rat and a human, respectively. Briefly, clozapine suspended in water at doses of 10, 30, and 100 mg/kg was individually administered to rats by oral gavage. Approximately 200  $\mu$ L of blood samples was withdrawn serially from the arterial cannula and placed into heparinized vials at 0, 5, 15, 30, 60, 90, 120, 240, 360 and 480 min. For quantitative analysis, each plasma sample (50  $\mu$ L) was vortex-mixed with acetonitrile (100  $\mu$ L) for protein precipitation. Data from these samples were used to construct the pharmacokinetic curves of clozapine and norclozapine. Plasma samples were diluted by blank plasma samples at an appropriate ratio before analysis if the clozapine or norclozapine concentrations exceeded 2500 ng/mL.

To investigate drug-drug interactions of rhein on the blood pharmacokinetics of clozapine, rhein at 10 mg/kg was orally administered to rats for 7 days. The day before the pharmacokinetic study, a PE50 tubing was implanted into the left carotid artery of rat for blood sampling. One hour after the 7<sup>th</sup> dose of rhein, clozapine at 100 mg/kg was given orally to rats for pharmacokinetic study.

#### Pharmacokinetic analyses

Pharmacokinetic calculations were performed on each individual data set by noncompartmental methods using WinNonlin Standard Edition, version 1.1 (Pharsight Corp., Mountain View, CA).

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### **norclozapine in the rat mPFC by *in vivo* microdialysis of freely moving rats**

The UPLC-MS/MS methods were performed as described above with some modifications. Briefly, for analysis of dialysate samples, the LC flow was diverted to waste from 0 to 2 min and then introduced to the mass spectrometer from 2.1 to 17 min using a six-port switching valve. The autosampler injection needle was washed with methanol to reduce the carry-over after each injection. An external standard was used in this study.

The dose of rhein for animals was chosen by two reasons. First, for adults, the powdered SHXXT formula of the pharmaceutical herbal product for clinical application is 1.5 g per time and 2-3 times daily. According to the dose translation (Reagan-Shaw et al., 2008) from human to animal, the calculated oral dose of SHXXT (0.5 g/kg) was equivalent to rhein administration dose of 1 mg/kg. Second, in our previous study (Hou et al., 2014a) on gene expression profiling in drug-metabolizing genes after SHXXT treatment, the results of microarray analysis demonstrate that Cyp7a1, Cyp2c6, Atp1b1, and Slc7a2 were up-regulated by approximately 2-fold; however, Ces2e was down-regulated by 19-fold post seven days of SHXXT treatment. Thus, based on the clinical application of SHXXT and our previous experimental evidences, rhein at 1 and 10 mg/kg were given orally for 7 days to explore the herb-drug interactions of rhein on the pharmacokinetics and pharmacodynamics of clozapine in the rat mPFC.

The microdialysis system consisted of a CMA400 microinjection pump, a CMA470 refrigerated fraction collector (CMA Microdialysis AB, Solna, Sweden) and

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microdialysis probes. A CMA Elite microdialysis probe (molecular weight cut-off of 20,000 Da; membrane of 4 mm in length, CMA Microdialysis AB, Solna, Sweden) was used for the brain medial prefrontal cortex sampling.

Surgery for implantation of microdialysis guide cannula was conducted under pentobarbital anesthesia (50 mg/kg, i.p.). The rats were mounted in a stereotaxic frame (David Kopf instruments, California, USA) and a CMA 12 guide cannula was inserted into the medial prefrontal cortex at + 3.2 mm anteroposterior, + 0.8 mm mediolateral (10° inclination), and -5.5 mm dorsoventral to bregma. The guide cannula and two anchor screws were affixed with dental cement (Hygenic Repair Acrylic kit, The Hygenic Corporation, Akron, Ohio, USA), and the wound sealed. Animals were individually housed in dialysis cages and allowed at least 2 days recovery from surgery.

For sampling, a CMA Elite microdialysis probe, with a 4 mm long dialysis membrane, was inserted into the guide cannula and the animal was then coupled to the equipment. The microdialysis probe was then perfused with Ringer's solution consisting of 147 mM sodium chloride, 2.2 mM calcium chloride, and 4 mM potassium chloride at 1.5  $\mu$ L/min and the animal was left to acclimatize at least two hours. Sample collection intervals were set to 20 min. After the 2 h stabilization period following the implantation of the microdialysis probe, four basal dialysates were obtained at 20 min intervals, and then the rat was administered with clozapine (100 mg/kg, p.o.) with or without rhein (1 and 10 mg/kg, p.o. x 7) pretreatment. One hour after the 7<sup>th</sup> dose of rhein, clozapine was given to the rat. The dialysates were collected into the vials containing 7.5  $\mu$ L of an antioxidant solution (100 mM acetic acid, 3.3 mM L-cysteine,

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0.27 mM disodium edetate and 12.5  $\mu$ M ascorbic acid) throughout the experiment. Samples were analyzed by HPLC-ECD for neurotransmitter evaluation and by UPLC-MS/MS for determination of clozapine and norclozapine concentrations.

*In vivo* recovery of clozapine and norclozapine through the microdialysis probe was estimated as described in the previous study (Lu et al., 2014). The microdialysis probe was inserted into the medial prefrontal cortex, and perfused with Ringer's solution containing clozapine or norclozapine at low, medium, and high concentrations (25, 100 and 250 ng/mL) at a flow rate of 1.5  $\mu$ L/min. *In vivo* recovery was evaluated in three individual experiments for each concentration with the brain microdialysis probe. The clozapine or norclozapine perfusate ( $C_{\text{perf}}$ ) and dialysate ( $C_{\text{dial}}$ ) concentrations were determined by UPLC-MS/MS. The *in vivo* recovery ( $R_{\text{dial}}$ ) of clozapine or norclozapine was calculated using the following equation:  $R_{\text{dial}} = (C_{\text{perf}} - C_{\text{dial}})/C_{\text{perf}}$ . The concentrations of clozapine or norclozapine were converted to free-form concentrations ( $C_f$ ) as follows:  $C_f = C_m/R_{\text{dial}}$ .

### **The drug-drug effects of rehin on extracellular neurotransmitter levels produced by clozapine in the rat mPFC by *in vivo* microdialysis and HPLC-ECD**

The HPLC-ECD system consisted of a BASi PM-92E LC pump (Bioanalytical Systems, West Lafayette, IN, USA), a CMA200 refrigerated micro sampler, a CMA240 sample injector with a 20  $\mu$ L loop (CMA, Stockholm, Sweden), and a Decade II electrochemical detector fitted with a SenCell<sup>TM</sup> electrochemical flow cell (2 mm glassy carbon working electrode and an in situ Ag/AgCl (ISAAC) reference electrode) (Antec, Zoeterwoude, The Netherlands). An RP-18e column (Merck

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Chromolith® Performance; 100 x 2 mm, i.d.; particle size, 2 µm) with a guard column (5 x 2 mm, i.d.) at 35 °C maintained by column heater in a Decade II amperometric detector with isocratic mobile phase (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.74 mM sodium 1-octanesulfonate, 0.027 mM EDTA, 2 mM KCl, and 8% methanol, pH 3.74, adjusting with 85% orthophosphoric acid) at a flow rate of 180 µL/min was used for neurotransmitter separation in brain dialysates. The buffer was filtered through a Millipore membrane (0.22 µm) and degassed by sonication prior to use. The analytes were detected at a detection potential of +700 mV versus the reference electrode, a filter value of 0.05 Hz, and range of 5 nA with an injection volume of 20 µL. Clarity chromatography software (DataApex, Prague, Czech Republic) was used for data processing.

