

Flavonoids Are Inhibitors of Breast Cancer Resistance Protein (ABCG2)-Mediated Transport

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ABSTRACT

Breast cancer resistance protein (BCRP) is a newly identified ATP-binding cassette transporter, shown to confer multidrug resistance (MDR) to a number of important anticancer agents and play an important function in governing drug disposition. Flavonoids are a class of polyphenolic compounds widely present in foods and herbal products. The interactions of flavonoids with P-glycoprotein and multidrug resistance-associated protein 1 have been reported; however, their interaction with BCRP is unknown. Our objective was to evaluate the effects of 20 naturally occurring flavonoids on the cellular accumulation and cytotoxicity of mitoxantrone in both BCRP-overexpressing and BCRP-negative human cell lines. BCRP-overexpressing and BCRP-negative human breast cancer cells (MCF-7) and large cell lung carcinoma cells (NCI-H460) were used in these studies. Many of the tested flavonoids (50 μ M) increased mitoxantrone accumulation in

BCRP-overexpressing cells, completely reversing mitoxantrone resistance, with no effect on the corresponding BCRP-negative cells, indicating that these flavonoids are BCRP inhibitors. The effects of these flavonoids on the cellular accumulation and cytotoxicity of mitoxantrone were flavonoid concentration dependent, and significant changes were produced at concentrations lower than 10 μ M for most of the flavonoids. Chrysin and biochanin A were the most potent BCRP inhibitors, producing significant increases in mitoxantrone accumulation at concentrations of 0.5 or 1.0 μ M and in mitoxantrone cytotoxicity at a concentration of 2.5 μ M. Flavonoid glycosides had no effects on the BCRP-mediated transport of mitoxantrone. The results obtained in this study could be clinically relevant in terms of both MDR reversal in cancer treatment and drug-flavonoid pharmacokinetic interactions.

The primary hurdle for effective cancer chemotherapy has been the intrinsic or acquired resistance of cancer cells to a variety of anticancer agents with distinct chemical structures or mechanisms of action, a phenomenon known as multidrug resistance (MDR). Among the cellular mechanisms proposed to mediate MDR, overexpression of a family of plasma membrane efflux transporters, namely ATP-binding cassette (ABC) transporters, has received extensive investigation (Litman et al., 2001). It is believed that overexpression of these ABC transporters, such as P-glycoprotein (P-gp) (Juliano and Ling, 1976) and multidrug resistance-associated protein 1 (MRP1) (Cole et al., 1992), in cancer cells limits intracellular accumulation of cytotoxic agents for efficient cell killing through the active extrusion of the cytotoxic agents by these ATP-dependent transporters. A strategy to reverse the transporter-mediated MDR is to inhibit the func-

tion of these transporters in the MDR tumor cells by coadministration of transporter inhibitors with the anticancer agents and thus increase the intracellular drug accumulation and restore the chemosensitivity. Breast cancer resistance protein (BCRP, MXR, ABCP, or ABCG2) is another member of the ABC transporter superfamily recently identified independently from drug selected human breast cancer cells (MCF-7) (Doyle et al., 1998), human colon carcinoma cells (S1-M1-80) (Miyake et al., 1999), and human placenta (Allikmets et al., 1998), respectively, by three different groups. Molecular characterization revealed that BCRP consists of 655 amino acids with a molecular mass of 72.1 kDa. In contrast to P-gp, which has 12 transmembrane domains and two ATP binding sites (Litman et al., 2001), BCRP is a half-ABC transporter and contains only six transmembrane domains and one ATP binding site (Allikmets et al., 1998; Litman et al., 2001). Overexpression of BCRP has been shown to cause cross-resistance to doxorubicin, topotecan, SN38, mitoxantrone, methotrexate and flavopiridol, as well as to nucleoside human immunodeficiency virus reverse

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ABBREVIATIONS: MDR, multidrug resistance; DMSO, dimethyl sulfoxide; ABC, ATP-binding cassette; P-gp, P-glycoprotein; MRP1, multidrug resistance-associated protein 1; BCRP, breast cancer resistance protein; PBS, phosphate-buffered saline; MX, mitoxantrone; FTC, fumitremorgin C; OD, optical density.

transcriptase inhibitors such as zidovudine and lamivudine (Doyle et al., 1998; Maliepaard et al., 1999; Robey et al., 2001b; Volk et al., 2002; Wang et al., 2003). Significant and variable expressions of BCRP have been detected in human tumors, such as acute myeloid leukemia and breast cancer (Ross et al., 2000; Kanzaki et al., 2001; van der Kolk et al., 2002). The contribution of BCRP to clinical MDR is receiving extensive investigation, and some association has been reported (Steinbach et al., 2002). In addition, BCRP, like P-gp, is also expressed in a number of normal tissues. BCRP is present in the canalicular membrane of liver hepatocytes, the apical membrane of the epithelium in the small and large intestine, the ducts and lobules of the breast, the luminal surface of brain capillaries, and human placenta (Maliepaard et al., 2001; Cooray et al., 2002) and plays an important role in the disposition of endobiotic and xenobiotic compounds, protecting the body or certain tissues from exposure to these toxic substrates (Jonker et al., 2000, 2002). Thus, analogous to the case of P-gp, inhibitors of BCRP could be used not only to reverse MDR mediated by this transporter but also to alter the pharmacokinetics of BCRP substrate drugs, including their intestinal absorption, biliary excretion, and brain penetration, causing beneficial or adverse drug interactions.

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. The daily intake of total flavonoids from the average U.S. diet was estimated to be 1 g (Kuhnau, 1976). In addition, a variety of flavonoid-containing dietary supplements and herbal products are now available in the market because of the proposed health-promoting activities of flavonoids, such as antioxidant, anticarcinogenic, anti-inflammatory, antiproliferative, antiangiogenic, and antiestrogenic (or estrogenic) effects and to the lack of toxicity associated with this class of compounds (Havsteen, 2002). Epidemiology and animal studies have suggested that a high intake of flavonoids may be linked to a reduced risk of cancer (Kohn et al., 2002), coronary disease (Hertog et al., 1993), and osteoporosis (Potter et al., 1998). Consistent with the increasing public interest in alternative medicine, consumption, including mega-dose intake of herbal products (Eisenberg et al., 2001; Ni et al., 2002), will probably increase, posing a serious potential for drug-flavonoid interactions.

To identify potential agents for MDR reversal and to predict potential drug-flavonoid pharmacokinetic interactions, the interaction of flavonoids with relevant transporters, such as P-gp and MRP1, have been investigated (Conseil et al., 1998; Leslie et al., 2001; Zhang and Morris, 2003). However, to our knowledge, the effects of flavonoids on BCRP-mediated transport have not been reported. Based on the structural similarity between flavonoids and estrogens (Fig. 1), which have been shown to be BCRP inhibitors (Sugimoto et al., 2003), we hypothesize that some naturally occurring flavonoids may also inhibit BCRP. In the present study, we examined the effects of 20 flavonoids on the accumulation of mitoxantrone, a well-known BCRP substrate, in both BCRP-negative and -positive cells and on mitoxantrone cytotoxicity in these cells to evaluate the potential interaction of these flavonoids with BCRP.

