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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Received May 9, 2015; accepted July 27, 2015

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 (PK) and pharmacodynamics (PD) of clozapine in brain when used to relieve clozapine-induced constipation. Here, we have investigated not only the PK of clozapine in blood but also the effects of rhein on the PK of clozapine in blood and in brain extracellular fluid together with the PD effects on neurotransmitters in extracellular fluid. The concentrations of clozapine and norclozapine in biologic samples were measured by ultra-performance liquid chromatography–tandem mass spectrometry. The drug-drug effects of rhein on extracellular neurotransmitter efflux in the rat medial prefrontal cortex (mPFC) produced by clozapine were assayed by high-performance liquid chromatography–electrochemical detection. The results demonstrate that the clozapine PK was nonlinear. Pretreatment with rhein for 7 days increased the 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 (3,4-dihydroxyphenylacetic acid) and altered the profile of homovanillic acid, another metabolite of dopamine, in the mPFC. In conclusion, rhein was found to substantially decrease clozapine and norclozapine concentrations in the mPFC dialysate, and 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.

Introduction

Antipsychotics are the cornerstone of the management of psychotic disorders and schizophrenia (De Hert et al., 2011), which is a severe mental illness characterized by positive symptoms, negative symptoms, and cognitive impairment. Clozapine is an atypical antipsychotic agent that is used for the treatment of schizophrenia. Numerous studies (Murray, 2006; Spina and de Leon, 2007; Fakra and Azorin, 2012) have demonstrated that clozapine, a D2–5-HT2 (serotonin) 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, a second-generation antipsychotic, is attributed to some degree to D2 antagonism, but more to the blockade of certain 5-HT receptors. The selective blockade of 5-HT receptors enhances the dopamine (DA) 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 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, which is a common gastrointestinal problem.
Laxatives and traditional Chinese medicine 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 has been reported that the single Chinese herb, rhizomes of *Rheum palmatum* L. (Rhubarb), is used as a remedy for constipation (Jong et al., 2010). Many active components found in *R. palmatum* L.—including aloe-emodin, emodin, and rhein—have pharmacologic 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 neurologic 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 (Gottäs et al., 2013), but also that there is no need for sample preparation because the dialysis membrane excludes proteins from the aqueous sample (Tsai, 2003). The use of ion-pair high-performance liquid chromatography–electrochemical detection (HPLC-ECD) is of great interest for the determination of monoamine neurotransmitters (i.e., norepinephrine; epinephrine; 3,4-dihydroxyphenylacetic acid (DOPAC); DA; 5-hydroxyindole-3-acetic acid (5-HIAA); homovanillic acid (HVA); 5-HT; and 3-methoxytyramine hydrochloride) in microdialysates (Bicker et al., 2013). Moreover, ultraperformance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) has been used to quantify the concentrations of drug and its metabolites in microdialysates (Cremers et al., 2012).

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 nonsmokers to achieve an equivalent plasma concentration due to induced metabolism (Tsuda et al., 2014). Fluoxetine and cimetidine, and to a lesser extent valproate, inhibit the activities of cytochrome P450 enzymes, which will increase the levels of clozapine and its metabolites (Watras and Taylor, 2013; Victoroff et al., 2014). Studies on the comparative pharmacokinetics (PK) of rhein in normal and constipated rats have demonstrated that 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 up-regulated by 2.11, 2.8, 3.6, 3.3, and 2.1 fold, 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 a drug-drug interaction perpetrator were investigated, except the in vitro data regarding inhibition of CYP enzymes by rhein in rat liver microsomes (Tang et al., 2009), emodin, a similar compound of rhein, has been reported to have inhibitory properties on P-glycoprotein (P-gp) based on in vitro studies (Liu et al., 2011). Therefore, alternations in drug-metabolizing genes may potentially impact the therapeutic window of drugs and cause herb-drug interactions. However, there are no reports on the PK of clozapine concerning brain distribution and the pharmacodynamics (PD) of extracellular neurotransmitter changes in the medial prefrontal cortex (mPFC) induced by concomitant rhein and clozapine use. Thus, the aims of this study are to investigate the PK of clozapine in freely moving rats by UPLC-MS/MS, to explore whether pretreatment with rhein affects the clozapine and norclozapine levels in the blood and mPFC of rats, and to evaluate whether pretreatment with rhein influences the PD of clozapine on the extracellular neurotransmitter efflux in rat mPFC.

