JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 751–758

Received December 3, 2004
Accepted March 2, 2005
DOI 10.1211/0022357056299
ISSN 0022-3573

Involvement of P-glycoprotein in regulating cellular levels of Ginkgo flavonols: quercetin, kaempferol, and isorhamnetin

Yi Wang, Jiang Cao and Su Zeng

Abstract

Quercetin, kaempferol, and isorhamnetin were the most important flavonoid constituents in extracts from *Ginkgo biloba* leaves. Transport studies of Ginkgo flavonols were performed in Caco-2 cell monolayers. Their apparent permeability in absorptive and secretion directions was determined, and quercetin, kaempferol and isorhamnetin displayed polarized transport, with the $P_{app,B-A}$ being higher than the $P_{app,A-B}$ ($P < 0.01$ for quercetin, $P < 0.001$ for kaempferol and isorhamnetin, Student's *t*-test). Bcap37/MDR1 cells, which were transfected with a P-glycoprotein (P-gp) gene construct, were treated with quercetin, kaempferol or isorhamnetin. The concentrations of Ginkgo flavonol in Bcap37/MDR1 cells were lower than those in parent cells ($P < 0.05$ for quercetin, $P < 0.01$ for isorhamnetin, Mann–Whitney U test). The concentrations of the flavonol in transfected cells increased when incubated with the P-gp inhibitor verapamil ($P < 0.05$ for kaempferol, Mann–Whitney U test). A colorometric assay for ATPase activity was applied to the detection of interaction of flavonol with P-gp. Quercetin and kaempferol inhibited the ATPase activity, and isorhamnetin stimulated the ATPase activity ($P < 0.05$ for isorhamnetin, Mann Whitney U test). The results indicated that Ginkgo flavonols quercetin, kaempferol and isorhamnetin were substrates of P-gp. The P-gp type efflux pump might limit the bioavailability of Ginkgo flavonols.

Department of Pharmaceutical Analysis and Drug Metabolism, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, Zhejiang, 310031, People's Republic of China

Yi Wang, Su Zeng

Clinical Research Institute, Sir Run Run Shaw Hospital, Zhejiang University, Hangzhou, Zhejiang, 310016, People's Republic of China

Jiang Cao

Correspondence: S. Zeng, Department of Pharmaceutical Analysis and Drug Metabolism, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, Zhejiang, 310031, People's Republic of China. E-mail: zengsu@zju.edu.cn

Funding: This project was supported by the National Natural Science Foundation of China (no. 30225047), Foundation for University Disciplinary by the Ministry of Education of China (no. 20040335013) and Zhejiang Provincial Natural Science Foundation of China (no. 302682).

Introduction

For centuries, extracts from the leaves of the *Ginkgo biloba* tree have been used in Chinese herbal medicine to treat a variety of medical conditions. In recent decades, Europe and some Asian countries have taken standardized extracts from *Ginkgo biloba* leaves to treat a wide range of disorders, including Alzheimer's disease, failing memory, age-related dementia, poor cerebral and ocular blood flow and congestive symptoms of premenstrual syndrome and also to prevent altitude sickness (McKenna et al 2001; Zimmermann et al 2002; Ahlemeyer & Krieglstein 2003; Lin et al 2003; Zhang et al 2004). Now it has become one of the best selling herbal products in the world. The medical benefits of *Ginkgo biloba* extract are attributed primarily to two groups of active constituents: the ginkgo flavone glycosides and the terpene lactones. Ginkgo flavone glycosides, which typically make up approximately 24% of the extract, mostly include quercetin, kaempferol and isorhamnetin (Oh & Chung 2004). Pharmacokinetic studies carried out in healthy subjects have shown that the extract has a low bioavailability, particularly with regard to the flavonoidic components (Ross & Kasum 2002).

In the past few years, it has become clear that transport proteins play an important role in preventing the uptake of toxic compounds from the gut into the body, including many drugs and food components. P-glycoprotein (P-gp) is the most thoroughly studied of these transporters and has received considerable attention recently. P-gp is an ATP-driven efflux pump capable of transporting a wide variety of structurally diverse compounds from the cell interior into the extracellular space (Borst & Elferink 2002; Hochman et al 2002; Loo & Clarke 2002). In addition to tumour cells, P-gp is present in normal tissues. Immunohistochemical studies detected P-gp in the adrenal glands and on the apical side of the epithelial liver cells (bile canaliculi), kidney (proximal tubule), pancreas and intestine. P-gp is also found in endothelial brain cells, where it is likely to participate in the blood–brain barrier function (Wacher et al 2001;

Lin 2003). The anatomical localization of P-gp suggests that it may play an important role in the processes of absorption, distribution, metabolism and excretion of drugs in man, and be a potential source for variability in drug pharmacokinetics and pharmacodynamics.

Quercetin and kaempferol are the most abundant polyphenolic compounds present in the human diet in fruits and vegetables (Michael et al 1992, 1993). Published studies suggest that quercetin and kaempferol interact with some clinical drugs (Mitsunaga et al 2000; Hsiu et al 2002; Ikegawa et al 2002; Dupuy et al 2003; Patel et al 2004). Despite evidence for a potential role of quercetin in modulation of P-gp, the conclusion was drawn from the interaction between P-gp substrates and flavonol (Phang et al 1993; Critchfield et al 1994; Shapiro & Ling 1997a; Zhou et al 2004). There is limited information available on the role of P-gp in transporting single Ginkgo flavonols. The role of P-gp in affecting the cellular level of Ginkgo flavonol bioavailability, however, has not been reported previously. In this study, we used monolayer cultures of a human colon adenocarcinoma cell line, Caco-2 cells (Fricker et al 1999; Mathieu et al 2003), which exhibit enterocyte-like characteristics, as an in-vitro model of intestinal absorption by epithelial cells. The effects of P-gp on the accumulation of Ginkgo flavonol were determined in Bcap37/MDR1 cells, which were transfected with a recombinant plasmid containing MDR1 cDNA and high expressing P-gp (Wang et al 2004). A colorometric assay for ATPase activity was applied to the detection of interaction of flavonol with P-gp. Herein, we report the results of this study and discuss implications for the bioavailability of Ginkgo flavonols.

