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Interaction of *Rhei Rhizoma* extract with cytochrome P450 3A and efflux transporters in rats

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Traditional Chinese herbal medicines are frequently prescribed in pharmacotherapy in Japan. In the present study, we evaluated the possible interaction of several herbal extracts including *Rhei Rhizoma* extract with cytochrome P450 (CYP) 3A and efflux transporters such as P-glycoprotein and multidrug resistance-associated protein (MRP) 2. *Rhei Rhizoma* extract (100 μg/ml) significantly suppressed the CYP3A-mediated 6β-hydroxylation of testosterone in hepatic microsomes, and increased the extent of bioavailability of midazolam, a typical CYP3A substrate, in rats. Also, *Rhei Rhizoma* extract (300 μg/ml) significantly suppressed P-glycoprotein-mediated efflux transport of rhodamine 123 (Rho123) in rat everted intestine. In an *in-vivo* study, *Rhei Rhizoma* extract added to intestinal perfusate at a concentration of 300 μg/ml significantly suppressed the intestinal exsorption of Rho123, though it exerted no effect on the biliary excretion of Rho123. Furthermore, the *in-vitro* and *in-vivo* MRP2-mediated intestinal efflux of 2,4-dinitrophenyl-*S*-glutathione was significantly suppressed by *Rhei Rhizoma* extract *(*1000 μg/ml). In conclusion, *Rhei Rhizoma* extract, which is taken orally at doses of 0.5–1 g each or 1–3 g daily in clinical practice, may cause pharmacokinetic herb-drug interactions in the process of the intestinal and/or hepatic CYP3A-mediated drug metabolism and P-glycoprotein- and/or MRP2-mediated efflux transport in the intestine.

1. Introduction

The small intestine and liver possess various detoxification/host defense systems including metabolizing enzymes and efflux transporters. Among the various enzymes, cytochrome P450 (CYP) 3A, responsible for phase I oxidative metabolic reactions, is abundantly expressed in the enterocytes and hepatocytes and contributes to the metabolism of more than 50% of therapeutic drugs, such as immunosuppressants, steroids, and calcium channel blockers (Guengerich 1992; Mahnke et al. 1997; Zhang et al. 1999). In addition to the metabolizing enzymes, ATP-dependent efflux transporters such as P-glycoprotein and multidrug resistance-associated protein (MRP) 2 are also expressed in the intestine and liver of humans and rodents (Takano et al. 2006; Murakami and Takano 2008). P-Glycoprotein and MRP2, expressed in the brush-border membrane of enterocytes and bile canalicular membranes, prevent intracellular accumulation of substrate compounds by limiting the influx to cells or facilitating the efflux from cells (Takano et al. 2006; Murakami and Takano 2008).

Traditional Chinese herbal medicines (*Kampo* medicines) are frequently used in Japan with synthetic prescribed (Western) drugs as complementary and alternative medicines in various clinical situations. However, the combined use of herbal medicines (or medical plants) with synthetic prescribed drugs may cause pharmacokinetic herb-drug interaction as reported previously by many researchers (Lown et al. 1997; Rau et al. 1997; Schmiedlin-Ren et al. 1997; Hashimoto et al. 1998; Kupferschmidt et al. 1998; Dresser et al. 2002; Bauer et al. 2003; Honda et al. 2004; Satoh et al. 2005; Kim et al. 2006; Bailey et al. 2007; Schwarz et al. 2007; de Castro et al. 2008). Natural products including herbal plants contain a great variety of components such as flavonoids, alkaloids and saponins, some of which are known to interact with CYP3A, P-glycoprotein, MRP2 and/or organic anion transporting polypeptides (OATPs) (Lown et al. 1997; Rau et al. 1997; Schmiedlin-Ren et al. 1997; Hashimoto et al. 1998; Kupferschmidt et al. 1998; Dresser et al. 2002; Bauer et al. 2003; Honda et al. 2004; Satoh et al. 2005; Kim et al. 2006; Bailey et al. 2007; Schwarz et al. 2007; de Castro et al. 2008). For example, it is well known that St. John's wort (*Hypericum perforatum L.*) can cause pharmacokinetic herb-drug interactions with various P-glycoprotein substrate drugs by interacting with intestinal P-glycoprotein (Bauer et al. 2003; Schwarz et al. 2007). Grapefruit juice is another example which markedly increases the oral bioavailability of various CYP3A and/or P-glycoprotein substrates by inhibiting intestinal CYP3A and P-glycoprotein (Lown et al. 1997; Rau et al. 1997; Schmiedlin-Ren et al. 1997; Hashimoto et al. 1998; Kupferschmidt et al. 1998; Honda et al. 2004; Kim et al. 2006; de Castro et al. 2008), though it decreases the oral bioavailability of OATP-mediated drugs (Dresser et al. 2002; Satoh et al. 2005; Bailey et al. 2007). A variety of Chinese herbal medicines are prescribed in clinical practice; however, the possible phar-

macokinetic herb-drug interactions with synthetic prescribed drugs are not yet fully understood. *In-vivo* studies would be required for analysing the mechanisms of pharmacokinetic herb-drug interactions in clinical practice, because natural compounds are mostly easily metabolized in the intestine and/or liver (Ravindranath and Chandrasekhara 1981; Andlauer et al. 2000). In the present study, we first evaluated the possibility of CYP3A-mediated herb-drug interaction by using four herbal extracts of *Paeoniae Radix*, *Ginseng Radix*, *Persicae Semen,* and *Rhei Rhizoma.* Among these herbal extracts, *Rhei Rhizoma* extract alone was found to significantly suppress CYP3A activity *in vitro* and *in vivo*. Hence, we further evaluated the possibility of interaction of *Rhei Rhizoma* extract with P-glycoprotein and MRP2 in rats.

2. Investigations and results

2.1. Effect of herbal extracts on CYP3A activity in rat hepatic microsomes

The effect of four herbal extracts on the metabolic rate of 6β-hydroxylation of testosterone as a marker of CYP3A activity was evaluated in rat hepatic microsomes (Fig. 1). The 6β-hydroxylation of testosterone was significantly suppressed by ketoconazole, a typical CYP3A inhibitor. *Rhei Rhizoma* extract also suppressed the hydroxylation in a concentration-dependent manner to the same extent as ketoconazole. In contrast, the three other herbal extracts (*Paeoniae Radix, Ginseng Radix* and *Persicae Semen*) showed no significant inhibitory effects even at a concentration of 400 μg/ml. *Rhei Rhizoma* extract alone also suppressed the metabolism of midazolam to 4- and 1′-hydroxymidazolam as follows: at 25 μg/ml of *Rhei Rhizoma* extract, the formation of 4-hydroxymidazolam and 1′-hydroxymidazolam decreased to approximately 60% of control, and at 50 μg/ml, to 15% and 30% of control, respectively.

