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CYP2C19 is involved in the effect of Wuzhi tablet (*Schisandra sphenanthera* extract) and its constituents on the pharmacokinetics of intravenous voriconazole

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The co-administration of voriconazole (VCZ) and Wuzhi tablet (WZ) is frequently prescribed for solid organ transplantation patients in China. However, the pharmacokinetic interactions between VCZ and WZ as well as its bioactive constituents, such as schisandrin A and schisandrol B, remain unknown. Therefore, the effects of WZ and the two lignans on the metabolism of VCZ and the potential role of cytochromeP450 (CYP450), especially cytochrome P450 2C19 (CYP2C19), were investigated. The results showed that WZ extensively inhibited the activities of CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4. Noteworthy, 2.5 mg/mL WZ almost completely inhibited the activity of 2C19, and the inhibition ratio reached 78.6±3% and 63.5±4.6% for schisandrin A and schisandrol B at concentrations 100 μM, respectively. In addition, rats were treated with a single or consecutive 14 day oral dose of WZ (250 mg/kg), schisandrol B (10 mg/kg) and schisandrin A (10 mg/kg). In rats treated with WZ, the $AUC_{0-\infty}$ value for intravenous VCZ dosing was increased by 80.2% (single dose, $p < 0.05$) and 66.4% (dosage for 14 day, $p < 0.05$) and the C_{max} was increased by 10.5% ($p < 0.05$) and (20.6%, $p< 0.05$), respectively, much greater than that when VCZ (28 mg/kg) was given alone. Unexpectedly, the AUC and C_{max} values after schisandrol B and schisandrin A treatment were significantly increased. However, the mRNA expression of liver CYP2C19 and the protein expression of liver CYP2C19 were surprisingly increased after treatment with WZ, schisandrol B and schisandrin A in rats. Therefore, attention should be paid to when WZ and VCZ are administered concomitantly, as dosage adjustment might become necessary. Further clinical study is warranted to validate the interaction between WZ and VCZ.

1. Introduction

The triazole antifungal voriconazole (VCZ) exhibits broad-spectrum antifungal activity and is the first-line treatment for invasive aspergillosis (Ruiz-Camps 2011; Mikulska et al. 2012). Recipients of solid organ transplantation are at high risk of developing invasive fungal disease after oral immunosuppressive agent such as tacrolimus (FK506). Therefore, VCZ has been used widely for the prevention and treatment of antifungal prophylaxis in solid organ transplantation patients (Mori et al. 2012).

VCZ is administered intravenously and orally in clinical setting. VCZ exhibits high oral bioavailability in healthy volunteers, and highly variable, non-linear pharmacokinetics (Dolton and McLachlan 2014). Additionally, the therapeutic window is narrow and a range of serious adverse events including hepatotoxicity and neurotoxicity complicate the clinical utility of VCZ (Mikulska et al. 2012).

VCZ is extensively metabolized by the polymorphic drug-metabolizing enzyme cytochrome 2C19 (CYP2C19), and marginally metabolized by 3A4 and 2C9 (Dolton and McLachlan 2014; Hamadeh et al. 2017). Therefore, drug or compounds that inhibit or induce the CYP2C19 may increase or decrease the blood level of VCZ (Dolton and McLachlan 2014). Clinically and pre-clinically, relevant interactions have been reported between VCZ and drugs, such as proton-pump inhibitors (Qin et al. 2014a; Blanco Dorado et al. 2020), imatinib (Lin et al. 2019) to cause serious fluctuation of VCZ exposure. Knowledge of pharmacological properties, metabolism, interactions, dosage indications is crucial to correctly dose VCZ and increase its clinical safety, concomitant medications need to be fully disclosed.

Wuzhi tablet is a preparation of ethanolic herb extract of *Schisandra chinensis*. Wuzhi tablet (WZ) (Registration number in China: WS-10557(ZD-0557)-2002) is a proprietary medication rather than an herbal supplement in the clinical practice, which contains 7.5 mg schisantherin A per tablet. Its major active chemical constituents include schisandrin A, schisandrin B, schisandrin C, schisandrol A, schisandrol B, and schisantherin A. Chemical structures are shown in Fig. 1 (Huyke et al. 2007; Hong et al. 2017).

