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Minor effects of the Citrus flavonoids naringin, naringenin and quercetin, on the pharmacokinetics of doxorubicin in rats

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We investigated the effects of naringin, naringenin and quercetin on the pharmacokinetics of doxorubicin in rats. These Citrus flavonoids are known as P-glycoprotein (P-gp) inhibitors and thus suspected to interact with doxorubicin, as shown by *in vitro* cell studies. Plasma concentrations, tissue distribution, and the urinary and biliary excretion of doxorubicin after intravenous infusion were investigated in rats followed by oral administration of Citrus flavonoids. To evaluate the impact of the biotransformation of Citrus flavonoids on the P-gp inhibition, the inhibitory effects of quercetin and its metabolite on P-gp were compared using *ex vivo* analysis. Contrary to previous *in vitro* results, the plasma concentration, biliary and urinary clearance, and tissue distribution of doxorubicin were not altered by pre-treatment with naringin and naringenin. Biliary clearance and urinary clearance were slightly decreased by quercetin, but there was no statistical difference. The minor effects of these flavonoids may relate to their low systemic concentration, due to the biotransformation *in vivo* situation. S9 stability assay and calcein accumulation assay showed that quercetin was a metabolically unstable compound, and the inhibitory effect of its metabolites on P-gp was negligible. In conclusion, naringin, naringenin and quercetin did not affect the *in vivo* pharmacokinetics of intravenously administered doxorubicin.

1. Introduction

The issue of herb-drug interactions has been receiving increased attention. Grapefruit juice has been known to inhibit the function of drug transporters such as P-glycoprotein (P-gp) and the organic anion transporting polypeptide (Oatp) and drug-metabolizing enzymes such as cytochrome P450 (CYP) 3A4 (Lown et al. 1997; Tian et al. 2002; Dresser et al. 2005). Especially, citrus flavonoids such as naringin, naringenin and quercetin (Fig. 1) are known to be involved in herb-drug interactions with various types of drugs *via* P-gp and/or CYP3A4 inhibition (Chieli et al. 1995). For example, quercetin inhibited the efflux of rhodamine-123 and restored sensitivity to doxorubicin in MCF-7 breast cancer cells (Scambia et al. 1994), and naringin and naringenin caused inhibition of colchicine secretion across Caco-2 cell monolayers (Dahan and Amidon 2009). In addition recent investigations showed that naringenin (0.7 mg/kg) and naringin (2.4 and 9.4 mg/kg) increased maximum concentration (C_{max}) and area under the plasma concentration-time curve (AUC) of orally administered talinolol by 1.5- to 1.8-fold, respectively (de Castro et al. 2008). However, little is known about the effects of these flavonoids on the pharmacokinetics of drug administered intravenously.

Doxorubicin is an antineoplastic agent used for the treatment of various types of cancer. Its oral bioavailability is very low, and thus, it is administered intravenously. Until now, results of many *in vitro* studies have suggested the occurrence of herb-drug pharmacokinetic interactions between plant flavonoids and doxorubicin *via* P-gp (Chieli et al. 1995; Zhang and Morris 2003; Nakatsuma et al. 2010). However it remains unclear whether these flavonoids affect the pharmacokinetics of doxorubicin *in vivo*. In this study, we evaluated the effect of 3 flavonoids — naringin, naringenin and quercetin — on the pharmacokinetics of doxorubicin, including excretion and tissue distribution, in rats.

2. Investigations and results

2.1. Effect of citrus flavonoids on doxorubicin plasma concentrations

The plasma concentration of doxorubicin was not altered by the oral administration of naringin, naringenin, or quercetin 30 min before the start of doxorubicin administration (Table, Fig. 2). However, administration of cyclosporin A at a dose of 10 mg/kg significantly increased the concentration of doxorubicin (Table, Fig. 2).

2.2. Effect of Citrus flavonoids on the excretion of doxorubicin

Major elimination routes of doxorubicin are known to be biliary and urinary excretion as well as metabolism (Tavoloni and Guarino 1980a). The biliary and urinary excretion of doxorubicin was measured up to 4 h after the initiation of the intravenous infusion. The percentage dose of biliary excretion was