### Materials and Methods

**Chemicals and Reagents.** The chemicals rhein, clozapine, norclozapine, carbamazepine, sodium 1-octanesulfonate monohydrate, sodium metabisulfite, 3-hydroxytyramine hydrochloride, DOPAC, 3-methoxytyramine hydrochloride, HVA, (-)-norepinephrine, (-)-epinephrine, 5-HT, and 5-HIAA were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Liquid chromatography/mass spectrometry–grade solvents were obtained from J.T. Baker, Inc. (Phillipsburg, NJ) and chromatographic reagents were obtained from Tedia Co., Inc. (Fairfield, OH). Sodium chloride, sodium dihydrogen phosphate, orthophosphoric acid (85%), hydrochloric acid, disodium edetate, potassium chloride, and sodium hydroxide were purchased from E. Merck (Darmstadt, Germany). T.e. deionized water (Millipore, Bedford, MA) was used for all preparations.

**Clozapine and Norclozapine Assay.** All of 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 tandem quadrupole mass spectrometer in electrospray ionization mode. The multiple-reaction monitoring mode was used for quantification. All ion transitions and collision energies were determined and optimized by using the MassLynx 4.1 software data platform (Waters). The mass spectrometry conditions were set as follows: electrospray ionization, positive mode; source temperature, 150°C; collision gas, argon; desolvation temperature, 400°C; desolvation gas flow, 800 l/h. The optimized cone voltages were 34 V for clozapine, 36 V for norclozapine, and 32 V for carbamazepine. The ion transitions monitored were 

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**Equipment.** Lipid standard (Biorad Laboratories, Hercules, CA) was used for quantification. All ion transitions and collision energies were determined and optimized by using the MassLynx 4.1 software data platform (Waters). The mass spectrometry conditions were set as follows: electrospray ionization, positive mode; source temperature, 150°C; collision gas, argon; desolvation temperature, 400°C; desolvation gas flow, 800 l/h. The optimized cone voltages were 34 V for clozapine, 36 V for norclozapine, and 32 V for carbamazepine. The ion transitions monitored were 

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**Chromatographic Separation.** Chromatographic separation was achieved using a C18 column (100 × 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 minutes, 60% A at 2.1–7 minutes, 52% A at 7.1–11 minutes, 20% A at 11.1–14 minutes, and 95% A at 14.1–17 minutes was used. The flow rate was set at 0.25 ml/min, and the injection volume was 5 μl. The MassLynx 4.1 software data platform was used for spectral acquisition, spectral presentation, and peak quantification.

**Methodology.** The method validation assays for quantification of clozapine and norclozapine in rat plasma and rat mPFC dialysates were conducted based on the current U.S. Food and Drug Administration bioanalytical method validation guidance (Zimmer, 2014). The specificity, matrix effects (MEs), and recovery were evaluated. The MEs 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 biologic matrix, such as plasma. The MEs result from coeluting
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matrix components that affect the ionization of the target analyte, resulting either in ion suppression or ion enhancement (Van Eckhaut et al., 2009). To evaluate the ME and recovery, six different lots of blank plasma were extracted and then spiked with clozapine or norclozapine at three concentrations. The corresponding peak areas of clozapine or norclozapine in the spiked biologic samples postextraction (A) were compared with those of the aqueous standards in mobile phase (B) at equivalent concentrations. The ratio (A/B) × 100) is defined as the ME. The corresponding peak areas of standards in the spiked biologic samples before extraction (C) were compared with those of standards in the spiked biologic samples postextraction (A) at equivalent concentrations. The ratio (C/A) × 100) is defined as the recovery. All linear calibration curves were required to have a coefficient of efficiency of at least 0.995. The intra- and interday variability, accuracy (bias %), and the relative S.D. were calculated.