Abbreviations
AUC, area under the blood concentration-time curve; CL_{iv}, total intravenous blood clearance; C_{max}, peak blood concentration; CYP2C19, cytochrome P450 2C19; DDI, drug-drug interaction; HLM, Human liver microsomes; IC_{50}: 50% inhibition concentration; IS, internal standard; LC/MS-MS, liquid chromatography-tandem mass spectrometry; MRT, mean residence time; PK: pharmacokinetic; qPCR, real-time quantitative PCR; RFP, rifampicin; SchE, *Schisandra sphenanthera* extract; WZ, Wuzhi Tablet; VCZ, voriconazole ; V_d, volume of distribution

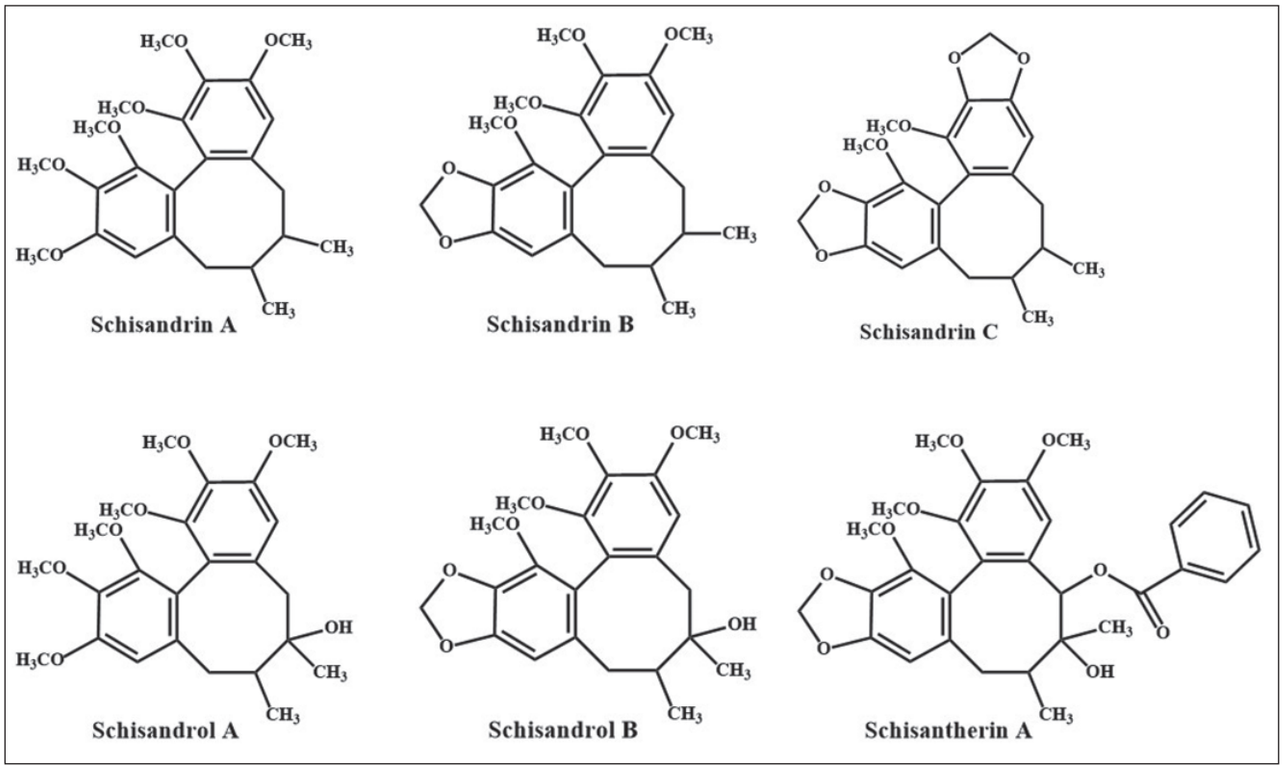

Fig. 1: Chemical structures of lignans in WZ.

WZ is often co-administered with FK506 for the treatment of drug-induced hepatitis or liver dysfunction in transplant patients (Feng, et al. 2019). In addition, WZ is also a promising FK506-sparing agent for transplant patients due to the increment of blood concentration of FK506 by metabolic pathway inhibition and therefore saves treatment costs (Qin et al. 2010). FK506 is a well-known potent immunosuppressant agent for the prevention and/or treatment of graft rejection in solid organ transplantation patients. However, the solid organ transplantation patients are at high risk of developing invasive fungal disease (IFD) and chronic hepatitis after oral FK506 causing compromised immune system (Mori et al. 2012; Qin et al. 2013). In this situation, VCZ is usually considered to be co-administered with FK506 and WZ. Therefore, the drug-drug interactions (DDI) among the triple medications and their pharmacokinetics profile is of great concern.

There are several studies related to DDI between WZ and FK506 as well as FK506 and VCZ (Xin et al. 2007; Wei et al. 2013).

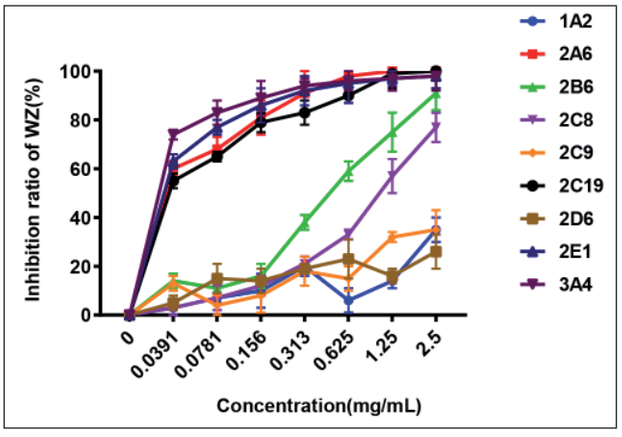

Fig. 2: Enzyme Inhibition ratio of WZ tablets on the activity of CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 in HLM (mean ± SD, n=5).