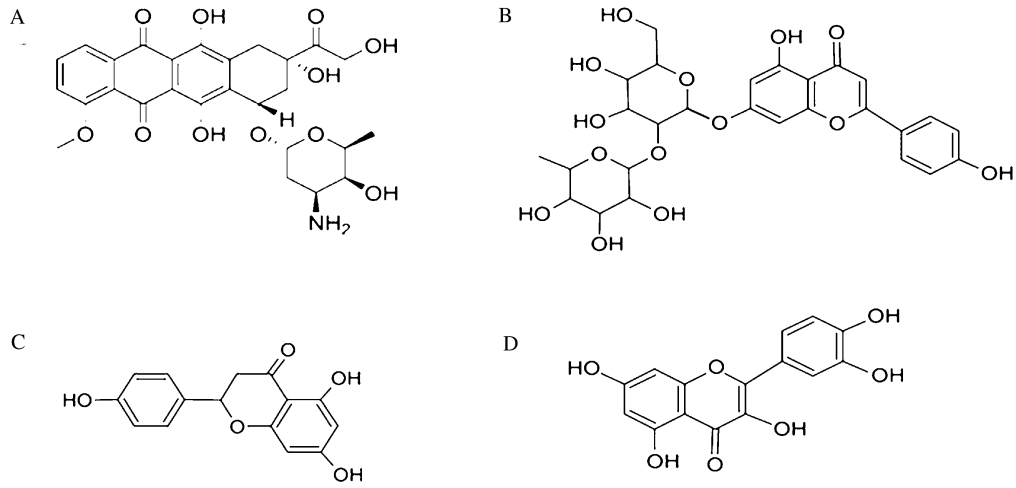

Fig. 1: Chemical structure of doxorubicin (A) and the 3 flavonoids: naringin (B), naringenin (C), and quercetin (D)

Table: Pharmacokinetic parameters of doxorubicin after intravenous infusion at a rate of 200 μg/h with a loading dose of 2 mg after pretreatment with saline, cyclosporin A (10 mg/kg), naringin (50 mg/kg), naringenin (50 mg/kg), or quercetin (100 mg/kg)

	Control	+ Cyclosporin A	+ Naringin	+ Naringenin	+ Quercetin
C_{ss} (ng/mL)	315.55 ± 100.29	506.56 ± 72.43 *	374.27 ± 98.76	340.20 ± 27.12	380.47 ± 126.91
Biliary excretion (% Dose)	20.33 ± 3.76	7.95 ± 1.94 *	23.29 ± 6.20	20.07 ± 3.89	25.93 ± 5.57
Urinary excretion (% Dose)	8.46 ± 2.23	7.8 ± 0.96	8.31 ± 1.08	10.02 ± 1.11	7.46 ± 2.54
CL_{total} (mL/h)	698.49 ± 166.74	377.22 ± 41.33	538.96 ± 37.35	588.55 ± 37.35	613.77 ± 191.47
$CL_{b/p}$ (mL/h)	368.24 ± 133.08	55.9 ± 7.77 *	313.6 ± 71.41	307.22 ± 22.64	246.40 ± 74.84
$CL_{u/p}$ (mL/h)	113.94 ± 41.85	61.76 ± 16.18 *	90.99 ± 19.12	112.92 ± 8.56	83.19 ± 1.15
$CL_{b/l}$ (g tissue/h)	4.78 ± 2.40	2.63 ± 0.14 *	7.1 ± 2.95	3.36 ± 0.60	3.76 ± 0.44
$CL_{u/k}$ (g tissue/h)	0.98 ± 0.21	0.59 ± 0.16 *	0.94 ± 0.21	0.94 ± 0.12	0.94 ± 0.07
K_p Brain (mL/g tissue)	1.09 ± 0.44	0.68 ± 0.09	0.91 ± 0.23	0.88 ± 0.04	0.64 ± 0.00
K_p Liver (mL/g tissue)	89.22 ± 49.21	21.58 ± 3.08 *	51.67 ± 25.76	91.35 ± 13.10	69.81 ± 15.76
K_p Kidney (mL/g tissue)	115.4 ± 36.38	108.49 ± 16.99	99.40 ± 28.29	118.85 ± 8.57	89.09 ± 4.13
K_p Heart (mL/g tissue)	51.04 ± 18.20	30.75 ± 6.81 *	38.42 ± 4.50	42.9 ± 3.94	31.89 ± 4.44

Data are represented as mean ± SD (n = 3 ~6)
* $p < 0.05$ vs. control

20.33 ± 3.76%, 7.95 ± 1.94%, 23.29 ± 6.20%, 20.07 ± 3.89%, and 25.93 ± 5.57% for cyclosporin A, naringin, naringenin, and quercetin, respectively (Table and Fig. 3). Except for cyclosporine A ($p < 0.05$), Citrus flavonoids did not make any difference. The percentage dose of urinary excretion showed similar results with biliary excretion. Only cyclosporine A decreased the urinary excretion of doxorubicin (Table). In addition, the biliary and urinary clearance of doxorubicin was not significantly affected by oral administration of naringin and naringenin (Fig 4). Biliary and urinary clearance was slightly decreased by quercetin, but there was no statistical difference (Fig 4). However, cyclosporin A treatment resulted in much lower biliary and urinary clearance than that observed in the

