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Efflux of baicalin, a flavone glucuronide of Scutellariae Radix, on Caco-2 cells through multidrug resistance-associated protein 2

Teruaki Akao, Masato Hanada, Yoko Sakashita, Keisuke Sato, Masashi Morita and Tsuneo Imanaka

Medical Pharmaceutics, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan

Teruaki Akao, Masato Hanada, Yoko Sakashita, Keisuke Sato

Molecular Cell Biology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan

Masashi Morita, Tsuneo Imanaka

Correspondence: T. Akao, Faculty of Pharmaceutical Sciences, University of Toyama 2630 Sugitani, Toyama 930–0194, Japan. E-mail: ta0113@pha.u-toyama.ac.jp

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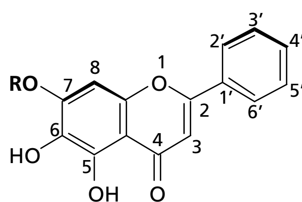
Abstract

Baicalin and its aglycone, baicalein, being are strong antioxidants and have various pharmacological actions. Baicalein has shown a unique metabolic fate in rat intestine, being excreted into the intestinal lumen from mucosal (epithelial) cells following glucuronidation of baicalein absorbed after oral administration. The purpose of this study was to examine the absorption and excretion of baicalin and baicalein in a Caco-2 cell monolayer model to evaluate the disposition of baicalin and baicalein in the human intestine. When baicalein at 5 μM was loaded on the apical side of the Caco-2 cell monolayer, baicalein was not transferred to the basolateral side, but more baicalin was excreted onto the apical side than was being absorbed onto the basolateral side. The amount of baicalin recovered on both sides accounted for more than 90% of the baicalein absorbed from the apical surface. This was supported by the fact that Caco-2 cell microsomes showed UDP-glucuronate glucuronosyltransferase activity towards baicalein to form baicalin. On the other hand, when baicalein was loaded at higher concentrations, baicalin excretion became saturated, and then baicalein was transferred to the basolateral side. Furthermore, baicalin efflux was not inhibited by MDR1/P-glycoprotein substrates such as ciclosporin and vinblastine, but significantly inhibited by multidrug resistance-associated protein 2 (MRP2, ABCC2) substrates such as probenecid and genistein. MRP2 was also detected in Caco-2 cells by Western blotting using specific antibodies. In addition, baicalin, but not baicalein, enhanced dose-dependently the vanadate-sensitive ATPase activity of human MRP2. These results indicated that, in Caco-2 cells, any baicalein absorbed after loading at low concentrations of baicalein was not transferred to the basolateral side, but was first transformed into baicalin in the cells and excreted through the action of MRP2, mainly to the apical side.

Introduction

Scutellariae Radix, the root of *Scutellaria baicalensis* Georgi (Labiatae), is used in combination with other herbs in Chinese traditional (Kampo) medicines, and contains baicalin (5,6,7-trihydroxyflavone-7-β-D-glucuronide; Figure 1) as its main active constituent. Baicalin and its aglycone, baicalein, have wide-ranging pharmacological effects, such as anti-allergic (Koda et al 1970), anti-inflammatory (Kubo et al 1984), antiviral (Baylor et al 1992; Li et al 2000), antiproliferative (Inoue & Jackson 1999), and antitumour (Motoo & Sawada 1994) effects, and also show strong antioxidant activity (Kimura et al 1981; Gao et al 1999). In addition, they inhibit prostaglandin E_2 production, and prevent inducible nitric oxide synthase and cyclooxygenase-2 gene expression (Chen et al 2001). Baicalein also has an anxiolytic-like effect (Liao et al 2003) and a protective effect against amnesia (Wang et al 2004).