Materials and Methods

Materials. Mitoxantrone and the flavonoids were purchased from Sigma-Aldrich (St. Louis, MO). Silymarin refers to, collectively, silibin (major component), silydianin, and silychristin (Kohn et al., 2002), and the molar concentration was calculated based on the molecular weight of silibin. RPMI 1640, fetal bovine serum, and phosphate-buffered saline (PBS) were purchased from Invitrogen (Carlsbad, CA). Human breast cancer MCF-7 sensitive, MCF-7 MX100, human large cell lung carcinoma NCI-H460 and NCI-H460 MX20 cells, and fumitremorgin C (FTC) were the kind gifts from Dr. Susan E. Bates (National Cancer Institute, Bethesda, MD).

Cell Culture. MCF-7 and NCI-H460 cells (both parent and mitoxantrone-selected subtype) were cultured in 75-cm² flasks with RPMI 1640 culture media supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere with 10% CO₂/90% air. The culture media also contained 100 units/ml penicillin and 100 µg/ml streptomycin. For mitoxantrone-selected MCF-7 MX100 and NCI-H460 MX20 cells, the culture media also contained 100 and 20 nM mitoxantrone, respectively.

Western Blot Analysis of BCRP, P-gp, and MRP1. Cells grown in 75-cm² flasks were washed with PBS 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, 200 µM sodium orthovanadate, 50 mM β-glycerolphosphate, 10 mM sodium pyrophosphate, 4 mM phenylmethylsulfonyl fluoride, 2 mM benzamide, 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 13,000g for 20 min. The protein concentrations of the soluble extracts were obtained by BCA protein assay (Pierce Chemical, Rockford, IL). Proteins (50 µg) were electrophoresed on 7.5% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes (Invitrogen, Grand Island, NY). 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) and then incubated first with primary antibody and then with secondary antibody at room temperature for 2 and 1.5 h, respectively. BXP21 (Kamiya Biomedical, Thousand Oaks, CA), C219 (DakoCytomation California Inc., Carpinteria, CA), and MRPm6 (Signet Laboratories, Inc., Dedham, MA) were used as primary antibodies to detect BCRP, P-gp, and MRP1, respectively. Anti-mouse IgG horseradish peroxidase (Amersham, Piscataway, NJ) was used as the secondary antibody. After incubation with the antibodies, membranes were washed and detected with ECL detection reagent (Amersham Biosciences Inc., Piscataway, NJ).

Mitoxantrone Accumulation Studies. The accumulation studies were performed using flow cytometric analysis as reported (Min-

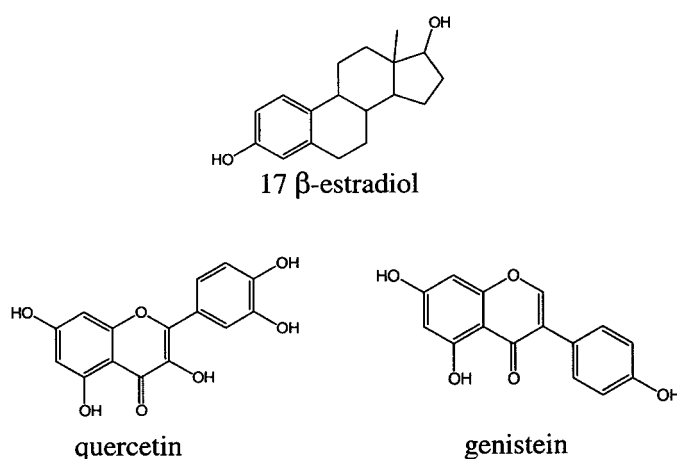


Fig. 1. The chemical structure of 17 β-estradiol and representative flavonoids quercetin and genistein.

derman et al., 2002) with some modification. In brief, the cells grown in 75-cm² flasks with about 90% confluence were trypsinized and washed with fetal bovine serum-free RPMI 1640 and resuspended in this medium with cell density of about 10⁶ cells/ml. The accumulation of mitoxantrone was performed by incubating the 1 ml of cells with various concentrations of flavonoids or the vehicle (0.1% DMSO) at 37°C for 15 min, followed by addition of 3 μ M of mitoxantrone. FTC (10 μ M) was used as a positive control. After incubation for another 30 min, the accumulation was stopped by adding 3 ml of ice-cold PBS and centrifugation. The cells were then washed with ice-cold PBS again, and the intracellular level of mitoxantrone was analyzed using the FACScan flow cytometer (BD Biosciences, San Jose, CA) equipped with a standard argon laser for excitation at 488 nm, and a bandpass filter at 670 nm was used to detect mitoxantrone fluorescence. Preliminary studies demonstrated that the fluorescence of all the tested flavonoids at this setting is negligible (data not shown). The accumulation of mitoxantrone was expressed as percentage of the control (in the presence of 0.1% DMSO).

Mitoxantrone Cytotoxicity Studies. Cytotoxicity studies were performed in 96-well plates. Cells (5×10^3) were seeded in each well of the 96-well plates. After cell attachment (24-h incubation), culture medium in each well was replaced with fresh medium containing 0 to 1 mM mitoxantrone and the specified concentrations of flavonoids or vehicle (0.1% DMSO). FTC (10 μ M) was used as a positive control. After a 24-h incubation, the drug-containing medium was aspirated off, and cells were washed twice with PBS buffer, followed by addition of fresh medium (without any drug). The incubation was then continued for an additional 24 h, and cell growth in each well was determined by sulforhodamine B assay (Skehan et al., 1990). The absorbance values (OD₅₇₀) from the sulforhodamine B assay indicate the cell number in each well of the 96-well plates. Growth inhibition by mitoxantrone (IC₅₀ value) either alone or with the flavonoids was obtained by fitting the percentage of cell growth (F) by the equation: $F = 100 \times (1 - (I_{\max} \times C^{\gamma}) / (IC_{50}^{\gamma} + C^{\gamma}))$ using WinNonlin (Pharsight, Mountain View, CA). The observed F values were calculated as 100 times the ratio of the cell growth [OD₅₇₀ - OD₅₇₀(1)] to the maximum cell growth [OD₅₇₀(0) - OD₅₇₀(1)]. OD₅₇₀(0) and OD₅₇₀(1) are the absorbance values from cells treated with 0 and 1 mM of mitoxantrone, respectively. C is the concentration of mitoxantrone. In each experiment, quadruplicate measurements were performed for each sample.

Statistical Analysis. Data were analyzed for statistically significant differences using an ANOVA test followed by a Dunnett's post hoc test or by a Student's t test. P values < 0.05 were considered statistically significant.