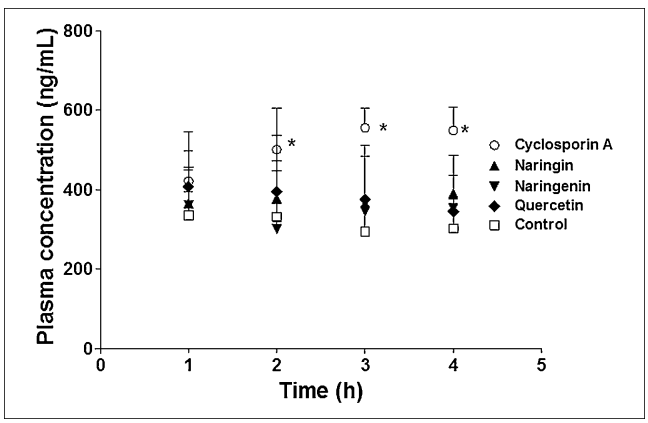

Fig. 2: Concentration-time profiles of doxorubicin after intravenous infusion and pretreatment with saline (□), cyclosporin A (○), naringin (▲), naringenin (▼), or quercetin (♦). Data are represented as mean ± SD ($n = 3-6$). * $p < 0.05$

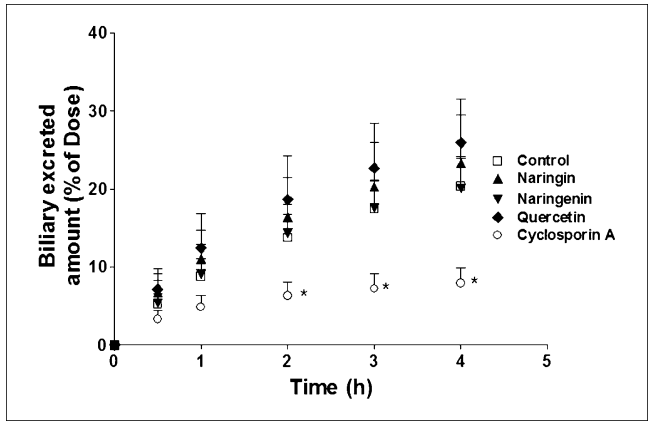

Fig. 3: Cumulative biliary excretion of doxorubicin after pretreatment with saline (□), cyclosporin A (○), naringin (▲), naringenin (▼), and quercetin (♦). Data are represented as mean ± SD ($n = 3-6$). * $p < 0.05$.