Baicalin has been detected in rat plasma after oral administration of baicalein (Wakui et al 1992), suggesting that it is absorbed directly from the gastrointestinal tract. However, our previous in-vitro and in-vivo studies have demonstrated rapid absorption of baicalein, but poor absorption of baicalin, from the rat intestinal tract (Akao et al 2000). Moreover, baicalin, but not baicalein, has been detected in the plasma of rats administered baicalein orally, and baicalein is conjugated efficiently to



Baicalin: R = β-glucopyranurosyl
Baicalein: R = H (5,6,7-trihydroxyflavone)

Figure 1 Structures of baicalin and baicalein.

baicalin in rat hepatic and intestinal microsomes. Those observations indicated that baicalin itself was poorly absorbed from the rat gut, but was hydrolysed to baicalein by intestinal bacteria and then reconverted to its original form in the body after absorption. Although the liver seems to be the main site for metabolism of xenobiotics, rat jejunal microsomes show a higher level of UDP-glucuronate glucuronosyl transferase (UGT) activity for baicalein than that of hepatic microsomes (Akao et al 2004). Moreover, an in-vitro absorption experiment using everted rat jejunal sacs showed that very little of the baicalein absorbed was transferred to the inner (serosal) side of the sac, whereas baicalin appeared outside the sac, and not inside it (Akao et al 2004). In germ-free rats a large amount (30.4%) of baicalin was recovered in the intestinal lumen 4 h after baicalein administration. Those results indicated that, in the rat, a large portion of any baicalein absorbed was retained, transformed into baicalin within the intestinal mucosal cells, and then excreted into the intestinal lumen. It seems that baicalin efflux was mediated by multidrug resistance-associated protein 2 (MRP2, ABCC2), as the rate of efflux in everted jejunal sacs from Eisai hyperbilirubinaemic rats (EHBR, hereditarily MRP2-defective) was significantly lower (by 56.4%) compared with similar preparations from Sprague-Dawley rats (a wild-type strain) (Akao et al 2004).

It is unclear whether the unique intestinal disposition of baicalin and baicalein observed in rats also occurs in man. The human colonic adenocarcinoma cell line Caco-2 is well established and has been used to study the absorption and disposition of drugs in the human intestine (Artursson 1990; Meunier et al 1995), though it shows limited expression of some transporters and metabolizing enzymes. Ng et al (2005) reported that baicalein was transported bi-directionally (both apical to basolateral and basolateral to apical directions) and then the formed glucuronides were very low on a Caco-2 cell monolayer model. Moreover, the baicalin formed was mainly accumulated at the basolateral side, suggesting participation of MRP3 for baicalin transport (Zhang et al 2005). Those results implied that man is quite different from rat on intestinal disposition of baicalin and baicalein.

The aim of this study was to investigate in detail the human intestinal disposition of baicalin and baicalein using the Caco-2 cell monolayer model, and to examine the transporter mediating baicalin efflux.

Materials and Methods

Materials

Baicalin and baicalein of standard grade, 1-chloro-2,4-dinitrobenzene (CDNB), ciclosporin, genistein, probenecid, and vinblastine sulfate were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). p-Nitrophenyl β-D-glucuronide was from Nacalai Tesque, Inc. (Kyoto, Japan). Human small-intestinal microsomes were from In Vitro Technologies (MD, USA). A pre-stained molecular weight marker mixture was purchased from Bio-Rad Laboratories Inc. (Hercules, CA). The solvents used for HPLC were of special HPLC grade. All other reagents were of the best quality available commercially.

Cell and culture

Caco-2 cells (TC7 strain) were donated from Professor T. Takano, Faculty of Pharmaceutical Sciences, Teikyo University. The cells (passage number 25–35) were cultured in HAM's F12 medium with L-glutamine (ICBN Biochemicals Inc., Aurora, OH), along with 10% fetal bovine serum, 0.25% $NaHCO_3$, 0.007% (w/v) benzyl penicillin, and 0.014% (w/v) streptomycin sulfate, and grown in an atmosphere of 5% CO_2 with 90% relative humidity at 37°C in a 75-cm^2 culture flask for three days. For transport studies, the cells were seeded on Transwell polycarbonate membranes (24 mm, 0.4-μm pore size, 4.7 cm^2, Corning Coaster Corp., Cambridge, UK) in 6-well plates at a density of 1.0×10^5 cells/cm^2. Cells were fed every other day and monolayers for transport experiments were used from 21 to 28 days after seeding. The integrity of the monolayer was checked by determination of the permeability of fluorescein isothiocyanate-dextran (FITC-dextran, average molecular weight 10 500, Sigma-Aldrich Co., Japan).

