Effect of the Flavonoids Biochanin A and Silymarin on the P-Glycoprotein-Mediated Transport of Digoxin and Vinblastine in Human Intestinal Caco-2 Cells

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Purpose. The purpose of this study was to investigate the effects of flavonoids biochanin A and silymarin on intestinal absorption of P-gp substrates by determining their effects on P-gp-mediated efflux in Caco-2 cells.

Methods. The cellular accumulation and bidirectional transport of digoxin and vinblastine in Caco-2 cells were determined in the presence and absence of flavonoids.

Results. The 1.5-h accumulation of digoxin and vinblastine in Caco-2 cells was significantly increased by 50 μM biochanin A or silymarin, and this effect was flavonoid-concentration dependent. The AP-to-BL transport of digoxin was significantly increased, whereas the BL-to-AP transport was significantly decreased by 50 μM biochanin A or 75 μM silymarin. At 150 μM concentrations of biochanin A or silymarin, mean transport ratios ($P_{app,B\text{-}A}/P_{app,A\text{-}B}$) of 1.62 and 4.48, respectively, compared with the control ratio of 43.4, were obtained.

Conclusion. These results indicate that biochanin A and silymarin can inhibit P-gp-mediated efflux in Caco-2 cells, suggesting they could potentially increase the absorption/bioavailability of coadministered drugs that are P-gp substrates.

KEY WORDS: P-glycoprotein; biochanin A; silymarin; intestinal transport; drug bioavailability.

INTRODUCTION

P-glycoprotein (P-gp) has received enormous attention from scientists in both cancer research and pharmaceutical sciences fields since its discovery in 1976 (1). It is now well accepted that this drug efflux transporter is one of the major mechanisms responsible for cancer multidrug resistance (2) and for drug–drug and drug–food interactions (3,4). In cancer cells, the ATP-dependent extrusion of a broad range of important chemotherapeutic agents, such as vinca alkaloids, anthracyclines, epipodophyllotoxins, and taxol (5), by P-gp results in inadequate intracellular accumulation of these cytotoxic agents for efficient cell killing, producing multidrug resistance. It has been well established that P-gp is also expressed in many normal tissues besides tumor cells, including the intestinal epithelium, blood–brain barrier, hepatocytes and renal tubular cells, and plays an important role in drug absorption, elimination, and distribution (6). It has been shown that the oral absorption and brain penetration of P-gp substrates are significantly lower in normal mice compared with mdr1a (–/–) mice (7) and oral bioavailability, brain penetration, biliary, and renal secretion of P-gp substrates can be significantly altered by simultaneous administration of P-gp modulators (4,8,9). Furthermore, the substrate specificity of P-gp extensively overlaps that of CYP3A4 (10), an important drug metabolizing enzyme whose substrate spectrum covers about 50% of drugs currently in the market (11). Therefore, P-gp may even act synergistically with CYP3A4 in limiting oral drug absorption/bioavailability because P-gp-mediated cellular efflux would result in repeated circulation of a P-gp substrate between intestinal lumen and enterocytes, and thus increase the chance of metabolism, leading to a reduced bioavailability (12).

Biochanin A and silymarin are flavonoids, a class of compounds currently attracting considerable scientific and therapeutic interest. Flavonoids are the most abundant polyphenols present in the human diet and are components found in vegetables, fruits, and plant-derived beverages, such as tea and red wine. Epidemiology and animal studies have suggested that a high intake of flavonoids may be linked to a reduced risk of cancer (13,14), coronary disease (15), and osteoporosis (16). Flavonoids have biochemical and pharmacological activities beneficial for human health, including antioxidant, anticarcinogenic, anti-inflammatory, antiproliferative, antiangiogenic, and antiestrogenic (estrogenic) effects (17), and ingestion produces no or very little toxicity (18). Because of the above-mentioned properties, a plethora of the over-the-counter dietary supplements or herbal products mainly containing flavonoids are now commercially available, with claims of various health benefits. Nowadays, there is an increasing interest in alternative medicine, and it is estimated that herbal products are ingested by about 10% or more of the general population and 30–70% of individuals with specific disease states (19–21). Because herbal products are usually marketed as dietary supplements and thus do not require FDA approval for their efficacy and safety before marketing, potential drug interactions are not extensively evaluated. However, there is increasing evidence indicating that the use of herbal products could result in clinically important and life-threatening interactions (22,23); therefore, careful evaluation of potential interactions between herbal remedies and conventional medicine is urgently needed. Biochanin A and silymarin are the major components of the marketed and widely used herbal products red clover extracts, such as Promensil (Novogen, Inc., Samford, CT, USA), and milk thistle, respectively. Red clover extracts are commonly used for the prevention or relief of postmenopausal symptoms, such as hot flashes, bone loss, and for maintaining men's prostate health. Commercially available products typically contain 40 mg of total isoflavones per tablet (24), of which approximately 26 mg is biochanin A (25). Milk thistle is currently used for the treatment of cirrhosis, chronic hepatitis, and liver disease associated with alcohol consumption and environmental toxin exposure (26), and commercially available products typically contain about 150–160 mg of silymarin per tablet (27). Upon consumption of these herbal products, high concentrations of biochanin A or silymarin in intestine could be expected, raising a question of potential drug-flavonoid interactions.

