Pharm Res (2010) 27:1296–1308
DOI 10.1007/s11095-010-0108-8

RESEARCH PAPER

Effects of Single and Multiple Flavonoids on BCRP-Mediated Accumulation, Cytotoxicity and Transport of Mitoxantrone *In Vitro*

Guohua An • Marilyn E. Morris

Received: 16 October 2009 / Accepted: 1 March 2010 / Published online: 6 April 2010

ABSTRACT

Purpose The objective of our study was to investigate the effect of single and multiple flavonoids on the accumulation and cytotoxicity of mitoxantrone in BCRP-overexpressing breast cancer cells and on the transport of mitoxantrone in BCRP-expressing normal cells.

Methods The effect of flavonoids on mitoxantrone accumulation and cytotoxicity was studied in the human breast cancer MCF-7 MX100 cell line. Mitoxantrone transport in the presence of flavonoids was studied in human and murine BCRP-transfected MDCK cell lines, and mitoxantrone concentrations were determined by HPLC.

Results Our results demonstrated that multiple flavonoid combinations act additively and exhibit strong BCRP inhibition for increasing mitoxantrone accumulation in breast cancer cells. Kaempferide, biochanin A, 5,7-dimethoxyflavone, and 8-methylflavone greatly increased the cytotoxicity of mitoxantrone in BCRP-overexpressing breast cancer cells. Additionally, the basolateral-to-apical membrane-directed transport of mitoxantrone in murine Bcrp1- and human BCRP-expressing MDCK cells, in the presence of 2.5 μM of these flavonoids, was also significantly decreased.

Conclusion The results indicate that flavonoids are potent BCRP inhibitors and that they exert additive effects when used in combination. Flavonoids demonstrate MDR-reversing effects, but also may influence the disposition of mitoxantrone and cause pharmacokinetic interactions.

KEY WORDS ABCG2 · cytotoxicity · flavonoids · mitoxantrone · multidrug resistance

ABBREVIATIONS

ABC	ATP binding cassette
BCRP	breast cancer resistance protein
FTC	fumitremogin C
MDCK	Madin-Darby canine kidney
MDR	multidrug resistance
SRB	sulforhodamine B

INTRODUCTION

Innate or acquired resistance of cancer cells to a variety of structurally and functionally distinct anticancer agents, a phenomenon known as multi-drug resistance (MDR), remains a major obstacle for the development of successful cancer therapy. An important mechanism for MDR is the enhanced cellular efflux of anticancer agents due to over-expression of ATP-binding cassette (ABC) transporter proteins (i.e. efflux transporters) (1). Among the 49 ABC transporters identified so far, P-glycoprotein (P-gp, ABCB1), Multidrug Resistance Protein1 (MRP-1, ABCC1) and Breast Cancer Resistance Protein (BCRP, ABCG2) are three main efflux transporters associated with MDR (2).

Compared to P-gp and MRP-1, which were discovered several decades ago, BCRP was first reported in 1998 (3). Although BCRP was called Breast Cancer Resistance Protein, the expression of this protein is not limited to breast cancer cells. In a study conducted by Diestra *et al.* (4), among 150 human solid tumors comprising 21 different

A detailed description of the mitoxantrone assay has been published (An G and Morris ME. HPLC analysis of mitoxantrone in mouse plasma and tissues: Application in a pharmacokinetic study. J Pharm Biomed Anal 51 (2010) 750–753).

G. An • M. E. Morris (✉)
Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, State University of New York,
517 Hochstetter Hall,
Amherst, New York 14260-1200, USA
e-mail: memorris@buffalo.edu

tumor types, the expression of BCRP was detected in all tumor types examined, with high frequency in colorectal carcinoma, gastric carcinoma, hepatocellular carcinoma, bladder carcinoma, ovarian carcinoma, small cell lung cancer, and melanoma. It has been reported that high levels of BCRP were associated with poor prognosis and response to clinical treatment (5,6). Many structurally distinct anticancer drugs, including mitoxantrone, topotecan, irinotecan, etoposide, and flavopiridol, are substrates of BCRP (7–9). Similar to P-gp, BCRP expression is not confined to tumor cells; it is also present in a number of normal human tissues. For instance, BCRP was found in the luminal surface of epithelial cells in the small intestine and colon, the bile canalicular membrane of hepatocytes, the proximal renal tubules, and the apical membrane of placenta (10). Therefore, in addition to causing resistance to anticancer agents through overexpression on cancer cells, BCRP may also influence the disposition of its substrates, due to its expression and apical orientation in tissues that are responsible for drug absorption, distribution, and elimination.

Mitoxantrone (MX), a topoisomerase II-targeting anthracenedione derivative, is a clinically well-established anticancer agent with high efficacy in breast cancer, acute leukemia, and non-Hodgkin's lymphoma. Mitoxantrone also has activity against non-small-cell lung cancer and cancer of the liver, prostate, bladder, and neck (11). Although its structure and activity are similar to the anthracycline derivatives, mitoxantrone has considerably lower cardiotoxicity than doxorubicin (12). Biliary excretion is the major route for elimination of mitoxantrone, and extensive metabolism has not been reported (13). Similar to other anti-tumor agents, the efficacy of mitoxantrone is greatly limited due to cellular resistance. Reported mechanisms involved in mitoxantrone resistance include altered topoisomerase II activities and overexpression of efflux transporters (14). Among those anticancer drugs which are substrates of BCRP, mitoxantrone has the highest affinity for the transporter. Most of the drug-selected cell lines that overexpress BCRP have been reported to display strong resistance to mitoxantrone (15). In addition to BCRP, mitoxantrone is also a substrate of P-gp, albeit with less affinity (16).