Transport experiments

The cell layers were washed twice with warm 20 mM phosphate-buffered saline (PBS, pH 7.4) on the apical and basolateral sides. PBS solution containing 30 mM glucose and 1 mg mL^{-1} bovine serum albumin (BSA) was added to the apical chamber (1.5 mL), and Hank's balanced salt solution (HBSS in mM; 0.952 $CaCl_2$, 0.441 KH_2PO_4, 5.36 KCl, 0.812 $MgSO_4$, 0.385 Na_2HPO_4, 136.7 NaCl, 30 D-glucose, 10 HEPES, pH 7.4, 315 mOsm kg^{-1}) with 1 mg mL^{-1} BSA was added to the basolateral chamber (2.6 mL). Baicalin or baicalein, dissolved in dimethyl sulfoxide (DMSO), was added to either chamber at an adequate concentration (5, 10, 25 or 50 μM) with a final concentration of 5% (v/v) DMSO. Samples (40 μL) were withdrawn from both chamberss for analyses of baicalin and baicalein by HPLC. An equal volume of blank solution was replaced in both chambers immediately after each sampling. The recovery percents were calculated in molar basis from amounts of baicalin and baicalein recovered and amount (100%) of baicalin or baicalein added. FITC-dextran was added to either chamber as a negative control, and at the end of each analysis samples were analysed by fluorometry.

The apparent permeability coefficient (P_{app}) was calculated using the following equation:

$P_{app} = (dQ/dt)/A \times C_0$

where dQ/dt is the linear appearance rate of drug in the receiver solution transported, A is the surface area of the membrane, and C_0 is the initial drug concentration.

Experiments involving inhibition of baicalin efflux were carried out by incubating 50 μM or 0.1 mM baicalein together with agents such as ciclosporin (50 μM), vinblastine (50 μM), genistein (0.1 mM), probenecid (1 mM) or 1-chloro-2,4-dinitrobenzene (CDNB, 1 mM) on the apical side (mucosal surface) of the monolayer for 1 h under similar conditions as above.

Caco-2 cell microsomes

Caco-2 cells, cultured on four dishes (15 cm i.d.) for nine days after seeding, were harvested and washed twice with saline. The cells were then homogenized with 1.15% KCl containing 1 mM EDTA. After centrifugation at 10 000 *g* for 15 min, the micosomal fractions were precipitated by centrifuging the supernatant at 100 000 *g* for 60 min. The resulting microsomes were washed once with 1.15% KCl containing 10 mM EDTA, suspended in 50 mM Tris-HCl buffer (pH 7.2) containing 20% glycerol, 1 mM EDTA, and 1 mM dithiothreitol, and stored at −80°C until use.

Measurement of microsomal UGT activity

To measure the activity toward baicalein as an acceptor, the reaction mixture contained a suitable amount (100–200 μg) of microsomal protein, 2 μmol UDP-glucuronide triphosphate, 2 μmol $MgCl_2$, and 0.2 μmol D-saccharic acid 1,4-lactone in 0.2 mL 50 mM Tris-HCl buffer (pH 7.4). The reaction was started by addition of 20 nmol baicalein dissolved in DMSO and stopped with 50 μL 1M HCl after incubation for 5 min at 37°C. The extract obtained with 0.4 mL methanol was analysed for determination of baicalin by HPLC as reported by Akao et al (2004). The activity towards 4-nitrophenol as an acceptor was measured under similar conditions as that towards baicalein. The produced glucuronide (4-nitrophenyl β–D-glucuronide, pNP-GlcUA) was also determined using an HPLC system consisting of a LC-10AD liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a Shimadzu SPD-M10AVP diode array detector; a column of Chemcosorb 7-ODS-H (4.6 mm i.d. × 150 mm, Chemco Scientific Co., Ltd, Osaka, Japan), a mobile phase of MeOH–H_2O–AcOH (30:70:4, v/v), and a flow rate of 0.7 mL min^{-1}. pNP-GlcUA and 4-nitrophenol were clearly separated at retention times of 5 and 12.5 min, respectively, and pNP-GlcUA was determined by spectrophotometric detection at 315 nm. Protein was determined by the method of Lowry et al (1951).