Results

BCRP, P-gp, and MRP1 Expression Levels. To investigate the effects of flavonoids on BCRP-mediated cellular efflux, we first characterized the expression of BCRP, P-gp, and MRP1 in the cells employed in this study using Western blot analysis. MCF-7/sensitive and MCF-7/Adr cells were used as the negative and positive control for P-gp (Fairchild et al., 1990); H69 and H69/AR cells were used as the negative and positive control for MRP1 (Cole et al., 1992). As shown in Fig. 2A, both parent NCI-H460 and MCF-7/sensitive cells had no detectable BCRP expression; however, BCRP is clearly overexpressed in mitoxantrone-selected NCI-H460 MX20 and MCF-7 MX100 cells, consistent with a previous report (Robey et al., 2001a). None of the NCI-H460 and MCF-7 cell lines (both parent and mitoxantrone-selected) had detectable P-gp or MRP1 expression (Fig. 2, B and C).

Effects of Flavonoids on Mitoxantrone Accumulation. To investigate the effects of flavonoids on BCRP-mediated efflux, the 30-min accumulation of mitoxantrone (a

model BCRP substrate) in BCRP-overexpressing MCF-7 MX100 and NCI-H460 MX20 cells and their corresponding BCRP-negative MCF-7/sensitive and NCI-H460 cells was evaluated in the presence or absence of the flavonoids (50 μ M) (Fig. 3). As shown in Table 1, all the tested flavonoids, except for epigallocatechin, epigallocatechin gallate, luteolin, morin, myricetin, naringin, and phloridzin, produced a significant increase in mitoxantrone accumulation in BCRP-overexpressing MCF-7 MX100 cells with no significant effects on mitoxantrone accumulation in BCRP-negative MCF-7/sensitive cells. These flavonoids also increased mitoxantrone accumulation in human lung carcinoma NCI-H460 MX20 cells, which also overexpress BCRP, with marginal effects in the BCRP-negative counterparts (NCI-H460 cells) (Table 1), indicating that these flavonoids may inhibit BCRP-mediated efflux of mitoxantrone in BCRP-overexpressing cells. The flavonoids apigenin, biochanin A, chrysin, genistein, hesperetin, kaempferol, naringenin, and silymarin increased mitoxantrone accumulation by more than 300% of the control value in both MCF-7 MX100 and NCI-H460 MX20 cells (Table 1), which is comparable with the accumulation in the presence of 10 μ M FTC ($439 \pm 89.8\%$ and $404 \pm 40.4\%$ of the control, respectively, $p < 0.001$ for both cell lines). Among these

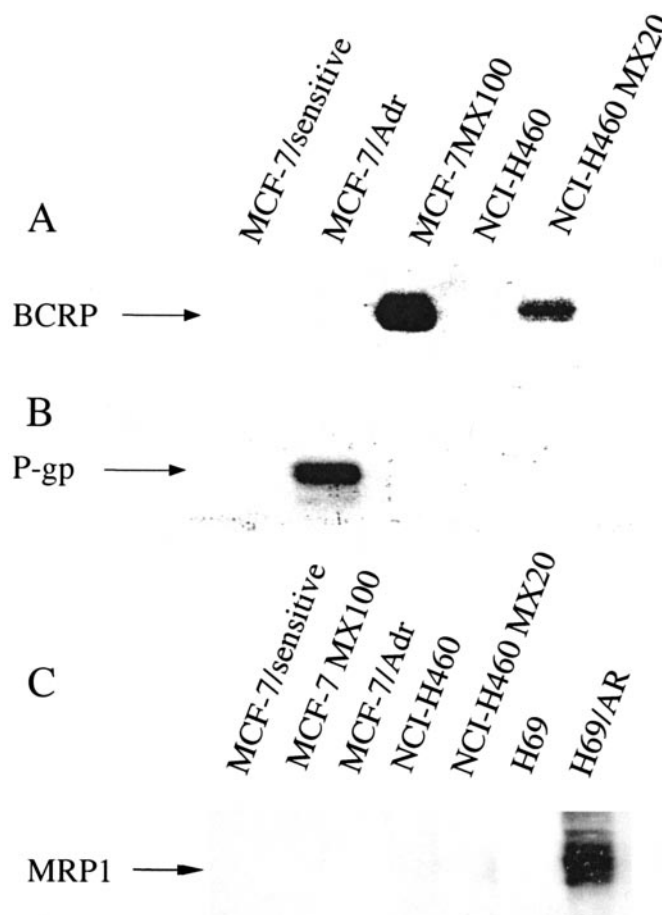


Fig. 2. Western blot analysis of BCRP, P-gp, and MRP1 expression. The cellular expression levels of BCRP (A), P-gp (B), and MRP1 (C) in the specified cell lines were determined as described under *Materials and Methods*. MCF-7/sensitive and MCF-7/Adr cell lysates were used as the negative and positive control for P-gp detection, respectively, and H69 and H69/AR cell lysates were used as the negative and positive control for MRP1 detection, respectively. The protein loading for each sample was 50 μ g, as determined by a BCA protein assay.