To investigate the drug-drug interaction effects of rhein on extracellular neurotransmitter release in mPFC produced by clozapine administration, an experiment was conducted by *in vivo* microdialysis sampling, and changes in extracellular neurotransmitter levels were measured by HPLC-ECD. The procedure of surgery for *in vivo* microdialysis of freely moving rats was as described above. Dialysates were collected into vials containing 7.5 µL of antioxidant reagent for an additional 320 min period and neurotransmitter content was analyzed by HPLC-ECD.

### **Statistical analysis**

Data were summarized as the mean ± SD or mean ± SEM. Comparisons among more than two groups were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test. Comparison between two groups was performed using the

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unpaired Student's t-test. Statistical significance was set at  $p < 0.05$ .



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## Results

### Optimization of the LC-MS/MS method

The standard solution (100 ng/mL) of clozapine, norclozapine, or carbamazepine was analyzed for optimization of MS conditions. The MRM mode provided high selectivity and sensitivity for the quantification assay was used for analyte identification. Chromatographic conditions were optimized for good sensitivity and peak shape. A combined organic solvent of acetonitrile and methanol with a volume ratio of 3:2, provided the best peak shape and was selected as the organic phase. Finally, the mobile phase consisting of acetonitrile- methanol- 5 mM ammonium formate solution (gradient elution) was used in the experiment.

UPLC-MS/MS method validation of clozapine and norclozapine in rat plasma was evaluated. Assay specificity was assessed by comparing the chromatograms of blank plasma samples and the results demonstrate that the UPLC-MS/MS conditions have no interference of clozapine, norclozapine, and carbamazepine (IS) from plasma. The matrix effects and recovery were evaluated for method validation (Van Eeckhaut et al., 2009). The matrix effects of clozapine, norclozapine, and carbamazepine (IS) were  $131 \pm 7$ ,  $129 \pm 10$ , and  $105 \pm 2\%$  in plasma, respectively. A value of 100% matrix effect indicated that the response in the mobile phase and in the plasma extracts was the same and there was no matrix effect. The mean recovery for clozapine, norclozapine, and IS were  $98 \pm 6$ ,  $108 \pm 9$ , and  $97 \pm 3\%$  in plasma, respectively. The variability (%) of recovery within 10% was acceptable. The calibration curves were linear over a concentration range of 50-2500 ng/mL for clozapine and norclozapine in rat plasma. Moreover, the calibration curves were linear over a concentration range of

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0.5-100 ng/mL for clozapine and norclozapine in rat brain cortical dialysate. The correlation coefficient of the calibration curves for clozapine and norclozapine were at least  $>0.995$ . The limit of quantification (LOQ) of clozapine and norclozapine in rat plasma was 50 ng/mL. Furthermore, the limit of quantification (LOQ) of clozapine and norclozapine in rat brain cortical dialysate was 0.5 ng/mL. The intra- and inter-day variability, accuracy (bias %), and the relative standard deviation (RSD) were within 15%. These results show that the UPLC-MS/MS method provides excellent quantitative analysis of clozapine and norclozapine in rat plasma extracts and in microdialysate samples.

### **Blood pharmacokinetics of clozapine and norclozapine in freely moving rats**

The mean plasma concentration-time profiles of clozapine and its metabolite after oral administration of clozapine at 10, 30, and 100 mg/kg ( $n=6$ ) are illustrated in Fig. 1 and pharmacokinetic parameters are listed in Table 1. Clozapine blood levels declined below the LOQ after 120 min following a 10 mg/kg dose. The  $C_{\max}$  for clozapine was  $169 \pm 88.2$ ,  $634 \pm 110$ , and  $644 \pm 96$  ng/mL for 10, 30, and 100 mg/kg oral clozapine, respectively, reflecting a nonlinear relationship for blood concentration. The  $T_{1/2}$  of clozapine in blood varied, and ranged from 86.3 to 212 min, indicating slow elimination of clozapine. Changes in the pharmacokinetic parameters of clozapine at 10, 30 and 100 mg/kg p.o. were determined. The AUC was increased by 5.9-fold and 20.3-fold with clozapine 30 and 100 mg/kg, respectively, compared with clozapine 10 mg/kg. The  $C_{\max}$  increased 3.8-fold with clozapine 30 mg/kg compared with clozapine 10 mg/kg; however, clozapine 100 mg/kg yielded a  $C_{\max}$  of  $644 \pm 96$  ng/mL, similar to clozapine 30 mg/kg. The MRT increased in a dose-dependent manner.

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As shown in Fig. 1, the norclozapine level in the blood was roughly 4.6-fold higher than clozapine following oral dosing with clozapine at 10 mg/kg. Clozapine 30 mg/kg orally yielded similar results. However, following oral administration of clozapine 100 mg/kg, the profiles of clozapine and norclozapine in the blood differed from those with clozapine at 10 and 30 mg/kg. The AUC of norclozapine was approximately 4.6-fold greater than that of clozapine following clozapine 10 and 30 mg/kg, indicating rapid metabolism of absorbed clozapine. Both  $C_{\max}$  and MRT values increased in a dose-dependent manner.

The effect of rhein on the blood pharmacokinetics of clozapine was investigated. With rhein 10 mg/kg for 7 days, the AUC of clozapine, but not norclozapine, increased by 2.3-fold compared with clozapine 100 mg/kg alone. In addition, the  $C_{\max}$  of clozapine significantly increased by 2.7-fold in combination with rhein pretreatment (Fig. 1 and Table 1).

### ***In vivo* microdialysis recovery**

The average values from the *in vivo* microdialysis recovery of the brain probe for low (25 ng/mL), medium (100 ng/mL) and high (250 ng/mL) clozapine concentrations were  $86.4 \pm 6.8$ ,  $89.3 \pm 3.8$ , and  $93.8 \pm 3.0\%$ , respectively, for clozapine and  $86.3 \pm 10.8$ ,  $87.8 \pm 3.4$ , and  $93.0 \pm 1.4\%$ , respectively, for norclozapine. There were no significant differences in the recovery of the brain microdialysis probe at the three concentrations of clozapine and norclozapine examined. Recovery of the microdialysis probe was independent of the clozapine and norclozapine concentration.

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Dialysis efficiency can be affected by factors including probe length and diameter, the diffusion coefficient of the analyte, perfusion solution composition, the perfusion flow rate, and the substance properties. Therefore, the recovery of each probe must be evaluated at the end of the *in vivo* experiment. The mean *in vivo* recovery was  $89.9 \pm 5.5\%$  for clozapine and  $89.2 \pm 6.5\%$  for norclozapine in the brain probe. There were no significant differences in the levels of recovery between the two substances.