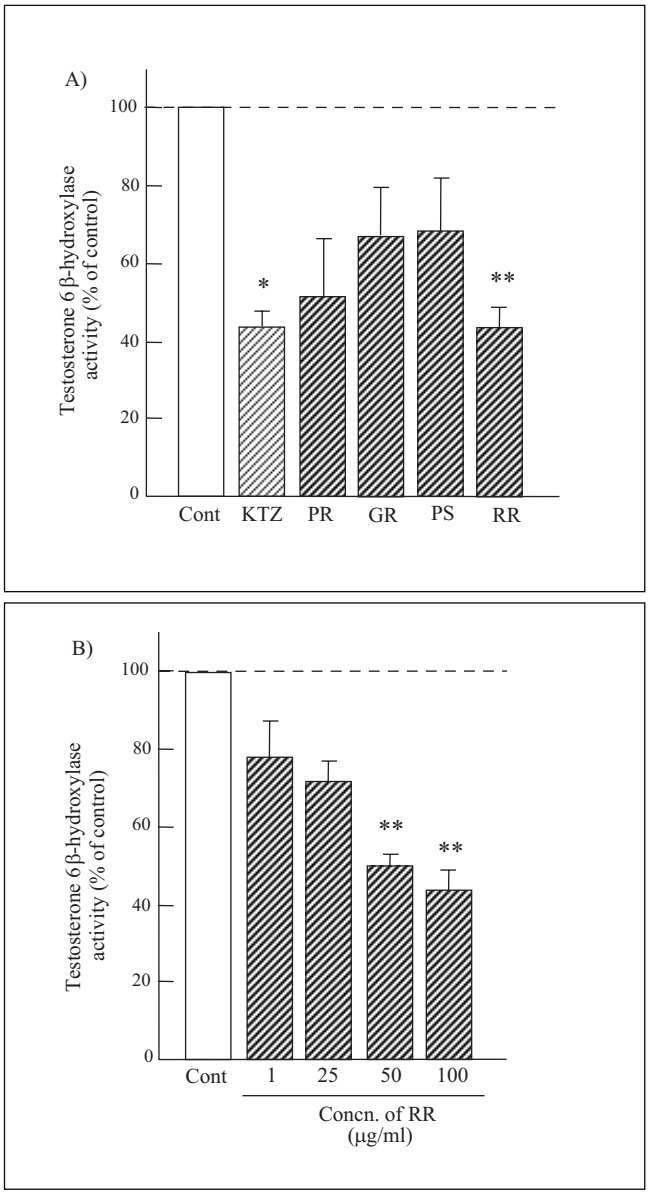


Fig. 1: Effect of herbal extracts (A) and concentration-dependent inhibitory effect of *Rhei Rhizoma* extract (B) on testosterone 6β-hydroxylase activity in rat hepatic microsomes. 6β-Hydroxylation of testosterone (250 μM) was determined for 10 min at 37 °C. Final concentrations of ketoconazole (KTZ), *Rhei Rhizoma* extract (RR) and the other 3 herbal extracts (*Paeoniae Radix*, PR; *Ginseng Radix* GR; *Persicae Semen*, PS.) were as follows: ketoconazole, 10 μM; *Rhei Rhizoma* extract, 100 μg/ml for (A) and 1-100 μg/ml for (B); other herbal extracts, 400 μg/ml. Each value represents mean ± S.E. of results from three to five rats. $^{**}P < 0.01$, $^{*}P < 0.05$: significantly different from value for control

2.2. Effect of Rhei Rhizoma extract on midazolam absorption in rats in vivo

The effect of *Rhei Rhizoma* extract on intestinal absorption of midazolam after intraluminal administration was examined (Fig. 2). By administering *Rhei Rhizoma* extract together with midazolam, the peak plasma concentration (C_{max}) and the area under the plasma concentration-time curve (AUC) of midazolam increased significantly to approximately twice that with midazolam alone.

2.3. Effect of herbal extracts on rhodamine123 and 2,4-dinitrophenyl-S-glutathione efflux transports in intestinal everted sac in vitro

The effect of herbal extracts on the serosal-to-mucosal efflux transport of rhodamine123 (Rho123) was evaluated using everted intestine prepared from the distal small intestine, since the distal small intestine possesses P-glycoprotein abundantly as compared with the proximal small intestine (Tian et al. 2002). The transport of Rho123 followed zero-order kinetics for 120 min with a small lag time and verapamil (300 μM), a potent P-glycoprotein inhibitor, significantly suppressed the efflux of Rho123 by approximately 35%. *Rhei Rhizoma* extract at a concentration of 300 μg/ml also suppressed the efflux of Rho123 to the same extent as verapamil, whereas other herbal extracts exerted no significant effect on Rho123 efflux at a concentration of 300 μg/ml (Figs. 3(A) and 4(A)). *Rhei Rhizoma* extract inhibited intestinal Rho123 efflux in a concentration-dependent manner, and the half-maximal inhibitory concentration (IC_{50}) value determined by the Hill equation was 81.9 ± 14.0 μg/ml. A mixture of *Rhei Rhizoma* extract (300 μg/ml) with verapamil (300 μM) did not increase the inhibitory effect any more compared with verapamil alone (Fig. 4(B)).