**Animal Experiments.** All animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (No. 1011202) of National Yang-Ming University. A total of 56 male, specific pathogen–free Sprague-Dawley rats weighing 220 ± 20 g were used in this study. For the PK study, six rats per group were anesthetized with pentobarbital (50 mg/kg i.p.) for cannulation. The detailed procedures of cannulation were performed as in a previous study (Hou et al., 2014a). Additionally, 10 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 U.S. Food and Drug Administration guidelines as follows: human equivalent dose (mg/kg) = animal dose (mg/kg) × (animal Km/human Km) (Reagan-Shaw et al., 2008). The Km factor, the body weight (kg) divided by the body surface area (m²), is used to convert the mg/kg dose in the study to the mg/m² dose. The Km factors are 6 and 37 for rat and 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 μl 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 minutes. For quantitative analysis, each dialysate sample (50 μl) was vortex mixed with acetonitrile (100 μl) for protein precipitation. Data from these samples were used to construct the PK 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 the drug-drug interactions of rhein on the blood PK of clozapine, rhein at 10 mg/kg was orally administered to rats for 7 days. The day before the PK study, PE50 tubing was implanted into the left carotid artery of the rat for blood sampling. One hour after the seventh dose of rhein, clozapine at 100 mg/kg was given orally to rats for the PK study.

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

**The Drug-Drug Effects of Rhein on the PK of Clozapine and Norclozapine in the Rat mPFC by In Vivo Microdialysis of Freely Moving Rats.** The UPLC-MS/MS methods were performed as described previously with some modifications. Briefly, for analysis of dialysate samples, the liquid chromatography flow was diverted to waste from 0 to 2 minutes and then introduced to the mass spectrometer from 2.1 to 17 minutes using a 6-port switching valve. The autosampler injection needle was washed with methanol to reduce the carryover after each injection. An external standard was used in this study.

The dose of clozapine for animals was chosen for 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 to 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 the 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 demonstrated that Cyp7a1, Cyp26f2, Atp1b1, and Slc7a2 were upregulated by approximately 2-fold; however, Ces2e was downregulated by 19-fold post–7 days of SHXXT treatment. Thus, based on the clinical application of SHXXT and our previous experimental evidence, rhein at doses of 1 and 10 mg/kg was given orally for 7 days to explore the herb-drug interactions of rhein on the PK and PD 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 microdialysis probes. A CMA Elite microdialysis probe (molecular mass cutoff of 20,000 Da; membrane 4 mm in length; CMA Microdialysis AB) was used for the brain mPFC sampling.

Surgery for implantation of the microdialysis guide cannula was conducted under pentobarbital anesthesia (50 mg/kg i.p.). The rats were mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) and a CMA 12 guide cannula was inserted into the mPFC at +3.2 mm anteroposterior, +0.8 mm mediolateral (10°C 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 Hygienic Corporation, Akron, OH) and the wound was 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 μL/min, and the animal was left to acclimatize at least 2 hours. Sample collection intervals were set to 20 minutes. After the 2-hour stabilization period following the implantation of the microdialysis probe, four basal dialysates were obtained at 20-minute 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. × 7) pretreatment. One hour after the seventh dose of rhein, clozapine was given to the rat. The dialysates were collected into the vials containing 7.5 μl of an antioxidant solution (100 mM acetic acid, 3.3 mM l-cysteine, 0.27 mM disodium edetate, and 12.5 μM 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 a previous study (Lu et al., 2014). The microdialysis probe was inserted into the mPFC, 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 μL/min. In vivo recovery was evaluated in three individual experiments for each concentration with the brain microdialysis probe. The clozapine or norclozapine perfusate (Cperf) and dialysate (Cdial) concentrations were determined by UPLC-MS/MS. The in vivo recovery (Rdial) of clozapine or norclozapine was calculated using the following equation: Rdial = Cperf − Cdial/Cperf. The concentrations of clozapine or norclozapine were converted to free-form concentrations (Cf) as follows: Cf = Rdial × Cdial.