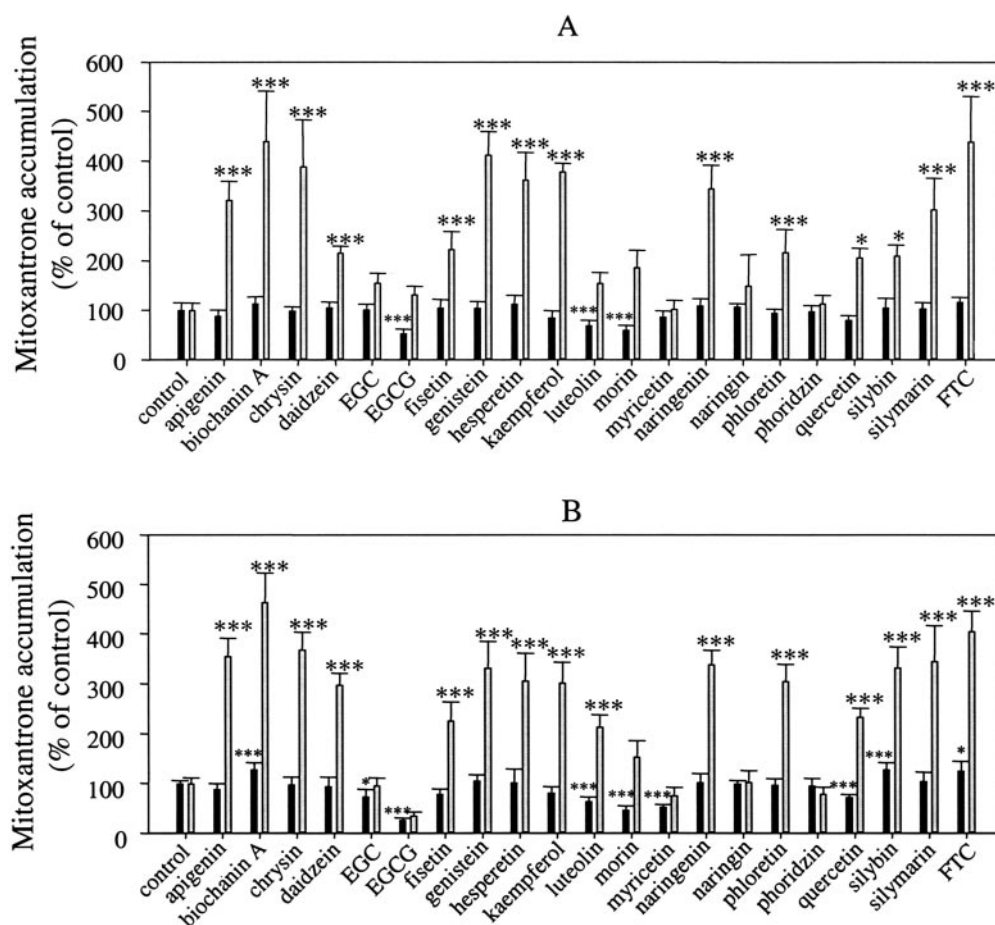


Fig. 3. Effects of flavonoids on the cellular accumulation of mitoxantrone. The 30-min accumulation of mitoxantrone in MCF-7/sensitive cells (A, solid bar), MCF-7 MX100 cells (A, shadowed bar), NCI-H460 cells (B, solid bar), and NCI-H460 MX20 cells (B, shadowed bar) in the presence of 50 μ M flavonoids or the vehicle (0.1% DMSO) was determined as described under *Materials and Methods*. FTC (10 μ M) was used as a positive control. Data are expressed as mean \pm S.D., $n = 6$ or 8. EGC, epigallocatechin; EGCG, epigallocatechin gallate. *, $p < 0.01$; ***, $p < 0.001$ compared with the control for BCRP-expressing cells; *, $p < 0.05$, ***, $p < 0.001$ compared with the control for sensitive cells.

TABLE 1

Effects of flavonoids on the accumulation of mitoxantrone in both parent and mitoxantrone-selected MCF-7 and NCI-H460 cells

The cellular accumulation of mitoxantrone in MCF-7 and NCI-H460 cells (both parent and drug-selected) in the absence or presence of flavonoids (50 μ M) was performed as described under *Materials and Methods*. FTC (10 μ M) was used as a positive control. The accumulation of mitoxantrone was expressed as percent of control (in the presence of the vehicle, 0.1% DMSO). The data were expressed as mean \pm S.D., $n = 6$ or 8.

	MCF-7/Sensitive	MCF-7 MX100	NCI-H460	NCI-H460 MX20
Control	100 \pm 13.4	100 \pm 14.8	100 \pm 4.42	100 \pm 11.0
Apigenin	88.6 \pm 9.85	321 \pm 36.1***	89.0 \pm 10.8	355 \pm 36.2***
Biochanin A	114 \pm 12.4	440 \pm 99.5***	129 \pm 14.2***	463 \pm 59.7***
Chrysin	98.5 \pm 8.03	389 \pm 94.1***	97.4 \pm 15.0	367 \pm 35.5***
Daidzein	106 \pm 10.9	215 \pm 13.1***	93.8 \pm 18.3	297 \pm 23.1***
EGC	101 \pm 10.6	155 \pm 19.1	73.5 \pm 11.9*	95.6 \pm 15.0
EGCG	52.9 \pm 8.78***	132 \pm 15.6	25.7 \pm 2.92***	34.4 \pm 8.00
Fisetin	105 \pm 16.1	223 \pm 33.2***	78.4 \pm 10.0	226 \pm 37.0***
Genistein	104 \pm 12.9	412 \pm 47.0***	105 \pm 10.1	331 \pm 54.0***
Hesperetin	112 \pm 15.9	362 \pm 55.2***	101 \pm 26.8	305 \pm 55.8***
Kaempferol	85.2 \pm 11.3	378 \pm 17.3***	80.5 \pm 10.4	301 \pm 42.5***
Luteolin	69.5 \pm 10.2***	155 \pm 19.5	63.7 \pm 8.48***	213 \pm 23.6***
Morin	60.2 \pm 9.25***	186 \pm 33.8	45.8 \pm 6.92***	153 \pm 30.3
Myricetin	86.5 \pm 11.8	102 \pm 17.5	52.0 \pm 5.40***	75.2 \pm 16.3
Naringenin	109 \pm 11.8	345 \pm 46.4***	102 \pm 17.05	338 \pm 28.0***
Naringin	107 \pm 6.09	149 \pm 62.1	98.7 \pm 6.66	102 \pm 23.2
Phloretin	94.8 \pm 6.84	216 \pm 44.1***	96.9 \pm 12.3	304 \pm 33.9***
Phloridzin	97.7 \pm 11.7	114 \pm 14.6	95.2 \pm 12.5	78.0 \pm 14.3
Quercetin	80.3 \pm 8.64	206 \pm 18.3*	71.6 \pm 5.52***	233 \pm 18.4***
Silibin	105 \pm 18.5	209 \pm 19.7*	128 \pm 13.6***	331 \pm 41.4***
Silymarin	103 \pm 11.9	302 \pm 60.9***	104 \pm 18.4	344 \pm 70.7***
FTC	116 \pm 9.30	439 \pm 89.8***	124 \pm 19.8*	404 \pm 40.4***

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

EGC, epigallocatechin; EGCG, epigallocatechin gallate.

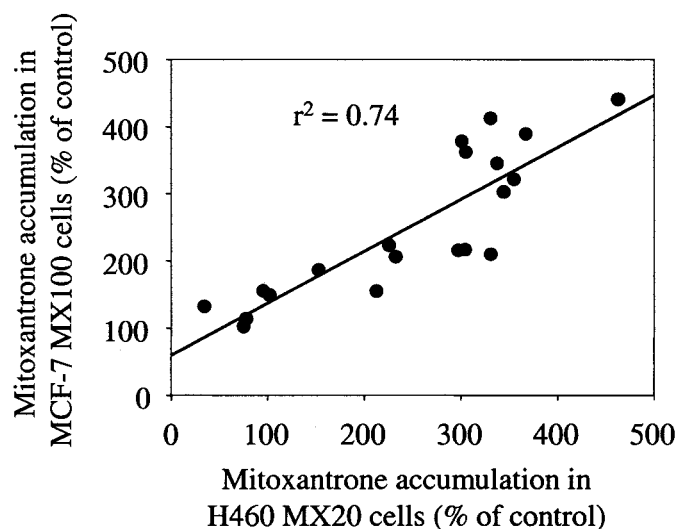


Fig. 4. Relationship between the effects of flavonoids in MCF-7 MX100 cells and NCI-H460 MX20 cells. The accumulation of mitoxantrone in both MCF-7 MX100 cells and NCI-H460 MX20 cells in the presence of 50 μ M flavonoids or the vehicle (0.1% DMSO) was determined as described under *Materials and Methods*. Regression analysis was performed using the mean values ($n = 6$ or 8) of mitoxantrone accumulation in MCF-7 MX100 cells and those in NCI-H460 MX20 cells.

flavonoids, biochanin A and the positive control FTC also produced a small but statistically significant increase of mitoxantrone accumulation in NCI-H460 cells (Table 1, $129 \pm 14.2\%$, $p < 0.001$; $124 \pm 19.8\%$, $p < 0.05$, respectively). On the other hand, epigallocatechin gallate, luteolin, and morin, at a 50 μ M concentration, resulted in a significant decrease in the apparent mitoxantrone accumulation in both MCF-7/sensitive and NCI-H460 cells (BCRP-negative) (Table 1). In NCI-H460 cells, a decrease in mitoxantrone accumulation was also observed in the presence of 50 μ M of epigallocatechin, myricetin, and quercetin (Table 1). The flavonoid glycosides naringin (naringenin 7-rhamnoglucoside) and phloridzin (phloretin 2'- β -D-glucoside) had no significant effects on mitoxantrone accumulation in both BCRP-negative and BCRP-positive cells (Table 1).