Materials and Methods

Materials

Quercetin, kaempferol, isorhamnetin, morin and (±)-verapamil hydrochloride (purity > 99.6%) were purchased from the China National Institute for the Quality Control of Pharmaceutical and Biological Products. Lucifer yellow CH dilithium salt was from J&K Chemical Limited Company. Mes hydrate, EGTA, L-ascorbic acid, sodium orthovanadate, ATP and Antifoam A concentrate were supplied by Sigma. Caco-2 cells (passage 18) were purchased from the Chinese Academy of Medical Sciences. Bcap37 human breast cancer cells were obtained from the Cancer Institute of Zhejiang University. Bcap37/MDR1 cells (Bcap37 cells infected with a recombinant plasmid containing MDR1 cDNA and high expressing P-gp) were developed by our laboratory (Wang et al 2004). Dulbecco's Modified Eagle's medium (DMEM) containing 4.5 g L^{-1} glucose, RPMI 1640 medium, nonessential amino acids (NEAA), fetal calf serum (FCS) and newborn calf serum were purchased from Invitrogen Life Technologies. Transwell clear polyester membrane inserts (0.4 μm pore diameter, 12 mm diameter) were purchased from Corning Inc. Millicell-ERS voltohmmeter was from Millipore Corp. Bio-Rad DC Protein Assay kit was purchased from Bio-Rad Laboratories. A Platinum EPS C_{18} 100A column (250 × 4.6 mm i.d., 5 μm) was purchased from Alltech Associates, Inc. All other reagents were obtained from commercial sources.

Cell culture

This study was approved by the Ethics Committee of the College of Pharmaceutical Sciences, Zhejiang University.

Caco-2 cells were cultured in DMEM supplemented with 10% heat-inactivated FCS, 1% NEAA, 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at 37°C in a humidified atmosphere of 5% CO_2–95% air. Cells were passaged every 3–4 days (at 70–80% confluence) by treatment with 0.25% trypsin and 0.02% EDTA (Hidalgo et al 1989; Deferme et al 2002).

Bcap37 cells and Bcap37/MDR1 cells were grown in RPMI 1640 medium supplemented with penicillin (100 IU mL^{-1}), streptomycin (100 μg mL^{-1}) and 10% heat-inactivated newborn calf serum. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 in air, and were subcultured every 3 days with 0.25% trypsin.

Transport experiments

For transport studies, Caco-2 cells were seeded in 12 mm i.d. Transwell inserts in 12-well plates at a density of 10^5 cells/cm^2 (Jeong et al 2004). The basolateral and apical compartments contained 1.5 and 0.5 mL of culture medium, respectively. Culture medium was replaced three times a week for 14 days and daily thereafter. Caco-2 cells in Transwells at passage 30–50 were used for transport experiments 21–25 days post seeding. The integrity of the monolayers was determined by measuring the transepithelial electrical resistance (TEER) with a Millicell-ERS voltohmmeter. Inserts with TEER values > 350 Ωcm^2 were used. The paracellular transfer of lucifer yellow CH was determined and served as an additional control for monolayer integrity. Lucifer yellow CH (50 μg mL^{-1}) in phosphate-buffered saline (PBS) was incubated on the apical side of the monolayers. The percentage of paracellular transfer through the monolayer was determined by measuring the fluorescence (excitation 485 nm, emission 530 nm) recovered on the basolateral side of the monolayers. The cell monolayers with apparent permeability coefficients of lucifer yellow CH flux of less than 0.5×10^{-6} cm s^{-1} were used in the transport experiments.

The inserts were washed twice for 30 min with warm transport buffer, phosphate-buffered saline (PBS), pH 7.4. The transepithelial permeability of quercetin, kaempferol and isorhamnetin was measured at a 50 μmol L^{-1} concentration. Transport buffer containing quercetin, kaempferol or isorhamnetin was added to either the apical (0.5 mL) or basolateral (1.5 mL) side of the inserts, whereas the receiving chamber contained the corresponding volume of transport buffer. Upon the end of the 2-h incubation at 37°C, samples were collected for immediate analysis.

Cellular uptake studies

To evaluate the effect of P-gp on Ginkgo flavonols' uptake by cells, Bcap37/MDR1 and Bcap37cells were separately

seeded at a density of 7.5×10^5/well to 35 mm i.d. tissue culture wells. After 24 h, the culture medium was aspirated and replaced by the medium containing 25 μmol L^{-1} quercetin, kaempferol or isorhamnetin. After 10, 20, 30 and 40 min, the medium was aspirated and the cells were washed 3 times with ice-cold PBS (pH 7.4) to stop further uptake. One millilitre of 0.1% Triton X-100 was added to each well to lyse the cells. The drug concentration in the lysis solution was determined by HPLC and normalized with cellular protein content. Protein content was determined using a Bio-Rad DC Protein Assay kit, with bovine serum albumin as the standard (Fukushima et al 1998; Takara et al 1999; Batrakova et al 2001).

Inhibition of cellular uptake

Bcap37/MDR1 and Bcap37 cells were separately seeded at a density of 7.5×10^5/well to 35 mm i.d. tissue culture wells. After 24 h, the culture medium was aspirated and replaced by the medium containing 25 μmol L^{-1} Ginkgo flavonols in the absence and presence of 6.6 μmol L^{-1} verapamil (Plumb et al 1990). After 30 min, the media were aspirated and the cells were washed 3 times with ice-cold PBS (pH 7.4) to stop further uptake. The drug concentration in lysis solution was determined by HPLC and normalized with cellular protein content.

Concentration measurements

All the concentrations of the compounds were determined by HPLC. To samples containing quercetin, kaempferol or isorhamnetin was added 50 μL of 0.02 mg mL^{-1} morin as an internal standard in transport experiments, and 50 μL of 0.01 mg mL^{-1} morin as an internal standard in uptake experiments. One millilitre of 6 mol L^{-1} hydrochloric acid was added to samples to precipitate proteins. The mixture was extracted with 3.0 mL of ether and acetone (14:1; v/v), vortexed for 2 min then centrifuged for 15 min at 600 g. The upper organic layer was quantitatively transferred into another test tube and evaporated to dryness with N_2. The residue was reconstituted in 80 μL mobile phase before analysis. Twenty microlitres of the sample was injected into the HPLC system. Chromatographic determination of the three Ginkgo flavonols was performed by using Agilent 1100 System equipped with a Platinum EPS C_{18} 100A column. The mobile phase consisted of phosphate buffer–tetrahydrofuran–methanol–isopropanol (65:15:10:20, v/v/v/v) at a flow rate of 0.5 mL min^{-1}, and the wavelength of the UV detector was set at 380 nm (Wang et al 2003). The concentration was determined by comparing with standard.