Flavonoids, a group of polyphenolic dietary components consisting of more than 8000 different compounds, are the most prevalent group of plant polyphenols present in fruits and vegetables. They are also the main components of many herbal supplements, such as *Ginkgo biloba*, *Sophora japonica*, and *Citrus grandis* (17,18). The basic structure of flavonoids consists of two aromatic rings (A and B) linked through three carbons that usually form a heterocyclic ring (C). Based on the variations in the patterns of hydroxylation and substitutions in ring C, flavonoids are divided into seven subclasses: flavones, flavonols, flavanones, flavanols, anthocyanidines, chalcones and isoflavones. The bioavailability of the dietary flavonoids is usually low, partially due to free hydroxyl groups that are subject to extensive phase II conjugation. In addition, flavonoids with methylated group(s) have been reported to have improved bioavailability (19). We reported previously that flavonoids can reverse BCRP-mediated mitoxantrone resistance in a breast cancer cell line: many flavonoids can increase mitoxantrone accumulation in BCRP-overexpressing MCF-7/MX100 cells, without affecting accumulation in BCRP-negative MCF-7 cells (20). Further concentration-dependent effects of flavonoids on mitoxantrone accumulation were investigated. The results indicated that among the 25 flavonoids screened, 12 flavonoids covering three subclasses (flavonols, isoflavones and flavones) demonstrated potent BCRP inhibition (EC_{50}<2.0 μM) in MCF7/MX100 cells (21). However, the effect of flavonoids on the transport of mitoxantrone in BCRP-expressing normal cells has not been investigated. In addition, the combined effect of multiple flavonoids is of interest because food and herbal products usually contain multiple flavonoids. Although the mechanism of inhibition of BCRP by flavonoids remains unclear, flavonoids have been reported to modulate MRP1 through multiple mechanisms (22). Thus, it is reasonable to consider flavonoids individually, rather than as a class of compounds. Therefore, it is possible that multiple flavonoid combinations may have additive or synergistic effects on BCRP inhibition and could reverse MDR more efficiently than individual flavonoids. In the present study, we first investigated the effect of multiple flavonoid combinations on the cellular accumulation of mitoxantrone in human breast cancer cells overexpressing BCRP (MCF-7/MX100 cells). Twelve flavonoids (apigenin, chrysin, galangin, 5,7-dimethoxyflavone, 7,8-benzoflavone, baicalein, 5,6,7-trimethoxyflavone, 6,4-dimethoxy-3-hydroxy-flavone, 2-hydroxy-a-naphthoflavone, kaempferide, biochanin A, and 8-methylflavone) with potent BCRP inhibition were selected, and their individual EC_{50} values were obtained from our previous publication (Table I) (21). Second, to further confirm the MDR-reversing effect of flavonoids on mitoxantrone resistance, the effects of flavonoids on the cytotoxicity of mitoxantrone were investigated. Four of the twelve flavonoids were selected, namely biochanin A, kaempferide, 5,7-dimethoxylflavone and 8-methylflavone (Fig. 1), which are from three structurally different subclasses and contain methylated groups on their skeleton, which is associated with improved *in vivo* bioavailability. The effects of three of these selected flavonoids on mitoxantrone cytotoxicity have not been previously investigated. Finally, we studied the effect of flavonoids on the transport of mitoxantrone in MDCK cells that were transfected with human BCRP or murine Bcrp1.

Table I The EC_{50} Values of Individual Flavonoids and Multiple Flavonoid Combinations for Increasing Mitoxantrone Accumulation in MCF7/MX100 Cells

Flavonoid candidates	EC_{50} (μM)
B: Biochanin A[a]	1.62 ± 1.02
K : Kaempferide[a]	1.02 ± 0.33
G : Galangin[a]	1.21 ± 0.27
A : Apigenin[a]	1.66 ± 0.55
B' : Baicalein[a]	1.21 ± 0.35
C : Chrysin[a]	0.39 ± 0.13
D : 5,7-Dimethoxyflavone[a]	1.41 ± 0.26
b : 7,8-benzoflavone[a]	0.07 ± 0.02
T : 5,6,7-Trimethoxyflavone[a]	1.09 ± 0.41
d : 6,4-dimethoxy-3-hydroxyflavone[a]	0.45 ± 0.25
H : 2-Hydroxy-a-naphthoflavone[a]	0.09 ± 0.05
M : 8-Methylflavone[a]	0.61 ± 0.15
BK	0.455 ± 0.035
ABK	0.406 ± 0.052
AB'DT	0.392 ± 0.098
AB'CDTdM	0.128 ± 0.036
AB'CDbTdHM	0.031 ± 0.001
BKGAB'CDTdM	0.091 ± 0.006

[a] Data for the individual flavonoids were obtained from our previous study (21).

MATERIALS AND METHODS

Materials

The flavonoids were purchased from Indofine (Hillsborough, NJ). Mitoxantrone, sulforhodamine B (SRB), sulfosalicylic acid, ascorbic acid, and triethylamine were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640, Dulbecco's modified Eagle's medium (DMEM), Hank's buffered salt solution (HBSS), phosphate-buffered saline (PBS), and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). [^{3}H]Mannitol (15 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Fumitremorgin C (FTC) was a kind gift from Dr. Susan E. Bates (National Cancer Institute, MD). Ametantrone was a kind gift from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD). All the other reagents or solvents used were commercially available and of reagent grade.

Cell Culture

Human breast cancer MCF-7 cells were used in mitoxantrone accumulation and cytotoxicity studies. MCF-7 and MCF-7/MX100 cells were kindly provided by Dr. Susan E. Bates (National Cancer Institute, MD, USA). The expression of human BCRP in mitoxantrone-selected MCF-7/MX100 cells was confirmed in our laboratory, and neither P-gp nor MRP1 expression was detected in the MCF-7 cell line. The MCF-7 cells were cultured in 75 cm^2 flasks with RPMI 1640 culture media supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. For the MCF-7/MX100 cells, 100 nM mitoxantrone was also included in the culture media.

The polarized Madin-Darby canine kidney cell line (MDCK-II) was used in the mitoxantrone transport study. The MDCK-II and its subclones that were transfected with murine Bcrp1 or human BCRP cells were obtained from Dr. Alfred Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands). The MDCK-II cells were grown in

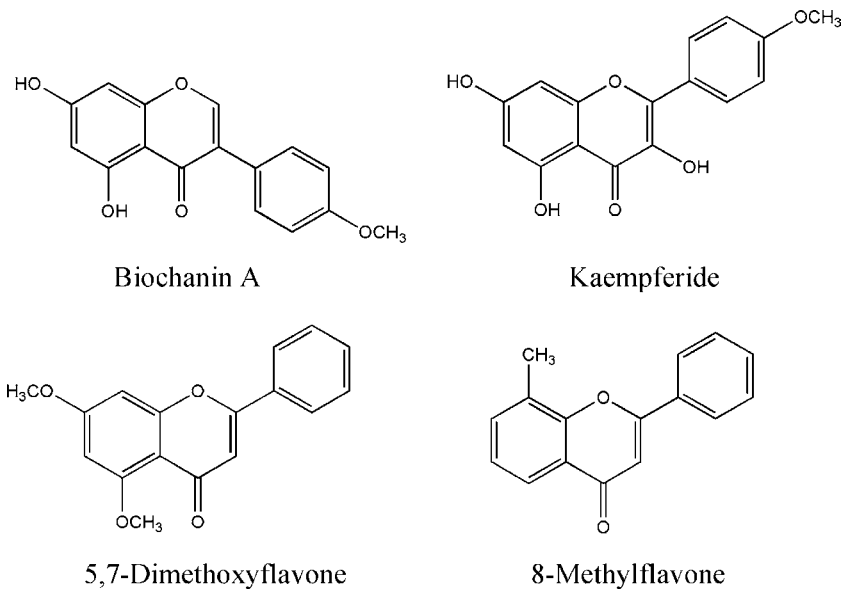


Fig. 1 Chemical structures of the flavonoids biochanin A, kaempferide, 5,7-dimethoxyflavone and 8-methylflavone.