However, DDI between WZ and VCZ have not been studied so far. Schisantherin A, schisandrin A, and schisandrol B were the top three bioactive lignans in WZ (Qin et al. 2013). Previous work has suggested that several constituents from *Schisandra sphenanthera* could affect the activity of CYP450 isozymes (Iwata et al. 2004). However, it is still unknown which active constituent in the WZ contributes to the holistic integrative effect. Therefore, the current study aimed to investigate the effect of WZ and its two major constituents, schisandrol B and schisandrin A et al., on the pharmacokinetics of intravenous VCZ in rats and the potential role of CYP2C19 *in vivo* and *in vitro*. All the samples were determined by LC-MS/MS.

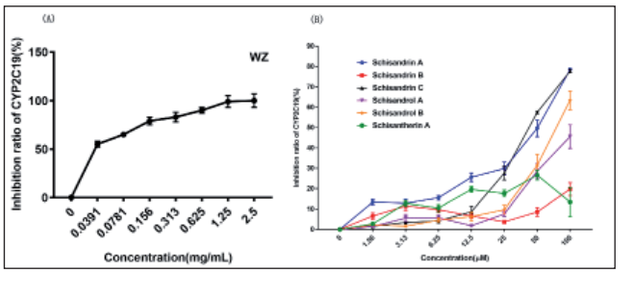

Fig. 3: Inhibition ratio of WZ tablets, schisandrin A, schisandrin B, schisandrin C, schisandrol A, schisandrol B and schisantherin A on the activity of CYP2C19 in HLM (mean ± SD, n=5).

2. Investigations and results

2.1. Enzyme inhibition on CYP450 activity in vitro

WZ inhibited all the CYP450 enzymes tested with an order of CYP3A4 > 2B6 > 2C19 > 2C8 > 2C9 > 1A2 > 2A6 > 2D6 > 2E1 for some extent (Fig. 2). Noteworthy, WZ obviously showed inhibitory activity towards CYP2C19 with IC50 around 39.1 μg/mL, CYP3A4 with IC50 around 20.3 ug/mL, respectively (Fig. 2).

WZ is a preparation of an ethanol extract of *Schisandra sphenanthera* which contains plenty of chemical compounds. To explore the individual compound's contribution to the inhibitory effect on CYP2C19, CYP3A4 and CYP2C9, we tested schisandrin A, schisandrin B, schisandrin C, schisandrol A, schisandrol B and schisantherin A on the activity of CYP2C19, CYP3A4 and CYP2C9 in HLM. WZ (2.5 mg/mL) almost completely inhibited the activity of 2C19 , and the inhibition ratio reached 78.6±3% and 63.5±4.6% for schisandrin A and schisandrol B at a concentration of 100 µM (Fig. 3).

2.2. *Metabolism of VCZ in vitro*

The effects of WZ and its constituents on the metabolism of VCZ in HLM are shown in Fig. 4. The IC_{50} of metabolism of VCZ was 39.1 µg/mL (WZ), 0.78 µM (schisantherin A) and 10.4 µM (schisandrol B), respectively, indicating a potent inhibition of VCZ metabolism by WZ.

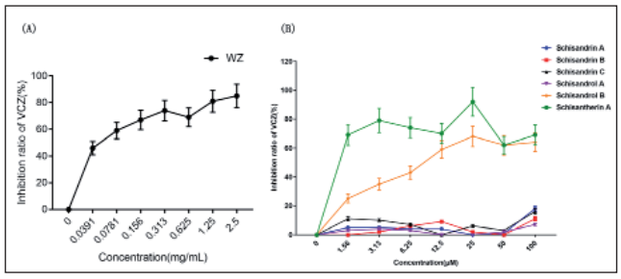

Fig. 4: Inhibition ratio of WZ and schisandrin A, schisandrin B, schisandrin C, schisandrol A, schisandrol B and schisantherin A on the metabolism of VCZ in HLM (mean ± SD, n=5).

2.3. *Pharmacokinetic (PK) study in rats*

The mean VCZ blood concentration versus time curves obtained after intravenous administration of VCZ with or without WZ, schisandrol B and schisandrin A, respectively, are shown in Fig. 5. The pharmacokinetic parameters of VCZ are presented in Tables 1 and 2.