Relationship between the Flavonoid Effects on Mitoxantrone Accumulation in MCF-7 MX100 and NCI-H460 MX20 Cells. If the effects of flavonoids on the accumulation of mitoxantrone in BCRP-overexpressing cells are caused by the modulation of BCRP, then for each individual flavonoid, similar effects should be observed in both MCF-7 MX100 and NCI-H460 MX20 cells because both cell lines

overexpress BCRP. As shown in Fig. 4, the accumulation of mitoxantrone in MCF-7 MX100 cells in the presence of flavonoids did positively correlate with that in NCI-H460 MX20 cells with a correlation coefficient (r^2) of 0.74.

Concentration-Dependent Effects of Flavonoids on Mitoxantrone Accumulation. The concentration-dependent effects of flavonoids on mitoxantrone accumulation in both MCF-7 MX100 and NCI-H460 MX20 cells were investigated for five flavonoids (apigenin, biochanin A, chrysin, genistein, and kaempferol), which demonstrated high BCRP inhibition activity when tested at 50 μ M concentrations. As shown in Fig. 5, the increase of mitoxantrone accumulation in both BCRP-overexpressing cell lines (MCF-7 MX100 and NCI-H460 MX20) by all these five flavonoids was flavonoid concentration dependent. Within the tested concentration range, the minimal concentrations that produced a statistically significant increase in mitoxantrone accumulation in MCF-7 MX100 cells were 1.0 μ M for chrysin ($246 \pm 16.3\%$, $p < 0.001$), 1.0 μ M for biochanin A ($216 \pm 12.2\%$, $p < 0.001$), 5.0 μ M for apigenin ($287 \pm 79.1\%$, $p < 0.001$), 5.0 μ M for genistein ($186 \pm 31.9\%$), and 5.0 μ M for kaempferol ($274 \pm 31.7\%$, $p < 0.001$) (Fig. 5A). This is, in general, consistent with the data obtained in NCI-H460 MX20 cells, in which the minimal effective concentrations for chrysin, biochanin A, apigenin, genistein, and kaempferol were 0.5 μ M ($207 \pm 19.7\%$, $p < 0.001$), 1.0 μ M ($166 \pm 2.98\%$, $p < 0.001$), 1.0 μ M ($187 \pm 11.3\%$, $p < 0.001$), 5.0 μ M ($166 \pm 2.10\%$, $p < 0.001$), and 5.0 μ M ($268 \pm 53.5\%$, $p < 0.001$), respectively (Fig. 5B).

Effects of Flavonoids on Mitoxantrone Cytotoxicity. To further confirm the BCRP-modulating activities of the flavonoids and to investigate the potential of using these flavonoids as chemosensitizing agents in the treatment of BCRP-mediated MDR, the effects of those flavonoids with the greatest effects in increasing mitoxantrone accumulation in BCRP-overexpressing cells (apigenin, biochanin A, chrysin, genistein, hesperetin, kaempferol, naringenin, and silymarin) on the cytotoxicity of mitoxantrone in MCF-7/sensitive and MCF-7 MX100 cells were characterized. As shown in Table 2 and Fig. 6, the IC_{50} value of mitoxantrone in MCF-7/sensitive cells is much lower than that in MCF-7 MX100 cells (5.30 ± 2.22 versus 199 ± 19.3 μ M, $p < 0.001$ by Student's t test), and a specific BCRP inhibitor, FTC (10 μ M), completely abolished the difference (2.30 ± 0.29 versus 1.79 ± 1.52 μ M, $p > 0.05$ by Student's t test) and resulted in IC_{50} values of mitoxantrone in both cell lines close to that in the sensitive MCF-7 cells, consistent with the overexpression

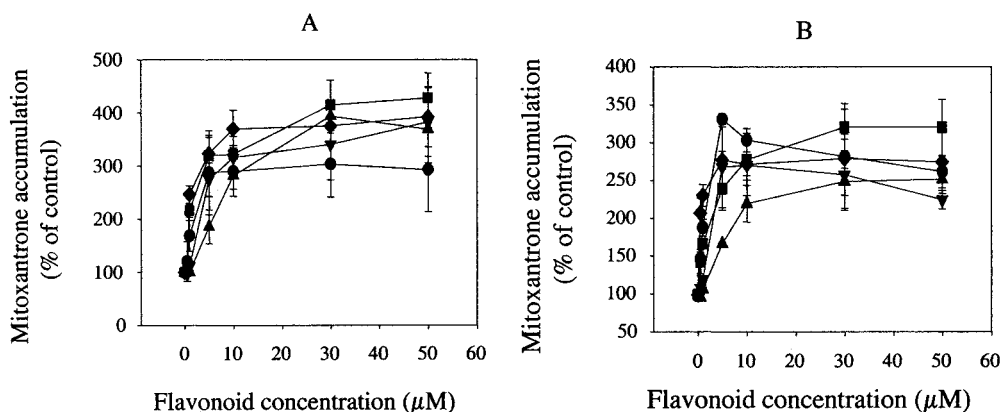


Fig. 5. Concentration-dependent effects of flavonoids on the cellular accumulation of mitoxantrone in BCRP-overexpressing cells. The 30-min accumulation of mitoxantrone in MCF-7 MX100 cells (A) and NCI-H460 MX20 cells (B) in the presence of different concentrations (range from 0.5–50 μ M) of flavonoids or the vehicle (0.1% DMSO) was performed as described under *Materials and Methods*. Data are expressed as mean \pm S.D., $n = 6$. \bullet , apigenin; \blacksquare , biochanin A; \blacklozenge , chrysin; \blacktriangle , genistein; \blacktriangledown , kaempferol.