a 5% CO_2 atmosphere at 37°C in DMEM which was supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. A solution of 0.25% trypsin-EDTA was used to detach the cells from the flasks.

Western Blot Analysis of BCRP/Bcrp1 in MDCK Cell Line

The expression of BCRP/Bcrp1 in MDCK cells was demonstrated using Western blot analysis. The cells were harvested using a rubber policeman and homogenized on ice in 250 μL of unbuffered lysis buffer containing 20 mM Tris, 120 mM NaCl, 0.5% Nonidet P-40, 2 mM benzamidine, and a combination of protease inhibitors (4 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin). The protein concentrations in the lysates were determined using a BCA protein assay kit (Pierce, Rockford, IL). The indicated amount of protein was electrophoresed on 4–15% SDS-PAGE and, subsequently, transferred onto a nitrocellulose membrane (Invitrogen, Carlsbad, CA). The membranes were then blocked overnight at 4°C in Tris-buffered saline that contained 0.2% (*v*/*v*) Tween-20 and 5% (*w*/*v*) non-fat milk. An immunoblot analysis was carried out with a monoclonal antibody raised against human BCRP (BXP-21) or murine Bcrp1 (BXP-9) at a dilution of 1:250 followed by horseradish peroxidase-linked secondary antibodies at a 1:2000 dilution. The proteins were visualized using an ECL Western blotting kit (Amersham, Piscataway, NJ).

Mitoxantrone Accumulation Studies

The accumulation studies were performed using flow cytometric analysis, as previously described (20). The stock solution of MX was prepared in double distilled water. Stock solutions of flavonoids were prepared in DMSO, and the final concentration of DMSO in the cell medium was 0.1%. Briefly, cells were trypsinized and resuspended in serum-free RPMI 1640 with a cell density of approximately 10^6 cells/ml. The cells (1 ml) were incubated with various concentrations of test compounds, vehicle (0.1% DMSO), or positive control (10 μM FTC, a specific BCRP inhibitor) at 37°C for 15 min, followed by the addition of 3 μM mitoxantrone. After incubation for another 30 min, the accumulation was stopped by ice cold PBS and centrifugation. The intracellular fluorescence of mitoxantrone was analyzed by a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) with a 488-nm standard argon laser and 670 nm bandpass filter. The cell debris and clumps were gated out, and a total of 5000 cells were analyzed per sample. The background fluorescence of each of the tested flavonoids was also checked and determined to be negligible under the assay conditions. The accumulation of mitoxantrone was expressed as percent of control (in the presence of the vehicle, 0.1% DMSO).

The EC50 values of flavonoid combinations for increasing mitoxantrone accumulation in MCF-7 MX/100 cells were obtained by fitting the fraction of maximal increase (F_1) by using the equation: $F_1 = C^\gamma/(EC_{50}{}^\gamma + C^\gamma)$ using WinNonlin (version 5.0, Pharsight Corporation, Palo Alto, CA). The observed F_1 values were calculated as the ratio of the net increase of mitoxantrone accumulation in the presence of the test compounds ($A–A_0$) to the maximal net increase, represented by the net increase of mitoxantrone accumulation in the presence of 10 μM FTC ($A_{ftc}–A_0$). A_{ftc}, A, and A_0 are the mitoxantrone accumulation in the presence of FTC, the test compounds, and 0.1% DMSO (the control), respectively.

Berenbaum's interaction index (I) was used to evaluate the potential interactions among multiple flavonoids as previously described (23). The '*I* value for each flavonoid combination was calculated by the following equation: $I = \sum \frac{D_{x,i}}{EC_{x,i}}$ where $D_{x,i}$ is the concentration of the individual flavonoid i in a flavonoid combination that produced x effect, and $EC_{x,i}$ is the concentration of flavonoid i which, when present alone, could also produce x effect. Loewe additivism, synergism, and antagonism are indicated when I values are not significantly different from 1, significantly less than 1, and significantly greater than 1, respectively.

Mitoxantrone Cytotoxicity Studies

Cytotoxicity studies were performed, as previously described, with some modification (20). The MCF-7/MX100 cells were seeded in 96-well plates, with a seeding density of 10^4 cells/well. After a 48-hour incubation period, various concentrations of mitoxantrone (ranging from 0–1000 μM), as well as the specified concentrations of individual flavonoids or the vehicle (0.1% DMSO), were added to each well. After another 24-hour incubation period, the cell growth in each well was determined using a sulforhodamine B (SRB) assay. The absorbance value at 570 nm (OD_{570}) from the SRB assay indicates the cellular protein in each well of the 96-well plates. The direct cytotoxicity of the tested flavonoids themselves (at 5 and 50 μM concentration) on cells was also investigated.

The growth inhibition of MCF-7/MX100 cells by mitoxantrone (IC_{50}) either alone or with the flavonoids was obtained by fitting the fraction of cell growth (F_2) by the equation: $F_2 = 100 \times \left(1 - \frac{I_{max} \times C^\gamma}{IC_{50}^\gamma + C^\gamma}\right)$.

The observed F_2 values were calculated as the ratio of the cell growth [$OD_{570}–OD_{570}(1000)$] to the maximum cell growth [$OD_{570}(0)–OD_{570}(1000)$]. $OD_{570}(0)$ and $OD_{570}(1000)$ are the absorbance values of cells treated

with 0 and 1000 μM mitoxantrone, respectively. OD_{570} is the absorbance value of cells treated with specified concentrations of mitoxantrone. C is the concentration of mitoxantrone. Two or three independent experiments were conducted, and quadruplicate measurements were performed in each experiment.