Previously, we demonstrated that both biochanin A and silymarin can inhibit P-gp activity and increase the accumu-

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lation of daunomycin in P-gp expressing breast cancer cells (28), therefore suggesting the possibility that coadministration of these compounds with drugs that are P-gp substrates may result in altered absorption/bioavailability of these drugs. The objective of this study was therefore to investigate the potential effects of biochanin A and silymarin on the intestinal absorption of conventional drugs that are P-gp substrates by examining their effects on the uptake and bi-directional transport of model P-gp substrates in human intestinal Caco-2 cells.

MATERIALS AND METHODS

Materials

Verapamil, biochanin A, and silymarin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Silymarin refers to collectively silybinin (major component), silydianin, and silychristin (14), and the molar concentration was calculated based on the molecular weight of silybinin. ^{3}H-digoxin (37 Ci/mmol), ^{3}H-vinblastine (VBL; 9 Ci/mmol), and ^{3}H-mannitol (15 Ci/mmol) were purchased from Perkin-Elmer Life Sciences, Inc. (Boston, MA, USA), Moravek Biochemicals, Inc. (Brea, CA, USA) and American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA), respectively. Eagle's Minimum Essential Medium (MEM), fetal bovine serum, 100× MEM nonessential amino acids solution, 100× antibiotic-antimycotic solution, and Hank's balanced salt solution (HBSS) were from Gibco BRL (Buffalo, NY, USA). Caco-2 cells were kindly provided by Dr. Amrita Kamath (Bristol Myers Squibb, Princeton, NJ, USA).

Cell Culture

Caco-2 cells were cultured in MEM culture medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids (Gibco BRL, Buffalo, NY, USA) and 1% antibiotic-antimycotic solution (Gibco BRL, Buffalo, NY, USA). All cells were incubated at 37°C in a humidified atmosphere with 5 % CO_2/95 % air. Culture medium for Caco-2 cells was replaced every other day and the cells were passaged at about 90% confluence, using trypsin–EDTA. Caco-2 cells with passage number 87–103 were used for experiments. For the uptake studies, Caco-2 cells were seeded onto 35-mm^2 dishes at a density of approximately 5×10^5 cells per dish and used for experiments 10–12 days postseeding. For transport studies, Caco-2 cells were seeded in Transwell polycarbonate inserts (6-well, 0.4-μm pore size, Corning Costar Co., Cambridge, MA, USA) at a density of approximately 10^5/cm^2 and used for experiments after 21–27 days postseeding.

Western Blot Analysis of P-gp

Cells grown in 35-mm^2 culture dishes were washed with HBSS and harvested using a rubber policeman. Total cell lysates were prepared by adding the lysis buffer (20 mM Tris pH 7.5, 120 mM NaCl, 100 mM NaF, 1% Nonidet P-40 (NP-40), 200 μM sodium orthovanadate (Na_3VO_4), 50 mM β-glycerolphosphate, 10 mM sodium pyrophosphate (NaPPi), 4 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM benzamidine, 10 μg/mL leupeptin, and 10 μg/mL aprotinin) to the harvested cells. The cells were kept on ice for 30 min. The soluble extracts were obtained by centrifuging the cell lysates at 13,000 g for 20 min. The protein concentrations of the soluble extracts were obtained by BCA protein assay (Pierce, Rockford, IL, USA). Predetermined amounts of proteins (50 μg) were electrophoresed on 7.5% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes (Invitrogen, Grand Island, NY, USA). Membranes were then blocked overnight at 4°C in Tris-buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) fat-free dry milk (Bio-Rad, Hercules, CA, USA), and then incubated first with the primary antibody C219 (DAKO, Carpinteria, CA, USA) and secondary antibody anti-mouse IgG HRP (Amersham, Piscataway, NJ, USA) at room temperature for 2 and 1.5 h, respectively. Membranes were then washed and detected with ECL detection reagent (Amersham, Piscataway, NJ, USA). Cell lysates from MCF-7/sensitive and MCF-7/ADR cells were used as the negative and positive control for P-gp, respectively.

Cellular Accumulation Studies

Cells grown in 35-mm^2 culture dishes for 10–12 days were washed twice with the assay buffer (137 mM NaCl, 54 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, pH 7.4) and then incubated with 11 nM ^{3}H-digoxin or 27 nM ^{3}H-VBL containing specified concentrations of biochanin A, silymarin or the vehicle only (0.3% DMSO, used as control) at room temperature for 1.5 h. Verapamil (100 μM), a known P-gp inhibitor, was used as a positive control. The accumulation of ^{3}H-digoxin or ^{3}H-VBL was stopped by rinsing the cells four times with ice-cold buffer (137 mM NaCl, and 14 mM Trisbase, pH 7.4). Cells were then solubilized using a solution of 0.3N NaOH and 1 % SDS, and aliquots were used to determine the radioactivity by liquid scintillation counting (1900 CA, Tri-Carb Liquid Scintillation Analyzer, Packard) and protein content using the BCA protein assay. Results were normalized for the protein content of the cells in each dish and ^{3}H-digoxin or ^{3}H-VBL accumulation values were expressed as percent accumulation in the control group.