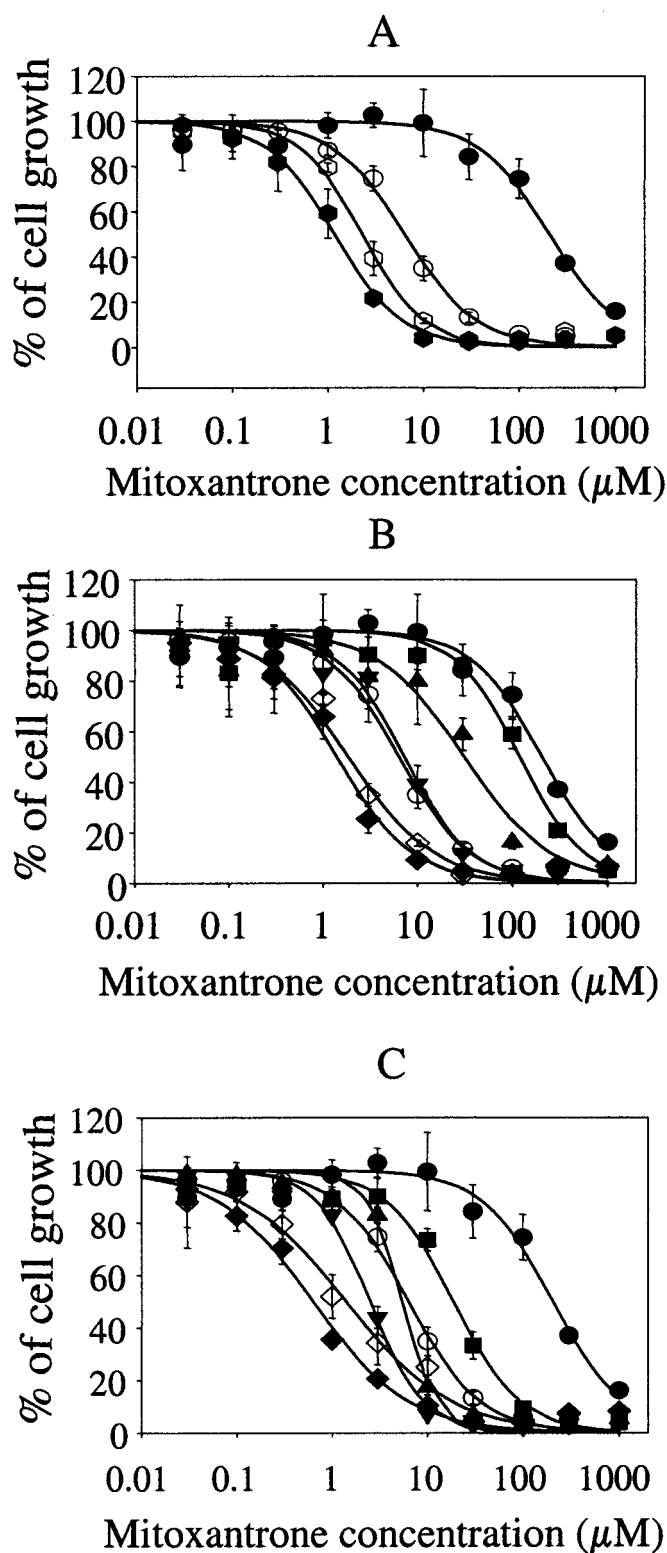


Fig. 6. Effects of flavonoids biochanin A and chrysin on the cytotoxicity of mitoxantrone in MCF-7/sensitive and MCF-7 MX100 cells. The cytotoxicity of mitoxantrone in MCF-7/sensitive cells (open symbols) and MCF-7 MX100 cells (closed symbols) in the presence of different concentrations of flavonoids (■, 2.5 μ M, ▲, 5 μ M, ▼, 10 μ M; ◇, 50 μ M) or the vehicle (0.1% DMSO, ○, ●) was evaluated as described under *Materials and Methods*. FTC (A, 10 μ M, open and closed hexagons) was used as a positive control. Presented are the data for the most potent flavonoids biochanin A (B) and chrysin (C) from a typical experiment. Data are expressed as mean \pm S.D., $n = 4$. Three separate quadruplicate experiments were performed for MCF-7 MX100 cells, and one quadruplicate experiment was performed for MCF-7/sensitive cells.

of BCRP in MCF-7 MX100 cells. In the presence of flavonoids (50 μ M), the IC_{50} value of mitoxantrone in MCF-7/sensitive cells was not significantly changed by apigenin, genistein, and kaempferol (3.44 ± 0.35 , 6.98 ± 0.81 , and 6.10 ± 0.96 μ M, respectively, versus control (5.30 ± 2.22 μ M, $p > 0.05$) (Table 2). A statistically significant decrease in the mitoxantrone IC_{50} value in MCF-7/sensitive cells was observed in the presence of 50 μ M of biochanin A and chrysin, although the changes were small (1.86 ± 0.35 and 0.95 ± 0.46 μ M, respectively, versus the control value of 5.30 ± 2.22 μ M, $p < 0.001$) (Table 2). In contrast, in the BCRP-overexpressing MCF-7 MX100 cells, flavonoids apigenin, biochanin A, chrysin, genistein, kaempferol, hesperetin, and naringenin (50 μ M for all the flavonoids) all dramatically lowered the IC_{50} value of mitoxantrone from 199 ± 19.3 μ M to 1.73 ± 1.42 , 2.19 ± 1.04 , 1.13 ± 1.11 , 2.29 ± 0.86 , 3.36 ± 1.84 , 0.95 ± 0.19 , and 1.23 ± 0.16 μ M ($p < 0.001$ for all of them), respectively, similar to or even lower than the IC_{50} value in the MCF-7/sensitive cells (Table 2), indicating a complete inhibition of BCRP in MCF-7 MX100 cells by all these flavonoids. Silymarin (50 μ M) also produced a significant decrease in the IC_{50} of mitoxantrone in MCF-7 MX100 cells (53.0 ± 7.27 versus 199 ± 19.3 μ M, $p < 0.001$), but the value is still much higher than that in MCF-7/sensitive cells (5.30 ± 2.22 μ M, $p < 0.001$) (Table 2), probably because of incomplete inhibition of BCRP.

Concentration-Dependent Effects of Flavonoids on Mitoxantrone Cytotoxicity in MCF-7 MX100 Cells. The concentration-dependent effects of flavonoids on mitoxantrone cytotoxicity in MCF-7 MX100 cells were also evaluated. As shown in Table 2 and Fig. 6, all the tested flavonoids demonstrated concentration-dependent mitoxantrone cytotoxicity-potentiating effects in MCF-7 MX100 cells. Chrysin and biochanin A showed the highest potency and produced a significant decrease in the IC_{50} of mitoxantrone (18.8 ± 0.06 and 107 ± 17.6 μ M, respectively, versus control 199 ± 19.3 μ M, $p < 0.001$ for both flavonoids) even at 2.5 μ M, which was the lowest tested flavonoid concentration. For other flavonoids, within the tested concentrations, the minimal concentrations to produce a significant decrease in the IC_{50} of mitoxantrone varied from 5 to 50 μ M (Table 2).