Immunoblotting

Total cell lysates were prepared by lysing harvested cells in 10 mM Tris-HCl (pH 7.4) with 10 mM KCl and 1.5 mM $MgCl_2$, and 0.5% (w/v) SDS supplemented with a protease inhibitor fluid composed of fluoro-4-(2-aminoethyl)benzensulfonyl chloroide, aprotinin, E-64, and leupeptin (Nacalai Tesque, Inc., Kyoto). After sonication, samples together with MRP2 vesicles (membrane vesicles from S*f*9, *Spodoptera frugiperda*, expressing human MRP2, SOLVO Biotechnology, Budaors, Hungary) were fractionated by SDS/7.5% (w/v) PAGE and then transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA) by electroblotting. The membrane was then blocked for 1 h in PBS containing 5% (w/v) milk powder and 2% (w/v) Tween 20, followed by incubation for 1 h with the primary antibody (mouse monoclonal antibody M_2III-6, Kamiyama Biochemical Co., Seattle, WA). Immunoreactivity was visualized with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Amersham Biosciences Co., Piscataway, NJ) followed by enhanced chemiluminescence detection (ECL plus/ECL Western Blotting Detection System, Amersham Biosciences Co., Piscataway, NJ).

Measurement of vanadate-sensitive ATPase activity

ATPase activity was measured basically by the method of Sarkadi et al (1992) by determining the liberation of inorganic phosphate from ATP colorimetrically according to an assay system developed by SOLVO Biotechnology (Budaors, Hungary). The reaction mixture contained (in mM) 8 $MgCl_2$, 40 3-(*N*-morpholino)propanesulfonic-Tris (pH 7.0), 40 KCl, 1.6 dithiothreitol, 0.08 EGTA, 4 Na-azide, 0.8 ouabain, and 5 Mg-ATP, and MRP2-S*f*9 membrane protein was used at a final concentration of 400 μg mL^{-1} in 0.1 mL. Each reaction was carried out for 60 min at 37°C in duplicate. The vanadate-sensitive activity of baicalin or baicalein, which was dissolved in DMSO adjusted to a final concentration of 2% (v/v) in the reaction mixture, was calculated from the values with and without 1.2 mM Na_3VO_4 in the presence of baicalin or baicalein. Probenecid was used at 1 mM as a positive control.

Determination of baicalin and baicalein

Samples from the apical and basolateral chambers were immediately extracted with two volumes of methanol after acidification with 1 M HCl. The determination of baicalin and baicalein was carried out by HPLC, as reported by Akao et al (2004).

Statistical analysis

One-way analysis of variance for multiple comparisons and unpaired two-tailed Student's *t*-test were used to determine the statistical significance ($P < 0.05$) of calculated results between the experimental groups.

Results

Baicalein uptake and baicalin efflux on the Caco-2 cell monolayer

After baicalin (5 μM) had been loaded on the apical and basolateral sides of the Caco-2 cell monolayer, most of it was retained throughout the 1-h incubation period (Figure 2). On the other hand, when baicalein at 5 μM was loaded on the apical side, it decreased in a time-dependent manner until 53.3% remained after 1 h of incubation (Figure 3a). Although only a faint amount of baicalein was detectable on the basolateral

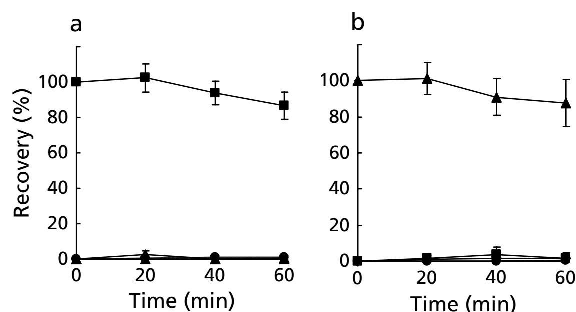


Figure 2 Recovery (%) of baicalin and baicalein on the apical and basolateral sides of the Caco-2 cell monolayer during 1-h incubation after baicalin loading at 5 μM on the apical (a) or basolateral (b) side. The amounts of baicalin (■) and baicalein (●) on the apical sides, and the amounts of baicalin (▲) and baicalein (◆) on the basolateral side, were measured at 0, 20, 40 and 60 min after baicalein loading. Each point represents the mean ± s.d. of three different experiments.