Preparation of purified native membrane vesicle

Bcap37/MDR1 cells were cultured in 150-cm^2 flasks. After being grown to confluence, cells were twice washed by PBS and harvested by PBS scraping. Cells were suspended in PBS with antiproteases, washed twice in PBS containing 1 mmol L^{-1} dithiothreitol (DTT) and 1 mmol L^{-1} phenylmethylsulfonyl fluoride (PMSF), and collected by centrifugation (1000 g, 10 min, 4°C). They were then separately suspended in PBS with DTT and PMSF and disrupted by sonication for 10 s at 10% maximum power. The resulting homogenate was centrifuged (1400 g, 15 min, 4°C). The supernatant was then layered on a 46% w/w sucrose cushion in PBS containing 2 mmol L^{-1} $MgCl_2$ and 1 mmol L^{-1} DTT, and centrifuged (7000 g, 20 min, 4°C). The layer at the sucrose interface was collected, diluted twice with PBS, $MgCl_2$, and DTT, and washed three times (95 000 g, 15 min, 4°C). Membranes were finally resuspended in PBS, $MgCl_2$, and DTT at a total membrane protein concentration of 2 mg mL^{-1} and passed through a tip to eliminate aggregates. Samples were prepared and frozen at −80°C (Sarkadi et al 1992; Garrigues et al 2002). Protein concentration was determined by using the Bio-Rad DC protein assay kit, with bovine serum albumin as a standard.

P-gp ATPase activity

Stimulation of P-gp ATPase catalytic activity was measured in membranes of Bcap37/MDR1 cells. The P-gp ATPase assay method used was a modification of the assay described by Sarkadi et al (1992) and as directed in the Gentest product insert. A 0.06-mL reaction mixture, containing 30 μg membranes, 20 μmol L^{-1} verapamil (positive control) or 100 μmol L^{-1} flavonol, and 4 mmol L^{-1} MgATP, in buffer containing 50 mmol L^{-1} Tris-MES, 2 mmol L^{-1} EGTA, 50 mmol L^{-1} KCl, 2 mmol L^{-1} dithiothreitol and 5 mmol L^{-1} sodium azide, was incubated at 37°C for 30 min. An identical reaction mixture containing 100 μmol L^{-1} sodium orthovanadate was assayed in parallel. The reaction was stopped by the addition of 30 μL 10% sodium dodecyl sulfate (SDS) + Antifoam A. Two additional reaction mixtures (+ and − orthovanadate), but without MgATP, were also prepared and incubated with the others, and then supplemented with SDS and MgATP, to represent time = 0 min of reaction. The incubations were followed with addition of 200 μL of 35 mmol L^{-1} ammonium molybdate in 15 mmol L^{-1} zinc acetate–10% ascorbic acid (1:4) and incubated for an additional 20 min at 37°C. The liberation of inorganic phosphate was detected by its absorbance at 650 nm and quantitated by comparing the absorbance with a phosphate standard curve (Drueckes et al 1995; Kim & Benet 2004).

Data analysis

Apparent permeability coefficients (P_{app}) were calculated using the following equation:

$$P_{app} = V/AC_0 \cdot dC/dt \ (\text{cm s}^{-1}) \quad (1)$$

Where V is the volume of the solution in the receiving compartment, A is the membrane surface area, C_0 is the initial concentration in the donor compartment and dC/dt is the change in drug concentration in the receiver solution over time.

The permeability ratio was calculated according to the following equation:

$$P_{ratio} = P_{app,B-A}/P_{app,A-B} \quad (2)$$

Where $P_{app,B\text{-}A}$ is the permeability from the basolateral to the apical side ($cm\,s^{-1}$) and $P_{app,A\text{-}B}$ the permeability from the apical to the basolateral side ($cm\,s^{-1}$).

All values were presented as a mean ± standard deviation (s.d.) throughout the paper. Differences between $P_{app,B\text{-}A}$ and $P_{app,A\text{-}B}$ of flavonol were evaluated using Student's *t*-test (Walgren et al 1998). Nonparametric data were analysed by the Kruskal–Wallis test followed by the Nemenyi test, or by the Mann–Whitney U test. $P < 0.05$ was considered to be statistically significant.

Results

Transepithelial transport of Ginkgo flavonols

Table 1 shows the permeability coefficients (P_{app}) of the Ginkgo flavonols (50 μmol L^{-1}) in the Caco-2 cell monolayers determined during 2 h incubation. The P_{app} of flavonols were significantly different from each other ($P < 0.001$, Nemenyi's test). Whether from the apical to basolateral transport or the basolateral to apical, the P_{app} of kaempferol was the highest, and isorhamnetin was the lowest. Secretion (basolateral to apical) rates of transport ($P_{app,B\text{-}A}$) of the three Ginkgo flavonols were higher than absorptive (apical to basolateral) rates of transport ($P_{app,A\text{-}B}$) ($P < 0.01$ for quercetin, $P < 0.001$ for kaempferol and isorhamnetin, Student's *t*-test). The P_{ratio} of quercetin, kaempferol and isorhamnetin were between 1.56 and 1.93.