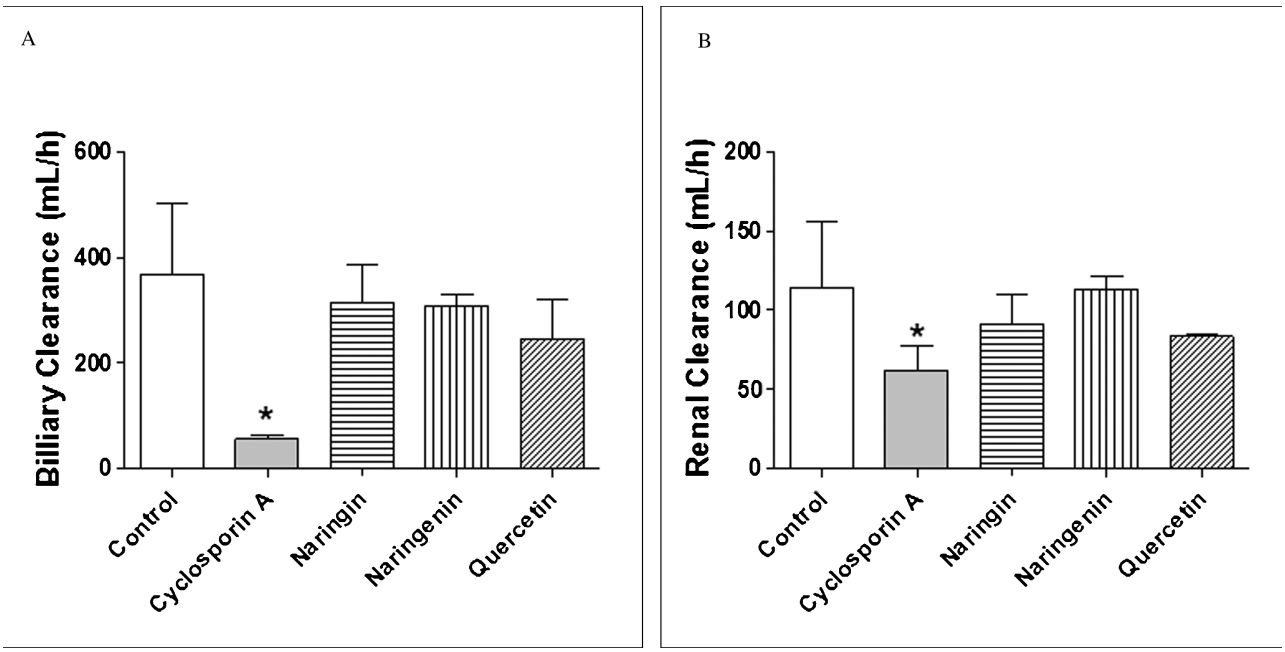

Fig. 4: Biliary (A) and renal (B) clearance of doxorubicin for each group. Data are represented as mean ± SD ($n = 3-6$). $^{*}p < 0.05$

control group (Fig. 4). Biliary and urinary clearance calculated from the concentration in the liver and kidney did not significantly differ between the flavonoids treatment and control groups (Table). There was no significant difference in systemic clearance between group except cyclosporin A treatment group (Table).

2.3. Effect of Citrus flavonoids on the tissue distribution of doxorubicin

Remarkably high levels of doxorubicin were observed in the liver, heart, and kidney, as previously reported (Tavoloni and Guarino 1980b). Treatment with flavonoids did not increase or decrease the K_p value of doxorubicin, whereas cyclosporin A treatment decreased the distribution of doxorubicin in the liver and heart (Table).

2.4. in vitro metabolism of quercetin

We measured a biotransformation of quercetin, because low clearances were observed apparently in the quercetin treatment group (Fig. 4). The percentages of quercetin remaining in the S9 fraction of the rat liver without and with coenzymes at 0, 5, 15, 30, 60, 90, and 120 min after the start of incubation are shown in Fig. 5. After the 120-min incubation, 92.3% of quercetin remained in the S9 fraction of the rat liver in the absence of coenzymes. However, in the S9 fraction of the rat liver in the presence of coenzymes, quercetin rapidly disappeared and little remained after the 120-min incubation.

2.5. Effect of quercetin metabolites on P-gp inhibition

Effect of quercetin metabolites on P-gp inhibition was measured by calcein accumulation assay using hMDR1 transfected cell line, MDCKII-MDR1. The accumulation of calcein in MDCKII-MDR1 cells treated for 30 min with 100 μM verapamil, a positive control for P-gp inhibition, and parent quercetin (S9 fraction without coenzymes) significantly increased approximately 3.5-fold and 1.7-fold, respectively, compared to that in vehicle-treated cells (Fig. 6). However, calcein accumulation in MDCKII-MDR1 cells decreased even when treated with quercetin metabolites (S9 fraction with coenzymes) suggesting quercetin metabolites did not inhibit P-gp function.

3. Discussion

As noted in the case of Saint John's wort, P-gp plays an important role in herb-drug interactions, and thus, P-gp–mediated interactions began to gain attention in the field of cancer chemotherapy (Bansal et al. 2009). Previous studies showed that flavonoids, plant-derived compounds with low toxicity, greatly potentiate the inhibition of P-gp and may be adjuvant compounds for cancer chemotherapy (Zhou et al. 2004). Among these flavonoids, quercetin is known to bind selectively to P-gp and efficiently inhibit its activity (Shapiro and Ling 1997). Extensive trials related to applications of these types of flavonoids for P-gp inhibition were performed (Boumendjel et al. 2002; Zhang and Morris 2003). Interesting related *in vitro* studies were also reported. For example, pretreatment with naringin before doxorubicin administration increased sensitivity to the drug (Ali et al. 2009). In addition, naringenin enhanced the antitumor effect of doxorubicin in tumor cells by