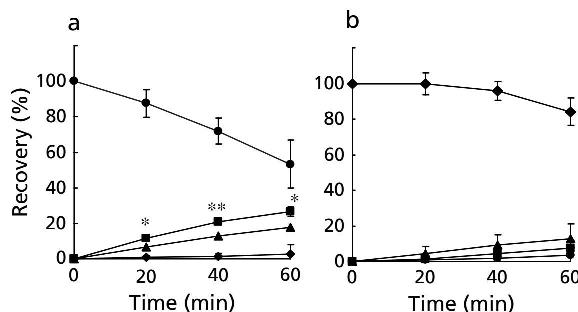


Figure 3 Recovery (%) of baicalin and baicalein on the apical and basolateral sides of the Caco-2 cell monolayer during 1-h incubation after baicalein loading at 5 μM on the apical (a) or basolateral (b) side. The procedure and symbols are the same as those in Figure 2. Each point represents the mean ± s.d. of three different experiments. *$P < 0.05$, **$P < 0.01$ compared with baicalin recovery on the basolateral side.

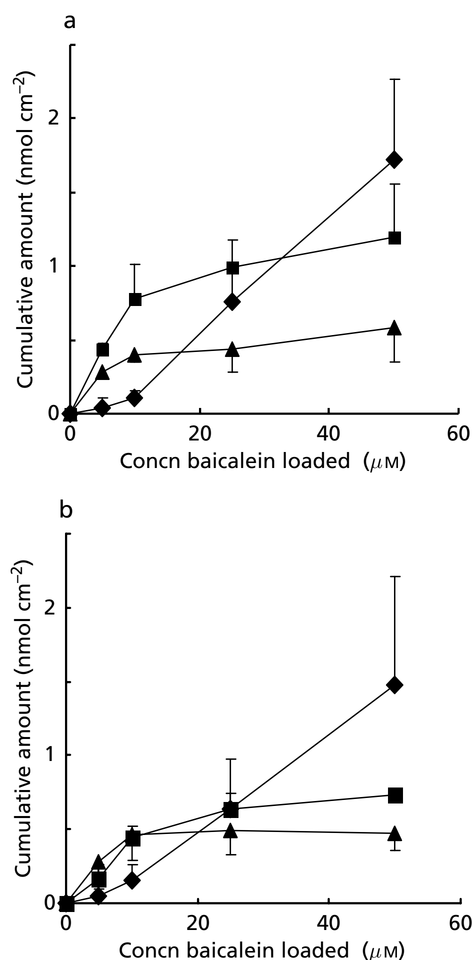


Figure 4 Effects of the concentration of baicalein on accumulation of baicalin and on transfer of baicalein. Baicalein was loaded on the apical (a) or the basolateral (b) side at concentrations of 5, 10, 25 or 50 μM. The amounts of baicalin accumulated onto the apical (■) and basolateral (▲) sides and the amount of baicalein transferred to the basolateral (◆) or apical (◆) side were measured 60 min after baicalein loading. Each point represents the mean ± s.d. of three different experiments.

side even after 1 h, baicalin was detectable on both sides and accounted for more than 90% of the baicalein taken up from the apical surface, the amount on the apical side being significantly larger than that on the basolateral side. Furthermore, when baicalein was loaded at higher concentrations of 10–50 μM, baicalin excretion from the apical surface was significantly (approximately 2-fold) higher and the amount accumulated onto either side showed a tendency to level off at 10 μM, suggesting saturation of baicalin excretion and absorption (Figure 4a). Meanwhile, the amount of baicalein transferred to the basolateral side increased markedly after baicalein was loaded at 10 μM. The P_{app} value of baicalein (at 50 μM) obtained from the apical to basolateral direction was $9.22 \pm 2.9 \times 10^{-6}$ cm s^{-1}, comparable with that reported by Ng et al (2005). These results indicated that, in Caco-2 cells, baicalein taken up was transformed to baicalin, which was then excreted mainly from the apical surface, before transfer of baicalein to the basolateral side.