Mitoxantrone Bidirectional Transport Studies

Transport studies using MDCK monolayers were carried out as previously described with minor modifications (24). MDCK-mock, MDCK-Bcrp1, and MDCK-BCRP cells were seeded in transwell polycarbonate inserts (0.4 μm pore size, 24 mm diameter; Transwell 3412; Costar, Corning, NY) at a density of approximately 10^6 cells per well. After 7 days of culture, the cells were ready to be studied. On the day of the transport study, the cell monolayers were first washed twice with HBSS for a total of 30 min. The transport buffer (HBSS, pH 7.2) containing 2.5 μM of the individual flavonoids or the control (0.1% DMSO) was then loaded in both the apical (A) (1.5 ml) and basolateral (B) (2.5 ml) chambers and incubated at 37°C for 30 min. After this time, mitoxantrone (5 μM) was added to either the apical or basolateral chamber (donor chamber). The samples (100 μl) were then taken from the opposite chamber (the receiver chamber) at 30, 60, and 90 min after the addition of mitoxantrone and replaced with the same volume of fresh HBSS. To prevent mitoxantrone oxidation, 5 μl of 20% (*w*/*v*) ascorbic acid in 0.9% saline was added to each sample. The samples were stored at −20°C until HPLC analysis. The apparent permeability coefficients (P_{app}) of [^{3}H]mannitol, a paracellular marker, across MDCK cell monolayers was measured to test the integrity of the monolayer, and these values were all lower than 1.0×10^{-6} cm/s.

The apparent permeability coefficients (P_{app}) of mitoxantrone across MDCK monolayers in both A-to-B and B-to-A directions was calculated by the equation

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

where $\Delta Q/\Delta t$ is the rate of mitoxantrone appearing in the receiver chamber, which is obtained from the slope of the regression line on the transport-time profile of mitoxantrone across the cell monolayers, C_0 is the initial concentration of mitoxantrone loaded in the donor chamber, and A is the cell monolayer surface area (4.71 cm^2).

HPLC Analysis

The concentrations of mitoxantrone in the buffer samples from the transport studies were analyzed by HPLC using a Nucleosil C18 (250 mm×4 mm, I.D.) column (Macherey-Nagel, Easton, WA). The isocratic mobile phase consisted of 16:84 (*v*/*v*) acetonitrile: sodium phosphate (pH 2.3 with 0.1% TEA), running at a flow rate of 1.0 ml/min. The calibration curve was linear over the concentration range of 2.5 to 1000 ng/ml. The system consisted of a Waters 1525 pump, 717 plus autosampler, a 2847 UV detector, and a Waters Breeze workstation. The autosampler was maintained at 4°C during the experiment. Mitoxantrone was detected at 610 nm with a retention time in the buffer sample of 6 min. The lower limit of quantification of mitoxantrone in the buffer sample was 2.5 ng/ml.

Statistical Analysis

The differences between the mean values were analyzed for significance using a Student's t-test or one-way analysis of variance, followed by Dunnett's test. The p-values were considered statistically significant when they were less than 0.05.

RESULTS

The Expression of BCRP/Bcrp1 in MDCK Cell Line

To investigate the effects of flavonoids on the BCRP-mediated efflux of mitoxantrone, we first characterized the expression of BCRP in the cells used in our study using a Western blot analysis. Since we have already confirmed the BCRP expression in MCF-7/MX100 cells, we characterized the expression of BCRP/Bcrp1 in the MDCK cells. A single band at about 72 kDa was clearly observed in both the MDCK/Bcrp1 and the MDCK/BCRP cells, indicating the over-expression of murine Bcrp1 and human BCRP in these two cell lines, respectively (Fig. 2). No detectable Bcrp1 or BCRP was found in the corresponding parental MDCK/mock cells.

Effects of Multiple Flavonoids on the Accumulation of Mitoxantrone in MCF-7 Cells

Our previous results demonstrated that many flavonoids can reverse BCRP-mediated mitoxantrone efflux in breast cancer cells, with EC_{50} values ranging from 0.07 to 183 μM (21). Since the bioavailability of flavonoids is usually poor and the plasma concentrations observed after dietary or dietary supplement consumption are in the nanomolar or lower micromolar range (25,26), the MDR-reversing effect of those flavonoids with high EC_{50} values may not produce inhibition *in vivo*. Therefore, all the flavonoid candidates selected in the current study are potent BCRP inhibitors with low EC_{50} values (less than 2 μM), as shown in Table I. Among the 12 flavonoid candidates, biochanin A belongs to

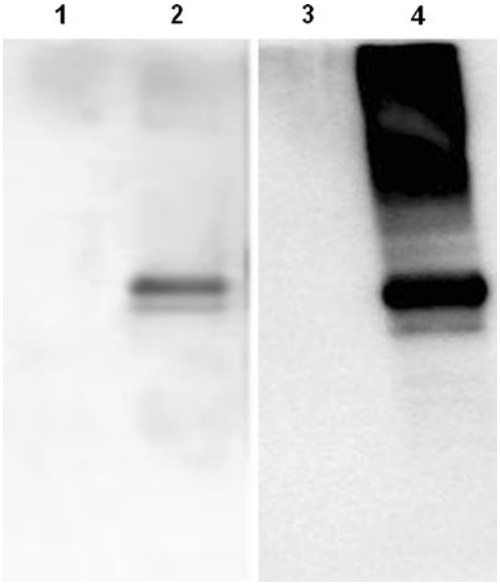


Fig. 2 Western blot analysis of human BCRP or murine Bcrp1 expression in MDCK cells. Lane 1 and lane 3 are mock-transfected MDCK cell lines. Lane 2 is human BCRP-transfected MDCK cell line. Lane 4 is murine Bcrp1-transfected MDCK cell line. Aliquots of whole cell lysates (17.5 μg) were electrophoresed on a 4–15% gradient gel and detected with anti-BCRP antibody BXP21 or anti-Bcrp1 antibody BXP-9.

the isoflavonoid subclass, kaempferide and galangin are members of the flavonol subclass, and the remaining candidates are all from the flavone subclass.

In order to test the potential interactions among multiple flavonoids on mitoxantrone accumulation, the EC_{50} values of the flavonoid candidates given in combination were first determined. During the study, equimolar concentrations of all the constituent flavonoids were used in all of the multiple flavonoid combinations. The concentration-dependent effects of flavonoid combinations on the mitoxantrone accumulation and the corresponding EC_{50} values, given as 2-, 3-, 4-, 7-, 9-, or 10-flavonoid combinations, were determined (Fig. 3 and Table I). The EC_{50} values, which refer to the concentration of each flavonoid in the tested multiple flavonoid combinations, ranged from 0.031 to 0.455 μM, indicating strong BCRP inhibition (Table I).