With the co-administration of a single oral dose of schisandrol B and schisandrin A and WZ, the $AUC_{0-\infty}$ of the intravenous VCZ was increased by 80.2% (p<0.05), 37.4% (p<0.05) and 47.5% (p<0.05), respectively, compared with that of the VCZ alone (Fig. 5A, 5B). WZ showed the strongest effect, with an increase of $AUC_{0-\infty}$ by 1.8 fold; schisandrol B and schisandrin A increased the $AUC_{0-\infty}$ by 1.37 and 1.47 fold, respectively. C_{max} of VCZ was increased by 10.5% (WZ), 9.18% (schisandrol B) and 18.1% (schisandrin A) respectively. Our previous studies suggest that co-administration's long-term effect for metabolism induction or inhibition can be distinguished from the short one (Xue et al. 2013; Qin et al. 2014b, 2016). When VCZ was co-administered with WZ, schisandrol B and schisandrin A for consecutive 14 days, the AUC of VCZ was increased by 66.4% (WZ), 29.3% (schisandrol B) and 39.0% (schisandrin A). C_{max} increased by 20.6% (WZ), 12.8% (schisandrol B), and 8.6% (schisandrin A), respectively. The total intravenous blood clearance decreased from 42.2±3.6 to 34.9±5.8 (WZ), from 38.3±5.2 to 39.8±2.3 (schisandrol B) and from 38.3±5.2 to 41.8±2.9 L/h/kg (schisandrin A), respectively (Fig 5C, D).

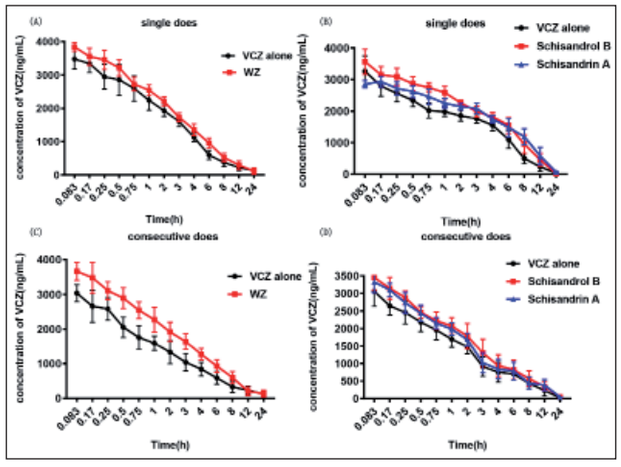

Fig. 5: Whole-blood concentration-time curves of VCZ (28mg/kg) after intravenous does of VCZ (28mg/kg) in rats with and without an oral single or consecutive dose (for 14days) of WZ (250 mg/kg), schisandrol B (10 mg/kg) and schisandrin A(10 mg/kg),respectively. (mean ± SD, n=5).

2.4. *Real-time quantitative PCR and Western blotting*

As shown in Fig. 6, compared with control gro up, after long-term treatment of WZ (250 mg/kg), schisandrol B (10 mg/kg) and schisandrin A (10 mg/kg) for 14 days, the mRNA expression of *cyp2c19* in liver increased by 2.58±0.87 (p<0.05), 1.76±0.15 (p<0.05) and 1.24±0.23 fold, respectively (Fig. 6D). Consistently, the protein expression of hepatic CYP2C19 increased by 76% (WZ, p<0.05) (Fig. 6A), 52% (Schisandrol B, p<0.05) (Fig. 6B) and 4% (Schisandrin A) (Fig. 6C), respectively, in rats.

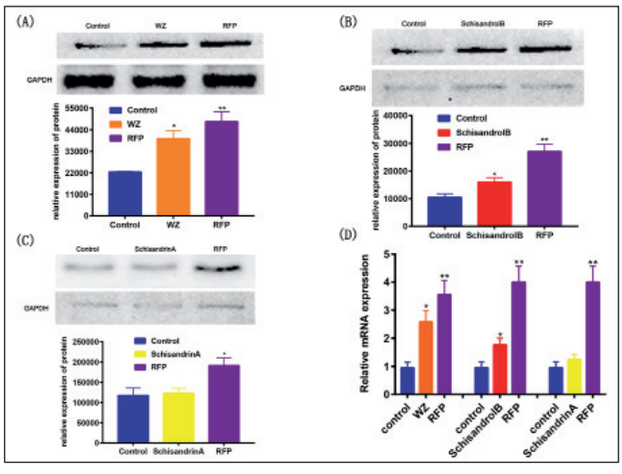

Fig. 6: Real-time quantitative PCR assay of liver Cyp2c19 mRNA expression (D) and western blotting assay of liver CYP2C19 protein expression (A~C) in rats. The control group received the control vehicle. The pretreatment group received oral WZ (250 mg/kg), schisandrol B (10mg/kg) or schisandrinA (10mg/kg) for 14 consecutive days, respectively, and the positive group received RFP at 100 mg/kg for 4 consecutive days. (mean ± SD, n=5) **: p<0.01; *: p<0.05 compared with control group.