Discussion

Transporter-mediated active efflux of cytotoxic agents is one of the best characterized mechanisms by which cancer cells develop MDR. BCRP has been shown to confer resistance to a number of important anticancer agents such as doxorubicin, mitoxantrone, topotecan, SN38, methotrexate, and flavopiridol (Doyle et al., 1998; Maliepaard et al., 1999; Robey et al., 2001b; Volk et al., 2002); thus, potent and nontoxic inhibitors of BCRP need to be identified for their potential clinical use in MDR reversal. Flavonoids are an integral component in our common diet with a long history of human consumption and exceptional safety records (Havsteen, 2002). In addition, these compounds have been associated with a variety of anticancer mechanisms (Havsteen, 2002), and related synthetic substances, such as flavone acetic acid and flavopiridol, have already been subjected to clinical trials (Siegenthaler et al., 1992; Senderowicz et al., 1998). Therefore, BCRP inhibitors derived from flavonoids should have the advantage of low toxicity. Furthermore, the

TABLE 2

Effects of flavonoids on the cytotoxicity of mitoxantrone in MCF-7/sensitive and MCF-7 MX100 cells

The cytotoxicity of mitoxantrone in MCF-7/sensitive and MCF-7 MX100 cells was determined in the presence or absence (0.1% DMSO) of specified concentrations of flavonoids as described under *Materials and Methods*. For MCF-7/sensitive cells, data are the mean \pm S.D. from a quadruplicate experiment; for MCF-7 MX100 cells, data are the mean \pm S.D. from three separate quadruplicate experiments.

Flavonoid Concentration	MCF-7/Sensitive		MCF-7 MX100			
	10 μ M	50 μ M	2.5 μ M	5 μ M	10 μ M	50 μ M
Control	5.30 \pm 2.22		199 \pm 19.3			
Apigenin	3.66 \pm 0.52	3.44 \pm 0.35		219 \pm 10.0	10.5 \pm 7.13*	1.73 \pm 1.42*
Biochanin A	2.40 \pm 0.27*	1.86 \pm 0.35*	107 \pm 17.6*	30.9 \pm 5.18*	9.23 \pm 2.07*	2.19 \pm 1.04*
Chrysin		0.95 \pm 0.46*	18.8 \pm 0.06*	6.25 \pm 2.13*	3.35 \pm 1.70*	1.13 \pm 1.11*
Genistein		6.98 \pm 0.81		148 \pm 23.2	29.3 \pm 6.76*	2.29 \pm 0.86*
Kaempferol		6.10 \pm 0.96		196 \pm 20.9	228 \pm 13.8	3.36 \pm 1.84*
Hesperetin				88.6 \pm 20.8*	11.6 \pm 0.83*	0.95 \pm 0.19*
Naringenin				189 \pm 11.6	163 \pm 29.5	1.23 \pm 0.16*
Silymarin				99.1 \pm 50.2*	104 \pm 35.5*	53.0 \pm 7.27*
FTC	2.30 \pm 0.29*				1.79 \pm 1.52*	

* $P < 0.001$.

coadministered flavonoids (as reversal agents) may be able to provide extra anticancer mechanisms (apart from MDR reversal) in addition to the cytotoxic agent, blocking multiple pathways by which the cancer cells can survive.

The similar function and tissue localization of BCRP to that of P-gp, a well-characterized and important detoxification mechanism for xenobiotic compounds, suggests that BCRP may also assume an important role in drug disposition. This notion is supported by the observation that the intestinal expression of BCRP is even higher than that of P-gp (Taipalensuu et al., 2001) and that the bioavailability and biliary excretion of topotecan (a BCRP substrate) can be greatly altered by the coadministration of GF120918, a potent inhibitor of both P-gp and BCRP, in P-gp-deficient mice (Jonker et al., 2000). Thus, the elucidation of flavonoid effects on BCRP-mediated transport also could help to predict potential drug-flavonoid pharmacokinetic interactions, which is an important issue considering the wide consumption of large amounts of flavonoids in foods or herbal preparations.

In the present study, we examined the effects of 20 flavonoids, representing all the chemical subclasses of flavonoids, on BCRP-mediated transport and demonstrated that the flavonoids apigenin, biochanin A, chrysin, genistein, hesperetin, kaempferol, naringenin, and silymarin and the positive control FTC all produced a more than 3-fold increase ($p < 0.001$) in mitoxantrone accumulation in the BCRP-overexpressing cells (MCF-7 MX100 and NCI-H460 MX20), with no or minimal effects on mitoxantrone accumulation in the corresponding BCRP-negative cell lines (MCF-7/sensitive and NCI-H460). The level of mitoxantrone accumulation achieved in the presence of 50 μ M of these flavonoids ($>300\%$ of the control in both BCRP-overexpressing cells) was comparable with those in the presence of 10 μ M FTC ($439 \pm 89.8\%$ and $404 \pm 40.4\%$ of the control in MCF-7 MX100 and NCI-H460 MX100 cells, respectively). These results indicate that the above-mentioned flavonoids strongly inhibited the BCRP-mediated efflux of mitoxantrone. The flavonoids daidzein, fisetin, phloretin, quercetin, and silibin produced a more than 2-fold increase ($p < 0.001$) in the mitoxantrone accumulation in the BCRP-overexpressing cells, with no or only slight effects in the BCRP-negative counterparts (MCF-7/sensitive and NCI-H460), suggesting that these flavonoids are also BCRP inhibitors. A very small but statistically significant increase in mitoxantrone accumulation in the BCRP-negative NCI-H460 cells was also observed in the presence of

the flavonoids biochanin A and silibin and in the positive control (FTC). This was most probably caused by a low level BCRP expression in the NCI-H460 cells because a low level of BCRP expression was detected in these cells in a study using Northern blot analysis (Robey et al., 2001a), although BCRP in these cells was not detectable by Western blot analysis in our hands. In addition, except for silibin, which resulted in mitoxantrone accumulation of $331 \pm 41.4\%$ in NCI-H460 MX20 cells, biochanin A and FTC at the tested concentrations did have the strongest effect on BCRP-mediated transport (mitoxantrone accumulation in NCI-H460 MX20 cells was $463 \pm 59.7\%$, $531 \pm 72.0\%$, and $404 \pm 40.4\%$, respectively). Therefore, it is plausible that, because of the potent inhibition of BCRP, a small increase of mitoxantrone accumulation can be produced in cells with very low BCRP expression. It might be argued that the observed effects of flavonoids on mitoxantrone accumulation possibly could be caused by the inhibition of P-gp or MRP1 instead of BCRP, but this possibility could be ruled out by the following facts: 1) neither P-gp nor MRP1 was detected in MCF-7 MX100 or NCI-H460 MX20 cells, but significant amounts of BCRP were clearly detected in both cell lines by Western blot analysis and 2) we have demonstrated in our cytotoxicity studies that the positive control FTC, a specific BCRP inhibitor without effects on P-gp or MRP1 (Rabindran et al., 1998), completely reversed the resistance of MCF-7 MX100 cells to mitoxantrone and resulted in similar IC_{50} values in both MCF-7/sensitive and MCF-7 MX100 cells (2.30 ± 0.29 versus 1.79 ± 1.52 μ M, $p > 0.05$ by Student's t test) when present at a 10 μ M concentration, indicating that the resistance of MCF-7 MX100 cells to mitoxantrone, in comparison with MCF-7/sensitive cells, can be totally ascribed to the overexpression of BCRP. Furthermore, although mitoxantrone has been shown to be a weak P-gp substrate (Schurr et al., 1989), it seems not to be a substrate for MRP1 (Cole et al., 1994), and depletion of cellular glutathione in MCF-7/VP cells (overexpress MRP1) failed to change mitoxantrone transport or sensitivity (Diah et al., 2001). Taken together, we believe that the increase of mitoxantrone accumulation in MCF-7 MX100 and NCI-H460 MX20 cells by these flavonoids is through the inhibition of BCRP instead of P-gp or MRP1. The correlation that could be established between the mitoxantrone accumulation levels in both BCRP-overexpressing cells in the presence of flavonoids ($r^2 = 0.74$) provided additional evidence.