The effect of herbal extracts on the mucosal efflux of 2,4-dinitrophenyl-*S*-glutathione (DNP-SG), a glutathione conjugated metabolite of 1-chroro-2,4-dinitrobenzene (CDNB) and an MRP2 substrate, after loading of CDNB into the membrane was examined using proximal small intestine (Fig. 3(B)), since the proximal small intestine expresses MRP2 abundantly as compared with the distal small intestine (Yokooji et al. 2007). CDNB was taken up rapidly by the everted intestine and rapidly metabolized to DNP-SG by glutathione *S*-transferase, as reported previously (Yokooji et al. 2005). The efflux of DNP-SG to the mucosal surface followed zero-order kinetics

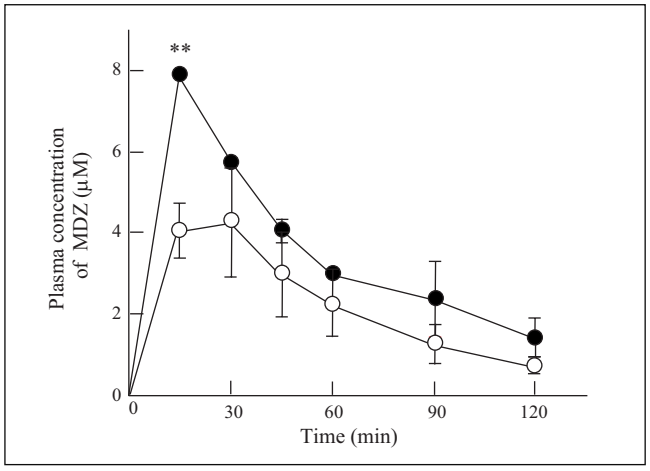


Fig. 2: Effect of *Rhei Rhizoma* extract on plasma concentration-time profile of midazolam given to intestinal loop in rats. Dose of midazolam to intestinal loop 50 μmol/kg. In an inhibition study, *Rhei Rhizoma* extract was administered into a separated loop at a dose of 100 mg/kg 15 min before midazolam administration. Opened and closed circles represent control and *Rhei Rhizoma* extract treatment, respectively. Each value represents mean ± S.E. of results from three to five rats. $^{**}P < 0.01$: significantly different from value for control

with no lag time (Fig. 5(A)). Probenecid (1 mM), an MRP2 inhibitor, significantly suppressed the mucosal efflux of DNP-SG by approximately 65%. *Rhei Rhizoma* extract did not affect DNP-SG efflux at a concentration of 300 μg/ml, but significantly suppressed it by approximately 40% of control at a concentration of 1,000 μg/ml (Fig. 5(B)). Other herbal extracts showed no significant inhibitory effect even at a concentration of 1000 μg/ml (Fig. 3(B)).

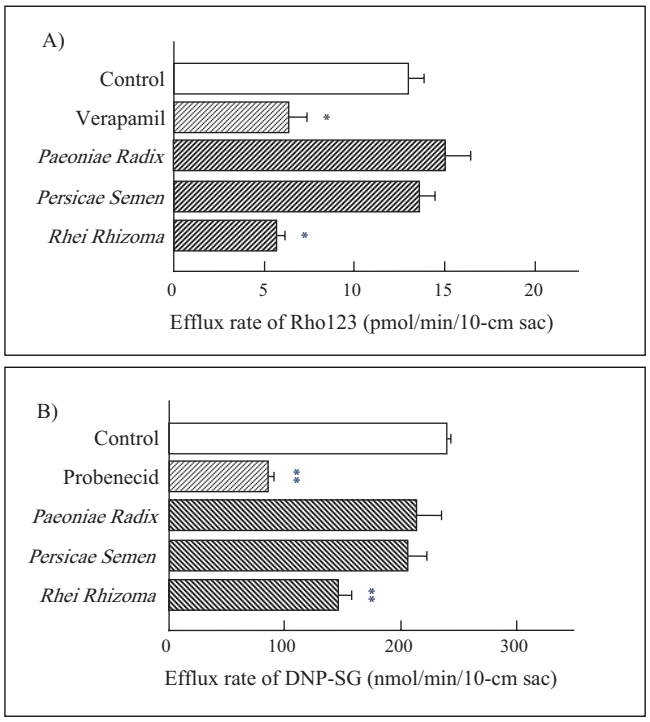
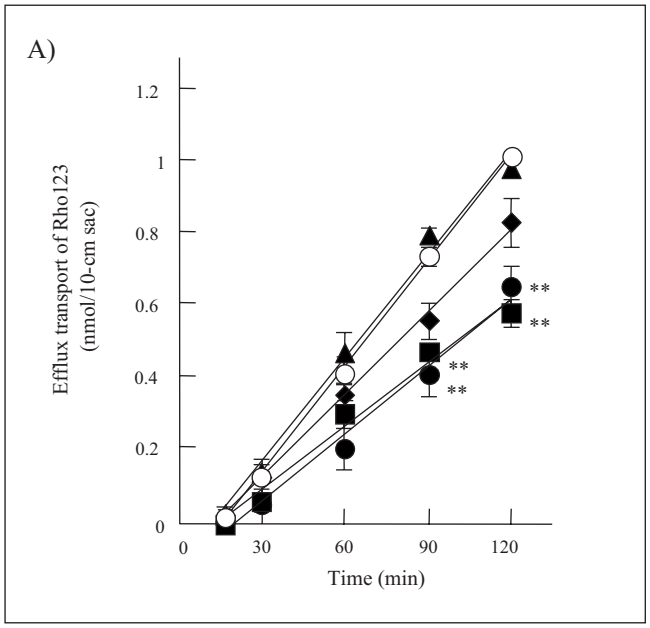


Fig. 3: Effect of herbal extracts on efflux transports of Rho123 (A) and DNP-SG after application of CDNB (B) in everted intestine *in vitro*. Rho123 and CDNB applied to serosal surface of 10-cm long everted sac at doses of 5 and 50 nmol, respectively. Efflux transport of Rho123 examined in absence and presence of verapamil (300 μM) or herbal extracts (300 μg/ml). Efflux transport of DNP-SG examined in presence and absence of probenecid (1 mM) or herbal extracts (1000 μg/ml). Each value represents mean ± S.E. of results from three rats. $^{**}P < 0.01$, $^{*}P < 0.05$: significantly different from value for control