Transport Studies

Transport experiments using Caco-2 cell monolayers were performed similarly as described previously (29). Briefly, Caco-2 cell monolayers with TEER values higher than 500 Ω·cm^2 were washed with warm HBSS twice for 30 min. Transport buffer (HBSS) containing 15.4 nM ^{3}H-digoxin was loading on the apical (1.5 mL) or basolateral (2.6 mL) chamber with HBSS (without ^{3}H-digoxin) added to the opposite chamber. Whenever the test compounds (biochanin A or silymarin) or verapamil (positive control) were used, they were added to both apical and basolateral chambers at specified concentrations 15 min before the addition of ^{3}H-digoxin. The same concentration of the vehicle (0.3% DMSO) was also added to the control group in a similar manner. All solutions were prepared immediately before each experiment. Samples (200 μL) were taken from the receiver side for scintillation counting after incubating the cell monolayers at 37°C for 30, 60, and 90 min and replaced with fresh transport buffer. In some transport experiments, Caco-2 cell monolayers were washed with ice-cold buffer (137 mM NaCl, and 14 mM Trisbase, pH 7.4) for four times and the cells solubilized using a solution of 0.3N NaOH and 1 % SDS at the end of the transport studies (90 min). The radioactivity and protein con-

tent of these cell lysates were then determined and ^{3}H-digoxin accumulation levels in the monolayer Caco-2 cells were obtained in a similar manner as that for the uptake studies. For all the transport experiments, the apparent permeability coefficients (P_{app}) of ^{3}H-mannitol, a paracellular marker, across Caco-2 cell monolayers in both apical to basolateral (AP-to-BL) and basolateral to apical (BL-to-AP) directions were also measured in triplicate in parallel experiments to ensure monolayer integrity. None of the apparent permeability coefficients of ^{3}H-mannitol across Caco-2 cells measured in all the experiments were greater than 1.00×10^{-6} cm/s and no significant differences between AP-to-BL and BL-to-AP transport of ^{3}H-mannitol were observed (data not shown).

Calculation of Transport Data

The apparent permeability coefficients (P_{app}) across Caco-2 cell monolayers in both AP-to-BL ($P_{app,A\text{-}B}$) and BL-to-AP ($P_{app,B\text{-}A}$) directions were calculated as follows:

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_0}$$

where $\Delta Q/\Delta t$ is the rate of the radiolabeled compounds (^{3}H-digoxin or ^{3}H-mannitol) appearing in the receiver chamber; C_0 is the initial concentration of the radiolabeled compound in the donor chamber; and A is the cell monolayer surface area (4.71 cm^2).

The apparent K_i values of biochanin A and silymarin for inhibiting P-gp-mediated efflux based on ^{3}H-digoxin BL-to-AP transport data were calculated by the previously reported equations (30):

$$K_{i,} = [(P_I / P_0) /(1 - P_I / P_0)] \cdot [I]$$

$$P_0 = P_{app,B\text{-}A,\,1} - P_{app,B\text{-}A,\,3}$$

$$P_I = P_{app,B\text{-}A,\,2} - P_{app,B\text{-}A,\,3}$$

$P_{app,B\text{-}A,\,1}$, $P_{app,B\text{-}A,\,2}$, and $P_{app,B\text{-}A,\,3}$ are the apparent permeability coefficients for the transport of ^{3}H-digoxin across Caco-2 cell monolayers in BL-to-AP direction in the absence of any inhibitors, in the presence of the test compound and in the presence of 100 μM verapamil (complete inhibition of P-gp), respectively. [I] is the concentration of the test compound. P_0 and P_I are the active efflux components of the permeability coefficient in the absence of any inhibitors and in the presence of the test compound, respectively. Based on the same principles of the above equations, the apparent K_i values were also calculated by using ^{3}H-digoxin AP-to-BL transport data (denoted as $K_{i,}'$) by the following equations:

$$K_{i,}' = [(P_I' / P_0') /(1 - P_I' / P_0')] \cdot [I]$$

$$P_0' = P_{app,A\text{-}B,\,3} - P_{app,A\text{-}B,\,1}$$

$$P_I' = P_{app,A\text{-}B,\,3} - P_{app,A\text{-}B,\,2}$$

$P_{app,A\text{-}B,\,1}$, $P_{app,A\text{-}B,\,2}$, and $P_{app,A\text{-}B,\,3}$ are the apparent permeability coefficients for the transport of ^{3}H-digoxin in the AP-to-BL direction in the absence of any inhibitors, in the presence of the test compound and in the presence of 100 μM verapamil (complete inhibition of P-gp), P_0' and P_I' are the active efflux components in the absence of any inhibitors and in the presence of the test compound, respectively.

Statistical Analysis

The differences between the mean values were analyzed for significance using one-way analysis of variance followed by Dunnett test. P values < 0.05 were considered statistically significant.