Cellular accumulation of Ginkgo flavonols

The stable over expression of P-gp in Bcap37/MDR1 cells was confirmed by western blot analysis (Wang et al 2004). The accumulation of Ginkgo flavonols in Bcap37 and Bcap37/MDR1 cells was measured at 10, 20, 30 and 40 min incubation time (Figure 1). During the investigation period, the three flavonols showed less accumulation in Bcap37/MDR1 than in Bcap37 cells. Especially, quercetin and isorhamnetin showed that the accumulation differences between the two cell lines were statistically significant at each investigated time point. In Bcap37 cells, the accumulations of Ginkgo flavonols was linear for up to 30 min and accumulation saturation was observed after 30 min for quercetin and kaempferol. In Bcap37/MDR1 cells, quercetin and kaempferol showed linear accumulation up to

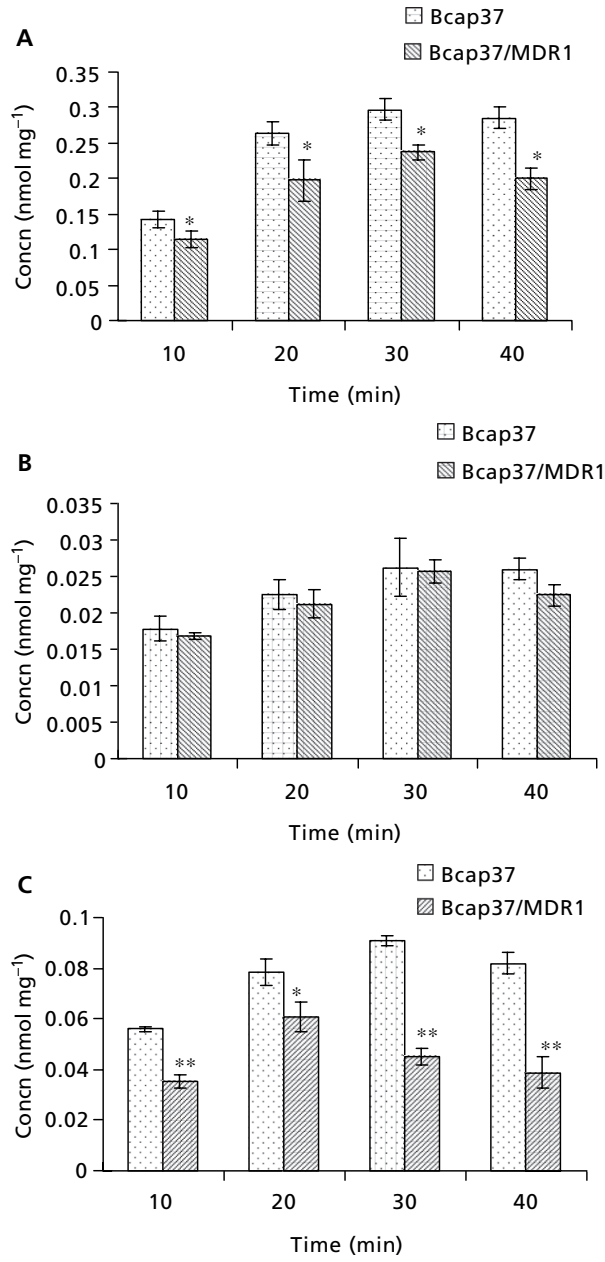

Figure 1 Accumulation of quercetin (A), kaempferol (B) and isorhamnetin (C) in Bcap37 and Bcap37/MDR1 cells. Bcap37 and Bcap37/MDR1 cells were separately incubated with quercetin (25 μmol L^{-1}), kaempferol (25 μmol L^{-1}) and isorhamnetin (25 μmol L^{-1}), individually, for 10, 20, 30, 40 min. Cell lysates were prepared as described in Materials and Methods; intracellular Ginkgo flavonols were analysed by HPLC. Each column represents the mean ± s.d. Data were analysed by using the non-parametric Mann–Whitney U test. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with Bcap37.

Table 1 Permeability coefficients of Ginkgo flavonols across Caco-2 cell monolayers

	Quercetin	Kaempferol	Isorhamnetin
$P_{app,B\text{-}A} \times 10^{-6}$ ($cm\,s^{-1}$)	$6.71 \pm 0.78^{**\dagger}$	$13.7 \pm 0.07^{***\dagger}$	$2.55 \pm 0.14^{***\dagger}$
$P_{app,A\text{-}B} \times 10^{-6}$ ($cm\,s^{-1}$)	$4.29 \pm 0.32^{\#}$	$7.11 \pm 0.57^{\#}$	$1.44 \pm 0.11^{\#}$
P_{ratio}	1.56	1.93	1.78

The bidirectional transport (n = 5) of Ginkgo flavonols (50 μmol L^{-1}) were examined across Caco-2 cell monolayers individually as described in Materials and Methods. Data are shown as mean ± s.d. $^{**}P < 0.01$, $^{***}P < 0.001$, compared with $P_{app,A\text{-}B}$ (Student's *t*-test); $^{\dagger}P < 0.001$, compared with unity (Nemenyi's test); $^{\#}P < 0.001$, compared with unity (Nemenyi's test).

30 min and isorhamnetin accumulation decreased after 20 min. The accumulation of quercetin was much more than that of the other Ginkgo flavonols in either Bcap37 or Bcap37/MDR1 cells.

Cellular accumulation of Ginkgo flavonols affected by P-gp inhibitor

In the presence of 6.6 μmol L^{-1} verapamil (a known P-gp inhibitor), the accumulation of the three Ginkgo flavonols was increased in Bcap37/MDR1 cells, and the uptake of kaempferol was significantly greater (Figure 2). In Bcap37 cells, 6.6 μmol L^{-1} verapamil did not cause a statistically significant increase in flavonols uptake in Bcap37 cells. When quercetin, kaempferol and isorhamnetin were added together to Bcap37/MDR1 cells, the pattern of accumulation caused by this P-gp inhibitor was similar to that when these compounds were added individually (data not shown).

Effect of Ginkgo flavonols on P-gp ATPase activity

Drug efflux associated with P-gp is ATP dependent and expression of P-gp results in the appearance of a vanadate-sensitive drug-stimulated ATPase activity. Membranes from Bcap37/MDR1 cells had a vanadate-sensitive ATPase activity, attributable to P-gp ATPase, of 6.9 nmol (mg membrane protein)$^{-1}$ min^{-1}. This activity was stimulated, with 3.0-fold increase, by incubating with verapamil. This showed membranes of Bcap37/MDR1 cells had P-gp ATPase activity (Garrigos et al 1997). Quercetin and kaempferol inhibited the ATPase activity of P-gp with a 0.8-fold of basal activity (Figure 3). Isorhamnetin achieved 1.9-fold stimulation of ATPase activity ($P < 0.05$, Mann–Whitney U test).