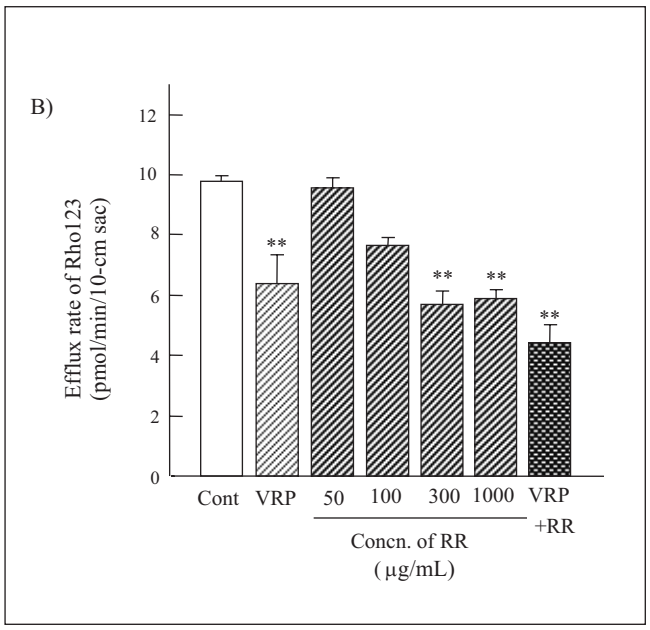


Fig. 4: Concentration-dependent inhibitory effects of *Rhei Rhizoma* extract on Rho123 efflux in everted intestine *in vitro*. A 10-cm long everted intestine was prepared from rat distal small intestine. In panel (A), concentration of *Rhei Rhizoma* extract added to transport media was as follows: 0 (○), 50 (▲), 100 (◆), 300 (●), and 1000 μg/ml (■). Panel (B) shows efflux rates of Rho123 in absence and presence of 300 μM verapamil (VRP) or *Rhei Rhizoma* extract (RR). Rho123 applied to serosal surface of everted sac at dose of 5 nmol. Each value represents mean ± S.E. of results from three rats. $^{**}P < 0.01$: significantly different from value for control

2.4. Effect of Rhei Rhizoma extract on P-glycoprotein function in the intestine and liver in vivo

The effect of orally administered *Rhei Rhizoma* extract on the intestinal exsorption (CL_{exp}) and biliary excretion (CL_{bile}) clearances of Rho123 was evaluated in rats at a steady-state plasma concentration (C_{pss}) of Rho123 (0.21 ± 0.01 μM) (Fig. 6 and Table). Cyclosporin A, a potent P-glycoprotein inhibitor, administered intravenously decreased both CL_{exp} (Fig. 6(A)) and CL_{bile} (Fig. 6(B)) of Rho123 by approximately 55% and 90% of control, respectively. *Rhei Rhizoma* extract administered into the intestinal perfusate at a concentration of 300 μg/ml also suppressed the CL_{exp} of Rho123 significantly. In contrast, CL_{bile}

Table: Effects of cyclosporin A (CsA) and *Rhei Rhizoma* extract (RR) on *in-vivo* Rho123 clearances under steady-state plasma concentration

	Cyclosporin A treatment			*Rhei Rhizoma* extract treatment		
	Control	+CsA	I/C ratio	Control	+RR	I/C ratio
C_{pss} (μM)	0.19 ± 0.10	0.21 ± 0.02		0.22 ± 0.01	0.24 ± 0.00	
CL_{total} (ml/min/kg)	61.5 ± 1.2	55.4 ± 3.4	0.90	64.4 ± 1.1	58.0 ± 2.3	0.90
CL_{exp} (ml/min/kg)	0.89 ± 0.06	0.40 ± 0.04**	0.45	0.96 ± 0.08	0.59 ± 0.04*	0.61
CL_{bile} (ml/min/kg)	3.71 ± 0.15	0.40 ± 0.02**	0.11	3.72 ± 0.13	3.69 ± 0.08	0.99

As inhibition phase, cyclosporin A (CsA) administered intravenously at dose of 30 mg/kg, or *Rhei Rhizoma* extract (RR) perfused in looped intestine at concentration of 300 μg/ml. I/C ratio represents value of + inhibitor/control. Each value represents mean ± S.E. of results from three rats

** $P < 0.01$,

* $P < 0.05$: significantly different from value for control

was not affected by *Rhei Rhizoma* extract administered into the intestinal perfusate at a concentration of 300 μg/ml.

2.5. Effect of Rhei Rhizoma extract on intestinal MRP2 function in vivo

The intestinal MRP2 function was evaluated in rats by measuring DNP-SG efflux into the lumen after intestinal application of CDNB in the presence or absence of *Rhei Rhizoma* extract (Fig. 7). The presence of probenecid and *Rhei Rhizoma* extract in the intestinal perfusate did not affect the rates of disappearance of CDNB from the intestinal perfusate, or the influx rates of CDNB into enterocytes. However, the presence of probenecid at a concentration of 1 mM in the intestinal perfusate significantly suppressed the intestinal efflux of DNP-SG by approximately 40% evaluated at 90 min. *Rhei Rhizoma* extract at a concentration of 1000 μg/ml also suppressed the intestinal efflux of DNP-SG by approximately 25% at 90 min.