After baicalein was loaded on the basolateral side at 5 μM, a similar phenomenon was observed, in spite of the low disappearance of baicalein from the basolateral side and the low accumulation of baicalin (Figure 3b), compared with that loaded on the apical side. Saturation of baicalin accumulation was also observed when baicalein was loaded at 5–50 μM on the basolateral side, and there was no significant difference between the baicalin amount on the apical side and that on the basolateral side (Figure 4b). The P_{app} value of baicalein (at 50 μM) obtained from the basolateral to apical direction was $7.88 \pm 3.9 \times 10^{-6}$ cm s^{-1}.

Glucuronidation of baicalein to baicalin by microsomes of Caco-2 cells

UDP-glucuronosyltransferase activity towards baicalein and 4-nitrophenol as acceptors was measured in microsomes of Caco-2 cells. The UDP-glucuronosyltransferase activity (V_{max}) towards baicalein to form baicalin was lower than that towards 4-nitrophenol, or that towards baicalein in microsomes

Table 1 UDP-glucuronosyltransferase activity (V_{max}) and apparent K_m value toward baicalin and 4-nirophenol in Caco-2 and human intestinal microsomes

	V_{max} (nmol min^{-1} (mg protein)$^{-1}$)	K_m (μM)
Caco-2 cell		
Baicalein	3.4 ± 0.78	22.4 ± 5.5
4-Nitrophenol	9.9 ± 0.74	87.8 ± 23.8
Human intestine (pooled)		
Baicalein	10.8	23.9

Values are expressed as mean ± s.d., n = 3 in Caco-2 cell microsomes and averages in duplicate in human intestinal microsomes (pooled).

of human small intestine (Table 1), the apparent K_m (22.4μM) being lower than that towards 4-nitrophenol (87.8μM) and almost the same as that towards baicalein in microsomes of human small intestine. Thus baicalein absorbed in Caco-2 cells was converted efficiently to baicalin.

Effects of MDR1 and MRP2 substrates (inhibitors) on baicalin efflux

Baicalin seemed to be accumulated onto either side of the Caco-2 cell monolayer through either a carrier or a transporter, as judged from the observed saturation of baicalin accumulation (Figure 4). Baicalin accumulation was completely inhibited by addition of both 10 mM NaN_3 and 25 mM 2-deoxy-glucose (data not shown), suggesting participation of an ATP-dependent transporter. However, the accumulation of baicalin onto either side was not suppressed, but rather enhanced by addition of MDR1/P-glycoprotein substrates such as ciclosporin or vinblastine (Sonneveld & Wiemer 1997) by 105 and 150%, respectively, on the apical side. Meanwhile, the baicalin accumulation was significantly inhibited by adding MRP2 substrates such as probenecid or genistein, and MRP2 presubstrate such as CDNB (Horikawa et al 2002), as shown in Figure 5.

Immunoblot analysis of Caco-2 cells

To demonstrate whether an MRP2 pump was present in Caco-2 cells, the cells were disrupted by sonication in the presence of a mixture of protease inhibitors, and subjected to immunoblot analysis, together with membrane vesicles from *Sf9* expressing human MRP2. Caco-2 cells showed a band that was immunostained using a MRP2-specific monoclonal antibody. This band showed a molecular weight of approximately 190 kDa, larger than a band from MRP2 membrane vesicles used as a positive control (Figure 6).

Stimulation of ATPase activity of human MRP2 by baicalin

By using high-level expression of human MRP2 in *Sf9* cells, we examined the effects of baicalin and baicalein on ATPase activity in a membrane environment. As shown in Table 2, the vanadate-sensitive ATPase activity of MRP2 was relatively low in the absence of baicalein and baicalin (4.67 nmol (mg protein)$^{-1}$ min^{-1}), but was stimulated by baicalin, but not by baicalein, in a dose-dependent manner. Baicalin at 1 mM showed activity close to that obtained with 1 mM probenecid, a well known substrate of MRP2. These results indicated that human MRP2 contributed to baicalin efflux.

