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Two potent cytochrome P450 2D6 inhibitors found in *Rhodiola rosea*

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Objectives: Throughout the world, in particular in Russia, Northern Europe and China, *Rhodiola* species are used as herb supplements. Previously, we found that the extract of *Rhodiola rosea*, one of the most widely used Rhodiola species, had an inhibitory effect on the catalytic activity of cytochrome P450 2D6. Here, its inhibitory components were identified. *Methods:* A human liver microsomal *in vitro* system was used with dextromethorphan as substrate. The production rate of destrorphan, a metabolite of dextromethorphan, was used to measure enzyme activity. The concentration of destrorphan in the samples was measured using LC-MS/MS. Inhibitory activity of eight main components from *Rhodiola rosea* was evaluated. *Results:* Rhodiosin and rhodionin showed inhibitory activity with IC_{50} values of 0.761 μM and 0.420 μM, respectively. The other components showed no obvious inhibition (with a residual enzyme activity of more than 90%). Both rhodiosin and rhodionin were determined to be non-competitive inhibitors with Ki values of 0.769 μM and 0.535 μM. *Conclusion:* Two of the main *Rhodiola rosea* compounds, rhodiosin and rhodionin, can inhibit cytochrome P450 2D6 non-competitively with high specificity which could have implications for interactions with co-administered drugs.

1. Introduction

Rhodiola species, which have been used historically as herb supplements in Europe and as herbal medicines in China, are valued for their improvement of human resistance to stress, fatigue, hypoxia, and their ability to induce longevity (Jafari et al. 2007; Spasov et al. 2000; Hohtola 2010).

As tonic drugs, *Rhodiola* species are often self-administered alone or in combination with other drugs. However, herb-drug interactions have not been well studied until it was recently reported that *Rhodiola rosea* displays inhibitory activity on CYP3A4 (one of the important enzymes involved in the metabolism of drugs in the human body) and P-glycoprotein (abbreviated as p-gp, an ATP-dependent efflux pump)(Hellum et al. 2010). The inhibition of drug-metabolizing enzymes and the efflux transporter P-glycoprotein may lead to an increased plasma concentration of a drug that is co-administered with *Rhodiola rosea* extract and could lead to an increase in the pharmacological effect and its duration and could also lead to an intensification of drug-induced toxicity.

To predict potential interactions of *Rhodiola rosea* with therapeutic drugs, the inhibitory effects of the extract on the catalytic activity of five main CYPs, including CYP3A4, CYP2C9, CYP2C19, CYP2D6 and CYP1A2, were evaluated in our preliminary experiment. Beside the inhibition on CYP3A4, the extract showed more inhibitory activity on the activity of CYP2D6, another important drug metabolism enzyme, which is involved in the metabolism of approximately 25% of all pharmaceutical drugs. It is possible for many substrate drugs of CYP2D6 (Guengerich 2001), such as psychotropics, antiarrhythmics and β-adrenergic receptor blockers, to be co-administered with *Rhodiola* species extracts.

Nearly 100 *Rhodiola* species are currently used as herbal remedies, but each of these may contain different constituents. An additional experiment showed that another widely used *Rhodiola* species, *Rhodiola sacra*, did not possess the ability to inhibit CYP2D6. The present study was conducted to identify CYP2D6-inhibiting compounds in *Rhodiola rosea* by using a human liver microsomal *in vitro* system. The results may help to predict herb-drug interactions.

2. Investigations and results

The inhibitory effects of eight components of *Rhodiola*, including rhodioloside, rhodiosin, kaempferol-3-sophoroside, rhodionin, kaempferol, rhodiolin, gallic acid and β-sitosterine, were tested at a final concentration of 10 μM. Rhodiosin and rhodionin showed an inhibitory activity while the others did not (residual enzyme activity was higher than 90% in these cases). The inhibitory activities of rhodiosin, rhodionin and quinidine (a positive control of CYP 2D6 inhibition) were subsequently measured at final concentrations of 0.05, 0.1, 0.25, 0.5, 0.75, 1, 1.5 and 2.5 μM. Figure 1 shows the concentration-dependent inhibition of dextromethorphan *O*-demethylation in CYP2D6 using various concentrations of rhodionin, rhodiosin and quinidine. The IC_{50} values for rhodiosin and rhodionin were 0.761 μM and 0.420 μM, respectively. The IC_{50} is categorized into classes with a high (IC_{50} below 1 μM), medium (IC_{50} between 1 μM and 10 μM), and low (IC_{50} above 10 μM) risk potentials for drug interactions (Krippendorff et al. 2007). Accordingly rhodiosin and rhodionin are two strong inhibitors of CYP2D6, which potentially could induce drug interactions when co-administered with primarily liver-metabolized drugs.