RESULTS

Effects on the Cellular Accumulation of ^{3}H-Digoxin and ^{3}H-VBL in Caco-2 Cells

To investigate the effects of biochanin A and silymarin on P-gp-mediated cellular efflux in Caco-2 cells, we first confirmed the presence of P-gp in the Caco-2 cells used in our experiments by Western blot analysis (data not shown). P-gp can be clearly detected in Caco-2 cells after cell growth for 8 days and in the positive control MCF-7/ADR cells (31). P-gp was not detectable in the negative control MCF-7/sensitive cells, consistent with a previous report (31). The 1.5-h cellular accumulation of ^{3}H-digoxin, a well-known P-gp substrate, in Caco-2 cells (Fig. 1A) was significantly increased by 150 μM biochanin A, 150 μM silymarin, or 100 μM verapamil (583 ± 39.6%, 442 ± 22.9%, and 1570 ± 52.9% of the control, respectively; $p < 0.001$ for all of the three groups). Similarly, the 1.5-h accumulation of ^{3}H-VBL, another well-known P-gp substrate, in Caco-2 cells (Fig. 1B) was also significantly increased by biochanin A, silymarin, and verapamil under the above-mentioned conditions (640 ± 48.9%, 692 ± 25.8% ,and 837 ± 61.5% of the control, respectively; $p < 0.001$ for all of the three groups). In addition, the increase of ^{3}H-digoxin accumulation in Caco-2 cells by biochanin A and silymarin was also shown to be flavonoid-concentration dependent (Fig. 2) with significant effects produced at the lowest tested concentration (50 μM) for both compounds. These results indicate that biochanin A and silymarin can inhibit P-gp-mediated cellular efflux in Caco-2 cells.

Effects on the Transport of ^{3}H-Digoxin across Caco-2 Monolayers

As indicated by the cellular accumulation studies, biochanin A and silymarin may inhibit P-gp-mediated cellular efflux in Caco-2 cells, suggesting their possible enhancing effects on the absorption of P-gp substrates upon coadministration. This possibility was then tested by examining the effects of biochanin A and silymarin on the transport of ^{3}H-digoxin across Caco-2 cell monolayers in both AP-to-BL and BL-to-AP directions. As shown in Fig. 3, both AP-to-BL and BL-to-AP transport values for ^{3}H-digoxin across Caco-2 cell monolayers were approximately linear with incubation times up to 1 h under all the tested conditions, and thus the P_{app} values for ^{3}H-digoxin transport were calculated using the 1-h transport data. As shown in Table I, the apparent permeability coefficient for the BL-to-AP transport of ^{3}H-digoxin ($P_{app,A\text{-}B}$: 12.6 ± 1.27 × 10^{-6} cm/s) was much higher than that

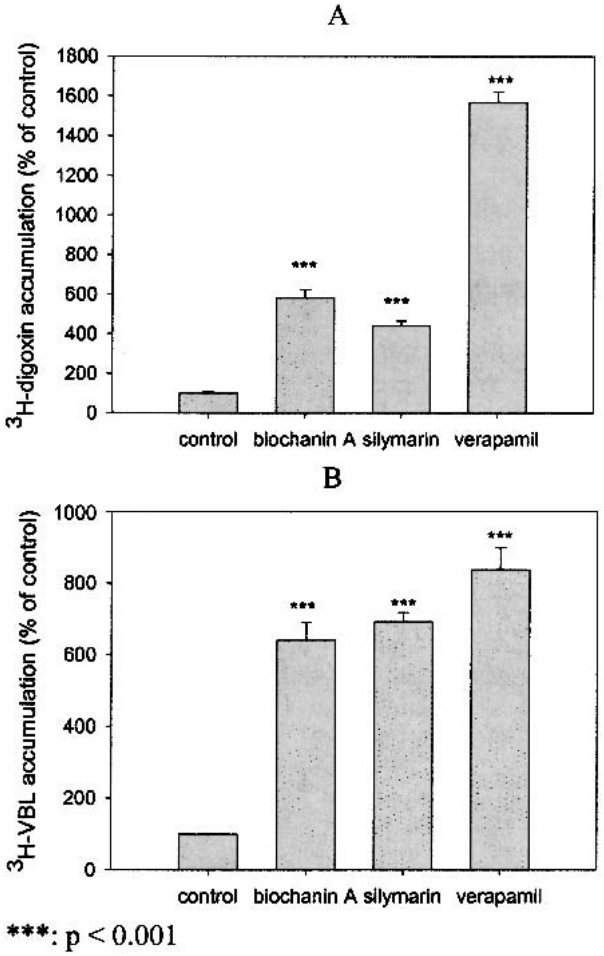

Fig. 1. Effects of biochanin A and silymarin on the cellular accumulation of ^{3}H-digoxin and ^{3}H-vinblastine (VBL) in Caco-2 cells. The 1.5-h cellular accumulation of ^{3}H-digoxin (11 nM; A) and ^{3}H-VBL (27 nM; B) in Caco-2 cells grown in 35-cm^2 culture dishes for 10–12 days in the presence of vehicle only (0.3% DMSO, control), 150 μM biochanin A, 150 μM silymarin, or 100 μM verapamil (positive control) was determined as described in the Materials and Methods section. Data are expressed as mean ± SE, n = 9, for ^{3}H-digoxin accumulation and n = 15 for ^{3}H-VBL accumulation.