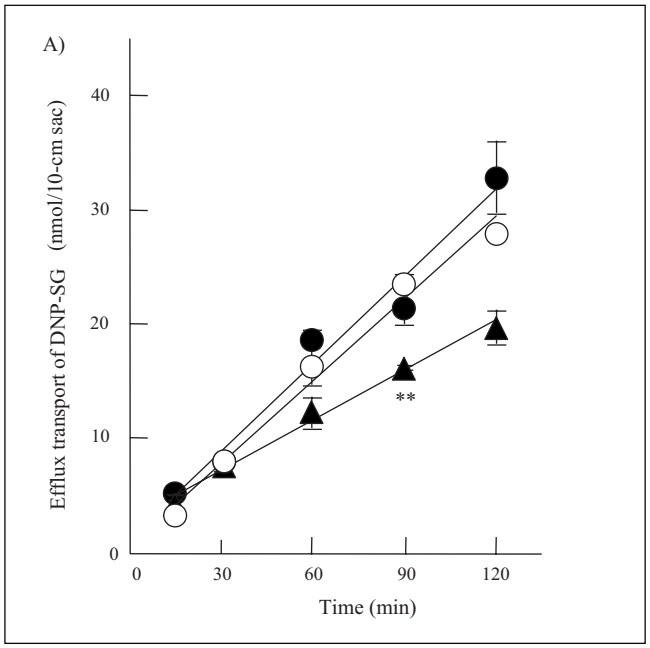
3. Discussion

Traditional Chinese herbal medicines (extracts) are frequently used in Japan together with synthetic prescribed drugs as complementary and/or alternative medicines in various clinical situations. Such combined use of herbal medicines and synthetic prescribed drugs may cause adverse events in various clinical situations (Ikegami et al. 2003; Skalli et al. 2007), in addition to those observed with St. John's wort and grapefruit juice (Lown et al. 1997; Rau et al. 1997; Schmiedlin-Ren et al. 1997; Hashimoto et al. 1998; Kupferschmidt et al. 1998; Dresser et al. 2002; Bauer et al. 2003; Honda et al. 2004; Satoh et al. 2005; Kim et al. 2006; Bailey et al. 2007; Schwarz et al. 2007; de Castro et al. 2008). St. John's wort (*Hypericum perforatum L.*) induces expression of intestinal CYP2C9, CYP3A4 and P-glycoprotein, and modifies intestinal bioavailability of various substrate drugs including cyclosporin A, midazolam, tacrolimus, digoxin, indinavir, warfarin, simvastatin, and so on (Skalli et al. 2007). A number of traditional Chinese herbal medicines also cause various drug interactions, depending on the combination of herbal medicines and synthetic prescribed drugs and the clinical situation (Izzo and Ernst 2001; Ikegami et al. 2003; Skalli et al. 2007; Di et al. 2008; Ulbricht et al. 2008).

In the present study, we employed four herbal extracts, *Paeoniae Radix*, *Ginseng Radix*, *Persicae Semen* and *Rhei Rhizoma*, which are used frequently in clinical practice, and evaluated their possible interactions with CYP3A in rat hepatic microsomes and P-glycoprotein and MRP2 in rat intestine. It is well recognized that there is a considerable overlap in substrate specificity between CYP3A and P-glycoprotein substrate drugs, indicating that most compounds metabolized by CYP3A4 may also interact with P-glycoprotein (Zhang et al. 1998; Pal and Mitra 2006). Among the four herbal medicines, only *Rhei Rhizoma* extract suppressed CYP3A activity in a concentration-dependent manner, and its inhibitory potency at a concentration of 100 μg/ml was almost comparable with that of ketoconazole (10 μM) (Fig. 1). Ketoconazole is known to be a potent inhibitor of CYP3A activity with a K_i value of 0.16 μM (Yamano et al. 1999). Also, in this *in-vivo* study, the plasma concentrations of midazolam given intra-luminally were significantly increased by the co-administration of *Rhei Rhizoma* extract (Fig. 2). Midazolam is known to be metabolized to 4-hydroxymidazolam and 1′-hydroxymidazolam by intestinal and hepatic CYP3A in humans and rats, where the CYP3A forms are reportedly different between the intestine and liver (Matsubara et al. 2004; Martignoni et al. 2006; Komura and Iwaki 2008). When administering *Rhei Rhizoma* extract together with midazolam, the plasma concentrations of midazolam increased significantly, suggesting the suppression of CYP3A-mediated first-pass metabolism of midazolam in the intestine and/or liver. Thus, *Rhei Rhizoma* was found to contain some components that interact with CYP3A *in vitro* and *in vivo*.

We then examined the possible interaction of herbal extracts with P-glycoprotein and MRP2 (Figs. 3–5). The inhibitory potency of *Rhei Rhizoma* extract at a concentration of 300 μg/ml against P-glycoprotein-mediated efflux transport of Rho123 was of almost the same magnitude as that of verapamil (300 μM), a typical P-glycoprotein inhibitor (Fig. 4). In contrast, the inhibitory effect of *Rhei Rhizoma* extract on MRP2 function was not strong, though it significantly suppressed MRP2-mediated efflux of DNP-SG at a concentration of 1000 μg/ml (Figs. 5 and 7). *Rhei Rhizoma* contains many and various components including anthraquinone, dianthrone, and catechin. It has been reported that anthraquinone components such as emodin, chrysophanol and rhein suppressed CYP1A-mediated *N*-hydroxylation of Trp-P-2, and that rhein is a substrate for MRP1 (Sun et al. 2000; van Gorkom et al. 2002). In addition, (−)-epicatechin is known to be effluxed by MRP2, P-glycoprotein and breast cancer resistance protein (BCRP) (Kadowaki et al. 2008). It will be necessary to clarify the main component(s) in *Rhei Rhizoma extract*, including their metabolites, that interact with intestinal CYP3A and efflux transporters under *in-vivo* conditions.

Rhei Rhizoma extract is administered orally at a dose of 0.5–1 g each (or 1–3 g daily) as a powder or *Daio-kanzo-to* (TJ-84, Tsumura & Co.), which is a *Kampo* medicine containing *Rhei Rhizoma* extract, to treat constipation in clinical practice. The physiological volume of small intestinal fluid is reportedly from 50 to 1100 ml with an average of 500 ml in the fasted condition (Löbenberg and Amidon 2000). In such a case, it may be speculated that the initial concentration of *Rhei Rhizoma* extract in the intestinal lumen reaches approximately 600–1000 μg/ml. In the present study, the estimated



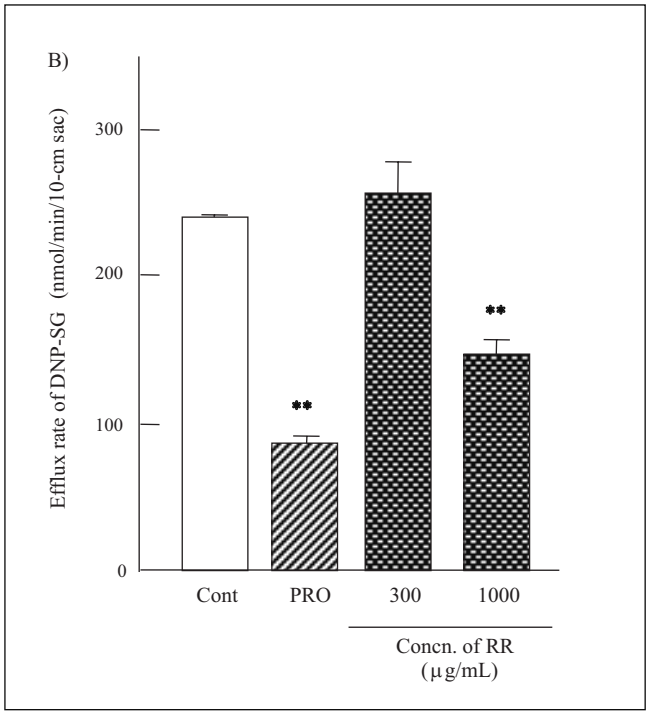


Fig. 5: Concentration-dependent inhibitory effects of *Rhei Rhizoma* extract on DNP-SG efflux after application of CDNB in everted proximal small intestine *in vitro*. In panel (A), concentration of *Rhei Rhizoma* extract was 0 (○), 300 (●) and 1000 μg/ml (▲). Panel (B) shows efflux rates of DNP-SG in absence or presence of 1 mM probenecid (PRO) or *Rhei Rhizoma* extract (RR). Each value represents mean ± S.E. of results from three rats. $^{**}P < 0.01$: significantly different from value for control