Discussion

The first study of the disposition of quercetin in man was reported in 1975 (Gugler et al 1975), in which quercetin was not detected in the plasma and urine of subjects receiving 4 g of quercetin orally, and quercetin was thought not to be absorbed. Hollman et al (1995) demonstrated that quercetin showed 24% absorption in ileostomy patients. Wang et al (2003) reported that the bioavailability of quercetin and kaempferol was low in man following an oral administration of Ginkgo biloba extract tablets. The researchers were concerned about degradation of quercetin in the stomach and small intestine, and tested this by placing quercetin in a solution of gastric juices in-vitro, which showed no loss of quercetin. However, there is still a possibility that the quercetin was degraded in-vivo by gastrointestinal secretions or by small-intestinal bacteria. For other flavonoid aglycones, there is very limited information on their absorption and bioavailability in man.

Caco-2 cells were derived from human colorectal adenocarcinoma. They are useful not only for prediction of absorption in man but also for the study of several transport systems. In culture, P-gp was expressed on the apical (luminal) membrane of polarized epithelial Caco-2 cells and transports compounds from the cytosolic compartment to the luminal side. P-gp transport poses resistance to

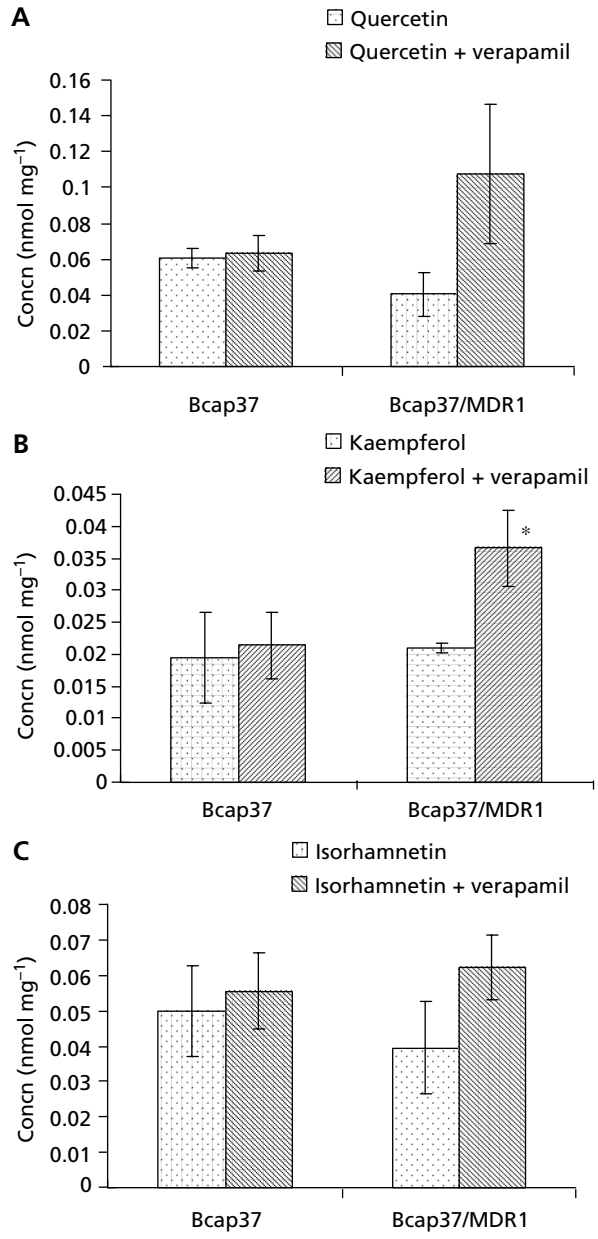

Figure 2 Effect of verapamil on the accumulation of quercetin (A), kaempferol (B) and isorhamnetin (C) in Bcap37 and Bcap37/MDR1 cells. Bcap37 and Bcap37/MDR1 cells were separately incubated with culture medium containing quercetin (25 μmol L^{-1}), kaempferol (25 μmol L^{-1}) and isorhamnetin (25 μmol L^{-1}), individually, with or without 6.6 μmol L^{-1} verapamil for 30 min. Cell lysates were prepared as described in Materials and Methods; intracellular Ginkgo flavonols were analysed by HPLC. Each column represents the mean ± s.d. Data were analysed using the non-parametric Mann–Whitney U test. The concentration of kaempferol in Bcap37/MDR1 cells incubated with verapamil was significantly higher than that without verapamil (*$P < 0.05$).

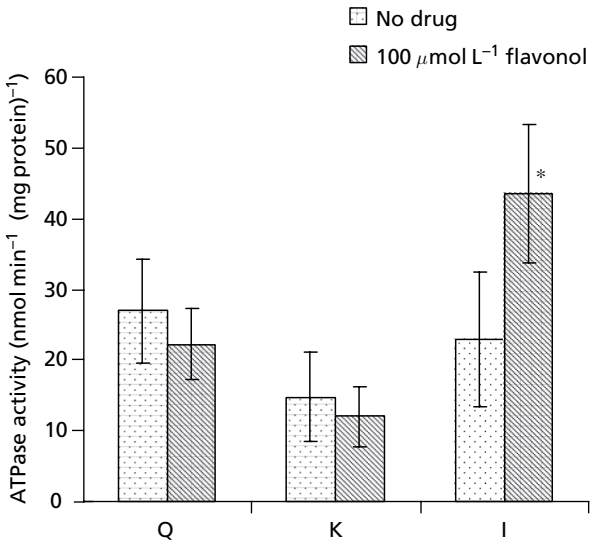

Figure 3 Effect of Ginkgo flavonols on P-gp ATPase activity. Vanadate-sensitive ATPase activity of 30 μg of membrane protein isolated from Bcap37/MDR1 cells was determined in the presence of (hatched bars) or absence (open bars) of 100 μmol L^{-1} flavonol (quercetin, kaempferol and isorhamnetin, individually). P-gp associated ATPase activity was measured as inorganic phosphate release from ATP as described in Materials and Methods. Each column represents the mean ± s.d. from three independent experiments. Data were analysed using the non-parametric Mann–Whitney U test. The difference between the ATPase activity in the absence and presence of isorhamnetin was statistically significant (*$P < 0.05$). Q, quercetin; K, kaempferol; I, isorhamnetin.