To evaluate the combinatory effects of multiple flavonoids with regards to BCRP inhibition, Berenbaum's interaction index method was used. The calculated interaction index (I) values for each flavonoid combination on mitoxantrone accumulation were determined (Table II). From the results, we can see that the interaction indices (I) for the 2-, 3-, 4-, 7-, 9-, or 10-flavonoid combinations were close to 1 (ranged from 0.89 to 1.22), indicating that all those flavonoid candidates, which demonstrate potent BCRP inhibition when present alone, may also act additively to inhibit BCRP, when present in either small or large combinations.

Effects of Flavonoids on the Cytotoxicity of Mitoxantrone in MCF-7 Cells

The concentration of mitoxantrone used in the cytotoxicity studies ranged from 0 to 1 mM. The stock solution of MX was prepared in double distilled water. To evaluate the potential use of flavonoids as chemosensitizers in the treatment of BCRP-mediated mitoxantrone resistance, four flavonoids from three different subclasses, namely biochanin A, kaempferide, 5,7-dimethoxylflavone, and 8-methylflavone, were selected and their effects on the cytotoxicity of mitoxantrone in BCRP-overexpressing MCF-7 cells were investigated. The chemical structures of these tested flavonoids are given in Fig. 1. The concentration-dependent effects of mitoxantrone on cell growth, either alone or in the presence of two concentrations (2.5 and 5 μM) of each tested flavonoid, were determined (Fig. 4). Compared to the cell growth inhibition by mitoxantrone alone, the growth inhibition curve is shifted to the left in the presence of each tested flavonoid. This indicates that these flavonoids can enhance mitoxantrone cytotoxicity and reverse BCRP-mediated resistance in MCF-7/MX100 cells. In addition, all of the tested flavonoids demonstrated concentration-dependent effects on mitoxantrone cytotoxicity (Fig. 4). The IC_{50} values of mitoxantrone in the absence and presence of tested flavonoids were also determined. The IC_{50} value of mitoxantrone in MCF-7/MX100 cells was 139 ± 13.3 μM (Table III). Significant decreases in its IC_{50} value were observed in the presence of the tested flavonoids: the IC_{50} values of mitoxantrone ranged from 19.2 to 60.0 μM in the presence of a flavonoid concentration of 2.5 μM and from 9.56 to 23.5 μM in the presence of concentrations of 5 μM. Additionally, the potential cytotoxicity of the flavonoids on MCF-7/MX100 cells, in the absence of mitoxantrone, was also investigated. Two different concentrations (5 and 50 μM) for each flavonoid were used, and our results showed that flavonoids themselves have no significant cytotoxicity at 5 μM and little cytotoxicity (<10%) at 50 μM (data not shown).

Effects of Flavonoids on the Bidirectional Transport of Mitoxantrone Across MDCK Cell Monolayers

To investigate the effects of flavonoids on the transport of mitoxantrone in BCRP-expressing normal cells, a mitoxantrone bi-directional transport study, in the presence or absence of the flavonoids, was conducted in MDCK cells transfected with human BCRP, murine Bcrp1, or an empty vector. The flavonoids tested in this study were the same as those used in the mitoxantrone cytotoxicity study (biochanin A, kaempferide, 5,7-dimethoxyflavone, and 8-methylflavone). The apparent permeability (Papp) values