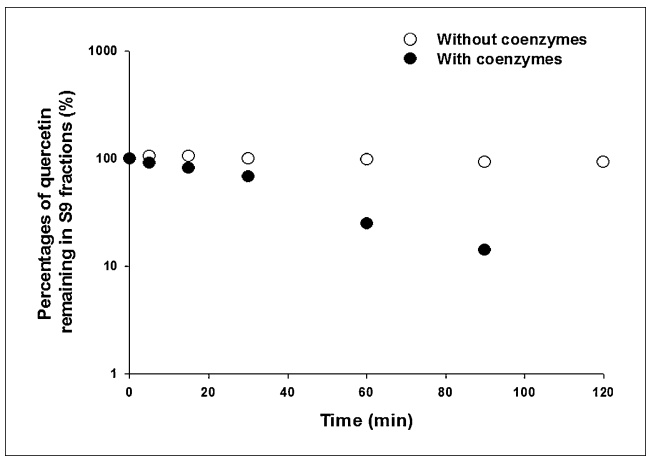

Fig. 5: Percentages of quercetin remaining in the S9 fraction of the rat liver in the presence (○) or absence of coenzymes (●) at 0, 5, 15, 30, 60, 90, and 120 min after the start of incubation

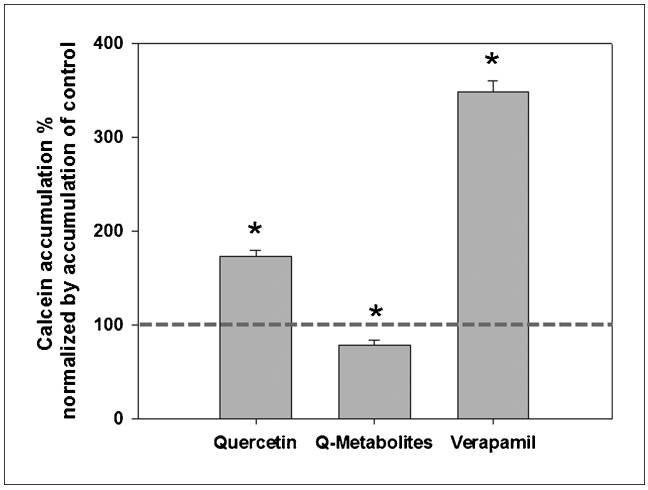

Fig. 6: Percentages of cellular calcein-AM accumulation vs. control after quercetin, quercetin metabolites (Q-Metabolites), and verapamil treatment in MDCKII-MDR1 cells. Data are represented as mean ± SD ($n = 3$). $^{*}p < 0.05$

selectively modulating drug efflux pathways (Zhang et al. 2009). Quercetin pretreatment significantly altered doxorubicin resistance of human myelogenous leukemia cells and doxorubicin accumulation in HCT-15 colon cells (Critchfield et al. 1994; Shen et al. 2008). However, *in vivo* evidence for the effects of these citrus flavonoids has not been published.

Because doxorubicin is administered intravenously and mainly excreted *via* bile and urine, we evaluated the effects of flavonoids on the pharmacokinetics of doxorubicin after intravenous infusion. The doses of naringin, naringenin, and quercetin were chosen according to conventional intake amounts of grapefruit juice for naringin and naringenin and daily intake amounts of high-end consumers of fruits and vegetables for quercetin (Erlund et al. 2001; Wanwimolruk and Marquez 2006; Harwood et al. 2007). The doses of naringin, naringenin, and quercetin (50 mg/kg, 50 mg/kg, and 100 mg/kg, respectively) were equivalent to 10-fold of human dose.

Unexpectedly, after the intravenous infusion of doxorubicin after pretreatment with naringin, naringenin, or quercetin, the concentrations of doxorubicin were not significantly different. The systemic, biliary and urinary clearance, and tissue distribution of doxorubicin were not altered clearly by pre-treatment with naringin and naringenin. Biliary clearance and urinary clearance were slightly decreased by quercetin, but there was no statistical significance. These findings were inconsistent with the *in vitro* observation of remarkable inhibitory activities of flavonoids. However, pretreatment with cyclosporin A, a potent P-gp inhibitor, significantly decreased the biliary excretion of doxorubicin, and it was coincident with the result from P-gp knockout mice (van Asperen et al. 2000). Cyclosporin A also decreased urinary excretion of doxorubicin (Shimizu et al. 2004).

In addition, the K_p values of doxorubicin in each organ were not affected by pretreatment with flavonoids. Considering the previously reported significant alteration of K_p values of doxorubicin in the liver and brain in P-gp knockout mice (van Asperen et al. 1999), naringin, naringenin, and quercetin did not affect the *in vivo* tissue distribution of doxorubicin *via* P-gp modulation.