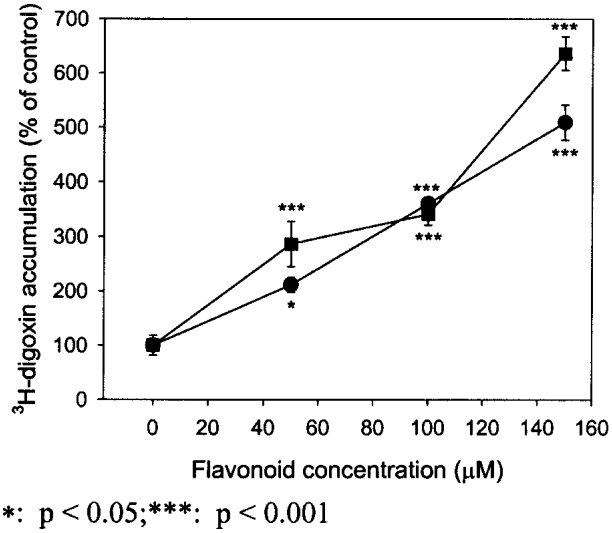

Fig. 2. Concentration-dependent effects of biochanin A and silymarin on ^{3}H-digoxin accumulation in Caco-2 cells. The 1.5-h cellular accumulation of ^{3}H-digoxin (11 nM) in Caco-2 cells grown in 35-cm^2 culture dishes for 10–12 days in the presence of varied concentrations of biochanin A (●) and silymarin (■) was determined as described in the Materials and Methods section. Data are expressed as mean ± SE, n = 3.

for the AP-to-BL transport ($P_{app,B\text{-}A}$: 0.29 ± 0.049 × 10^{-6} cm/s) with a mean transport ratio ($P_{app,B\text{-}A}$ / $P_{app,A\text{-}B}$) of 43.4, consistent with the involvement of P-gp-mediated efflux of ^{3}H-digoxin in these cells. In the presence of 150 μM biochanin A (Table I), the $P_{app,A\text{-}B}$ was significantly increased (from 0.29 ± 0.049 × 10^{-6} cm/s to 3.14 ± 0.199 × 10^{-6} cm/s, $p < 0.001$), whereas the $P_{app,B\text{-}A}$ was significantly decreased (from 12.6 ± 1.27 × 10^{-6} cm/s to 5.16 ± 0.48 × 10^{-6} cm/s, $p < 0.001$), resulting in a mean transport ratio of 1.64. In the presence of 150 μM silymarin (Table I), the $P_{app,A\text{-}B}$ was also significantly increased (from 0.29 ± 0.049 × 10^{-6} cm/s to 1.15 ± 0.15 × 10^{-6} cm/s, $p < 0.05$) and $P_{app,B\text{-}A}$ significantly decreased (from 12.6 ± 1.27 × 10^{-6} cm/s to 5.13 ± 0.95 × 10^{-6} cm/s, $p < 0.001$), resulting in a mean transport ratio of 4.48. The positive control verapamil (100 μM; Table I), increased $P_{app,A\text{-}B}$(from 0.29 ± 0.049 × 10^{-6} cm/s to 3.77 ± 0.42 × 10^{-6} cm/s, $p < 0.001$), and decreased $P_{app,B\text{-}A}$(from 12.6 ± 1.27 × 10^{-6} cm/s to 3.53 ± 0.48 × 10^{-6} cm/s, $p < 0.001$) as expected, and resulted in a mean transport ratio of 0.94, indicating complete inhibition of P-gp. The calculated K_i values of biochanin A and silymarin for inhibiting P-gp-mediated cellular efflux of ^{3}H-digoxin are listed in Table I. The K_i value of biochanin A based on the AP-to-BL transport data is similar to that based on the BL-to-AP transport data (33.2 μM and 32.7 μM, respectively). However, the K_i value of silymarin based on the AP-to-BL transport data is much higher than that based on the BL-to-AP transport data (457 μM and 30.4 μM, respectively), indicating its stronger effects on the BL-to-AP transport of ^{3}H-digoxin than on the AP-to-BL transport. The accumulation of ^{3}H-digoxin in Caco-2 monolayer cells measured at the end of the AP-to-BL (apical loading) and BL-to-AP transport studies (Fig. 4) were significantly elevated by 150 μM biochanin A (307 ± 12.9% and 155 ± 11.7% of the control, respectively; $p < 0.05$ for both apical and basolateral loading) and 100 μM verapamil (685 ± 83.1% and 217 ± 14.9% of the control, respectively; $p < 0.001$ for both apical and basolateral loading). Silymarin (150 μM) also increased ^{3}H-digoxin accumulation (Fig. 4) (159 ± 10.1% and 124 ± 10.1% of the control after apical and basolateral loading, respectively), although the effects are not statistically significant. All these results indicate that both biochanin A and silymarin can inhibit P-gp-mediated cellular efflux and thus increase the cellular accumulation, AP-to-BL transport, and decrease BL-to-AP transport of ^{3}H-digoxin. Biochanin A had stronger effects than silymarin, when used at the same concentration; however, it seems not to be able to completely block P-gp at least at the concentrations tested. The concentration-dependent effects of biochanin A and silymarin on ^{3}H-digoxin transport were also investigated. As shown in Tables II and III, the effects of both flavonoids were shown to be concentration-dependent and significant effects can be observed at 50 μM of biochanin A (Table II) for both AP-to-BL and BL-to-AP transport, and at 150 μM (Table I) and 75 μM (Table III) of silymarin for the AP-to-BL and BL-to-AP transport, respectively.