3. Discussion

Although VCZ is regularly co-administered with FK506 and WZ, DDI between WZ and VCZ have not been studied so far. Several reports, including our previous studies, have indicated that several constituents from *Schisandra sphenanthera* could affect the activity of CYP450 isozymes (Iwata et al. 2004; Qin et al. 2014a). Due to the important role of the induction or inhibition of metabolizing enzymes in DDI, it is necessary to investigate the effects of WZ and its main active lignans on the activity of CYP450 isozymes. Our results show that the activity of CYP2C19 and CYP3A4 can be inhibited by WZ, schisandrin A and schisandrol B, respectively. WZ inhibited all the CYP450 enzymes tested in the following order: CYP3A4 > 2B6 > 2C19 > 2C8 > 2C9 > 1A 2> 2A6 > 2D6 > 2E1 for some extent. The main metabolic pathway of VCZ is mediated by CYP2C19, with marginal effects of 3A4 and 2C9 (Dolton and McLachlan 2014; Lamoureux et al. 2016; Hamadeh et al. 2017). Therefore, it is easily inferred that WZ, schisandrin A and schisandrol B could affect the metabolism of VCZ in HLM. Further, the DDI might occur if VCZ is co-administered with WZ.

Table 1: Pharmacokinetic parameters of VCZ after a single intravenous dose (28mg/kg) with and without an single oral dose or consecutive 14 days of WZ (250 mg/kg). (mean ± SD, n=5)

Parameter	Single dose administration		Consecutive 14 days administration	
	VCZ alone	With WZ	VCZ alone	With WZ
$AUC_{0\text{-}12h}$ (ng·h/mL)	6873.7±254.1	13119.87±452.6	7750.8±425.7	12899.1±398.5
$AUC_{0\text{-}\infty}$ (ng·h/mL)	7316.1±264.5	13175±421.9	7760.7±412.3	12914.2±369.4
C_{max} (ng/mL)	3475.8±300.1	3843.1±129.3	3036.7±244.2	3663.4±256.7
t1/2 (h)	4.21±0.25	3.7±0.95	3.66±0.75	3.43±0.82
MRT(h)	1.16±0.85	3.45±0.78	3.08±0.58	3.69±0.26
CL/iv((L/h/kg)	52.2±5.6	45.9±3.4	42.2±3.6	34.9±5.8

Table 2: Pharmacokinetic parameters of VCZ after a single intravenous dose (28mg/kg) with and without a single oral dose or consecutive 14 days of Schisandrol B (10 mg/kg) or Schisandrol A (10 mg/kg). (mean ± SD, n=5)

Parameter	Single dose administration			Consecutive 14 days administration		
	VCZ alone	With Schisandrol B	With Schisandrin A	VCZ alone	With Schisandrol B	With Schisandrin A
$AUC_{0\text{-}24h}$ (ng·h/mL)	15035.2±254.3	20899.6±567.8	22116.4±481.5	17546.3±331.3	20152.8±482.6	23179.9±469.7
$AUC_{0\text{-}\infty}$ (ng·h/mL)	15239.1±105.6	20947.2±502.7	22491.6±416.1	17653.8±378.2	22834.7±468.5	24541.0±337.4
C_{max} (ng/mL)	3265.2±480.5	3565.4±392.5	3856.2±118.3	3059.4±423.7	3452.7±357.2	3323.4±297.8
t1/2 (h)	4.18±0.54	3.58±0.16	3.86±0.25	3.71±1.9	4.57±0.4	4.50±0.25
MRT(h)	5.19±0.75	5.38±0.35	6.48±0.64	5.86±0.68	6.24±0.42	6.30±0.6
CL/iv ((L/h/kg)	43.4±1.5	37.6±4.8	35.6±7.4	38.3±5.2	39.8±2.3	41.8±2.9

To confirm whether WZ and its six lignans (schisandrin A, schisandrin B, schisandrin C, schisandrol A, schisandrol B and schisantherin A) are influencing the metabolism of VCZ, their effects on the metabolism of VCZ in HLM were investigated *in vitro*. The IC_{50} of metabolism of VCZ was 39.1 μg/mL (WZ), 0.78 μM (schisantherin A) and 10.4 μM (schisandrol B), respectively, indicating a potent inhibition of VCZ metabolism by WZ. The results was in agreement with the inhibition of WZ, schisandrin A and schisandrol B on the activity of CYP2C19. In light of these observations, it may be reasonable that WZ and the two lignans inhibit the activity of CYP2C19, and also the CYP2C19-mediated VCZ metabolism. Furthermore, the blood concentration of VCZ may be increased in the presence of WZ and its lignans. Thus, to confirm the results from the *in vitro* experiments, an in-vivo study in rats is necessary.

In clinical practice, the patients were commonly treated with three capsules of WZ Tablets twice daily. Therefore, the dosage amounts to 45 mg/d (Xin et al. 2007). Normally, the regiment of intravenous VCZ was 6 mg/kg twice a day on the first day, followed by 3 mg/kg twice daily for 6-27 days (Donnelly and De Pauw 2004). In the investigation, the dosages of VCZ, WZ, schisandrol A, schisandrol B were extrapolated from the clinical practice according to our previously published papers (Qin et al. 2010b). Due to the single dose widely used in clinical practice, we choose a single dose for the investigation and the dose-dependency need to be investigated further.