IC_{50} value of *Rhei Rhizoma* extract for P-glycoprotein function was approximately 80 μg/ml. Also, *Rhei Rhizoma* extract suppressed MRP2 function almost completely at a concentration of 1000 μg/ml (Figs. 5 and 7), suggesting the induction of herb-drug interaction between *Rhei Rhizoma* and various substrate drugs for P-glycoprotein, MRP2 and/or CYP3A in the intestine. In contrast, the systemic effects of *Rhei Rhizoma* extract on the function of P-glycoprotein and MRP2 expressed in the liver and kidney are considered to be small, if any, in view of its inhibitory potencies. Most components of *Rhei Rhizoma* extract could be metabolized when they are absorbed from the intestine, and the

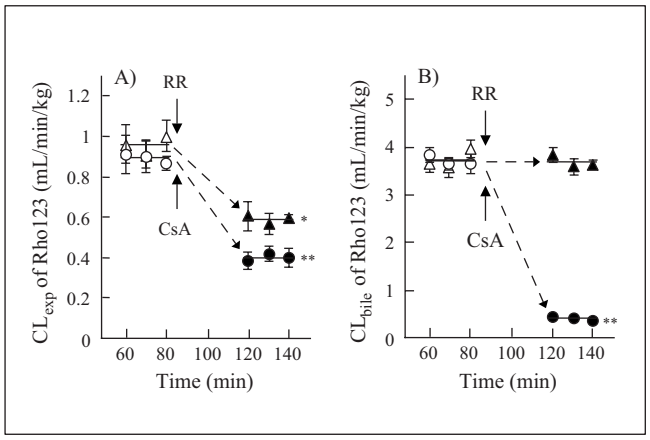


Fig. 6: Effect of *Rhei Rhizoma* extract on intestinal exsorption (CL_{exp}, A) and biliary excretion (CL_{bile}, B) clearances of Rho123 under steady state in rats. Plasma concentration of Rho123 kept at constant level, and intestinal efflux and biliary excretion of Rho123 measured periodically before (opened symbols) and after (closed symbols) administration of cyclosporin A (CsA, ●) or *Rhei Rhizoma* extract (RR, ▲). In inhibition phase, cyclosporin A administered intravenously at dose of 30 mg/kg, or *Rhei Rhizoma* extract perfused at concentration of 300 μg/ml (allows). Each value represents mean ± S.E. of results from three rats. $^{**}P < 0.01$, $^{*}P < 0.05$: significantly different from value for control

absorbed components would be diluted in the body, resulting in a low systemic concentration. Further detailed experiments are necessary regarding the effect of *Rhei Rhizoma* extract on CYP3A in the liver, because the oral bioavailability of midazolam, a typical CYP3A substrate, was significantly increased by coadministration of *Rhei Rhizoma* extract (Fig. 2).

In conclusion, we evaluated the possible interaction of herbal extracts of *Paeoniae Radix*, G*inseng Radix*, *Persicae Semen* and *Rhei Rhizoma* with CYP3A, P-glycoprotein and MRP2. Of these, *Rhei Rhizoma* extract alone showed an inhibitory effect on rat hepatic CYP3A activity. *Rhei Rhizoma* extract also inhibited *in-vitro* and *in-vivo* P-glycoprotein and MRP2 function in rat intestine. These results suggest that oral administration of *Rhei Rhizoma* extract at a clinically administered dose together with CYP3A and/or P-glycoprotein substrate drugs could cause pharmacokinetic herb-drug interactions in the intestine. Careful observation will be necessary when *Rhei Rhizoma* extract is used together with pharmacologically potent CYP3A and/or P-glycoprotein-related drugs in clinical practice.

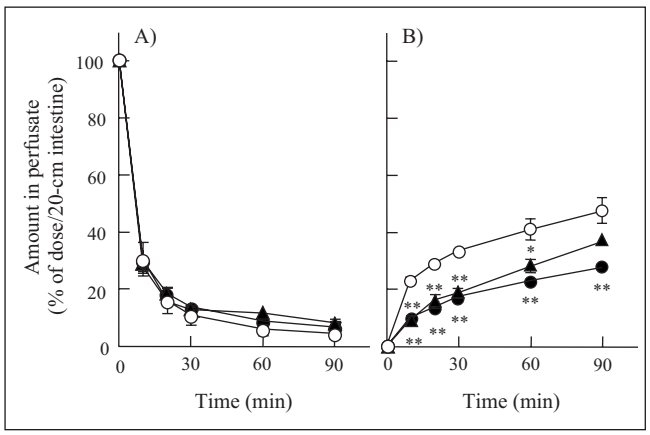


Fig. 7: Influx of CDNB into enterocytes (A) and efflux of DNP-SG from enterocytes to intestinal lumen (B) during re-circulation of CDNB in absence or presence of probenecid or *Rhei Rhizoma* extract. Initial concentration of CDNB 20 μM and recirculation rate 3.0 ml/min. Concentrations of probenecid (●) and *Rhei Rhizoma* extract (▲) 1 mM and 1000 μg/ml, respectively. Each value represents mean ± S.E. of results from three rats. $^{**}P < 0.01$, $^{*}P < 0.05$: significantly different from value for control

4. Experimental

4.1. Chemicals and reagents

Rho123 was obtained from Acros Organics (Geel, Belgium). CDNB, reduced glutathione (GSH), cyclosporin A and verapamil hydrochloride were purchased from Wako Pure Chemicals (Osaka, Japan). 1-Fluoro-2,4-dinitrobenzene (FDNB) and testosterone were obtained from Tokyo Kasei (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. 6β-Hydroxytestosterone, ketoconazole and probenecid were from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Midazolam (Dormicum®) was obtained from Astellas Pharma Inc. (Tokyo, Japan). Herbal extracts of *Paeoniae Radix (Paeonia lactiflora* Pallas*)*, *Ginseng Radix* (*Panaxa ginseng* C.A. Meyer*), Persicae Semen (Prunus persica batsch)* and *Rhei Rhizoma (Rheum palmatum* Linne*)* were kindly supplied by Tsumura & Co. (Tokyo, Japan). All other chemicals used were of the highest purity available.