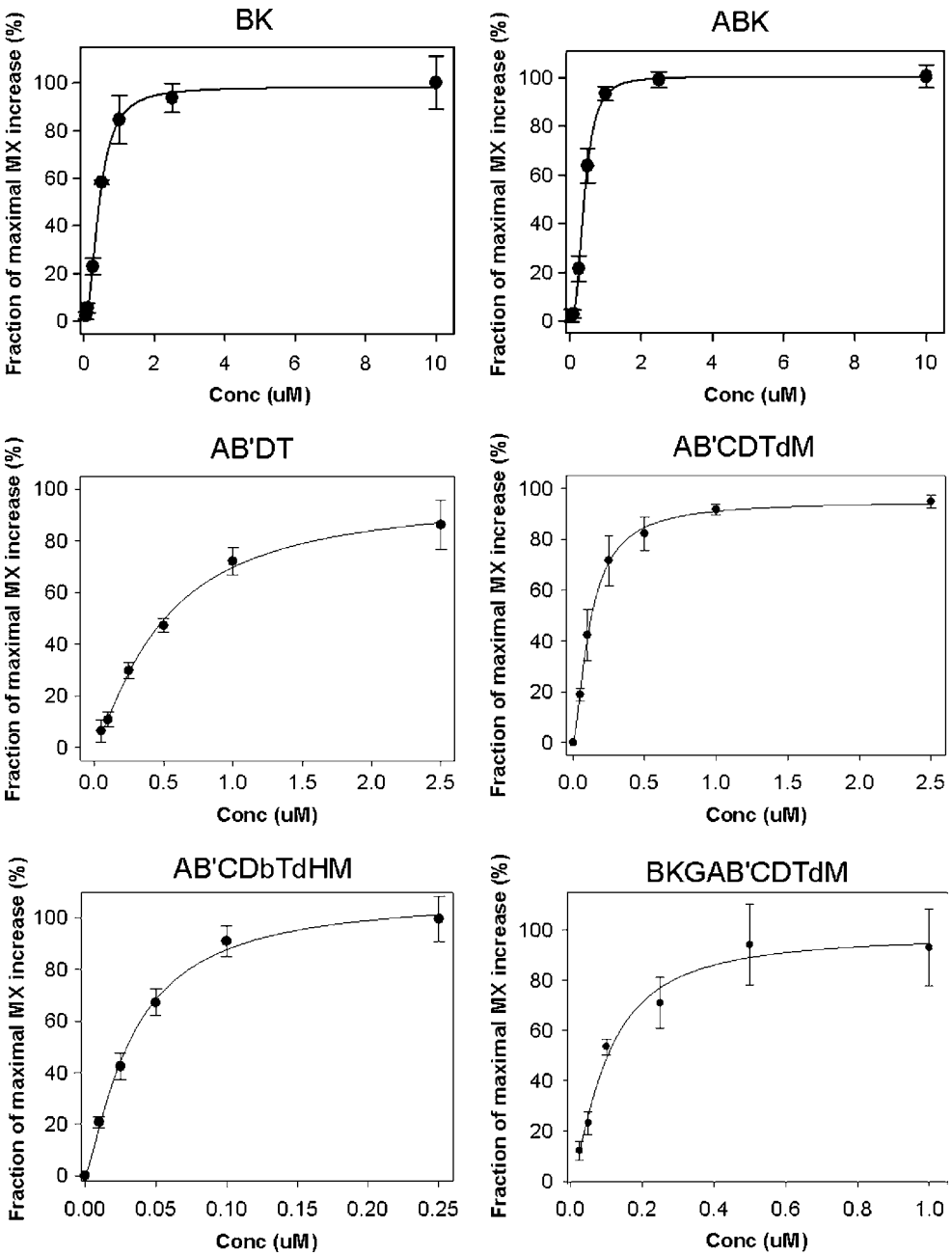


Fig. 3 Concentration-dependent effects of multiple flavonoid combinations on mitoxantrone accumulation in MCF-7/MX100 cells. Equimolar concentrations of all flavonoids were used in the flavonoid combinations. Data are presented as mean ± SD from three independent triplicate experiments. The solid lines represent the predicted value by fitting F1 with the equation as described in "Materials and Methods."

of mitoxantrone across three different MDCK cell line variants in the absence of flavonoids are shown in Table IV. The Papp of mitoxantrone (5 μM) in the basolateral (B)-to-apical (A) direction across Bcrp1- or BCRP-expressing MDCK cell monolayers was much higher than in the A-to-B direction, with a transport ratio (B-A/A-B) of 13.1 and 5.13, respectively. The $P_{app,\ B\text{-}A}$ *versus* $P_{app,A\text{-}B}$ of mitoxantrone across MDCK/mock cells were $5.68 \pm 0.86 \times 10^{-7}$ cm/s and $2.14 \pm 0.81 \times 10^{-7}$ cm/s, respectively, with a transport ratio of 2.65. Although the transport ratio is lower in MDCK/mock cells compared to that in MDCK/BCRP and MDCK/Bcrp1 cells, mitoxantrone was transported more efficiently in the B-to-A than in the A-to-B direction in all cases. This finding suggests the presence of endogenous, apically directed efflux transporter expression.

The transport of mitoxantrone across the BCRP- or Bcrp1-expressing MDCK cell monolayers, either alone or in the presence of flavonoids, is shown in Fig. 5 and Table IV. In MDCK/Bcrp1 cells, the B-to-A directional transport of mitoxantrone was significantly decreased in the presence of flavonoids, indicating that Bcrp1-mediated transport was inhibited. The efflux ratios in MDCK/Bcrp1 cells were decreased from 13.1 (without flavonoids) to 2.19 (with 2.5 μM biochanin A), 3.38 (with 2.5 μM kaempferide), 2.32 (with 2.5 μM 5,7-dimethoxyflavone), and 3.29 (with 2.5 μM 8-methylflavone), resulting in a transport pattern

Table II The Interaction Index (I) and its 95% Confidential Interval (CI) for Flavonoid Combinations on Mitoxantrone Accumulation in MCF7/MX100 Cells

Multiple flavonoid combinations	I	CI
BK	0.91 ± 0.21	[0.38, 1.44]
ABK	0.89 ± 0.12	[0.61, 1.18]
AB'DT	1.19 ± 0.20	[0.45, 1.95]
AB'CDTdM	1.22 ± 0.34	[0.38, 2.07]
AB'CDbTdHM	1.10 ± 0.05	[0.98, 1.21]
BKGAB'CDTdM	1.09 ± 0.08	[0.90, 1.28]

The interaction index (I) for each flavonoid combination was calculated as described in "Materials and Methods." The I values are given as mean ± SD from three independent experiments.

Table III The Effects of Flavonoids on the Cytotoxicity of Mitoxantrone (IC_{50}) in MCF7/MX100 Cells

Flavonoids	IC_{50} of mitoxantrone (μM)
Control	139 ± 13.3
Biochanin A (2.5 μM)	60.0 ± 16.0*
Biochanin A (5 μM)	23.5 ± 2.89*
Kaempferide (2.5 μM)	19.2 ± 4.30*
Kaempferide (5 μM)	9.56 ± 2.15*
5,7-Dimethoxyflavone (2.5 μM)	26.4 ± 2.56*
5,7-Dimethoxyflavone (5 μM)	11.3 ± 1.11*
8-Methylflavone (2.5 μM)	19.7 ± 4.61*
8-Methylflavone (5 μM)	10.3 ± 2.05*

*$P < 0.05$ compared with control (without flavonoids)

similar to that of the MDCK/mock cells. Similar results were also observed in MDCK/BCRP cells: efflux ratios decreased from 5.13 (without flavonoids) to 1.27 (with 2.5 μM biochanin A), 2.59 (with 2.5 μM kaempferide), 3.03 (with 2.5 μM 5,7-dimethoxyflavone), and 1.99 (with 2.5 μM 8-methylflavone). These results suggest that the flavonoids used in this investigation can inhibit both murine and human BCRP-mediated transport of mitoxantrone in MDCK cells.

DISCUSSION

Many compounds have been identified as BCRP inhibitors *in vitro*, including elacridar, fumitremogin C, Ko134, novobiocin, saquinavir, flavonoids, and estrogen agonists and antagonists (27–30). Among the reported BCRP inhibitors, flavonoids represent promising chemosensitizing agents since they are dietary compounds with little or no toxicity. Our previous results demonstrated that many