transcellular transport in the apical to basolateral direction and facilitates transport in the basolateral to apical direction. Thus, P-gp efflux resulted in higher basolateral to apical transport than apical to basolateral transport. This assay, where the P_{ratio} was compared with a value of 1, was regarded as the standard for identifying P-gp substrates. Involvement of a P-gp-mediated efflux mechanism was indicated if the P_{ratio} was > 2. Additional experiments would be carried out on a compound with a P_{ratio} of 1.5–2.0 (Polli et al 2001). In this study, the secretion rate of transport of the three flavonols was 1.5–1.9 fold greater than the absorptive rates of transport ($P < 0.01$ for quercetin, $P < 0.001$ for kaempferol and isorhamnetin, Student's t-test). As Caco-2 cells were also expressing other membrane transporters (i.e. multidrug resistant associated protein (MRP) and lung cancer associated resistance protein (LRP)), Ginkgo flavonols transported by P-gp did not manifest. Comparing the P_{app} of the Ginkgo flavonols, the P_{app} of kaempferol was the highest ($P < 0.001$, Nemenyi's test). Based on previous observations by Yee (1997), examining the relationship between P_{app} values obtained from Caco-2 cells and human in-vivo oral absorption for a number of drugs, our finding would suggest that oral absorption of kaempferol was more in man than that of other flavonols. Recently, it was shown that kaempferol was absorbed more efficiently than quercetin in man, even at low oral doses (DuPont et al 2004), which was in agreement with what we reasoned.

In cellular drug accumulation assays, the accumulation of P-gp substrate in cells that over-express P-gp was restricted by P-gp-mediated efflux of the compound back into the extracellular fluid. Drug accumulation increased under conditions where P-gp was inhibited, such that the difference in accumulation in P-gp deficient cells and P-gp over-expressing cells became smaller. In this research, Bcap37/MDR1 cells were Bcap37cells transfected with recombinant plasmid containing MDR1 cDNA and stable high expressing P-gp. As in the case of drug transport, the influence of P-gp on drug accumulation is not only a function of P-gp activity but is also influenced by passive diffusion of the molecule. Under steady-state conditions, the rate of drug entering the cell by passive membrane diffusion equals the rate of drug leaving the cell by passive diffusion plus the rate of active drug efflux by P-gp. Thus, the relative contribution of P-gp decreases with increasing passive diffusion. An incubation concentration of 25 μmol L^{-1} was chosen to decrease the influence of passive diffusion. After 30 min, the concentration of flavonols in cells did not increase and it went into a steady state. So a 30-min incubation time was chosen in the cellular uptake inhibition study. The accumulation concentrations of Ginkgo flavonols in Bcap37 cells were more pronounced than those in Bcap37/MDR1 cells at 10, 20, 30 and 40 min. Concentrations of quercetin and isorhamnetin in the two cell lines showed the differences were statistically significant. When Bcap37/MDR1 cells were incubated with the P-gp inhibitor verapamil, the uptake of kaempferol was significantly greater. The results suggest that quercetin, kaempferol and isorhamnetin were substrates of P-gp. Interestingly, kaempferol had the highest permeable rate for transport across cell monolayers in the transport experiment, but in the cellular accumulated assay it had the lowest accumulated rate in cells. In addition to the different contents of cell membranes, it was possible that kaempferol transport through Caco-2 cell monolayers involved paracellular transport. Since the molecular weight of kaempferol was the lowest among the three flavonols, the possibility was that the effect on paracellular transport of flavonol was most remarkable. The mechanism is to be explored in the future.

Orthovanadate inhibits P-gp by trapping MgADP in the nucleotide binding site. Thus, ATPase activity measured in the presence of orthovanadate represents non-P-gp ATPase activity and can be subtracted from the activity generated without orthovanadate to yield vanadate-sensitive ATPase activity. Preliminary studies using phosphate buffer standards demonstrated that 100 μmol L^{-1} flavonol did not interfere with the phosphate assay. Our results showed that quercetin and kaempferol inhibited the ATPase activity of P-gp, but that isorhamnetin stimulated the ATPase activity of P-gp. The difference between the ATPase activity in the absence and presence of isorhamnetin was statistically significant. However, these differences for quercetin and kaempferol were not statistically significant ($P > 0.05$, Mann–Whitney U test). On the other hand, we detected the prominent effect of flavonols on P-gp constitutive ATPase activity when the concentration of flavonol was increased to 100 μmol L^{-1}. This may be due to the limited sensitivity of the assay because the P-gp constitutive

ATPase activity is not very high. Of known P-gp substrates, some are reported to stimulate the ATPase activity of P-gp (e.g., calcein-AM, demecolcine) and some can inhibit the ATPase activity of P-gp (e.g., ciclosporin, Hoechst 33342). Loo et al (2003) have explained why a substrate is a stimulator or inhibitor of P-gp. Shapiro & Ling (1997a) found that quercetin bound to purified P-gp and efficiently inhibited its activity, which is in agreement with our findings. Mitsunaga et al (2000) addressed the mechanism of the concentration-dependent biphasic action of quercetin and found that low concentrations of quercetin (10 μM) enhanced the phosphorylation and hence the activity of P-gp, whereas high concentrations of quercetin (50 μM) inhibited P-gp. At the same time, kaempferol had a similar biphasic effect. But in our experiment, we did not observe this phenomenon. They found the biphasic effect of quercetin by measuring the quantity of a P-gp substrate, and did not determine the effect of the flavonols on the ATPase activity of P-gp. These results illustrate the limitations of using one type of assay system to extrapolate another type of activity.