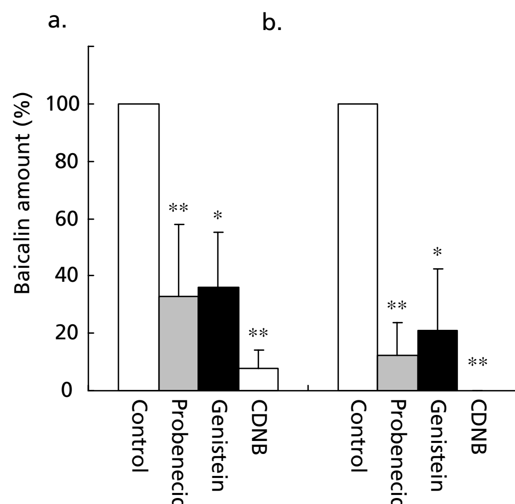


Figure 5 Effects of MRP2 substrates on baicalin accumulation. Probenecid, genistein or CDNB was added on the apical side of the Caco-2 cell monolayer at a concentration of 1, 0.1 or 1 mM, respectively, together with 25 μM baicalein. The amounts of baicalin accumulated onto the apical (a) and basolateral (b) sides were measured 60 min after baicalein loading. The amounts were expressed as percentages against control (100%). Each value represents the mean ± s.d. of three different experiments. *$P < 0.05$, **$P < 0.01$ compared with control.

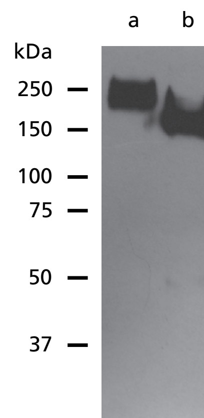


Figure 6 Immunoblot analysis of Caco-2 cells using a MRP2-specific monoclonal antibody. Caco-2 cells (lane a, 22 μg protein) and *Sf9* membrane vesicles expressing human MRP2 (lane b, 0.25 μg protein) were analysed by SDS-PAGE with 7.5% polyacrylamide gel. Molecular weight (kDa) standards are shown to the left.

Table 2 Effects of baicalin, baicalein and probenecid on vanadate-sensitive ATPase of *Sf*9 membrane vesicles expressing human MRP2

	Control	Baicalin				Baicalein		Probenecid
		4 μM	20 μM	100 μM	1000 μM	20 μM	100 μM	1000 μM
ATPase activity (nmol min^{-1} mg^{-1})	4.67	5.50	9.25	9.83	14.2	4.83	5.25	18.5

Membrane ATPase activity was measured for 60 min at 37°C in the presence of 4 mM ATP. Baicalin (4, 20, 100 and 1000 μM), baicalein (20 and 100 μM) or probenecid (1000 μM) were added to the reaction mixture.

Discussion

When administered orally, baicalin, a predominant flavone glucuronide of Scutellariae Radix, shows a unique metabolic fate in rats. It is poorly absorbed, but absorbed as the aglycone baicalein, transformed by intestinal bacteria in the intestinal tract, and then restored to its original form in rat tissues (Akao et al 2000). Accordingly, when baicalein is administered orally, baicalin, and not baicalein, is detected in rat plasma (Akao et al 2000). Meanwhile, a surprisingly large amount of baicalin, which has been excreted from intestinal epithelial cells via glucuronidation, has also been recovered in the intestinal tract after administration of baicalein (Akao et al 2004), indicating an important role of the intestine in the first-pass effect of baicalein absorbed. Thus, in this study, it was clarified that a similar process was involved in the absorption and excretion of baicalein even in the human intestine, from which the Caco-2 cell line is derived.