4.2. Chemical synthesis of DNP-SG

DNP-SG, a typical substrate for MRP2, was synthesized according to the method reported previously by using FDNB as a lead compound (Yokooji et al. 2006). The compound synthesized was chromatographically pure, as was that in our previous study (Yokooji et al. 2005).

4.3. Animals

Experiments with animals were performed in accordance with the "Guide for Animal Experimentation" from the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima International University, which is in accordance with the "Guidelines for proper conduct of animal experiments" from the Science Council of Japan. Male Sprague-Dawley (SD) rats 7 to 9-weeks-old were fasted overnight with free access to water before the experiments.

4.4. Effect of herbal extracts on CYP3A activity in rat hepatic microsomes

The CYP3A-mediated drug interaction with herbal extracts was evaluated by measuring the metabolizing activity of testosterone 6β-hydroxylation in rat hepatic microsomes. Hepatic microsomal suspension was prepared in the same manner as reported previously (Yumoto et al. 2001). Briefly, the 105,000 × *g* pellet of supernatants obtained after centrifugation of liver homogenates at 18,000 × *g* for 15 min was re-suspended in solution A (10 mM HEPES-NaOH buffer containing 150 mM KCl, pH 7.4). All the procedures were performed at 4 °C. Protein concentration of microsomal suspension was measured by the Bradford method using bovine γ-globulin as the standard, and the suspension was diluted with solution A to make a final protein concentration of 2 mg/ml (Bradford 1976). Testosterone, $MgCl_2$ and NADPH were dissolved in solution A containing 1% DMSO, DMSO being used to aid the solubility of testosterone. A mixture of the drug solution (0.725 ml) and solution A with or without ketoconazole or herbal extracts (0.025 ml) was added to microsomal suspension (0.25 ml) to initiate the metabolic reaction (final concentrations: testosterone, 250 μM; ketoconazole, 10 μM; *Rhei Rhizoma* extract, 1–100 μg/ml; the other herbal extracts, 400 μg/ml; microsomal protein, 0.5 mg/ml; NADPH, 1 mM; $MgCl_2$, 5 mM). The enzyme reaction was performed in a shaking water-bath at 37 °C for 10 min, and was terminated by adding ice-cold acetonitrile (1 ml). The suspension was centrifuged at 3,000 rpm for 10 min, and the supernatants were subjected to determination of the concentration of 6β-hydroxytestosterone by HPLC. The effect of herbal extracts on CYP3A activity was also evaluated in rat hepatic microsomes by using midazolam, which is also widely accepted as a CYP3A probe (Kronbach et al. 1989).

4.5. Effect of Rhei Rhizoma extract on CYP3A activity in rats in vivo

CYP3A activity in vivo was evaluated by measuring the intestinal absorption of midazolam. Rats were anaesthetized with pentobarbital (30 mg/kg, *i.p.,* injection) and affixed supine on a surface kept at 37 °C to maintain the body temperature at approximately 36 °C. In a control study (without *Rhei Rhizoma* extract*)*, midazolam (50 μmol/kg) was administered into a 20-cm long intestinal loop, which was prepared at a region of 10 cm below the bile duct opening, and blood was sampled from the jugular vein at a designated time interval. In an inhibition study, two intestinal loops (each 20-cm long) were made in a row from 10 cm below the bile duct opening. *Rhei Rhizoma* extract solution (100 mg/2 ml/kg) was administered into the second loop, and 15 min later, midazolam (50 μmol/kg) was administered into the first loop, to avoid putative herb-drug direct interactions such as micelle formation. Blood (0.25 ml each) was collected at intervals for 2 h to measure plasma midazolam concentrations.

4.6. Effect of herbal extracts on P-glycoprotein and MRP2 function in intestinal everted sac in vitro

P-glycoprotein and MRP2 function was evaluated by measuring the serosal-to-mucosal efflux transport of the substrate compounds, Rho123 and DNP-SG, across the intestinal everted sac, respectively. The effects of herbal extracts on P-glycoprotein-mediated Rho123 transport and MRP2-mediated DNP-SG transport were examined in intestinal everted sac in a similar manner to that previously reported, with small modifications (Yokooji et al. 2005; Yumoto et al. 2003). Briefly, a 10-cm long intestinal everted sac was prepared using the proximal and distal small intestine to evaluate the P-glycoprotein and MRP2 functions. In evaluating the P-glycoprotein function, 1 ml of Rho123 (5 μM) dissolved in pH 7.4 isotonic Dulbecco's phosphate buffered saline (D-PBS) was applied to the serosal side of the closed everted sac. The sac containing Rho123 was immersed in 8 ml of pH 7.4 D-PBS containing 4% DMSO prewarmed at 37 °C and preoxygenated with 5% CO_2/95% O_2. The negligible effect of DMSO at a concentration of 4% on efflux transporter function and/or membrane integrity has been observed in P-glycoprotein-mediated efflux transport of Rho123 across rat intestine and Caco-2 cell monolayers (Takano et al. 1998; Yumoto et al. 1999). CO_2/O_2 gas was bubbled through the incubation medium continuouly throughout the efflux transport study. The MRP2 function was examined in a similar manner as for P-glycoprotein function, using CDNB (50 μM) and pH 6.0 isotonic PBS instead of Rho123 and pH 7.4 D-PBS, respectively. The mucosal efflux of Rho123 or DNP-SG across the everted intestine was measured by sampling the mucosal medium periodically for 120 min. In an inhibition study, verapamil (300 μM) or probenecid (1 mM) was added to the mucosal medium as an inhibitor of P-glycoprotein or MRP2, respectively. Herbal extracts were also added to the mucosal medium at appropriate final concentrations to evaluate their inhibitory effects.