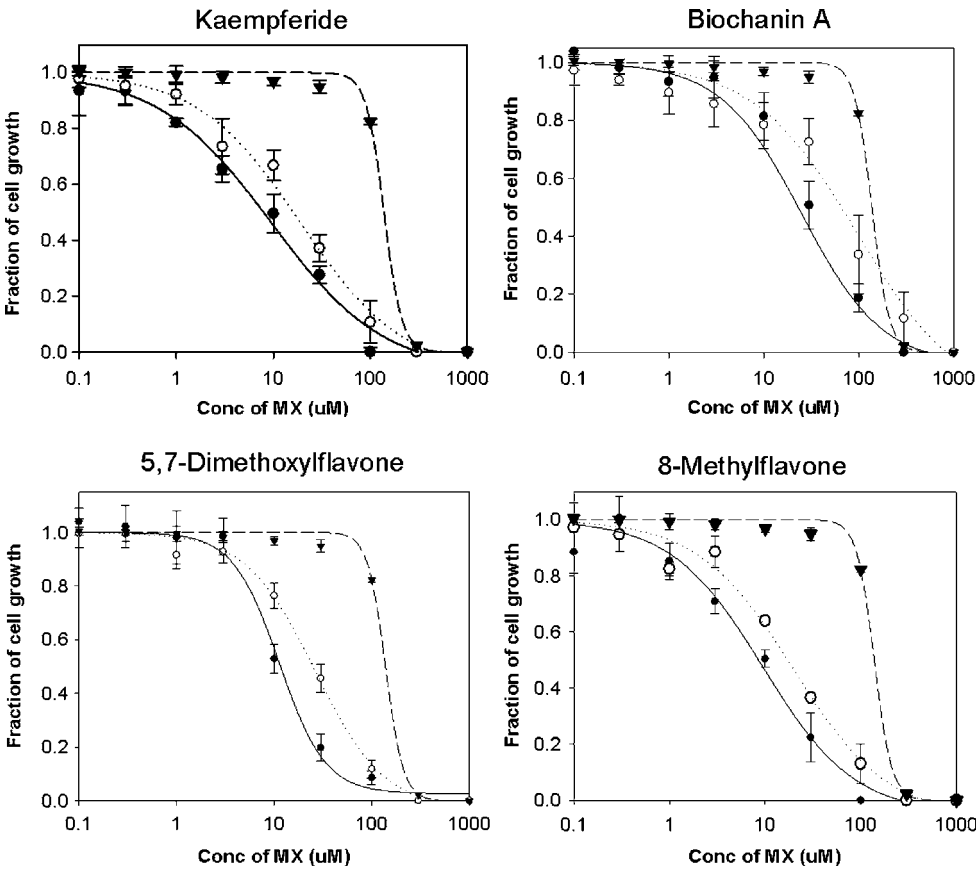


Fig. 4 The effects of the flavonoids biochanin A, kaempferide, 5,7-dimethoxyflavone and 8-methylflavone on the cytotoxicity of mitoxantrone in MCF-7/MX100 cells. Cell growth inhibition by mitoxantrone was determined in the absence (▼) and presence of 2.5 μM (○) or 5 μM (●) concentrations of each flavonoid. Data are presented as mean ± SD, $n = 4$. Two or three independent quadruplicate experiments were performed.

Table IV The Apparent Permeability (Papp) of Mitoxantrone Across Three MDCK Cell Monolayers in the Absence or Presence of Flavonoids

Cell line	Treatment	Papp,A-to-B (cm/s, 10^{-7})	Fold increase in A-to-B	Papp,B-to-A (cm/s, 10^{-7})	% decrease in B-to-A	Transport ratio
MDCK/mock	MX (5 μM)	2.14 ± 0.81	–	5.68 ± 0.86	–	2.65
MDCK/Bcrp1	MX (5 μM)	2.1 ± 0.16	–	27.5 ± 7.8	–	13.1
	+B (2.5 μM)	2.28 ± 0.82	1.03	5.03 ± 0.39*	81.7	2.19
	+K (2.5 μM)	1.44 ± 0.23	0.65	4.88 ± 1.9*	82.3	3.38
	+D (2.5 μM)	3.22 ± 0.43*	1.46	7.48 ± 2.49*	72.8	2.32
	+M (2.5 μM)	1.13 ± 0.15	0.51	3.73 ± 1.0*	86.4	3.29
MDCK/BCRP	MX (5 μM)	3.59 ± 2.1	–	18.4 ± 3.2	–-	5.13
	+B (2.5 μM)	3.7 ± 1.6	1.03	4.69 ± 1.4*	74.5	1.27
	+K (2.5 μM)	2.04 ± 0.29	0.57	5.28 ± 2.3*	71.3	2.59
	+D (2.5 μM)	3.43 ± 1.04	0.96	10.4 ± 4.7*	43.5	3.03
	+M (2.5 μM)	3.35 ± 0.27	0.93	6.69 ± 2.2*	63.3	1.99

*$p < 0.05$ compared with the corresponding controls

flavonoids exhibit BCRP-mediated inhibition of mitoxantrone accumulation in breast cancer cells, with EC_{50} values ranging from 0.07 to 183 μM (21). Interestingly, there are several reports indicating that a total herb extract shows an enhanced activity when compared to equivalent doses of its individual constituents (31,32). Since the human diet and many herbal products usually contain multiple flavonoids, instead of a single flavonoid, we investigated the effect of multiple flavonoids on the mitoxantrone accumulation in BCRP-overexpressing breast cancer cells. Berenbaum's interaction index was used to evaluate the potential interactions among multiple flavonoids with respect to BCRP inhibition. Although there are several methods available to assess drug interactions, we chose Berenbaum's interaction index because this method could be used to characterize very complex mixtures of three or more agents (33). Among the twelve flavonoid candidates with EC_{50} values less than 2.0 μM, we tried several combination patterns including 2-,3-,4-,7-,9-, or 10-flavonoid combinations. With equimolar concentration of all constituents, these combinations showed very potent BCRP inhibition, with EC_{50} values ranging from 0.031 to 0.455 μM. The calculated interaction indices (I) of all of these tested combinations were all close to 1, indicating additive effects of the multiple flavonoid combinations. Due to the poor solubility and low bioavailability of flavonoids, it may be difficult for individual flavonoids to reach high enough concentrations that are needed for BCRP inhibition *in vivo*, even with the consumption of high doses. The additive nature of flavonoid combinations may solve this problem: BCRP-mediated MDR may be reversed by multiple flavonoids present at low concentrations.