The pharmacokinetic data showed that the blood concentration of VCZ when co-administration with WZ, schisandrol B and schisandrin A increased with a slower elimination, but the extent was slighter than that of single administration. WZ showed significant inhibition of the metabolism of VCZ, which might be attributed to the synergism effects of the different constituents in WZ including schisandrol B and schisandrin A. Due to the narrow therapeutic window of VCZ (1.5–4 μg/mL) (Mikulska et al. 2012), the blood concentration of VCZ should be closely monitored after co-administration of WZ and VCZ.

To explore the mechanism of the inhibitory effect on CYP2C19 activity, the effects of WZ, schisandrol B and schisandrin A on the mRNA and protein expression of CYP2C19 in rats are assayed. The investigation demonstrated that consecutive intragastric administration of WZ, schisandrol B and schisandrin A induced the mRNA expression of *cyp2c19* and unregulated CYP2C19 protein level in rat liver. On the other hand, WZ is more potent compared with single schisandrol B and schisandrin A, in both *in viv*o and *in vitro* experiments. Therefore, it is not difficult to understand that synergistic effects may exist among the constituents in WZ. This phenomenon also has been reported before (Qin et al. 2014b).

It is interesting that WZ and its constituents' effect on CYP2C19 in transcription, translation and activity points into an opposite direction. However, other examples are known. Aqueous extract of *Schisandra chinensis* Baill or WZ could inhibit the activity of CYP3A, while increasing the mRNA and protein expression of CYP3A during long-term administration (Chen et al. 2010). Erythromycin can induce the expression of CYP450, while its metabolite forms an inactive complex with the iron (II) of CYP450. Thus, the self-induction of CYP450 by erythromycin leads to the reduction of the activity of CYP450 (Danan et al. 1981; Zhang et al. 2010). Similar to triacetyloleandomycin, it can induce CYP450 expression, meanwhile inhibit activity of protein (Watkins et al. 1985; Wrighton et al. 1985). To our knowledge, the activity of CYP450 can be modulated by xenobiotics at the level of transcription, translation, and post-translation protein modification. Therefore, upregulation or downregulation of the protein at the transcriptional and translational level might not necessarily result in the increase or decrease of the protein activity. Further investigation are warranted to clarify the underlying mechanism at the post-translational level involved WZ's effect on CYP2C19 activity.

In conclusion, WZ and its consituents schisandrol B and schisandrin A, could inactivate CYP2C19 and therefore inhibit the metabolism of VCZ. The inhibition contributes to the increased $AUC_{0\text{-}\infty}$ and C_{max} of VCZ while co-administered in both short and long time treatment. Therefore, attention should be paid to the concomitant administration of WZ and VCZ in clinical practice, where dose adjustment might be necessary. Further clinical study is warranted to validate the observational results.

4. Experimental

4.1. Chemicals and reagents

Voriconazole powder injection was from Pfizer (America). The crude extract of *Schisandra sphenanthera* was manufactured and supplied by FangLue Pharmaceutical Company (Guangxi, China) under GMP guidelines. The final product meets the China SFDA standard (YBZ14932006) and has been quantified to 7.5 mg schisantherin A per tablet by HPLC analysis. Human liver microsomes were purchased from Xenotech Co., Ltd (#H2640, USA, batch NO.: 1210223). D3-voriconazole (as internal standard, IS) was synthesized and provided by ANPEL Laboratory Technologies (Shanghai) Inc. Schisandrin A, schisandrin B, schisandrin C, schisandrol A, schisandrol B and schisantherin A were all produced by Shanghai Winherb Medical Science and Technology Development Co.,Ltd (China). Positive inhibitors of CYP450s such as α-naphthoflavone and tranylcypromine, tranylcypromine, ticlopidine, quercetin, fluconazole, (S)-(+)-N-3-benzylnirvanol, quinidine, ketoconazole, and Hank's balanced salt solution (HBSS) were from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), Williams' E medium, fetal bovine serum (FBS), L-glutamine, minimum essential medium nonessential amino acids (MEMNAA), penicillin G, and streptomycin were from Invitrogen (Carlsbad, CA, USA). The BCA protein assay kit was purchased from Beyotime (Jiangsu, China). All other solvents and regents were of either high-performance liquid chromatography (HPLC) or analytical grade. Trizol reagent from Invitrogen (Carlsbad, CA). M-MLV and Taq enzymes were obtained from Promega (Madison, WI) and Takara Medical Co., Ltd. (Kyoto, Japan), respectively. Oligonucleotide primers were synthesized by Sangon Co. (Shanghai, China). Antibodies of rabbit anti-rat CYP2C19 and horseradish peroxidase-labeled anti-goat IgG and anti-rabbit IgG were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies of mouse anti-GADPH Mouse mAb (6C5) were obained from Calbiochem (CA).