### **The drug-drug effects of rhein on the brain ECF pharmacokinetics of clozapine and norclozapine**

The mean concentration-time profiles of clozapine and its metabolite in rat mPFC dialysate after administration of clozapine (100 mg/kg, p.o.) with or without rhein (1 and 10 mg/kg, p.o. for 7 days, respectively) pretreatment (n=10) are illustrated in Fig. 2 and their pharmacokinetic parameters were calculated (Table 2). As shown in Fig. 2, the drug concentration versus time curve of clozapine and norclozapine in rat mPFC after oral administration of clozapine at 100 mg/kg with or without rhein pretreatment indicated trace amounts of clozapine and norclozapine in rat mPFC, with a lower concentration of norclozapine relative to clozapine. Brain clozapine concentrations exceeded those of norclozapine by approximately 6.7-fold, with AUC values of  $3706 \pm 1159$  min ng/mL for clozapine and  $557 \pm 297$  min ng/mL for norclozapine. The  $C_{\max}$  of clozapine in mPFC yielded similar results. As shown in Fig. 2, the disposition of clozapine in rat mPFC remained at very low levels following coadministration with rhein at 1 or 10 mg/kg for 7 days; however, norclozapine concentrations were undetectable. Seven day of rhein at 1 or 10 mg/kg decreased the distribution of clozapine and norclozapine in rat mPFC (Fig. 2).

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Following 7 days of oral rhein 1 or 10 mg/kg pretreatment, the AUC of clozapine reduced by approximately 3-fold and the  $C_{max}$  decreased approximately 2-fold compared to clozapine alone (Table 2). Additionally, the elimination half-life decreased significantly in a dose-dependent manner. The total body clearance (CL) of clozapine significantly increased approximately 3-fold following pretreatment with rhein at 1 and 10 mg/kg for 7 days. The distribution of brain-to-plasma clozapine ( $AUC_{brain}/AUC_{plasma}$ ) for 100 mg/kg clozapine only was 0.021, indicating that the penetration of clozapine into brain was low. However, pretreatment with rhein significantly declined the penetration rate of clozapine (AUC ratio = 0.007).

Trace amounts of norclozapine were present in the mPFC and the AUC was  $557 \pm 297$  min ng/mL, approximately 7 times less than that of clozapine. Following rhein pretreatment norclozapine concentrations were not detectable, showing that pretreatment influenced the distribution of norclozapine in the mPFC. Following clozapine 100 mg/kg alone, the AUC ratio of norclozapine was 0.001, indicating that the penetration of norclozapine into the brain was less than that of clozapine with an AUC ratio of 0.021.

**The drug-drug effects of rhein on extracellular neurotransmitter release in the mPFC produced by oral administration of clozapine and assayed using *in vivo* microdialysis and HPLC-ECD**

Basal cortical extracellular DA, DOPAC, HVA, and 5-HIAA levels in the dialysates obtained from all rats used in this study were  $0.20 \pm 0.03$ ,  $0.29 \pm 0.03$ ,  $1.12 \pm 0.16$ ,

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and  $1.99 \pm 0.17$  pmol/20  $\mu$ L (mean  $\pm$  SEM; N=40), respectively. Extracellular levels of DA in the mPFC were significantly increased by administration of clozapine to a maximum value of  $168 \pm 23\%$  of preinjection levels (Fig. 3A). The DA efflux in the mPFC began at 20 min after dosing with clozapine at 100 mg/kg; the increase reached the maximum value of 168% of baseline at 60 min, and then returned to baseline 180 min post dosing. In contrast, rhein pretreatment reduced the extracellular level of DA produced by clozapine (100 mg/kg, p.o.) administration in a dose-dependent manner. As shown in Fig. 3A, the influence of rhein on cortical DA efflux induced by clozapine was complete.

Clozapine 100 mg/kg induced a significant and long-lasting increase of approximately 300% of baseline DOPAC and HVA levels in the mPFC (Fig. 3B and C). The extracellular level of DOPAC in the mPFC began to increase 40 min after dosing; the increase reached a maximum value of 300% of baseline at 80 min and was maintained at high levels throughout the experiment. However, rhein pretreatment reduced the extracellular levels of DOPAC and HVA produced by clozapine. Notably, rhein pretreatment abolished the DOPAC efflux in the mPFC produced by clozapine.

The clozapine-induced HVA efflux in the mPFC started 60 min post dose, increased to a maximum value of 300% of baseline, and was maintained throughout the experiment (Fig. 3C). However, following rhein pretreatment, the profile of the HVA efflux in mPFC produced by clozapine were changed. Following pretreatment with rhein, the HVA efflux began to increase at 60 min post dose, reached a maximum value of 250% of baseline at 120 min, and then slowly declined to baseline levels. Clozapine 100 mg/kg failed to affect the extracellular levels of 5-HIAA; however, the

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decline could be significantly observed when pretreated with rhein (Fig. 3D).

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## Discussion

According to the previous results of comparative pharmacokinetic study concerning the pharmacokinetics of rhein, it is indicated that the herbal formulae with multiple constituents significantly increased the absorption rate of rhein (Hou et al., 2014b). Additionally, our results demonstrate that only rhein existed in the unconjugated form after oral administration of the herbal formulae. Thus, the pure rhein compound was chosen to elucidate the drug-drug interaction effects on the pharmacokinetics and pharmacodynamics of clozapine. In the present study, validated LC-MS/MS methods were applied to the pharmacokinetics of clozapine and norclozapine in rat plasma and brain dialysate. The MRM data demonstrated that the quantitative mass transitions of these analytes are consistent with previous reports (Rao et al., 2009; Patteet et al., 2014).

Clozapine and its metabolite norclozapine were determined after oral dosing. Blood pharmacokinetics of oral clozapine at low (10 mg/kg), medium (30 mg/kg), and high (100 mg/kg) doses in freely moving rats was investigated; the results demonstrate that the clozapine and norclozapine levels in rat plasma rose with dose, and the norclozapine levels in plasma were greater than those of clozapine, indicating that the pharmacokinetics of clozapine in blood was nonlinear. Consistent with the previous studies, norclozapine concentrations exceeded those of clozapine at 15 min after drug application, suggesting that the metabolism of clozapine was rapid once clozapine was absorbed (Baldessarini et al., 1993; Weigmann et al., 1999). The effects of dosing regimen (1, 3, 10, 20, 30, 60 mg/kg) on serum and brain concentrations of clozapine and its metabolites in the rat have been investigated (Baldessarini et al., 1993). The



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results demonstrated that clozapine and its metabolites levels in rat serum and striatal brain were highly dose dependent. Following a 10 mg/kg intraperitoneal dose of clozapine, peak clozapine levels were reached very rapidly in serum (10 min) and somewhat later in brain (30 min). The ratio of norclozapine to clozapine in serum ranged from very low to trace values of norclozapine at a low dose of clozapine (1 mg/kg).

Our goals in this work were to investigate whether pretreatment with rhein alters the exposure of clozapine and norclozapine in the conscious rat using brain microdialysis. In order to get the maximal therapeutic window of drugs, clozapine at 100 mg/kg was chosen to investigate the drug-drug interaction effects of rhein on the pharmacokinetics and pharmacodynamics of clozapine in rat brain. Although studies of clozapine and norclozapine pharmacokinetics in the rat mPFC by *in vivo* microdialysis and HPLC-MS/MS have been reported (Cremers et al., 2012; Li et al., 2014), there are no studies of drug-drug interactions of rhein on clozapine pharmacokinetics in the rat mPFC. Thus, pharmacokinetics of clozapine and norclozapine in rat mPFC was conducted by *in vivo* microdialysis in conscious rats.