Recently naringin and quercetin were known as *in vitro* inhibitors of Oatps and naringenin was reported to be a substrate of Oatp (Chabane et al. 2009; Bailey 2010; Mandery et al. 2010). However, considering that doxorubicin acts as weak base (pKa 8.34) (Kleeberger and Rottinger 1993) and there has been no reports that doxorubicin is a substrate of Oatps, it is likely that Oatps-mediated interaction has little impact on results of this study.

The apparently contradictory effects of these flavonoids between *in vitro* and *in vivo* studies may be explained by the biotransformation of flavonoids *in vivo*. As shown in Fig. 5, the metabolic half-life of quercetin in the S9 fraction was 29.7 min, suggesting severe biotransformation of quercetin. Many flavonoids, including naringenin and naringin, also undergo conjugation when the compounds permeate the intestinal membrane or liver (Ameer et al. 1996; Peng et al. 1998). Naringenin and quercetin are also converted into their conjugated metabolites by UDP-glucuronosyltransferase (Ameer et al. 1996; Peng et al. 1998; Murota and Terao 2003). In general, when flavonoids are orally administered, the plasma concentrations of their conjugates are much higher than those of their aglycones (Walle et al. 2007; Bredsdorff et al. 2010). Thus, we suppose that the actual concentrations of parent compounds in the canalicular membrane of the liver, luminal side of proximal tubule, and brain-blood barrier are too low to inhibit P-gp function because of extensive metabolism. If the compound is orally administered, it may come in contact with high concentrations of flavonoids in the intestinal mucus, especially on the apical side of the lumen, and oral absorption of the compound may be influenced by the P-gp inhibitory effect produced by flavonoids as previously reported (Choi and Kang 2008; Dahan and Amidon 2009). In the quercetin and etoposide interaction study, the pharmacokinetic parameters of etoposide were significantly altered by quercetin in the oral group but not in the i.v. group (Li and Choi 2009). Although naringin was reported to decrease the excretion of paclitaxel that was intravenously administered, the difference was not substantial (30%), and it may be mainly related to the inhibition of CYP3A1/2 (Lim and Choi 2006).

To evaluate the inhibitory effect of quercetin metabolite on P-gp, the intracellular accumulation of calcein after quercetin parent or quercetin metabolite treatment was measured in MDCKII-MDR1 cells. Quercetin itself produced a similar inhibitory effect on P-gp as verapamil, a potent P-gp inhibitor. However, this inhibitory effect on the function of P-gp was not produced by its metabolite (Fig. 6). These results suggested that the inhibitory effect of quercetin on P-gp function disappeared during biotransformation.

In summary, no significant effects of naringin, naringenin, and quercetin on the *in vivo* pharmacokinetics of intravenously administered doxorubicin were observed. This finding is in contrast to the reported effect of these citrus flavonoids on P-gp substrates *in vitro* and may be because of the low plasma and tissue concentrations of flavonoids resulting from extensive biotransformation. Evidently, *in vivo* studies *via* clinically used administration routes that involve biotransformation processes would be necessary to prevent overestimation of herb-drug interaction potential based on *in vitro* investigation.

4. Experimental

4.1. Animals

Male Sprague-Dawley rats (275–330 g, Samtaco, Osan, Korea) were used. The rats were maintained under 12-h light/dark cycle at 25 °C (Animal Center for Pharmaceutical Research, College of Pharmacy, Kyung Hee University, Seoul, Korea). The rats were randomly divided into groups (4–6 animals in each group) and used for pharmacokinetic studies after acclimation with free access to water and food. All experiments were conducted according to the guidelines of the Committee on Care and Use of Laboratory Animals of the Kyung Hee University.

4.2. Drug and chemicals

Doxorubicin hydrochloride supplied by Boryung Pharm Co. (Seoul, Korea) was diluted in normal saline (CJ, Seoul, Korea) to obtain a concentration of 200 μg/mL and 5 mg/mL for intravenous infusion and loading dose administration, respectively. Zoletil 50, which is used to induce anesthesia, was purchased from Virbac Laboratories

(Carros, France). Naringin, naringenin, quercetin, calcein-AM, verapamil, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Hank's Buffered Salt Solution (HBSS), D-glucose, Triton X-100, uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA), adenosine 3′-phosphate 5′-phosphosulfate lithium salt hydrate (PAPS), S-(5′-adenosyl)-L-methionine chloride (SAM), β-nicotinamide adenine dinucleotide phosphate, reduced tetra(cyclohexylammonium) salt (NADPH), L-ascorbic acid, and magnesium chloride ($MgCl_2$) were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Dimethylsulfoxide (DMSO), L-glutamine, and the lyophilized Sprague-Dawley rat liver 9000 g supernatant (S9) fraction were products of Cellgro by Mediatech Inc. (Manassas, VA), Invitrogen Inc. (Carlsbad, CA), and MoltoxInc. (Boone, NC), respectively. DMEM, FBS, and penicillin/streptomycin were purchased from Hyclone (Logan, UT). Cyclosporin A was obtained from Norvatis Pharma (Tokyo, Japan). All chemicals were of analytical grade, and solvents were of high-performance liquid chromatography grade.