**The Drug-Drug Effects of Rhein 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 FM-92E LC pump (Bioanalytical Systems, West Lafayette, IN), a CMA240 refrigerated microsampler, a CMA240 sample injector with a 20 μL loop (CMA Microdialysis AB), and a Decade II electrochemical detector fitted with a SenCell electrochemical flow cell (2 mm glassy carbon working electrode and an in situ Ag/AgCl reference electrode) (Antec, Zoeterwoude, The Netherlands). An RP-18e column (Merck Chromolith Performance; 100 × 2 mm, i.d.; particle size, 2 μm (Merck KGaA, Darmstadt, Germany)) with a guard column (5 × 2 mm, i.d.) at 35°C maintained by a column
plasma was 50 ng/ml. Furthermore, the limit of quantification of clozapine and norclozapine in rat brain cortical dialysate was 0.5 ng/ml. The intra- and interday variability, accuracy (bias %), and the relative S.D. 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 PK 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 the PK parameters are listed in Table 1. Clozapine blood levels declined below the limit of quantification after 120 minutes following a 10 mg/kg dose. The $C_{\text{max}}$ values for clozapine were 169 ± 88.2, 634 ± 110, and 644 ± 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 minutes, indicating slow elimination of clozapine. Changes in the PK parameters of clozapine at 10, 30 and 100 mg/kg p.o. were determined. The area under the curve (AUC) was increased by 5.9- and 20.3-fold with clozapine 30 and 100 mg/kg, respectively, compared with clozapine 10 mg/kg. The $C_{\text{max}}$ increased 3.8-fold with clozapine 30 mg/kg compared with clozapine 10 mg/kg; however, clozapine 100 mg/kg yielded a $C_{\text{max}}$ of 644 ± 96 ng/ml, similar to clozapine 30 mg/kg. The mean residence time increased in a dose-dependent manner.

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 the $C_{\text{max}}$ and mean residence time values increased in a dose-dependent manner.

The effect of rhein on the blood PK 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 $C_{\text{max}}$ of clozapine significantly increased by 2.7-fold in combination with rhein pretreatment (Fig. 1; 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% ± 6.8%, 89.3% ± 3.8%, and 93.8% ± 3.0%, respectively, for clozapine and 86.3% ± 10.8%, 87.8% ± 3.4%, and 93.0% ± 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. Dialysis efficiency can be affected by factors including the probe length and diameter, diffusion coefficient of the analyte, perfusion solution composition, perfusion flow rate, and 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% ± 5.5% for clozapine and 89.2% ± 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 Extracellular Fluid 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 PK 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 ± 1159 min/ng per ml for clozapine and 557 ± 297 min/ng per 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 days of rhein at 1 or 10 mg/kg decreased the distribution of clozapine and norclozapine in rat mPFC (Fig. 2).

After 7 days of oral rhein 1 or 10 mg/kg pretreatment, the AUC of clozapine was reduced by approximately 3-fold and the C_{max} decreased approximately 2-fold compared with clozapine alone (Table 2). Additionally, the elimination half-life decreased significantly in a dose-dependent manner. The total body clearance 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 ± 297 min/ng per ml, approximately 7× 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.** The basal cortical extracellular DA, DOPAC, HVA, and 5-HIAA levels in the dialysates obtained from all rats used in this study were 0.20 ± 0.03, 0.29 ± 0.03, 1.12 ± 0.16, and 1.99 ± 0.17 pmol/20 μl (mean ± S.E.M.; N = 40), respectively. Extracellular levels of DA in the mPFC were significantly increased by administration of clozapine to a maximum value of 168% ± 23% of preinjection levels (Fig. 3A). The DA efflux in the mPFC began at 20 minutes after dosing with clozapine at 100 mg/kg; the increase reached the maximum value of 168% of baseline at 60 minutes, and then returned to baseline 180 minutes postdosing. In contrast, rhein pretreatment reduced the extracellular level of DA produced by clozapine (100 mg/kg p.o.) administration.

![Fig. 1. Mean plasma concentration-time profile of (A) clozapine and (B) norclozapine after oral administration of clozapine (10, 30, and 100 mg/kg, respectively) with or without rhein (10 mg/kg p.o. × 7) pretreatment. (C) The blood AUC values of clozapine and its metabolite after oral administration of clozapine with or without rhein (10 mg/kg p.o. × 7) pretreatment. Each point represents mean ± S.E.M. (N = 6 per group).](image-url)
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. 3, B and C). The extracellular level of DOPAC in the mPFC began to increase 40 minutes after dosing; the increase reached a maximum value of 300% of baseline at 80 minutes 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 minutes postdose, increased to a maximum value of 300% of baseline, and was maintained throughout the experiment (Fig. 3C). However, following rhein pretreatment, the profiles of the HVA efflux in mPFC produced by clozapine were changed. Following pretreatment with rhein, the HVA efflux began to increase at 60 minutes postdose, reached a maximum value of 250% of baseline at 120 minutes, and then slowly declined to baseline levels. Clozapine 100 mg/kg failed to affect the extracellular levels of 5-HIAA; however, the decline could be significantly observed when pretreated with rhein (Fig. 3D).