Drug exposure can be measured using an animal model by microdialysis at the target site (Gottas et al., 2013). As the dialysate contained a considerable amount of nonvolatile salts, the ionization of analytes would be suppressed when microdialysate was directly analyzed by UPLC-MS/MS without prior sample preparation. Thus, two methods were simultaneously used to reduce the influence of non-volatile salts in microdialysates. First, a gradient elution with a mobile phase containing a high proportion of aqueous phase was used to yield early elution of the non-volatile salts in

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the microdialysis samples in the initial separation process. In addition, a divert valve guiding the eluent to waste during the first 2 min of analysis was applied to prevent the salts from entering the ion source. These approaches were successful in minimizing ion suppression and reducing ion source contamination.

Our pharmacokinetic results demonstrated that the brain clozapine concentrations exceeded those of norclozapine by approximately 6.7-fold. Previously norclozapine was detected only at doses greater than or equal to 10 mg/kg and clozapine-N-oxide was undetectable in the brain (Baldessarini et al., 1993). The AUC of clozapine in the mPFC was significantly reduced by approximately 33% following rhein pretreatment (1 and 10 mg/kg, p.o. for 7 days); additionally, norclozapine levels in the mPFC were undetectable in combination with rhein pretreatment (1 and 10 mg/kg, p.o. for 7 days), indicative of decreased distribution of clozapine and norclozapine in the mPFC and an influence on the absorption of clozapine from the gastrointestinal system. Contrary to brain pharmacokinetics of clozapine, the effects of rhein on the blood pharmacokinetics of clozapine demonstrate that rhein pretreatment enhanced the absorption of clozapine and increased the concentrations of clozapine in the blood. On the contrary the blood pharmacokinetic profile of norclozapine in combination with rhein was not different from clozapine alone.

Although investigations on extracellular neurotransmitters efflux in brain using in vivo microdialysis and HPLC-ECD have been reported (Ferry et al., 2014; Gough et al., 2014; Matsumoto et al., 2014), this is the first study to investigate the effects of the drug-drug interaction of rhein on central nervous system pharmacodynamics of clozapine. Clinically, anthraquinones derivatives present in various drugs of plant

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origin are used all over the world for constipation remedy (Muller-Lissner, 2013). The best characterized compounds are sennoside and its aglycone (rhein anthrone) found in senna leaves and senna pods (Matsumoto et al., 2012; Kon et al., 2014). After oral administration, sennoside is degraded only in the lower parts of the gastrointestinal tract, releasing its active metabolite rhein anthrone. The main laxative constituents, sennosides, are prodrugs that are converted to an active component, rhein, by intestinal microflora. However, any factors, especially antibiotics, damaging the intestinal microflora affect the therapeutic effects of sennosides. For the reason, we used rhein, the pure compound, to investigate the drug-drug interactions with clozapine.

Consistent with a previous study (Kuroki et al., 1999), our results demonstrate that clozapine causes a robust increase in dopamine release in the medial prefrontal cortex of freely moving rats. In addition, clozapine elevated cortical DOPAC, indicating drug effects on cortical dopamine metabolism as extracellular DOPAC is considered to be a marker for cytoplasmatic dopamine synthesis. Likewise, clozapine increased dialysate HVA levels, possibly reflective of rapid conversion of most extracellular DOPAC to HVA by catechol-*O*-methyltransferase. Therefore, a preferential increase of dopamine release in medial prefrontal cortex seems to be a common mechanism of action of atypical antipsychotic drugs, which may be relevant for their therapeutic action on negative symptoms of schizophrenia. Notably, our results found that pretreatment with rhein (1 and 10 mg/kg, p.o. for 7 days) reduced the extracellular levels of dopamine and its metabolites (DOPAC and HVA) produced by clozapine (100 mg/kg, p.o.) administration (Fig. 3A-D) in a dose-dependent manner.

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The inhibitory effect of rhein on mPFC clozapine, dopamine and metabolite levels suggests some inhibition of the transport of clozapine in mPFC. In respect to the metabolic rate of clozapine, it is known that clozapine is primarily metabolized by CYP1A2 into two main metabolites (Spina and de Leon, 2007). In humans, clozapine has a complex hepatic metabolism with multiple CYP isoforms involved in its biotransformation. The major metabolic pathways are N-demethylation and N-oxidation to form noreclozapine, which has limited pharmacological activity, and clozapine N-oxide. Currently available *in vitro* and *in vivo* evidence clearly indicate that CYP1A2 plays a major role in the metabolism of clozapine, although other CYP isoforms, including CYP2C19, CYP2D6, CYP3A4 and CYP2C9, also contribute to its biotransformation (Spina and de Leon, 2007). Furthermore, it is reported that rhein weakly inhibits CYP1A2 and CYP2D6 (Tang et al., 2009), which is consistent with our findings on pharmacokinetics of clozapine in plasma. Thus, it is not likely that the inhibition of extracellular DA and its metabolites efflux is through facilitation of the clozapine metabolism.

Generally, low brain penetration can be due to low blood-brain barrier (BBB) permeability, P-glycoprotein (P-gp) efflux, or high plasma protein binding (Di et al., 2008). The major difference between microdialysis and conventional blood sampling is that only the unbound compound can be determined. However, 94.5% of clozapine binds to serum proteins in humans (Schaber et al., 1998), indicating that the relatively low amounts of unbound clozapine can be quantified in blood by means of microdialysis sampling. A clinical study has been reported that drug concentrations in cerebrospinal fluid (CSF) are assumed to roughly equal to unbound concentrations in plasma (Nordin et al., 1995). The positive correlations between serum and CSF levels

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of clozapine in schizophrenic patients has been investigated (Nordin et al., 1995); the results demonstrated that serum clozapine levels were between 43 and 165 ng/mL, and CSF clozapine concentrations ranged from 2 to 39 ng/mL, corresponding to  $23 \pm 14\%$  of the levels in serum. In our study, contrary to brain pharmacokinetics of clozapine, rhein enhanced the concentrations of clozapine in the blood, suggesting that the delivery of clozapine in the mPFC was diminished. It has been reported that schizophrenic patients respond poorly to antipsychotic treatment could be explained by inefficient drug transport across the BBB due to P-gp mediated efflux (Moons et al., 2011). Additionally, emodin, a similar compound of rhein, has been reported to have inhibitory properties on P-gp based on in vitro studies (Liu et al., 2011). Thus, it is possible to speculate that drug-drug interaction of rhein might contribute to attenuate clozapine-induced dopamine and dopamine metabolites release in the mPFC by reducing the transport of clozapine in mPFC. Study on prediction of clozapine exposure in the ECF of human brain using a translational pharmacokinetic (PK) modeling approach demonstrated that a PK model that relates clozapine and norclozapine disposition in rat plasma and brain, including BBB transport, was developed to be successfully translated to predict clozapine and norclozapine concentrations accordant receptor occupancy of both agents in human brain (Li et al., 2014). In our study, rhein significantly increased total plasma clozapine  $C_{max}$  and AUC; on the contrary, rhein significantly decreased the unbound AUC and  $C_{max}$  of clozapine in the mPFC. Thus, monitoring the therapeutic effective plasma levels of clozapine may be not an ideal approach for prediction of clozapine concentrations in brain.