4.3. In vivo pharmacokinetic study

Right external femoral veins, arteries, bile ducts and urinary bladders of rats were cannulated using polyethylene tube (Natsume, Tokyo, Japan) under anesthesia. The cannulas were filled with heparinized saline (50 IU/mL) to prevent blood clotting. Flavonoid compounds (naringin [50 mg/kg], naringenin [50 mg/kg], and quercetin [100 mg/kg]) that were dispersed in distilled water were orally administered 30 min before infusion of doxorubicin. Doxorubicin was dissolved in normal saline and continuously infused intravenously at a rate of 200 μg/h with a loading dose of 2 mg of doxorubicin. The same procedures described above were performed for the negative and positive controls. Saline was orally administered for negative controls, and cyclosporin A (10 mg/kg) was intraperitoneally administered for positive controls (Morjani and Madoulet 2010). Plasma samples were collected at 0, 1, 2, 3, and 4 h, and samples of urine and bile were collected during 0 to 1-(0 to 30 min, 30 to 60 min in case of bile), 1 to 2-, 2 to 3-, 3 to 4-h intervals after initiation of the doxorubicin infusion. The blood volume was replaced with an equal volume of saline to compensate for fluid loss. For the organ distribution study, brains, livers, kidneys, and hearts were collected immediately after the rats were euthanized at the end of study. Blood samples were centrifuged immediately, and 100-μL aliquots of the plasma and collected organs were stored in the freezer (–80 °C) for analysis.

4.4. In vitro metabolism of quercetin

The procedures used were similar to a reported method (van der Woude et al. 2004). Incubation mixtures (final volume 1 mL) consisted of 0.1 M potassium phosphate, pH 7.5, 2 mM vitamin C, 3 mg/mL S9 homogenate protein, 4 mM nicotinamide adenine dinucleotide phosphate-oxidase, 4 mM S-adenosylmethionine, 4 mM uridine diphosphate glucuronic acid, and 0.2 mM 3'-phosphoadenosine-5'-phosphosulfate, and 16 mM $MgCl_2$. The control (final volume of 1 mL) consisted of only 0.1 M potassium phosphate, pH 7.5, 2 mM vitamin C, and 3 mg/mL S9 homogenate protein without any coenzymes. The reaction was started by the addition of 1 mM quercetin from a 500 mM stock solution in DMSO. The samples were incubated in a 37 °C at 120 revolutions per min. At 0, 5, 15, 30, 60, 90, and 120 min after the start of incubation, duplicate 100-μL aliquots were collected, added to an Eppendorf tube containing 300-μL of ice-cold acetonitrile, and vortex-mixed to terminate the reaction. The samples were then centrifuged at 15,000 rpm for 10 min, and a 300-μL aliquot of the supernatant was stored at − 80 °C until use in the LC-MS/MS analysis of quercetin. For the uptake assay in the MDCKII-MDR1 cell line, a 300-μL aliquot of the supernatant was evaporated to dryness by using a Speed-Vac concentrator (Centra Vac; Vision Scientific Co., Bucheon, Korea) and stored at − 80 °C.

4.5. Effect of quercetin metabolites on P-gp inhibition

Intracelluar calcein accumulation was measured using calcein acetoxymethyl ester, a non-fluorescent substrate for P-gp that is converted to fluorescent calcein inside cells by esterase (Holló et al. 1994). The MDCKII-MDR1 cell line was kindly provided by Dr. Piet Borst (The Netherlands Cancer Institute, Amsterdam, Netherlands) and grown in DMEM supplemented with 10% FBS, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 4 mM L-glutamine. The cells were incubated in at 37 °C in a humidified 5% CO_2 atmosphere (Bakos et al. 1998).