Discussion

Previous results of a comparative PK study concerning the PK of rhein have indicated that herbal formulas with multiple constituents significantly increase 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 formulas. Thus, the pure rhein compound was chosen to elucidate the drug-drug interaction effects on the PK and PD of clozapine. In the present study, the validated LC-MS/MS methods were applied to the PK of clozapine and norclozapine in rat plasma and brain dialysate. The multiple-reaction monitoring 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 PK 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 demonstrated 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 PK of clozapine in blood was nonlinear. Consistent with the previous studies, norclozapine concentrations exceeded those of clozapine at 15 minutes after drug application, suggesting that the metabolism of clozapine was rapid once clozapine was absorbed.
Drugs entering the ion source. These approaches were successful.

2 minutes of analysis was applied to prevent the salts from

a divert valve guiding the eluent to waste during the first

phase containing a high proportion of aqueous phase was used

simultaneously used to reduce the influence of nonvolatile

out prior sample preparation. Thus, two methods were

microdialysate was directly analyzed by UPLC-MS/MS with-

salts, the ionization of analytes would be suppressed when

microdialysis at the target site (Gottås et al., 2013). Because

studies of drug-drug interactions of rhein on clozapine PK in

100 mg/kg was chosen to investigate the drug-drug interaction

treatment with rhein alters the exposure of clozapine and

values of norclozapine at a low dose of clozapine (1 mg/kg).

somewhat later in brain (30 minutes). The ratio of norcloza-

Following a 10 mg/kg i.p. dose of clozapine, peak clozapine

the rat have been investigated (Baldessarini et al., 1993). The

and brain concentrations of clozapine and its metabolites in

PK Parameter | Clozapine (100 mg/kg p.o.) | + Rhein (1 mg/kg p.o. × 7) | + Rhein (10 mg/kg p.o. × 7)
---|---|---|---
AUC<sub>0-220 min</sub> (min ng/ml) | 3706 ± 1159 | 1238 ± 290<sup>+</sup> | 1136 ± 196<sup>+</sup>
T<sub>1/2</sub> (min) | 253 ± 106 | 50.5 ± 12.9 | 25.2 ± 3.26<sup>+</sup>
C<sub>max</sub> (ng/ml) | 21.4 ± 4.94 | 11.5 ± 2.40 | 9.30 ± 2.05<sup>+</sup>
T<sub>max</sub> (min) | 235 ± 24.3 | 203 ± 21.1 | 280 ± 14.6
V<sub>c</sub> (l/kg) | 2510 ± 597 | 4281 ± 1492 | 2307 ± 408
CL (l/min/kg) | 22.9 ± 10.5 | 59.1 ± 10.9<sup>+</sup> | 63.2 ± 3.08<sup>+</sup>
MRT (min) | 209 ± 6.29 | 177 ± 10.1 | 200 ± 4.47
AUC ratio | 0.021 | 0.007 | 0.006

AUC<sub>0-220 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
AUC ratio | 0.001 | ND | ND

AUC, area under the concentration versus time curve; AUC ratio (AUC<sub>mPFC</sub>/AUC<sub>plasma</sub>), distribution of brain-to-blood clozapine or

norclozapine; CL, total body clearance; MRT, mean residence time; ND, not detected; volume of distribution.

<sup>+</sup>Significantly different from clozapine alone at P < 0.05.

(Baldessarini et al., 1993; Weigmann et al., 1999). The effects

doing the dosing regimen (1, 3, 10, 20, 30, and 60 mg/kg) on serum

and brain concentrations of clozapine and its metabolites in

the rat have been investigated (Baldessarini et al., 1993). The

results demonstrated that clozapine and its metabolite levels

in rat serum and striatal brain were highly dose dependent.

Following a 10 mg/kg i.p. dose of clozapine, peak clozapine

levels were reached very rapidly in serum (10 minutes) and

somewhat later in brain (30 minutes). The ratio of norcloza-

pino 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 pre-
treatment with rhein alters the exposure of clozapine and norclozapine in the conscious rat using brain microdialysis. To

get the maximal therapeutic window of drugs, clozapine at

levels exceeded those of norclozapine by approxi-

mately 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 the brain PK of

clozapine, the effects of rhein on the blood PK of clozapine

demonstrate that rhein pretreatment enhanced the absorp-
tion of clozapine and increased the concentrations of clozapine

in the blood. On the other hand, the blood PK profile of

norclozapine in combination with rhein was not different from

clozapine alone.