## Conclusion

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A validated LC-MS/MS method was applied to investigate the pharmacokinetics of clozapine and norclozapine in freely moving rats. The pharmacokinetic results demonstrate that pharmacokinetic profile of clozapine at 100 mg/kg was dramatically different from that of clozapine at 10 or 30 mg/kg. The same analytical method was also used to explore the drug-drug interaction of rhein on brain ECF pharmacokinetics of clozapine and norclozapine. The pharmacokinetic results demonstrate that pretreatment with rhein for 7 days significantly reduced the levels of clozapine and norclozapine in the mPFC. Furthermore, pretreatment with rhein for 7 days totally diminished the efflux of DA and its metabolite (DOPAC) and altered the profile of HVA (metabolite of DA) in the mPFC. Since clozapine is an atypical antipsychotic agent used for the treatment of schizophrenia, co-administration of rhein for treating constipation, the major side effect of clozapine, may potentially modulate the therapeutic effects of clozapine, consequently does not effectively treat schizophrenia.

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### **Authorship contributions**

*Participated in research design:* M.L. Hou, and T.H. Tsai

*Conducted experiments:* M.L. Hou

*Contributed new reagents or analytic tools:* C.H. Lin, L.C. Lin, and T.H. Tsai

*Performed data analysis:* M.L. Hou, and T.H. Tsai

*Wrote or contributed to the writing of the manuscript:* M.L. Hou, and T.H. Tsai

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### **Footnotes**

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### Figure legends

Fig. 1. Mean plasma concentration-time profile of clozapine (A) and norclozapine (B) after oral administration of clozapine (10, 30, and 100 mg/kg, respectively) with or without rhein (10 mg/kg, PO x 7) pretreatment. The blood AUC values of clozapine and its metabolite after oral administration of clozapine with or without rhein (10 mg/kg, PO x 7) pretreatment (C). Each point represents mean  $\pm$  SEM (N=6 per group).

Fig. 2. Mean concentration-time profile of clozapine and norclozapine in the rat mPFC after oral administration of clozapine (100 mg/kg) with or without rhein (1 and 10 mg/kg, PO x 7, respectively) pretreatment. Each point represents mean  $\pm$  SEM (N=10).

Fig. 3. Time course effects of clozapine (100 mg/kg, PO) on extracellular neurotransmitter levels (A) dopamine; (B) DOPAC; (C) HVA; (D) 5-HIAA in the mPFC with or without rhein (1 and 10 mg/kg, PO x 7, respectively) pretreatment. The arrow indicates the time of clozapine or vehicle (water, 10 mL/kg, PO, N=2) injection. Data are mean  $\pm$  S.E.M. of the dialysate neurotransmitter levels, expressed as a percentage of each pre-drug baseline neurotransmitter value (N=10 per group).

\*Significantly different from clozapine alone at  $p < 0.05$ .

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Table 1. Pharmacokinetic parameters of clozapine and norclozapine in rat plasma after oral administration of clozapine with or without rhein pretreatment

PK	Clozapine			Clozapine (100 mg/kg)
	10 mg/kg	30 mg/kg	100 mg/kg	+ Rhein (10 mg/kg, PO x7)
<b>Clozapine</b>				
AUC <sub>0-480 min</sub> (min μg/mL)	8.89 ± 3.54	52.7 ± 10.6	180 ± 30.8	422 ± 135
T <sub>1/2</sub> (min)	149 ± 32.1	86.3 ± 8.90	212 ± 42.6	134 ± 14.5
C <sub>max</sub> (ng/mL)	169 ± 88.2	634 ± 110	644 ± 96.0	1730 ± 420*
T <sub>max</sub> (min)	15 ± 0	17.5 ± 2.50	75 ± 55.1	214 ± 72
Vd (L/kg)	109 ± 29.2	71.1 ± 13.4	132 ± 28.1	70.2 ± 26
CL (mL/min/kg)	489 ± 46.1	596 ± 128	428 ± 45.5	380 ± 167
MRT (min)	44.7 ± 1.10	80.4 ± 10.3	236 ± 4.91	239 ± 24
<b>Norclozapine</b>				
AUC <sub>0-480 min</sub> (min μg/mL)	41.2 ± 8.10	237 ± 27.2	582 ± 73.1	600 ± 131
T <sub>1/2</sub> (min)	56.3 ± 8.07	98.3 ± 14.1	-	-
C <sub>max</sub> (ng/mL)	699 ± 103	959 ± 58.5	1608 ± 221	1606 ± 330
T <sub>max</sub> (min)	15 ± 0	22.5 ± 3.35	368 ± 113	334 ± 110
MRT (min)	50.8 ± 4.50	147 ± 14.3	263 ± 2.80	268 ± 8.31

Data expressed as mean ± SEM (N = 6). AUC, area under the concentration versus time curve; t<sub>1/2</sub>, elimination half-life; C<sub>max</sub>, the peak plasma concentration of a drug after administration; T<sub>max</sub>, the time point of maximum plasma concentration curve; Vd, volume of distribution; CL, total body clearance; MRT, mean residence time.

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\*Significantly different from clozapine (100 mg/kg) alone at  $p < 0.05$ .



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Table 2. Pharmacokinetic parameters of protein unbound form of clozapine and norclozapine in rat mPFC after oral administration of clozapine (100 mg/kg) with or without rhein (1 and 10 mg/kg, PO x 7, respectively) pretreatment

PK parameters	Clozapine (100 mg/kg, PO)	Clozapine (100 mg/kg, PO) + Rhein (1 mg/kg, PO x 7)	Clozapine (100 mg/kg, PO) + Rhein (10 mg/kg, PO x 7)
<b>Clozapine</b>			
AUC <sub>0-320 min</sub> (min ng/mL)	3706 ± 1159	1238 ± 290*	1136 ± 196*
T <sub>1/2</sub> (min)	253 ± 106	50.5 ± 12.9	25.2 ± 3.26*
C <sub>max</sub> (ng/mL)	21.4 ± 4.94	11.5 ± 2.40	9.30 ± 2.05*
T <sub>max</sub> (min)	235 ± 24.3	203 ± 21.1	280 ± 14.6
Vd (L/kg)	2510 ± 597	4281 ± 1492	2307 ± 408
CL (L/min/kg)	22.2 ± 10.5	59.1 ± 10.9*	63.2 ± 3.08*
MRT (min)	209 ± 6.39	177 ± 10.1	200 ± 4.47
AUC ratio	0.021	0.007	0.006
<b>Norclozapine</b>			
AUC <sub>0-320 min</sub> (min ng/mL)	557 ± 297	ND	ND
T <sub>1/2</sub> (min)	83.6 ± 39.5	ND	ND
C <sub>max</sub> (ng/mL)	3.24 ± 1.26	ND	ND
T <sub>max</sub> (min)	282 ± 14.4	ND	ND
MRT (min)	220 ± 10.6	ND	ND