4.2. Animals

Male Sprague-Dawley rats (250–330 g) were supplied by the Laboratory Animal Service Center at Guangdong pharmaceutical university (Guangzhou, China). The animals were kept in a room at 22-24 °C with a light/dark cycle of 12/12 h and 55-60% relative humidity. They had free access to standard rodent chow and clean tap water. The rats were fasted for 12 h before the experiments.

4.3. Fingerprint analysis of Wuzhi tablet

The crude extract of *Schisandra sphenanthera* was manufactured and supplied by FangLue Pharmaceutical Company (Guangxi, China) under GMP guidelines. The final product meets the China SFDA standard (YBZ14932006) and has been quantified to 7.5 mg schisantherin A per tablet by HPLC analysis.

4.4. Quantification of VCZ by UPLC-MS/MS analysis

UPLC-MS/MS method was developed and validated for the analysis of VCZ in all samples (blood and microsomes). The blood samples were prepared using a single-step liquid-liquid extraction procedure. Briefly, for the extraction procedure, 10 μL of D3-voriconazole as internal standard (IS), 100 μL of ammonium acetate, and 1000 μL of extraction solvent acetic ether: dichloromethane (4:1,v/v) were added to 100 μL of plasma, or QC material. As for samples from microsomes metabolism experiment, 800 μL of extraction solvent acetic ether: dichloromethane (4:1,v/v) were added directly. After vortex-mixing for 1min and standing at room temperature for 10 min, the mixtures were centrifuged at 2500×g for 5 min. After centrifugation, the organic phase was then transferred to a clean centrifuge tube and evaporated to dryness. The residues were dissolved in 50 μL of methanol:water (1:1, v/v) of the re-constituent solution and injected onto the UPLC/MS/MS for analysis.

A Waters ACQUITY-UPLC (Waters Inc., America) was used for solvent and sample delivery. The chromatographic separation was achieved on an ACQUITY UPLC BEH C18 (2.1×100 mm, 1.7 μM) with 0.1% formic acid (A) and acetonitrile containing 0.2% formic acid (B). The flow rate was 0.4 mL/min. The VCZ was eluted with 5 min isocratic run with 45% solvent B. The auto-sample was conditioned at 15 °C, and the sample injected for analysis was 10 μL.

A XEVO-TQD triple quadrupole mass spectrometer (Waters Inc., America) equipped with a Turbo Ion Spray ionization (ESI) source was used for mass analysis and detection. Mass spectrometric analysis was performed in the positive-ion mode (ESI^+) and set up in the multiple reaction monitoring (MRM) mode. The mass transitions for MRM were m/z 349.9→m/z126.9 for VCZ, 352.6→129.6 for IS.

The extraction recoveries were 56.1-60.2% and 64.6-72.3% for VCZ in blood and microsomes buffer, respectively. The linearity of the calibration curves over the concentrations of 5 – 4000 ng/mL and 5 – 40 ng/ mL for VCZ in blood and microsomes, respectively, were all good, with a correlation coefficient $\gamma^2 > 0.99$. The lower limit of quantification (LLOQ) of the analytical method was 5 ng/mL for VCZ. The intra and interbatch precision and accuracy were less than 15% for all quality control samples in blood and microsomes buffer, respectively. No matrix effect was observed to influence the determination of each analyte in rat plasma and microsomes.

4.5. Enzyme inhibition on CYP450 activity

CYP450 activity was determined based on our preliminary tests with some modification (He et al. 2007). Briefly, the mixture (60 μL) containing 0.1 M potassium phosphate buffer (PBS, pH7.4), 4.8 mM $MgCl_2$, 0.17 mg/mL HLM, probe substrate and various concentrations of WZ (39.1 – 2500 μg/mL) or and six lignans (1.56 – 100 μM) were pre-incubated for 10 min at 37 ºC in a water bath with gentle shaking. The reaction was initiated by addition of 40 μL NADPH (3 mM) and terminated with 200 μL ice-cold acetonitrile containing 100 ng/mL tolbutamide (IS) after 10 min incubation. The concentrations of the metabolites of CYP450s probe substrates were determined by LC-MS/MS.

4.6. Metabolism of voriconazole (VCZ) assay in vitro

The metabolism of VCZ was assayed by measuring the reduction of VCZ. Taking the VCZ therapeutic range of 0.5–5 mg/L into consideration, 1 μM of VCZ was chosen as substrate concentration in the metabolism study. Briefly, the incubation system, with a total volume of 200 μL, contained 0.1 M potassium phosphate buffer (PBS, pH7.4), 4.8 mM $MgCl_2$, Human liver microsomes (HLM) (final concentration 0.2 mg/mL) and 1 μM VCZ with or without WZ (39.1 – 2500 μg/mL) or six lignans (1.56 – 100 μM). The reaction was started by addition of 40 μL NADPH (3 mM) to the system. The mixture was incubated for 10 min at 37 °C and terminated with 200 μL ice-cold acetonitrile containing 600 ng/mL D3-voriconazole (IS). VCZ was determined by LC-MS/MS. The metabolism ratio was calculated by compared the initial drug concentration with the concentration after incubation.