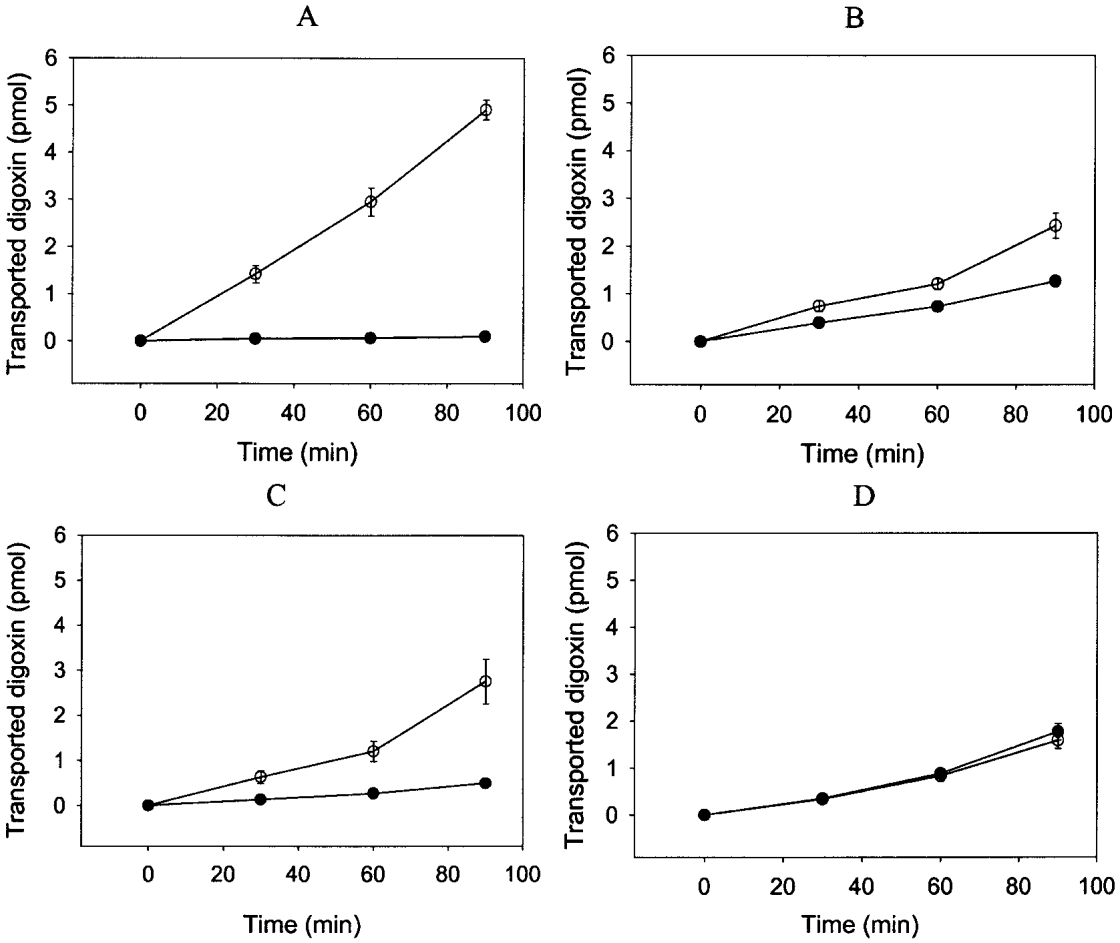

Fig. 3. The time profile of bidirectional ^{3}H-digoxin transport across Caco-2 cell monolayers. The AP-to-BL (●) and BL-to-AP (○) transport of ^{3}H-digoxin (15.4 nM) across Caco-2 cell monolayers in the presence of vehicle only (0.3% DMSO, control; A), 150 μM biochanin A (B), 150 μM silymarin (C), or 100 μM verapamil (D) was evaluated as described in the Materials and Methods section. Data are expressed as mean ± SE, n = 6.