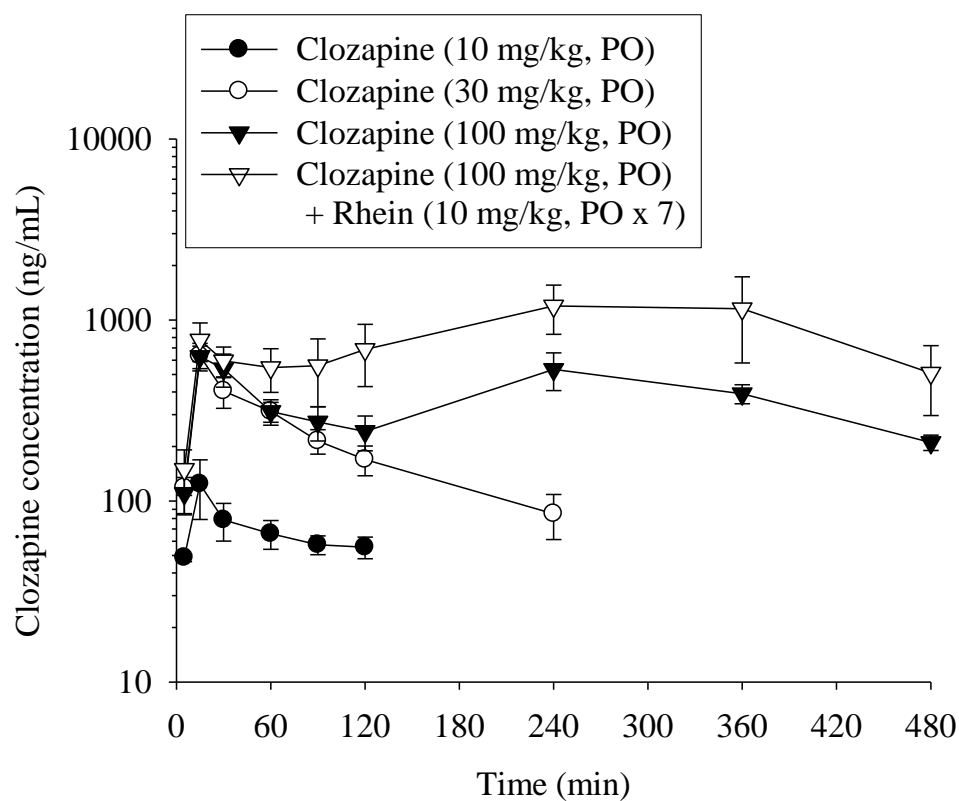
JPET #225763

AUC ratio	0.001	ND	ND
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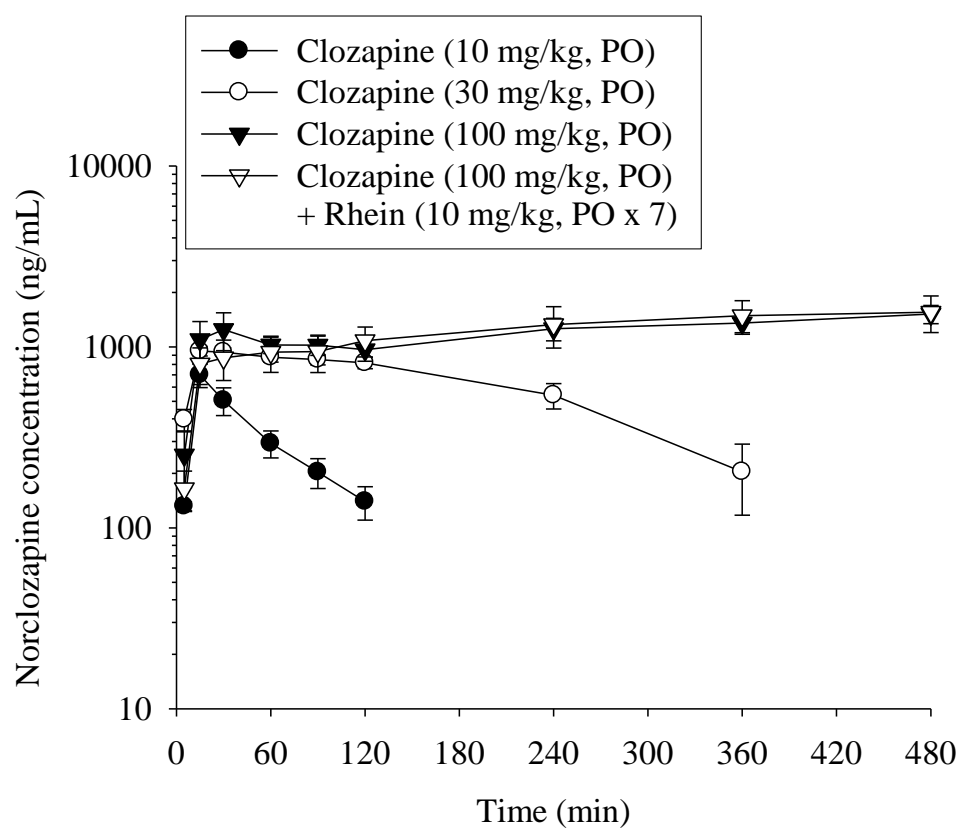
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Data expressed as mean  $\pm$  SEM (N=10). AUC, area under the concentration versus time curve;  $t_{1/2}$ , elimination half-life;  $C_{\max}$ , the peak plasma concentration of a drug after administration;  $T_{\max}$ , the time point of maximum plasma concentration curve;  $V_d$ , volume of distribution; CL, total body clearance; MRT, mean residence time; AUC ratio ( $AUC_{\text{mPFC}}/AUC_{\text{plasma}}$ ), the distribution of brain-to-blood clozapine or norclozapine. \*significantly different from clozapine alone at  $p < 0.05$ . ND, not detected.

(A)



(B)



(C)