Although investigations on extracellular neurotransmitter
eflux 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 the central
nervous system PD of clozapine. Contrary to the brain PK of

clozapine, the effects of rhein on the extracellular neurotransmitter

eflux 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 the central
nervous system PD of clozapine. Clinically, anthraquinone
derivatives present in various drugs of plant origin are used
all over the world for constipation remedy (Müller-Lissner,
2013). The best characterized compounds are sennoside and
norclozapine; CL, total body clearance; MRT, mean residence time; ND, not detected; volume of distribution.

<sup>+</sup>Significantly different from clozapine alone at P < 0.05.
therapeutic effects of sennosides. For this 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 DA release in the mPFC of freely moving rats. In addition, clozapine elevated cortical DOPAC, indicating drug effects on cortical DA metabolism because extracellular DOPAC is considered to be a marker for cytoplasmatic DA 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 DA release in mPFC 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 DA and its metabolites (DOPAC and HVA) produced by clozapine (100 mg/kg p.o.) administration (Fig. 3, A–D) in a dose-dependent manner.

The inhibitory effect of rhein on mPFC clozapine, DA, and metabolite levels suggests some inhibition of the transport of clozapine in mPFC. With 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 norclozapine, which has limited pharmacological activity, and clozapine N-oxide. Currently

Fig. 3. Time course effects of clozapine (100 mg/kg p.o.) on extracellular neurotransmitter levels (A) DA; (B) DOPAC; (C) HVA; (D) 5-HIAA in the mPFC with or without rhein (1 and 10 mg/kg p.o. ×7, respectively) pretreatment. The arrow indicates the time of clozapine or vehicle (water, 10 ml/kg p.o., N = 2) injection. Data are mean ± S.E.M. of the dialysate neurotransmitter levels, expressed as a percentage of each predrug baseline neurotransmitter value (N = 10 per group). *Significantly different from clozapine alone at P < 0.05.
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 the PK of clozapine in plasma. Thus, it is not likely that the inhibition of extracellular DA and its metabolite efflux is through facilitation of the clozapine metabolism.

Generally, low brain penetration can be due to low blood-brain barrier permeability, 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 reported that drug concentrations in cerebrospinal fluid are assumed to be roughly equal to unbound concentrations in plasma (Nordin et al., 1995). The positive correlations between serum and cerebrospinal fluid levels 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 cerebrospinal fluid clozapine concentrations ranged from 2 to 39 ng/ml, corresponding to 23% ± 14% of the levels in serum. In our study, contrary to the brain PK 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 responding poorly to antipsychotic treatment could be explained by inefficient drug transport across the blood-brain barrier due to P-gp-mediated efflux (Moons et al., 2011). Additionally, emodin, a similar compound to 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 the drug-drug interaction of rhein might result in the transport of clozapine in the mPFC. A study on the prediction of DA metabolite exposure in the extracellular fluid of human brain using a translational PK modeling approach demonstrated that a PK model, which relates clozapine and norclozapine disposition in rat plasma and brain (including blood-brain barrier transport), was developed and can be successfully translated to predict clozapine and norclozapine concentration according receptor occupancy of both agents in human brain (Li et al., 2014). In our study, rhein significantly increased total plasma clozapine Cmax and AUC; on the other hand, rhein significantly decreased the unbound AUC and Cmax of clozapine in the mPFC. Thus, monitoring the therapeutic effective plasma levels of clozapine may not be an ideal approach for prediction of clozapine concentrations in brain.

In conclusion, a validated LC-MS/MS method was applied to investigate the PK of clozapine and norclozapine in freely moving rats. The PK results demonstrate that the PK 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 the brain extracellular fluid PK of clozapine and norclozapine. The PK 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, coadministration of rhein for treating constipation, the major side effect of clozapine, may potentially modulate the therapeutic effects of clozapine, which consequently does not effectively treat schizophrenia.

Acknowledgments

The authors thank Dr. Kun-Po Chen (Taipei City Hospital, Taipei, Taiwan) for generously supplying clozapine.

Authorship Contributions

Participated in research design: Hou, Tsai.
Conducted experiments: Hou.
Contributed new reagents or analytic tools: C.-H. Lin, L.-C. Lin, Tsai.
Performed data analysis: Hou, Tsai.
Wrote or contributed to the writing of the manuscript: Hou, Tsai.

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