It should be noted that the apparent accumulation of mi-

toxantrone was significantly decreased in both BCRP-negative cell lines (MCF-7/sensitive and NCI-H460) by the flavonoids epigallocatechin gallate, luteolin, and morin. Similarly, a significant decrease of apparent mitoxantrone accumulation by the flavonoids epigallocatechin, myricetin, and quercetin was also observed in NCI-H460 cells. The exact reason(s) for this seemingly decreased mitoxantrone accumulation is currently unknown but might be possibly caused by the quenching effects of these flavonoids on mitoxantrone fluorescence, resulting in a decreased fluorescence reading and thus an apparent reduction in mitoxantrone accumulation. However, the increased mitoxantrone accumulation in the BCRP-overexpressing cells in the presence of luteolin and morin, in contrast to the significantly decreased mitoxantrone accumulation in BCRP-negative cells, suggests that luteolin and morin also may be BCRP inhibitors.

Interestingly, both naringin (naringenin 7-rhamnoglucoside) and phloridzin (phloretin 2'- β -D-glucoside), the only flavonoid glycosides included in this study, had no significant effect on mitoxantrone accumulation in either BCRP-negative or -positive cells, indicating that naringin and phloridzin are not BCRP inhibitors. However, their corresponding aglycone naringenin and phloretin were shown to be potent BCRP inhibitors. Thus, it is reasonable to speculate that attachment of a sugar moiety may markedly attenuate or totally abolish BCRP-inhibitory activity of the flavonoids.

To investigate the concentration-dependent effects of flavonoids on BCRP-mediated transport, the effects of five flavonoids with strong BCRP-inhibitory activities when tested at 50 μ M concentrations, namely, apigenin, biochanin A, chrysin, genistein, and kaempferol, were evaluated. These five flavonoids demonstrated concentration-dependent inhibition of BCRP. The minimal effective concentrations of these flavonoids in both MCF-7 MX100 cells and NCI-H460 MX20 cells were very consistent. Chrysin and biochanin A seem to be the most potent BCRP inhibitors among these flavonoids and significant inhibition of BCRP by these flavonoids could be produced at concentrations as low as 0.5 or 1.0 μ M.

To confirm their BCRP-inhibitory effects and to investigate the potential of using flavonoids as chemosensitizing agents, nine compounds, which were able to produce substantial increases in mitoxantrone accumulation in BCRP-overexpressing cells (namely, apigenin, biochanin A, chrysin, hesperetin, genistein, kaempferol, naringenin, and silymarin) were tested in cytotoxicity studies to examine their effects on mitoxantrone cytotoxicity. The positive control FTC (10 μ M) and all the tested flavonoids at 50 μ M concentrations, except silymarin, completely sensitized MCF-7 MX100 cells and markedly reduced the IC₅₀ value of mitoxantrone from the control value of 199 ± 19.3 μ M to a value similar to or even lower than that in the BCRP-negative MCF-7/sensitive cells (5.30 ± 2.22 μ M). The complete sensitization of MCF-7 MX100 cells by FTC, a specific BCRP inhibitor, indicates that the resistance of these cells to mitoxantrone can be attributed totally to the overexpression of BCRP; therefore, the complete sensitization of these cells by the flavonoids is a result of the complete inhibition of BCRP in these cells. In the MCF-7/sensitive cells, apigenin, genistein, and kaempferol, at 50 μ M concentrations, consistently had no significant effects on mitoxantrone cytotoxicity; on the other hand, the flavonoids biochanin A and chrysin, at 50 μ M concentrations, significantly decreased the IC₅₀ of mitoxantrone in MCF-7/

sensitive cells, although the decreases were small (1.86 ± 0.35 and 0.95 ± 0.46 μ M, respectively, versus control 5.30 ± 2.22 μ M, $p < 0.001$). In addition, when the mitoxantrone IC₅₀ values in the presence of these flavonoids in both sensitive and resistant MCF-7 cells were compared, no significant differences were observed (biochanin A, 1.86 ± 0.35 versus 2.96 ± 1.04 μ M; chrysin, 0.95 ± 0.46 versus 1.13 ± 1.11 μ M, $p > 0.05$), also suggesting that BCRP in MCF-7 MX20 cells was completely inhibited by 50 μ M concentrations of these flavonoids. The reduction of mitoxantrone IC₅₀ in MCF-7/sensitive cells by the flavonoids could be caused by the inhibition of the small amount of BCRP expressed in these cells, because a very low expression of BCRP in MCF-7/sensitive cells was detected by Northern blot analysis (Robey et al., 2001a), although we did not detect BCRP in these cells by Western blot analysis. In addition, the positive control FTC, which is a specific inhibitor of BCRP, also produced a small reduction of mitoxantrone IC₅₀ in MCF-7/sensitive cells (2.30 ± 0.29 versus 5.30 ± 2.22 μ M, $p < 0.001$), providing support for this hypothesis. The enhancing effects of all the tested flavonoids on mitoxantrone cytotoxicity in MCF-7 MX100 cells were shown to be flavonoid concentration dependent, and most of the flavonoids (apigenin, biochanin A, chrysin, genistein, hesperetin, and silymarin) produced a significant reduction of mitoxantrone IC₅₀ value at concentrations lower than 10 μ M. The most potent flavonoids in terms of reversing mitoxantrone resistance were chrysin and biochanin A, which enhanced mitoxantrone cytotoxicity at concentrations as low as 2.5 μ M.

In conclusion, many naturally occurring flavonoids can inhibit BCRP-mediated efflux and thus increase the cellular accumulation of BCRP substrates and restore the sensitivity of MDR cells. Some of these flavonoids such as chrysin and biochanin A can be effective at very low concentrations and potentially could be used alone or in combination to reverse BCRP-mediated MDR or as lead compounds for the development of optimal derivatives for clinical application. From the drug interaction point of view, considering the wide consumption of large amounts of flavonoids in flavonoid-containing foods or herbal products, pharmacokinetic interactions of these flavonoids with drugs that are BCRP substrates, resulting in alterations in bioavailability and clearance, could take place after coadministration.

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