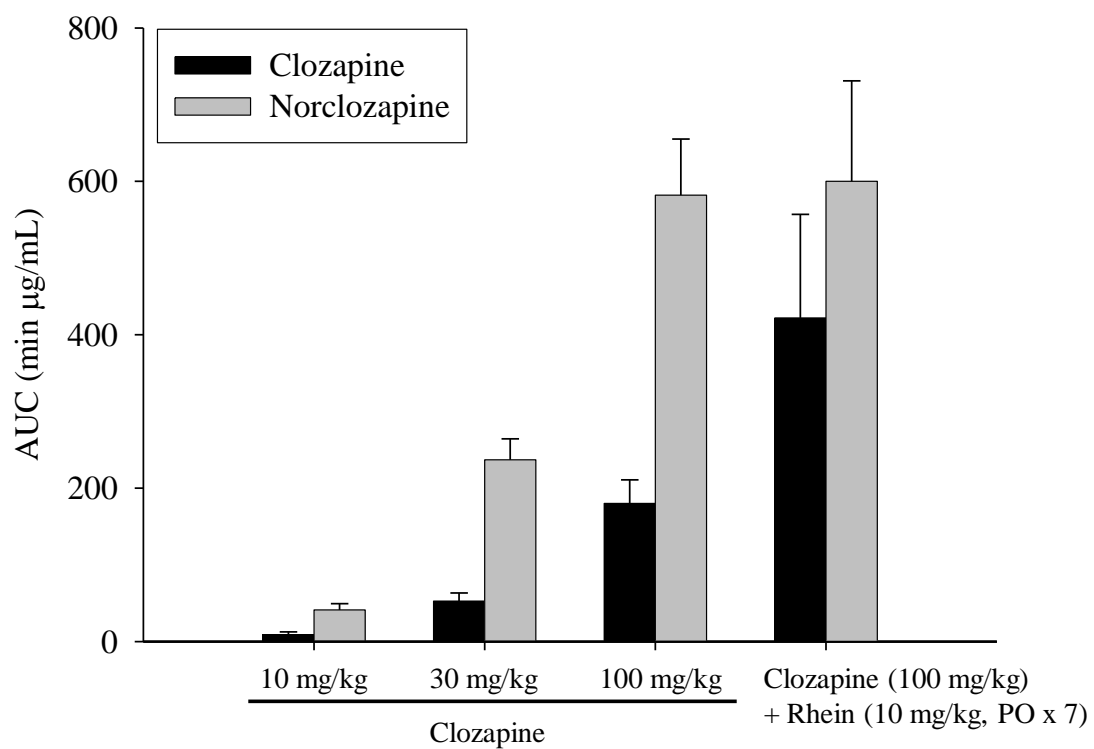


Fig. 1.

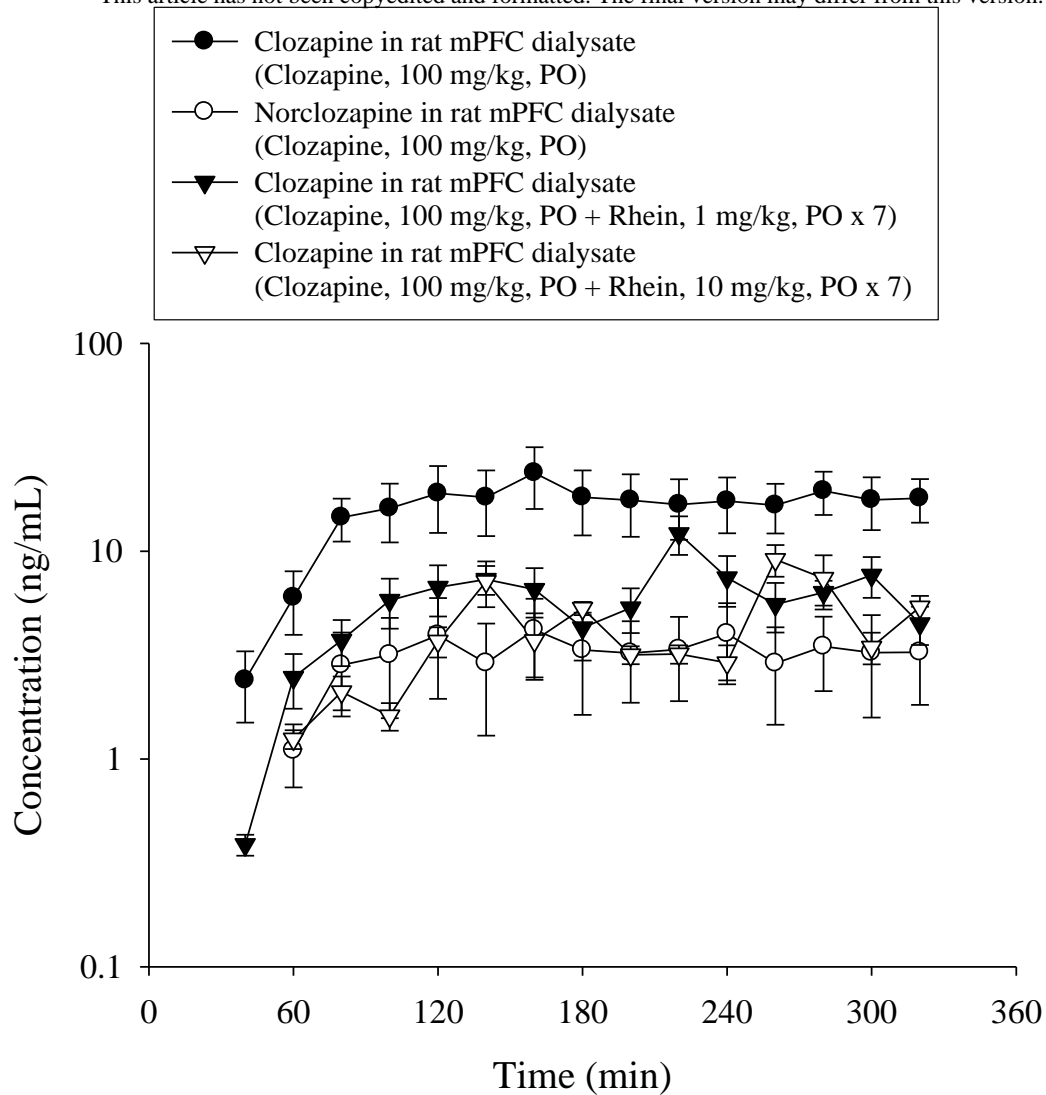
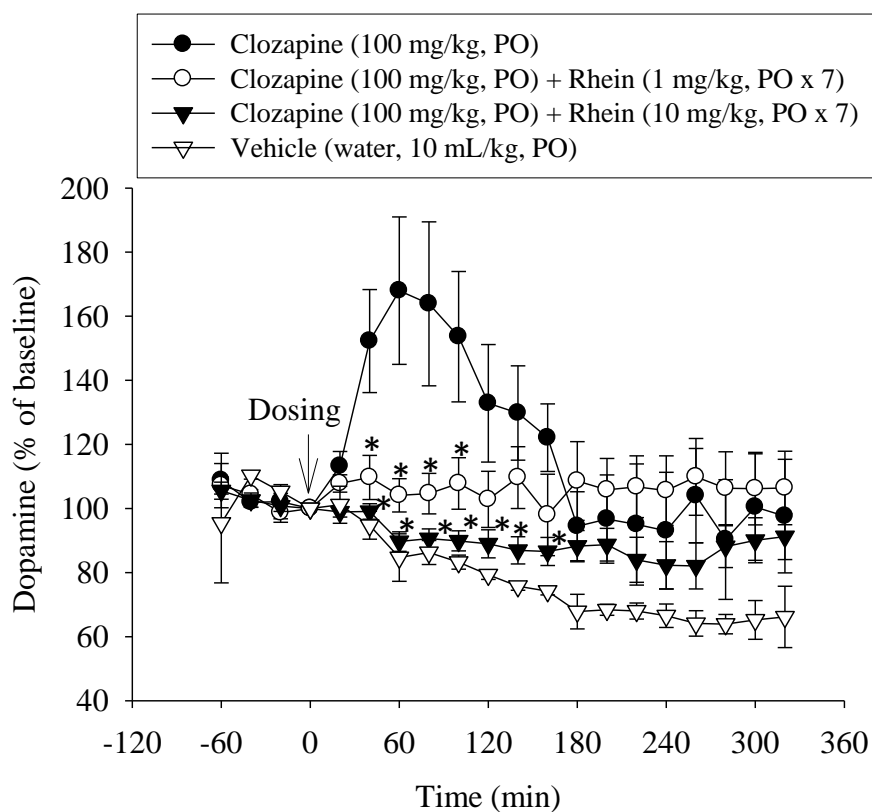
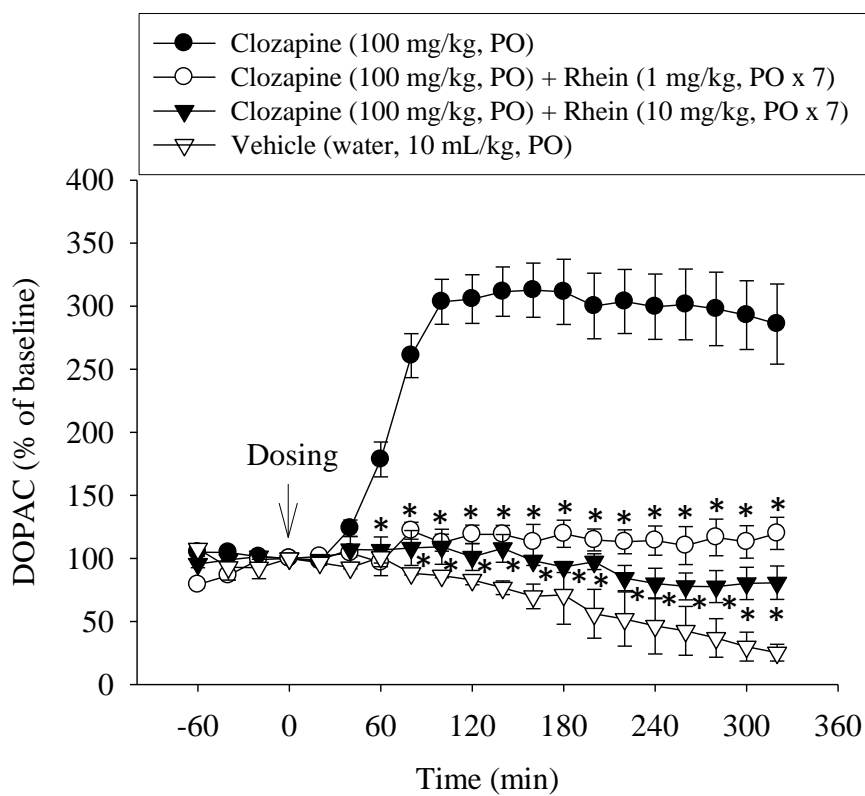