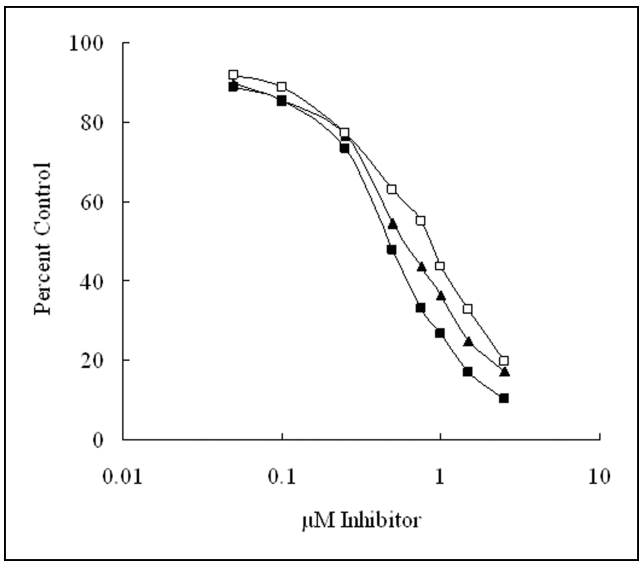

Fig. 1: Concentration-dependent inhibition of dextromethorphan O-demethylation in human liver microsome by various concentrations of rhodionin (■), rhodiosin(□) and quinidine (▲). Each data point represents the average of duplicate determinations.

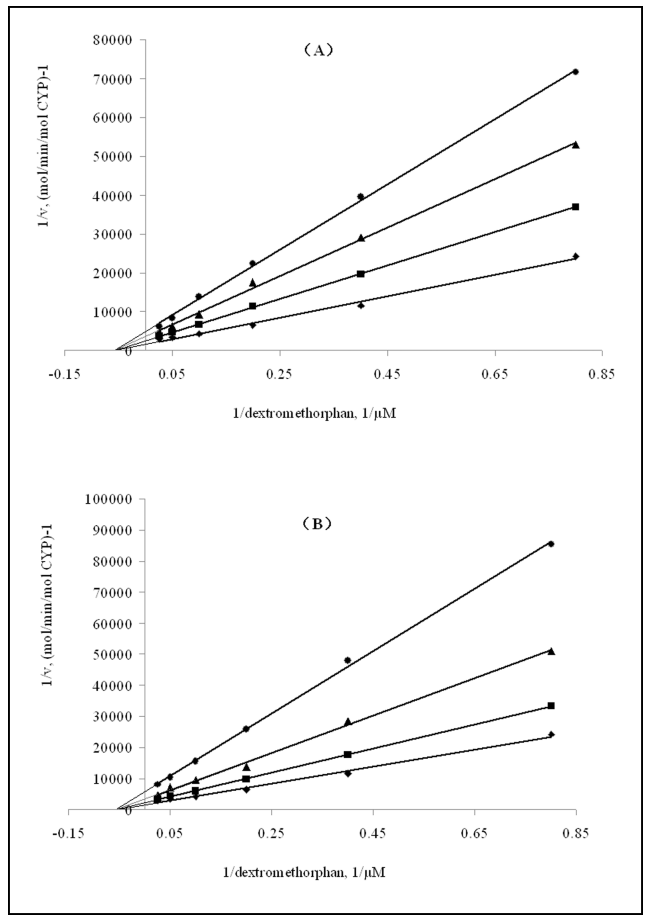

Fig. 2: Lineweaver-Burk plots of the effect of rhodionin (A) and rhodiosin (B) on dextromethorphan O-demethylation in human liver microsome. Rhodionin concentrations: 0 μM (♦), 0.3 μM (■), 0.6 μM (▲) and 1.2 μM (●). Rhodiosin concentrations: 0 μM (♦), 0.4 μM (■), 0.8 μM (▲) and 1.6 μM (●). Lines represent results of linear regression of transformed data. Each data point represents the average of duplicate determinations.