DISCUSSION

A number of studies have looked at the effects of some naturally occurring flavonoids on P-gp-mediated drug efflux, but conflicting results have been reported. For example, the flavonoids quercetin and kaempferol have been shown to decrease adriamycin accumulation in HCT-15 colon cells by stimulation of P-gp (32). However, in another study, quercetin was shown to inhibit P-gp in MCF-7/ADR cells (33). We have shown in this study that both biochanin A and silymarin can significantly increase the 1.5-h cellular accumulation of digoxin and VBL, well-known P-gp substrates, in Caco-2 cells, and their effects on digoxin accumulation were shown to be flavonoid-concentration dependent. These results suggest that both biochanin A and silymarin may inhibit P-gp-mediated cellular efflux in Caco-2 cells. It is interesting that,

Table I. Effects of Biochanin A and Silymarin on the Transport of ^{3}H-Digoxin across Caco-2 Monolayers

	$P_{app,A-B}$ (10^{-6} cm/s)	$P_{app,B-A}$ (10^{-6} cm/s)	Mean transport ratio ($P_{app,A-B}/P_{app,B-A}$)	Mean apparent K_i (μM)	
				Based on AP-to-BL data	Based on BL-to-AP data
Control	0.29 ± 0.049	12.6 ± 1.27	43.4		
Biochanin A	3.14 ± 0.199***	5.16 ± 0.48***	1.64	33.2	32.7
Silymarin	1.15 ± 0.15*	5.13 ± 0.95***	4.48	457	30.7
Verapamil	3.77 ± 0.42***	3.53 ± 0.48***	0.94		

Note: The AP-to-BL and BL-to-AP transport of ^{3}H-digoxin (15.4 nM) across Caco-2 cell monolayers in the presence of vehicle only (0.3% DMSO, control), 150 μM biochanin A, 150 μM silymarin, or 100 μM verapamil was examined, and the apparent permeability coefficient (P_{app}) and K_i values were calculated as described in the Materials and Methods section. Data are expressed as mean ± SE for P_{app} values, n = 6.
* $p < 0.05$; *** $p < 0.001$.

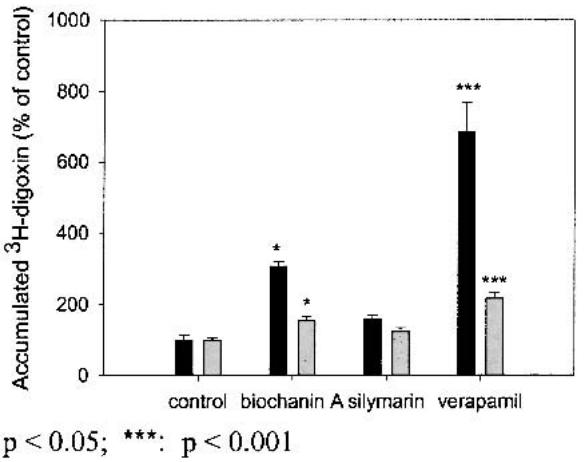

Fig. 4. The effects of biochanin A and silymarin on the accumulation of ^{3}H-digoxin in Caco-2 monolayer cells. The AP-to-BL (black bar) and BL-to-AP (grey bar) transport of ^{3}H-digoxin (15.4 nM) across Caco-2 cell monolayers in the presence of vehicle only (0.3% DMSO, control), 150 μM biochanin A, 150 μM silymarin, or 100 μM verapamil was determined. After transport studies (1.5 h), Caco-2 monolayer cells were washed and lysed. The monolayer cell-associated radioactivity was determined, normalized by the protein content, and the obtained values were further normalized by the control value to get relative ^{3}H-digoxin accumulation levels in Caco-2 monolayer cells. Data are expressed as mean ± SE, n = 3.

with respect to the effects of verapamil, both flavonoids seem to have stronger effects on VBL accumulation than on digoxin accumulation; the average increase in the cellular accumulation of digoxin and VBL produced by 150 μM biochanin A and silymarin was 32.9% and 23.3% (for digoxin), and 73.3% and 80.3% (for VBL), respectively, of that produced by 100 μM verapamil. The exact reason(s) for this apparent substrate-dependent effect is currently unknown but could be related to the exact binding site(s) of these substrates on P-gp or caused by the inhibition of multiple transporters in Caco-2 cells by these flavonoids. Digoxin has been shown to be a relatively specific substrate for P-gp, but VBL is a substrate for both P-gp and MRP-2 (34,35). Therefore, it is possible that the flavonoids may have inhibited both P-gp and MRP-2 in the Caco-2 cells and thus resulted in apparent stronger effects in increasing VBL accumulation.