In the Caco-2 cell monolayer, baicalin was hardly absorbed (Figure 2), similar to results obtained using rats in-vitro and in-vivo (Akao et al 2004), also as reported by Zhang et al (2005). Baicalein loaded on the apical side of the monolayer at a low concentration of 5 μM decreased on the same side, but was hardly transferred to the basolateral side (Figure 3a), similar to a study using rat everted jejunal sacs in which less than 1% of baicalein absorbed from the mucosal surface was transferred to the serosal side (Akao et al 2004). Most (more than 90%) of the baicalein taken up in Caco-2 cells was glucuronidated into baicalin, as confirmed from the UGT activity in Caco-2 cell microsomes (Table 1), and the baicalin formed was excreted mainly from the apical surface. Even when baicalein was loaded at high concentrations (more than 10 μM), the amount of baicalin excreted from the apical surface was 2-fold larger than that absorbed to the basolateral side, but became saturated at a baicalein concentration of 10 μM (Figure 4a). However, Ng et al (2005) and Zhang et al (2005) reported that baicalin was preferentially absorbed to the basolateral side after baicalein loading at the apical side. Although reasons for the discrepancy between their and our results were obscure, one might be due to differences in the type of solution in the basolateral chambers, where they have used PBS solution but we used Hank's balanced salt solution. The use of the Hank's balanced salt solution also might cause poor uptake of baicalein from the basolateral surface (Figure 3b), because divalent heavy metal ionic species involved in the solution seemed to let baicalein be insoluble. When baicalein was loaded at the basolateral side, baicalin was accumulated onto the both sides at an almost similar amount, with the saturation at 10 μM baicalein. This might have been caused by the uneven distribution (insolubility) of baicalein taken in the cells. On the other hand, baicalein was transferred with both apical-to-basolateral and basolateral-to-apical directions at baicalein concentrations of more than 10 μM, and the transferred amount increased linearly in a concentration-dependent manner (Figure 4). The P_{app} values of baicalein (at 50 μM) obtained from apical-to-basolateral and basolateral-to-apical directions were $9.22 \pm 2.9 \times 10^{-6}$ and $7.88 \pm 3.9 \times 10^{-6}$ cm s^{-1}, respectively, comparable with those reported by Ng et al (2005). As Ng et al (2005) and Zhang et al (2005) used only baicalein concentrations of 17 and 50 μM, respectively, in the transport experiments using Caco-2 monolayer, they reported that baicalein was transported well. On the transport experiment using the Caco-2 cell monolayer model, especially for compounds glucuronidated in the cells such as baicalein, it seems to be important to study at the low concentrations. These results indicated that baicalein taken up by Caco-2 cells was glucuronidated before transfer through the basolateral surface and that the baicalin formed was excreted, but after saturation of baicalin excretion or baicalein glucuronidation, the remaining baicalein was transferred to the basolateral side. Thus, even in man, it was suggested that after oral intake of baicalein at low daily doses, only baicalin, and not baicalein, would be detected in the blood, similar to the situation in rats (Akao et al 2000). Intestinal disposition and enteric recycling, even in man, seems to be more important than hepatic disposition and enterohepatic cycling, respectively, during first-pass metabolism of baicalein, in view of the metabolism of flavonoids such as genistein and apigenin via enteric recycling (Chen et al 2003).

In rat everted jejunal sacs, baicalin formed in mucosal cells after baicalein loading was excreted mostly from the mucosal surface through the mediation of MRP2 (Akao et al 2004). Also in the Caco-2 cell monolayer, twice as much baicalin that had formed in the cells was excreted from the apical surface than was being absorbed from the basolateral surface over a concentration range of 10–50 μM (Figure 4a). The efflux of baicalin from the apical surface was not inhibited, but rather tended to be stimulated by MDR1/P-glycoprotein substrates such as ciclosporin and vinblastine, coincident with the observation that in rats, co-administration of ciclosporin or quinidine promoted the active transport of baicalin into bile and reduced its level in blood (Tsai & Tsai 2004). Meanwhile, the efflux of baicalin was inhibited by MRP2 substrates such as probenecid and genistein and MRP2 presubstrate such as CDNB. Further-

more, baicalin, but not baicalein, enhanced MRP2 ATPase activity in a concentration-dependent manner; 1 mM baicalin showed almost a similar level of stimulation to 1 mM probenecid (Table 2). The Caco-2 cells used in these experiments expressed MRP2, as revealed by Western blotting analysis (Figure 6). As MRP2 is found in the apical side of the intestine epithelium (Borst et al 1999), these results indicated that the baicalin formed in Caco-2 cells was excreted from the apical surface through the mediation of MRP2, as is the case in rat intestine, suggesting that the metabolic fate of baicalein and baicalin was similar to that in the human intestine. Caco-2 cells also express MRP3 at the basolateral side (Borst et al 1999), similar to the gut. As baicalin was also absorbed to the basolateral side to some extent, baicalin might be a substrate of transporters at the basolateral side, such as MRP3.

Conclusions

The results obtained using a Caco-2 cell monolayer model clearly showed that baicalein taken in the cells was glucuronidated to baicalin, which in turn was excreted mainly from the apical surface through MRP2, similar to the disposition of baicalin and baicalein in rat intestine (Akao et al 2004).

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