Flavonoids have been described as modulators, but contradictory effects have been reported with different cells: quercetin increased the accumulation of daunorubicin in GLC4/ADR cells (Versantvoort et al 1993) and exhibited a remarkable inhibition of P-gp-mediated efflux of ritonavir by increasing its cellular uptake in MDR1-MDCK cells (Patel et al 2004), whereas quercetin and kaempferol were found to increase adriamycin efflux from HCT-15 colon cells (Critchfield et al 1994) and to be potent stimulators of P-gp-mediated efflux of 7,12-dimethylbenz(a)-anthracene (Phang et al 1993). From our results, the phenomenon that quercetin inhibited the P-gp substrate accumulation in P-gp high expressing cells was caused not only by its inhibition of P-gp ATPase activity, but also by its competitive effects of P-gp substrates. Shapiro and colleagues found that P-gp contained two distinct sites for drug binding and transport, and these sites interact in a positively cooperative manner (Shapiro & Ling 1997a, b; Walgren et al 1998). They showed that quercetin stimulated rhodamine-123 transport and inhibited Hoechst 33342 transport in isolated P-glycoprotein-rich plasma membrane vesicles from Chinese hamster ovary CH(R) B30 cells. Because each substrate stimulated the P-gp-mediated transport of the other, the effect that quercetin and kaempferol increased adriamycin and 7, 12-dimethylbenz(a)-anthracene efflux was explained. This is consistent with what we concluded.

Conclusion

These results provide evidence that quercetin, kaempferol and isorhamnetin are transported by P-gp. Quercetin and kaempferol may inhibit the ATPase activity of P-gp, and isorhamnetin may stimulate the ATPase activity. The efflux of these compounds by P-gp might play a vital role in limiting the bioavailability of Ginkgo flavonols in-vivo. Further, the results suggest that co-administration of the three flavonols with P-gp substrate agent may alter drug pharmacokinetics and pharmacodynamics.

References

Ahlemeyer, B., Krieglstein, J. (2003) Neuroprotective effects of Ginkgo biloba extract. *Cell. Mol. Life Sci.* **60**: 1779–1792

Batrakova, E., Miller, D.W., Li, S., Alakhov, V. Y., Kabanov, A. V., Elmquist, W. F. (2001) Pluronic P85 enhances the delivery of digoxin to the brain: I vitro and in vivo studies. *J. Pharmacol. Exp. Ther.* **296**: 551–557

Borst, P., Elferink, R. O. (2002) Mammalian ABC transporters in health and disease. *Annu. Rev. Biochem.* **71**: 537–592

Critchfield, J. W., Welsh, C. J., Phang, J. M., Yeh, G. C. (1994) Modulation of adriamycin accumulation and efflux by flavonoids in HCT-15 colon cells. *Biochem. Pharmacol.* **48**: 1437–1445

Deferme, S., Van, G. J., Augustijns, P. (2002) Inhibitory effect of fruit extracts on P-glycoprotein-related efflux carriers: an in-vitro screening. *J. Pharm. Pharmacol.* **54**: 1213–1219

Drueckes, P., Schinzel, R., Palm, D. (1995) Photometric microtiter assay of inorganic phosphate in the presence of acid-labile organic phosphates. *Anal. Biochem.* **230**: 173–177

DuPont, M. S., Day, A. J., Bennett, R. N., Mellon, F. A., Kroon, P. A. (2004) Absorption of kaempferol from endive, a source of kaempferol-3-glucuronide, in humans. *Eur. J. Clin. Nutr.* **58**: 947–954

Dupuy, J., Larrieu, G., Sutra, J. F., Lespine, A., Alvinerie, M. (2003) Enhancement of moxidectin bioavailability in lamb by a natural flavonoid: quercetin. *Vet. Parasitol.* **112**: 337–347

Fricker, G., Gutmann, H., Droulle, A., Drewe, J., Miller, D. S. (1999) Evidence for different ABC-transporters in Caco-2 cells modulating drug uptake. *Pharm. Res.* **16**: 402–407

Fukushima, T., Inoue, H., Takemura, H., Kishi, S., Yamauchi, T., Inai, K., Nakayama, T., Imamura, S., Urasaki, Y., Nakamura, T., Ueda, T. (1998) Idarubicin and idarubicinol are less affected by topoisomerase II-related multidrug resistance than is daunorubicin. *Leuk. Res.* **22**: 625–629

Garrigos, M., Mir, L. M., Orlowski, S. (1997) Competitive and influence of propafenone analogs on Pgp ATPase activity non-competitive inhibition of the multidrug-resistance-associated P-glycoprotein ATPase—further experimental evidence for a multisite model. *Eur. J. Biochem.* **244**: 664–673

Garrigues, A., Nugier, J., Orlowski, S., Ezan, E. (2002) A high-throughput screening microplate test for the interaction of drugs with P-glycoprotein. *Anal. Biochem.* **305**: 106–114

Gugler, R., Leschik, M., Dengler, H. J. (1975) Disposition of quercetin in man after single oral and intravenous doses. *Eur. J. Clin. Pharmacol.* **9**: 229–234

Hidalgo, I. J., Raub, T. J., Borchardt, R. T. (1989) Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* **96**: 736–749

Hochman, J. H., Yamazaki, M., Ohe, T., Lin, J. H. (2002) Evaluation of drug interactions with P-glycoprotein in drug discovery: in vitro assessment of the potential for drug-drug interactions with P-glycoprotein. *Curr. Drug Metab.* **3**: 257–273

Hollman, P. C. H., deVries, J. H. M., van Leeuwen, S. D., Mengelers, M. J. B., Katan, M. B. (1995) Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am. J. Clin. Nutr.* **62**: 1276–1282

Hsiu, S. L., Hou, Y. C., Wang, Y. H., Tsao, C. W., Su, S. F., Chao, P. D. (2002) Quercetin significantly decreased cyclosporin oral bioavailability in pigs and rats. *Life Sci.* **72**: 227–235

Ikegawa, T., Ohtani, H., Koyabu, N., Juichi, M., Iwase, Y., Ito, C., Furukawa, H., Naito, M., Tsuruo, T., Sawada, Y. (2002) Inhibition of P-glycoprotein by flavonoid derivatives in adriamycin-resistant human myelogenous leukemia (K562/ADM) cells. *Cancer Lett.* **177**: 89–93

Jeong, E. J., Lin, H., Hu, M. (2004) Disposition mechanism of raloxifene in the human intestinal Caco-2 model. *J. Pharmacol. Exp. Ther.* **310**: 376–385

Kim, W. Y., Benet, L. Z. (2004) P-glycoprotein (P-gp/MDR1)-mediated efflux of sex-steroid hormones and modulation of P-gp expression in vitro. *Pharm. Res.* **21**: 1284–1293