4.7. Pharmacokinetic (PK) study in rats

Pharmacokinetics of VCZ were determined after co-administration of an oral dose WZ, schisandrol A, schisandrol B, single or consecutively for 14 days. In the single dose study: (1) WZ (250 mg/kg), schisandrin A (10 mg/kg) or schisandrol B (10 mg/kg) dissolved in CMCC-Na solvent were given to rats by gavage at a single dose. After 2 h, the contents of VCZ powder injection dissolved in pure water was given to rats into the caudal vein at a dose of 28 mg/kg. (2) The control group received an equivalent volume of vehicle by gavage 2 h before the injection of VCZ (28 mg/kg). About 0.22 mL of blood samples were withdrawn from the caudal vein before and at 0.083, 0.17, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 12, 24 h after dosing. Blood samples (200 μL) were immediately collected and stored at -20 °C until analysis. In consecutive14 days of administration of WZ, schisandrin A, schisandrol B were given once a day, on the 15th day, before 2 h the injection of VCZ, respectively, other procedures are same as the single dose study.

4.8. Preparation of rat hepatic microsomes

Rats were randomized to five groups (n = 5 per group).The control group received the control vehicle. The pretreatment group received oral WZ (0.25 mg/kg), schisandrol B (10 mg/kg) or schisandrol A (10 mg/kg) for 14 consecutive days, the positive group received rifampicin (RFP) at 100 mg/kg/day for 4 consecutive days. All the rats were intragastrically treated. The dosages of WZ, schisandrol B and schisandrin A were converted from the clinical dosage. The time to continue intragastric administration depended on the result of pre-experiment. Rats treated with various compounds were sacrificed and the livers were immediately excised. A small portion of liver samples was snap-frozen in liquid nitrogen and stored at -80 °C before extraction of total RNA and microsomal proteins.

4.9. Real-time quantitative PCR

Approximately 100–200 mg of liver sample was homogenized in 8 mL TRIzol reagent to extract total RNA. Reverse-transcribed to complementary DNA(cDNA) using Prime script RT reagent kit, and amplified with SYBR premix tag reagent using SYBR Greener qPCR Universal System (Applied Biosystems 7500). Each sample was analyzed in triplicate. Relative mRNA levels were calculated as described previously (Yuan et al. 2010, 2013). The quantification of 18S rRNA was carried out for each sample as well as for normalization of target mRNA results. Oligonucleotide primers for CYP2C19 and 18s RNA were CYP2C19: 5'-CAA CAA CCC TCG GGA CTT TA-3'; R: 5'-GTC TCT GTC CCA GCT CCA AG-3' and 18s RNA were 5'-GGG ATG GGA AAG AGG AGT-3' (S), 5'-ATGGAG CAG ATG ATG TTG G-3'(A), respectively.

4.10. Western blotting

For Western blotting analysis, liver proteins (10 μg) for each sample were separated by 10% sodium dodecyl sulfate poly-acrylamide gel (SDS-PAGE) and then transferred onto PVDF membranes (Millipore, USA). After blocking in 5% non-fat milk at room temperature for 1 h, and the membranes were incubated with primary antibodies against CYP2C19 (1:1000), and GAPDH (1:2000) overnight at 4 °C. The membranes were washed three times by PBS, and then were incubated with 1:5000 diluted HRP-conjugated second antibodies (1:5000) at room temperature for 2 h. Finally, the membranes were detected using the Bio-rad ChemiDoc MP System (Bio-rad; USA). GAPDH levels were normalized to relative abundance of the protein.

4.11. Statistical analysis

The half inhibitory concentration (IC_{50}) was calculated by fitting the Hill equation to the data using non-linear regression (least squares best fit modeling) with GraphPad Prism (version 6.01, GraphPad Software Inc., San Diego, CA, USA). All data are expressed as the mean±standard deviation (S.D.) The significant difference between the data was determined using SPSS software (version 16.0 for Windows, SPSS Inc., Chicago, IL, USA). P_{values} of < 0.05 were regarded as statistically significant. Drug and Statistics (V3.0, Shanghai University of Traditional Chinese Medicine Clinical Research Center, Shanghai Bo Jia Pharmaceutical Technology Co., Ltd, China) was used to calculate the pharmacokinetic parameters with a non-compartmental model. All data are expressed as the mean±standard deviation (S.D.).

Ethics statement: All aspects of this study were approved by the medical ethics committee of Guangdong pharmaceutical university. All procedures were in accordance with the Regulations of Experimental Animal Administration issued by the Ministry of Science and Technology of the People's Republic of China (http://www.most.gov.cn).

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