The effects of biochanin A and silymarin on the transport of digoxin across Caco-2 cells were also shown to be in agreement with their P-gp inhibitory activities. The P_{app} value of the AP-to-BL transport of digoxin was significantly increased, whereas the P_{app} value of the BL-to-AP transport was significantly decreased by 150 μM of biochanin A and silymarin. In addition, the amount of digoxin accumulated in the Caco-2 monolayer cells, measured at the end of the transport studies, was shown to be elevated by both biochanin A and silymarin (not significant for silymarin). These results are consistent with the P-gp inhibitory effects of these flavonoids. The apparent K_i values of these flavonoids for P-gp inhibition based on the digoxin BL-to-AP transport data were calculated using the equations developed by Gao et al. (30). Because the AP-to-BL transport data may be physiologically more relevant in terms of drug absorption, the apparent K_i values based on the digoxin AP-to-BL transport data were also calculated using equations derived in a similar manner as described by Gao et al. (30). The apparent K_i values of biochanin A, based on both AP-to-BL and BL-to-AP transport data of digoxin, are almost the same (33.2 vs. 32.7 μM); however, the apparent K_i value of silymarin based on the digoxin AP-to-BL data is much higher than that based on the BL-to-AP transport data (457 vs. 30.4 μM), indicating that silymarin has stronger effects on digoxin BL-to-AP transport than on AP-to-BL transport. There is no satisfactory explanation for this "direction-dependent" effect available at this time, but similar observations have been reported (36). As indicated by their apparent K_i values (33.2 vs. 457 μM), biochanin A is more effective than silymarin in increasing digoxin AP-to-BL transport. We have also demonstrated, in this study, that the effects of biochanin A and silymarin on the transport of digoxin across Caco-2 cell monolayers are concentration dependent, with significant effects produced at concentrations of 50 μM biochanin A and 150 μM silymarin for digoxin AP-to-BL, and 50 μM biochanin A and 75 μM silymarin for BL-to-AP transport, respectively. Therefore, biochanin A appears to be more potent than silymarin in terms of digoxin AP-to-BL transport inhibition, as suggested by its lower effective concentration and its lower K_i value. It should be pointed out that the mean transport ratio ($P_{app,B-A}$ / $P_{app,A-B}$) of digoxin in the presence of 150 μM silymarin (4.48, Table I) determined in our early sets of experiments was lower than that in the presence of 300 μM silymarin (8.37, Table III) determined in our later sets of

Table II. Concentration-Dependent Effects of Biochanin A on the Transport of ^{3}H-Digoxin across Caco-2 Cell Monolayers

Biochanin A (μM)	$P_{app,A-B}$ (10^{-6} cm/s)	$P_{app,B-A}$ (10^{-6} cm/s)	$P_{app,B-A}/P_{app,A-B}$
0	0.29 ± 0.049	12.6 ± 1.27	43.4
20	0.435 ± 0.118	10.1 ± 1.17	23.3
50	1.66 ± 0.175***	8.67 ± 0.67*	5.21
100	3.23 ± 0.362***	8.59 ± 0.92*	2.66
150	3.14 ± 0.199***	5.16 ± 0.48***	1.64

Note: The AP-to-BL and BL-to-AP transport of ^{3}H-digoxin (15.4 nM) across Caco-2 cell monolayers in the presence of varied concentrations of biochanin A was evaluated, and the apparent permeability coefficients for both AP-to-BL ($P_{app,A-B}$) and BL-to-AP ($P_{app,B-A}$) transport were calculated as described in the Materials and Methods section. Data are expressed as mean ± SE. n = 6.
* p < 0.05; ***p < 0.001.

Table III. Concentration-Dependent Effects of Silymarin on the Transport of ^{3}H-Digoxin across Caco-2 Cell Monolayers

Silymarin (μM)	$P_{app,A-B}$ (10^{-6} cm/s)	$P_{app,B-A}$ (10^{-6} cm/s)	$P_{app,B-A}/P_{app,A-B}$
0	0.212 ± 0.051	13.6 ± 0.51	64.1
75	0.211 ± 0.009	11.3 ± 0.40*	53.6
225	0.678 ± 0.037***	9.37 ± 0.42***	13.8
300	0.991 ± 0.075***	8.29 ± 0.49***	8.37

Note: The AP-to-BL and BL-to-AP transport of ^{3}H-digoxin (15.4 nM) across Caco-2 cell monolayers in the presence of varied concentrations of silymarin was evaluated, and the apparent permeability coefficients for both AP-to-BL ($P_{app,A-B}$) and BL-to-AP ($P_{app,B-A}$) transport were calculated as described in the Materials and Methods section. Data are expressed as mean ± SE, n = 6 (for the control (0 μM silymarin), n = 3).
* p < 0.05; ***p < 0.001.

experiments. This discrepancy is probably due to the changes in P-gp expression and / or membrane characteristics in the different batches of Caco-2 monolayers. This explanation is also supported by the higher mean transport ratio of digoxin in the absence of any inhibitors determined in those later studies (64.1, Table III) than that determined in those early experiments (43.4, Table I).

The *in vivo* intestinal concentrations of biochanin A and silymarin upon consumption of herbal products containing these flavonoids are not known, but it is likely that the concentrations we used in this study could be achievable *in vivo* after the ingestion of herbal preparations. The calculated maximum clinical intestinal concentrations of biochanin A and silymarin are about 366 μM and 1.2 mM, respectively, assuming a tablet of red clover extracts or milk thistle was ingested along with 250 ml water. Even if we assume a 50–75% lower concentration because of poor solubility and/or intestinal metabolism, the P-gp inhibitory effects of biochanin A and silymarin demonstrated in this study could be clinically relevant and their potential interaction with the absorption / bioavailability of drugs that are P-gp substrates should be further tested *in vivo*.

In conclusion, the flavonoids biochanin A and silymarin can inhibit P-gp-mediated efflux in Caco-2 cells. Since the intestinal concentrations of biochanin A and silymarin could be high when herbal products containing these flavonoids are ingested, it is possible that they may increase the absorption/ bioavailability of coadministered drugs that are P-gp substrates.

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