Lin, J. H. (2003) Drug-drug interaction mediated by inhibition and induction of P-glycoprotein. *Adv. Drug Deliv. Rev.* **55**: 53–81

Lin, C. C., Cheng, W. L., Hsu, S. H., Chang, C. M. (2003) The effects of Ginkgo biloba extracts on the memory and motor functions of rats with chronic cerebral insufficiency. *Neuropsychobiology* **47**: 47–51

Loo, T. W., Clarke, D. M. (2002) Location of the rhodamine-binding site in the human multidrug resistance P-glycoprotein. *J. Biol. Chem.* **277**: 44332–44338

Loo, T. W., Bartlett, M. C., Clarke, D. M. (2003) Drug binding in human P-glycoprotein causes conformational changes in both nucleotide-binding domains. *J. Biol. Chem.* **278**: 1575–1578

Mathieu, F., Galmier, M. J., Nicolay, A., Pognat, J. F., Lartigue, C. (2003) Transepithelial transport of Bepridil in the human intestinal cell line, Caco-2, using a dynamic model. *Eur. J. Drug Metab. Pharmacokinet.* **28**: 155–160

McKenna, D. J., Jones, K., Hughes, K. (2001) Efficacy, safety, and use of ginkgo biloba in clinical and preclinical applications. *Altern. Ther. Health Med.* **7**: 70–86, 88–90

Michael, G. L. H., Peter, C. H. H., Martijn, B. K. (1992) Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. *J. Agric. Food Chem.* **40**: 2379–2383

Michael, G. L. H., Peter, C. H. H., Betty, V. D. P. (1993) Content of potentially anticarcinogenic flavonoids of tea infusions, wines, and fruit juices. *J. Agric. Food Chem.* **41**: 1242–1246

Mitsunaga, Y., Takanaga, H., Matsuo, H., Naito, M., Tsuruo, T., Ohtani, H., Sawada, Y. (2000) Effect of bioflavonoids on vincristine transport across blood-brain barrier. *Eur. J. Pharmacol.* **395**: 193–201

Oh, S. M., Chung, K. H. (2004) Estrogenic activities of Ginkgo biloba extracts. *Life Sci.* **74**: 1325–1335

Patel, J., Buddha, B., Dey, S., Pal, D., Mitra, A. K. (2004) In vitro interaction of the HIV protease inhibitor ritonavir with herbal constituents: changes in P-gp and CYP3A4 activity. *Am. J. Ther.* **11**: 262–277

Phang, J. M., Poore, C. M., Lopaczynska, J., Yeh, G. C. (1993) Flavonol-stimulated efflux of 7,12-dimethylbenz(a)anthracene in multidrug-resistant breast cancer cells. *Cancer Res.* **53**: 5977–5981

Plumb, J. A., Milroy, R., Kaye, S. B. (1990) The activity of verapamil as a resistance modifier in vitro in drug resistant human tumour cell lines is not stereospecific. *Biochem. Pharmacol.* **39**: 787–792

Polli, J. W., Wring, S. A., Humphreys, J. E., Huang, L., Morgan, J. B., Webster, L. O., Serabjit-Singh, C. S. (2001) Rational use of in vitro P-glycoprotein assays in drug discovery. *J. Pharmacol. Exp. Ther.* **299**: 620–628

Ross, J. A., Kasum, C. M. (2002) Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu. Rev. Nutr.* **22**: 19–34

Sarkadi, B., Price, E. M., Boucher, R. C., Germann, U. A., Scarborough, G. A. (1992) Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. *J. Biol. Chem.* **267**: 4854–4858

Shapiro, A. B., Ling, V. (1997a) Effect of quercetin on Hoechst 33342 transport by purified and reconstituted p-glycoprotein. *Biochem. Pharmacol.* **53**: 587–596

Shapiro, A. B., Ling, V. (1997b) Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities. *Eur. J. Biochem.* **250**: 130–137

Takara, K., Tanigawara, Y., Komada, F., Nishiguchi, K., Sakaeda, T., Okumura, K. (1999) Cellular pharmacokinetic aspects of reversal effect of itraconazole on P-glycoprotein-mediated resistance of anticancer drugs. *Biol. Pharm. Bull.* **22**: 1355–1359

Versantvoort, C. H., Schuurhuis, G. J., Pinedo, H. M., Eekman, C. A., Kuiper, C. M., Lankelma, J., Broxterman, H. J. (1993) Genistein modulates the decreased drug accumulation in non-P-glycoprotein mediated multidrug resistant tumour cells. *Br. J. Cancer* **68**: 939–946

Wacher, V. J., Salphati, L., Benet, L. Z. (2001) Active secretion and enterocytic drug metabolism barriers to drug absorption. *Adv. Drug Deliv. Rev.* **46**: 89–102

Walgren, R. A., Walle, U. K., Walle, T. (1998) Transport of quercetin and its glucosides across human intestinal epithelial Caco-2 cells. *Biochem. Pharmacol.* **55**: 1721–1727

Wang, F. M., Yao, T. W., Zeng, S. (2003) Disposition of quercetin and kaempferol in human following an oral administration of Ginkgo biloba extract tablets. *Eur. J. Drug Metab. Pharmacokinet.* **28**: 173–177

Wang, Y., Cao, J., Zeng, S. (2004) Establishment of a P-glycoprotein substrate screening model and its preliminary application. *World J. Gastroenterol.* **10**: 1365–1368

Yee, S. (1997) In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man – fact or myth. *Pharm. Res.* **14**: 763–766

Zhang, C., Zu, J., Shi, H., Liu, J., Qin, C. (2004) The effect of Ginkgo biloba extract (EGb 761) on hepatic sinusoidal endothelial cells and hepatic microcirculation in CCl4 rats. *Am. J. Chin. Med.* **32**: 21–31

Zhou, S., Lim, L. Y., Chowbay, B. (2004) Herbal modulation of P-glycoprotein. *Drug Metab. Rev.* **36**: 57–104

Zimmermann, M., Colciaghi, F., Cattabeni, F., Di Luca, M. (2002) Ginkgo biloba extract: from molecular mechanisms to the treatment of Alzhelmer's disease. *Cell. Mol. Biol. (Noisy-le-grand)* **48**: 613–623