4.7. Effect of Rhei Rhizoma extract on P-glycoprotein function in the intestine and liver in vivo

Rats were anesthetized with pentobarbital (30 mg/kg, *i.p.*, injection) and affixed supine on a surface kept at 37 °C to maintain their body temperature above 36 °C. The *in-vivo* CL_{exp} and CL_{bile} of Rho123 under steady states in the absence or presence of cyclosporin A, a typical P-glycoprotein inhibitor, or *Rhei Rhizoma* extract were measured in the same manner as reported previously (Yumoto et al. 1999). Briefly, cannulation (polyethylene tubing) was made at a femoral vein (PE-50) for administration of drugs, at a femoral artery (PE-50) for sampling blood, and at a bile duct (PE-10) for sampling bile, respectively. The distal intestinal lumen was flushed with a sufficient amount of saline prewarmed at 37 °C, and both ends were catheterized with silicone cannulae to make a 20-cm long intestinal loop. C_{pss} of Rho123 was established by a bolus injection (436 nmol/kg), followed by a constant rate infusion at 200 nmol/h. The intestinal lumen was perfused with isotonic D-PBS containing 5 mM glucose (pH 7.4) in a single perfusion mode at a rate of 1 ml/min. After the C_{pss} of Rho123 was achieved (55 min after the initiation of constant rate infusion), intestinal effluent and bile were collected every 10 min on several occasions, blood being collected at the intermediate time of each biological fluid collection as a control phase. After the control-phase clearance study, cyclosporin A dissolved in 50% ethanol was injected intravenously at a dose of 30 mg/ml/kg, or the intestinal perfusate was changed to D-PBS containing *Rhei Rhizoma* extract (300 μg/ml) as an inhibition-phase clearance study. After perfusing for 30-min for stabilization, intestinal effluent and blood were further collected at intervals. Values of CL_{exp} and CL_{bile} were estimated by dividing the exsorption rate from blood to the intestinal lumen and the biliary excretion rate by the C_{pss} of the drug, respectively. These clearance values were normalized with the body weight (kg) of each rat.

4.8. Effect of Rhei Rhizoma extract on intestinal MRP2 function in vivo

Rats were anaesthetized with pentobarbital (30 mg/kg, *i.p.* injection) and affixed supine on a surface kept at 37 °C to maintain the body temperature above 36 °C. Jejunum (a 20-cm long segment from 5 cm below the bile duct opening) was used to evaluate the effect of *Rhei Rhizoma* extract on the MRP2-mediated efflux transport of DNP-SG, a conjugated metabolite of CDNB, from enterocytes into the intestinal perfusate after loading of CDNB in the rats. Each intestinal segment was perfused with 20 ml pH 6.5 PBS (20.4 mM Na_2HPO_4, 6.3 mM NaH_2PO_4, 129 mM NaCl, 1.5 mM KCl, 14 mM glucose, 1.14 mM GSH, 1 mM $CaCl_2$) containing 4% DMSO and 20 μM CDNB in re-circulating perfusion mode at 3 ml/min. In the inhibition study, the intestinal segment was perfused with pH 6.5 PBS containing 20 μM CDNB and 1000 μg/ml *Rhei Rhizoma* extract in the same manner as described above. The intestinal perfusate was sampled periodically to determine the concentrations of CDNB and DNP-SG.

4.9. Data analysis

The biological fluid samples containing 6β-hydroxytestosterone, Rho123 or DNP-SG were diluted with acetonitrile or 20% perchloric acid as appropriate. The deproteinized samples were kept on ice for at least for 30 min, and centrifuged at 3,000 rpm for 10 min. Concentrations of 6β-hydroxytestosterone in the supernatants of various biological samples were determined by HPLC using a YMC-pack ODS-AM column (50 × 4.6 mm; YMC Inc., Wilmington, NC, USA). Mobile phase used was a mixture of 10 mM phosphate buffer (pH 6.8) and acetonitrile (78:22, v/v). The flow rate of mobile phase was 1 ml/min, and detection of 6β-hydroxytestosterone was at a wavelength of 254 nm. Concentrations of Rho123 in the supernatants of various biological samples were determined by HPLC using a YMC-pack ODS-AM column (50 × 4.6 mm; YMC Inc., Wilmington, NC, USA). Mobile phase used was a mixture of 1% acetic acid and acetonitrile (75:25, v/v). The flow rate of mobile phase was 1 ml/min and detection of Rho123 was at a wavelength of 485 nm for excitation and 546 nm for emission. Concentration of DNP-SG was also determined by HPLC using a Mightysil RP-18 column (Kanto Kagaku, Tokyo, Japan) with a mixture of 1% acetic acid and acetonitrile (85:15, v/v) as mobile phase at a flow rate of 1 ml/min. Detection was at a wavelength of 365 nm. Concentrations of midazolam in plasma samples were also determined by HPLC using an Inertsil ODS-3 column (150 × 4.6 mm; GL Sciences, Inc., Tokyo, Japan). Briefly, sampled blood was centrifuged at 3,000 rpm for 15 min to obtain plasma samples, and these plasma samples were mixed with an equal volume of saturated sodium phosphate. The midazolam in plasma samples was then extracted with ethyl acetate (3 ml). The organic solvent was evaporated to dryness under reduced pressure and the residue was dissolved with methanol (100 μl). Mobile phases used were a mixture of 10 mM phosphate buffer (pH 7.4), methanol and acetonitrile (50:30:20, v/v), and the flow rate of mobile phase was 1 ml/min. Detection was at a wavelength of 240 nm.

The IC_{50} value of *Rhei Rhizoma* for P-glycoprotein-mediated efflux of Rho123 across everted intestine was estimated by using the following Hill equation (Nagai et al. 2006):

$$v - v_0 = (v' - v_0) / [1 - ([I] / IC_{50})^n]$$

where v is the observed transport rate of Rho123; v' is the transport rate of Rho123 in the absence of an inhibitor; v_0 is the transport rate of Rho123 in the presence of verapamil (non-specific transport); [I] is the initial concentration of inhibitor (*Rhei Rhizoma* extract*)*, and n is the Hill coefficient. Curve fitting analysis was performed using KaleidaGraph™ program (Version 3.501, Synergy Software, PA, USA).

Differences among group mean values were assessed by the Kruskal-Wallis test or ANOVA followed by a post-hoc test (Dunn's test) or Student's *t*-test. A difference of $P < 0.05$ was considered statistically significant.

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