Fig. 2.

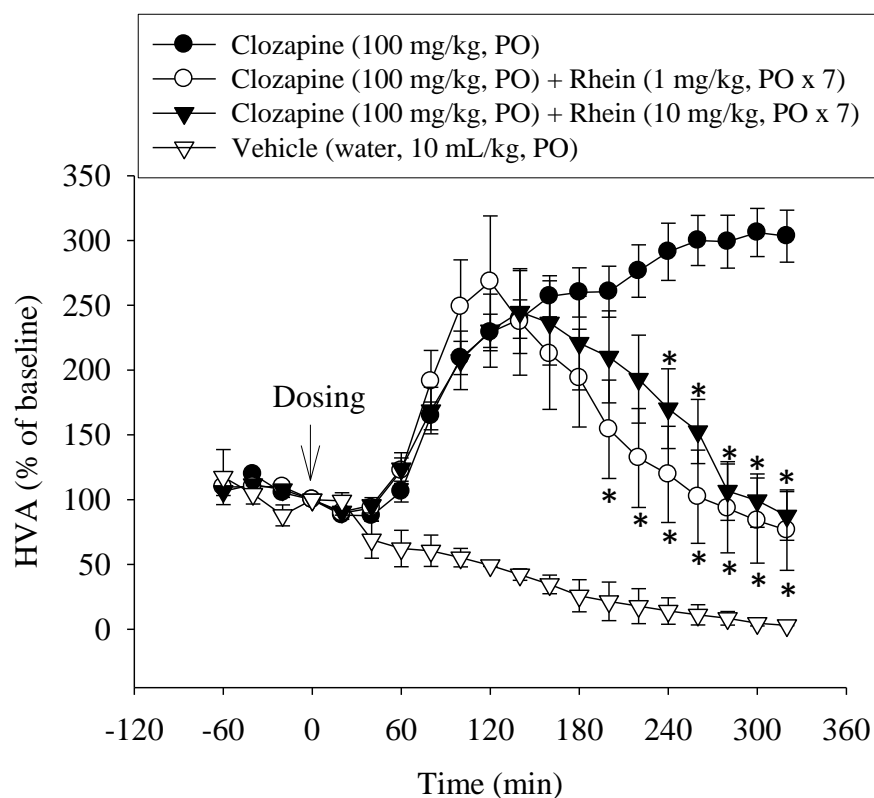
(A)



(B)



(C)



(D)

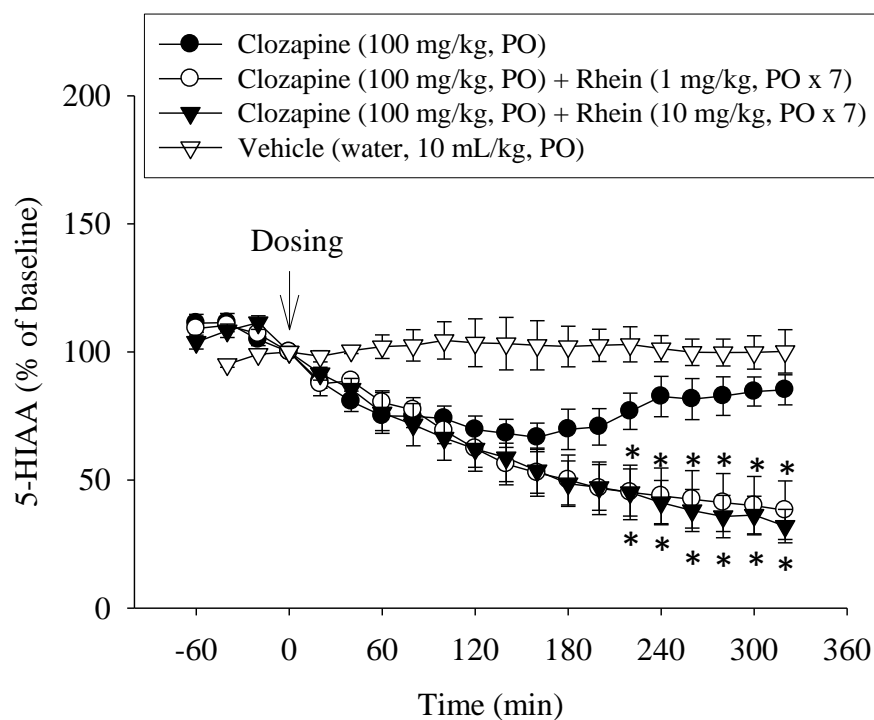


Fig. 3.