The cells were seeded into 12-well cell culture plates (Falcon, BD Biosciences) at a density of 5×10^4 cells/mL, and the medium was replaced every 24 h. After a 48-h incubation, the cells were washed twice with 37 °C phosphate buffered saline (PBS) consisting 140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 and were incubated in transport media consisting 9.7 g/L HBSS, 10 mM HEPES, 4 mM $NaHCO_3$ and 10 mM glucose for 30 min. Subsequently each well was treated with the above evaporated S9 fraction sample (quercetin parent: incubated without coenzyme, quercetin metabolized: incubated with coenzyme, both reconstituted with transport media containing DMSO), verapamil, or each vehicle for 15 min. Concentrations of DMSO were less than 0.2%. Calcein-AM solution was loaded onto cells at a final concentration of 1 μM. After 30 min of loading, the cells were washed twice with ice-cold PBS and kept in 1% Triton X-100 overnight at room temperature. The amount of calcein in the cells was measured using a spectrofluorometer (FP-750, Jasco Corporation, Tokyo, Japan) at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

4.6. HPLC and LC-MS/MS analyses

Concentrations of doxorubicin in plasma, bile and urine were determined using a reverse-phase HPLC system consisting of an Agilent Technologies 1200 series coupled to a fluorescence detector (Agilent, Santa Clara, CA). In briefly, 200 μL of acetonitrile were added to 100 μL of plasma and 100 μL of the organ homogenates for protein precipitation. Thereafter, the mixture was vortex-mixed for 2 min and, centrifuged at 15,000 rpm for 2 min, and an aliquot (200 μL) of the supernatant was evaporated to dryness by using a Speed-Vac concentrator (Centra Vac; Vision Scientific Co., Bucheon, Korea). The residue was reconstituted with 200 μL of mobile phase (see below), and an aliquot (50 μL) was injected directly into the HPLC system. A reversed-phase YMC C18 column (250 × 4.6 mm; particle size, 5 μm) was used for the analysis of doxorubicin. The mobile phase consisted of 50 mM sodium phosphate buffer (A) and acetonitrile (B). The solvent flow through the column was 1.0 mL/min with the condition of 70% (A) and 30% (B) and the column effluent was monitored by the FLD detector at λ_{ex}480/λ_{em}560. A calibration study indicated that the detector response was linear over the doxorubicin concentration range of 50 ng/mL to 1000 ng/mL, 10 μg/mL to 200 μg/mL and 10 μg/mL to 400 μg/mL in plasma, urine and bile, respectively.

Concentrations of quercetin were determined using LC-MS/MS (Agilent Technologies 1200 series, Agilent, Santa Clara, CA) coupled to a Waters Quattro micro^{TM} API mass spectrometer (Waters, Milford, MA) equipped with an electrospray ionization interface used to generate negative ions $[M-H]^-$. A 30-μL aliquot from the above samples was directly injected onto a Sepax GP-C18 column (50 × 2.1 mm internal diameter; particle size, 3 μm; Sepax Technologies, Inc., Newark, DE). The mobile phase, 2% formic acid:acetonitrile (20:80, v/v), was run with a flow rate of 0.3 mL/min. Quercetin was quantified using multiple reaction monitoring (MRM) of the transitions of *m/z* 301 → 150.871. The optimal mass parameters obtained were as follows: capillary voltage, 3.0 kV; cone voltage, 47 V; collision energy, 24 V; source temperature, 120 °C; and desolvation temperature, 350 °C. Nitrogen was used as the desolvation gas and cone gas with flow rates of 800 and 50 L/h, respectively. The analytical data were processed using MassLynx V 4.1 software (Waters, Milford, MA). A calibration study indicated that the detector response was linear over the quercetin concentration range of 10–1000 μM ($r^2 = 0.999$).

4.7. Data analysis

Pharmacokinetic parameters were calculated using a non-compartmental pharmacokinetic analysis method. AUC was calculated using the linear trapezoidal method. Mean steady-state concentration was determined from the plasma concentration time profile of doxorubicin. Other pharmacokinetic parameters, including hepatic clearance ($CL_{b/p}$), biliary clearance ($CL_{b/l}$), renal clearance ($CL_{u/p}$), and urinary clearance ($CL_{u/k}$) were calculated using the following equations:

$$CL_{b/p} \text{ and } CL_{u/p} = \frac{\frac{dX_{\text{urine or bile}}}{dt}}{\text{Plasma concentration}}$$

$$CL_{b/l} \text{ and } CL_{u/k} = \frac{\frac{dX_{\text{urine or bile}}}{dt}}{\text{Tissue concentration}}$$

Tissue distribution was represented by K_p, defined as the ratio of the tissue concentration to the corresponding plasma concentration.

A *P*-value less than 0.05 was considered to be statistically significant using an one-way ANOVA followed by Dunnett's multiple comparisons test. All data are expressed as mean ± standard deviation (SD).

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