Lineweaver-Burk plots of the effects of rhodionin and rhodiosin on dextromethorphan *O*-demethylation in human liver microsomes are shown in Fig. 2. Km was found to be equal to Km', showing that rhodionin was a non-competitive inhibitor. The Ki value was calculated according to Eq. (2) and the result showed that rhodionin was a strong inhibitor of CYP2D6 with a Ki of 0.535 μM. Rhodiosin too was a non-competitive inhibitor (Fig. 2-B), and a strong inhibitor of CYP2D6 with a Ki value of 0.769 μM.

Quinidine, a positive control for CYP2D6 inhibition, showed an inhibitory activity with IC_{50} and Ki values of 0.447 μM and 0.542 μM, respectively.

3. Discussion

Traditional Chinese medicines (TCMs) have been used in China and other countries for thousands of years and proved to be effective and safe. However, their pharmacological activities are moderate and they sometimes fail to relieve disease symptoms rapidly. Thus, patients tend to take TCMs in combination with chemical medicines (CMs). It was reported that 57.3% of Chinese patients take TCMs while taking CMs (Li et al. 2011). We found that 81.8% of the inpatients in our hospital did so. Combination of TCMs with CMs does not always benefit the patient because adverse drug-drug interactions (DDIs) may happen. TCMs usually contain hundreds of components and some of them may influence the catalytic activities of enzymes that are important for the metabolism of the co-administered CMs, changing the metabolism and pharmacokinetics of CMs. This could lead to reduced (or increased) pharmaceutical activities and increased toxicity (Foti et al. 2007; Lee et al. 2006).

Rhodiola species are used as tonic drugs and are usually used concomitantly with chemical agents, such as beta-blockers, class I antiarrhythmics, antidiabetics, antianginal agents, etc. Many of them are substrates of CYP2D6 and therefore, their metabolism and pharmacokinetics may be altered by CYP2D6 inhibitors (Flockhart 2007). Considering that two strong inhibitors of CYP2D6 were found in *Rhodiola* species, more attention should be paid when *Rhodiola* species are co-administrated with substrates of CYP2D6.

More than 100 species of *Rhodiola* exist in the world and 73 of them have been found in China. *Rhodiola rosea* is a popular TCM and recorded in the Pharmacopoeia of the PR of China (Editorial Committee of the Pharmacopoeia of the Ministry of Health, the PR of China 2010). Salidroside, one main active compound of *Rhodiola* species, is used to control the quality of crude extracts. However *Rhodiola* species from different sources contain different compounds and show various pharmacological activities. In preliminary experiments the extract of *Rhodiola rosea* could inhibit the activity of CYP 2D6, while extract of *Rhodiola sacra*, another widely used *Rhodiola* species, could not. Accordingly, for the rational use of Rhodiola species, contents of rhodiosin and rhodionin should be assayed.

In summary, the extract of *Rhodiola rosea* has the potential to inhibit CYP 2D6 activity, as the two active components rhodiosin and rhodionin, possess CYP 2D6 inhibition activity.

4. Experimental

4.1. Chemicals and reagents

Salidroside (>98% purity), gallic acid (>98% purity), and tinidazole (>99% purity) were purchased from the National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). *Rhodiola* extract was purchased from Shanghai Sunny Biotech Co., Ltd. (Shanghai, China). Rhodiosin (>98% purity), rhodionin (>98% purity) and quebrachol (>98% purity) were purchased from Shanghai Sunny Biotech Co., Ltd. (Shanghai, China). Dextromethorphan hydrobromide, quinidine, destrorphan, nicotinamide-adenine dinucleotide phosphate ($NADP^+$), glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). HPLC-grade acetonitrile and methanol were purchased from Merck KGaA (Darmstadt, Germany). HPLC-grade formic acid was purchased from Tedia Company Inc. (Fair-

field, USA). All other reagents were of analytical grade. Purified water was produced in-house using a Milli-Q system (Millipore, Bedford, MA, USA). Human liver microsomes were obtained from 12 healthy individuals (7 males and 5 females) in the Department of Hepatobiliary Surgery, at the Affiliated Hospital of Qingdao University Medical College (Qingdao, China) and stored at −80 °C until use. The protocol was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University Medical College (Qingdao, China).

4.2. Equipment

An Agilent 6410B triple quadrupole LC-MS system (Agilent Corporation, MA, USA) equipped with G1312B quaternary pump, G1322A vacuum degasser, G1329B autosampler and G1316B therm Column compartments were used for all destrorphan concentration measurements. The system was controlled by MassHunter software (Agilent Corporation, MA, USA).

4.3. Inhibition of CYP 2D6 activity in human liver microsomes

Results of cytochrome P450 inhibition measurements *in vitro* depend on the selection of the substrate as well as on incubation conditions. Therefore the commonly used dextromethorphane was selected as substrate for the CYP2D6 activity assays (Bertelsen et al. 2003; Yu et al. 2001). The total incubation volume was 200 µl, and the sample (5 µM) was incubated in 100 mM potassium phosphate buffer (pH 7.4) at 37 °C and in a NADPH-generating system in the absence (negative control) or presence of various concentrations of test compounds for 30 min. The concentrations of microsomes, $NADP^+$, glucose 6-phosphate, glucose 6-phosphate dehydrogenase and magnesium chloride were 1 mg/ml, 0.05 mM, 1.25 mM, 0.8 uint/ml and 0.625 mM, respectively. After being preincubated at 37 °C for 5 min, each reaction was initiated by the addition of the NADPH-generating system and terminated with 0.4 ml of acetonitrile containing 500 ng/ml of tinidazole (as internal standard). This was followed by the centrifugation of the samples at 3000 × g for 10 min. The supernatant was stored at −20 °C until analysis.
A preliminary test with each test compound at a concentration of 10 µM was carried out to access their inhibitory activity. If the catalytic activity decreased below 90%, a series of tests with different concentrations of the test compound was conducted. The inhibitary activity of these compounds was further quantified. The IC_{50} value was calculated from dose-response curves consisting of 7–8 points determined in two independent experiments. The half maximal inhibitory concentration of quinidine, the positive control of CYP 2D6 inhibition, was assayed along with the test substances.
Lineweaver-Burk plots were used to determine the inhibition type and Ki value. Final concentrations of dextromethorphan were 1.25, 2.5, 5, 10, 20 and 40 µM. The final concentrations of rhodiosin were 0, 0.3, 0.6 and 1.2 µM and 0.4, 0.8 and 1.6 µM for rhodionin. The Km (Michaelis-Menten constant) and Vmax (the maximum reaction velocity) for the uninhibited reaction are extractable from the 1/Vo vs 1/[S] plot. Km' (apparent Km for the inhibited enzyme) and Vmax' (apparent Vmax for the inhibited enzyme) can be determined using the same graph for the inhibited reaction. If Vmax = Vmax', the inhibitor is of the competitive type and the Ki value can be calculated using equation 1 and the known [I] (substrate concentration). If Km = Km', the inhibitor is of non-competitive type and the Ki value can be calculated using Eq. (2) and the known [I].

$$\mathrm{Km} = \mathrm{Km} \times (1 + [\mathrm{I}]/\mathrm{Ki}) \quad (1)$$

$$\mathrm{Vm} = \mathrm{Vm'} \times (1 + [\mathrm{I}]/\mathrm{Ki}) \quad (2)$$

4.4. Dextromethorphan O-demethylation assay

O-Demethylated dextromethorphan, the main metabolite of dextromethorphan, was measured using LC-MS/MS. Separation was performed using a ZORBAX SB-C_{18} column (3.5 µm, 2.1 × 100 mm, Agilent Corporation, Massachusetts, USA) with an isocratic mobile phase consisting of acetonitrile-water-formic acid (35:65:0.05, v/v/v) at a flow rate of 0.25 ml/min. The column was maintained at 35 °C and the injection volume was 10 µl. Electrospray ionization was carried out in the positive mode with a spray voltage of 4000 V. Nitrogen was used as nebulizer gas at a nebulizer pressure of 40 psi with a source temperature of 105 °C. The desolvation gas (nitrogen) was heated to 350 °C and delivered at a flow rate of 10 L/min. For collision-induced dissociation (CID), high purity nitrogen was used as collision gas at a pressure of 0.1 MPa. Quantification was performed using multiple reaction monitoring (MRM) mode at *m/z* 258.3→157.1 for dextromethorphan O-demethylation and *m/z* 248.2→121.1 for tinidazole (internal standard, IS). The fragmentor energies of MS1 for the metabolite and IS were both 140 V. Optimized collision energies of 45 eV and 15 eV were used for the metabolite and IS, respectively.

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