To further confirm the MDR-reversing effect of flavonoids on mitoxantrone resistance, we investigated the effect of flavonoids on the cytotoxicity of mitoxantrone in breast cancer cells. Compared to the mitoxantrone control group, the IC_{50} value of mitoxantrone decreased more than 10-fold when mitoxantrone was co-incubated with 5 μM kaempferide, 8-methylflavone and 5,7-dimethoxyflavone. This finding suggests that these flavonoids may be used as chemosensitizing agents. Biochanin A also significantly reduced the mitoxantrone IC_{50} values, but the effect was less than observed with the other three tested flavonoids. In the presence of 5 μM and 2.5 μM biochanin A, the IC_{50} values of mitoxantrone were 23.5 and 60.0 μM, respectively. These values were slightly lower than our previously reported data, which provided a mitoxantrone IC_{50} value of 30.9 μM (with 5 μM biochanin A) and 107 μM (with 2.5 μM biochanin A) (20). This small discrepancy might be explained by the change of BCRP expression levels among different batches of MCF-7/MX100 cells. The cells used in the current study have slightly lower BCRP expression than those cells used previously. This explanation is also supported by comparing the mitoxantrone IC_{50} in the absence of flavonoids: the IC_{50} value of mitoxantrone in the control group examined in the current study is also lower than the one previously determined (139 μM vs. 199 μM, respectively). Although the cytotoxicity studies involved longer incubation times (24 h) than the accumulation studies (30 min), the chemosensitizing effect of flavonoids on mitoxantrone cytotoxicity in BCRP-overexpressing cancer cells is likely the result of inhibition of BCRP function, instead of a reduction in BCRP expression. In a study conducted by Imai *et al.* (34), using K562/BCRP cells treated with the flavonoids genistein, naringenin, and acacetin, all of which can sensitize K562/BCRP cells to mitoxantrone, none of these flavonoids changed the expression level of BCRP compared to the control group. The expression of BCRP in MCF-7 cells after the treatment of flavonoids has been studied by other members of our laboratory (Yang X, Moon YJ, Viswanathan L and Morris ME, unpublished data). They tested the time-dependent

effect of several flavonoids, including biochanin A, on BCRP expression, and their results showed that that none of the flavonoids changed the mRNA and protein expression of BCRP after a 24-hr treatment. Some flavonoids can induce BCRP expression after 5 days treatment: this is concentration dependent and typically requires higher concentrations than we used in our study. Since we used a 24-hr incubation period in the cytotoxicity study using MCF-7 cells, the down-regulation of BCRP by flavonoids would not be expected. Moreover, many flavonoids have been identified as ligands of the aryl hydrocarbon receptor (AhR) (35). Since BCRP expression is inducible and induction can be mediated by AhR, it is more common for flavonoids to up-regulate rather than down-regulate BCRP expression after long-term incubation or administration.

Due to their low EC_{50} values, it is possible that some flavonoids, even after oral administration, may reach a systemic concentration that is sufficient for reversing BCRP-mediated drug resistance. For example, Walle *et al.* (36) investigated the pharmacokinetics and tissue distribution of 5,7-dimethoxyflavone in rats. Their results revealed that the peak plasma concentration of 5,7-dimethoxyflavone was 2.5 ± 0.8 μM at 1 h after the oral dosage of 5 mg/kg. Moreover, the concentrations of 5,7-dimethoxyflavone in most tissues investigated in their study were higher than that in plasma, with concentrations in the liver exceeding those in

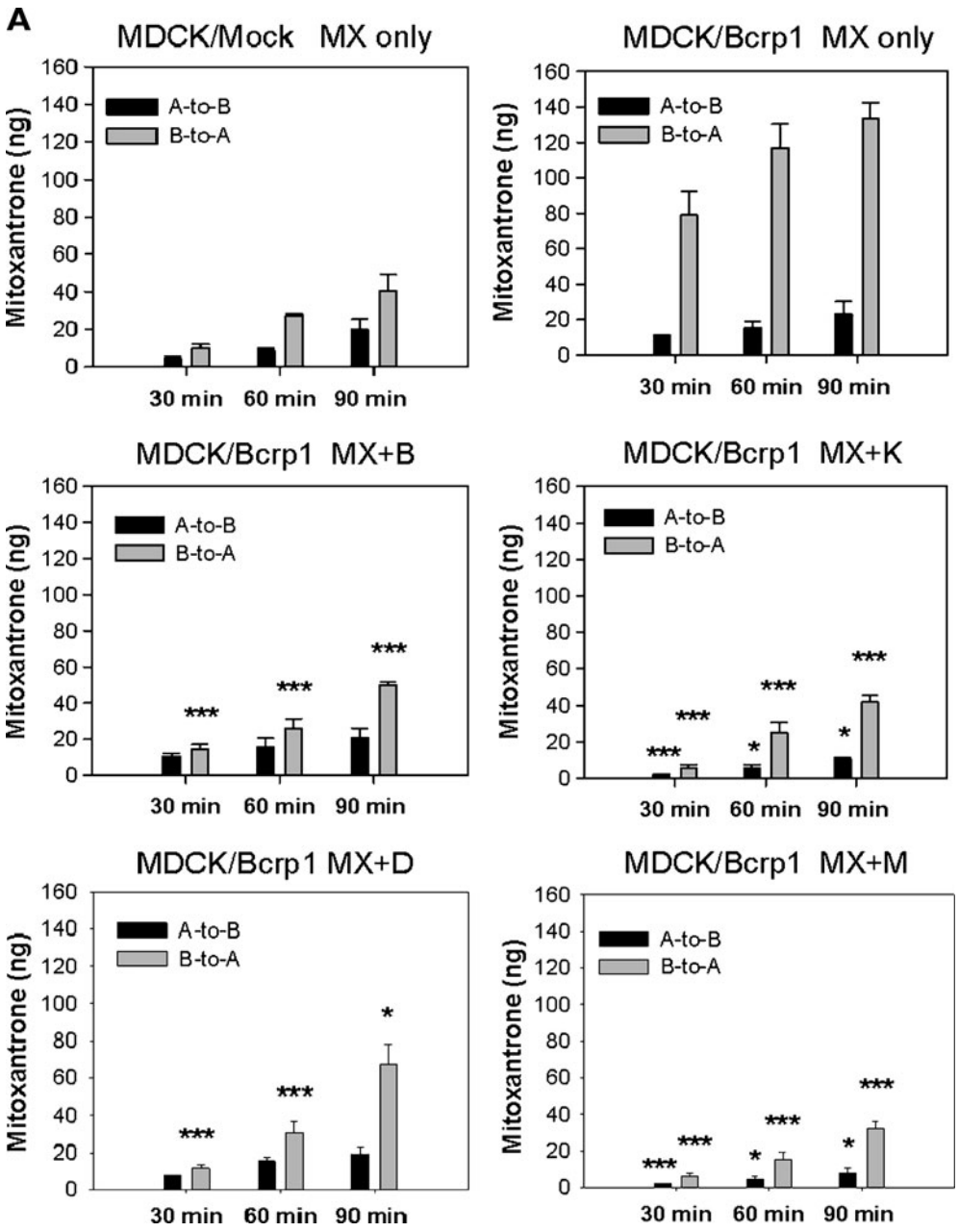
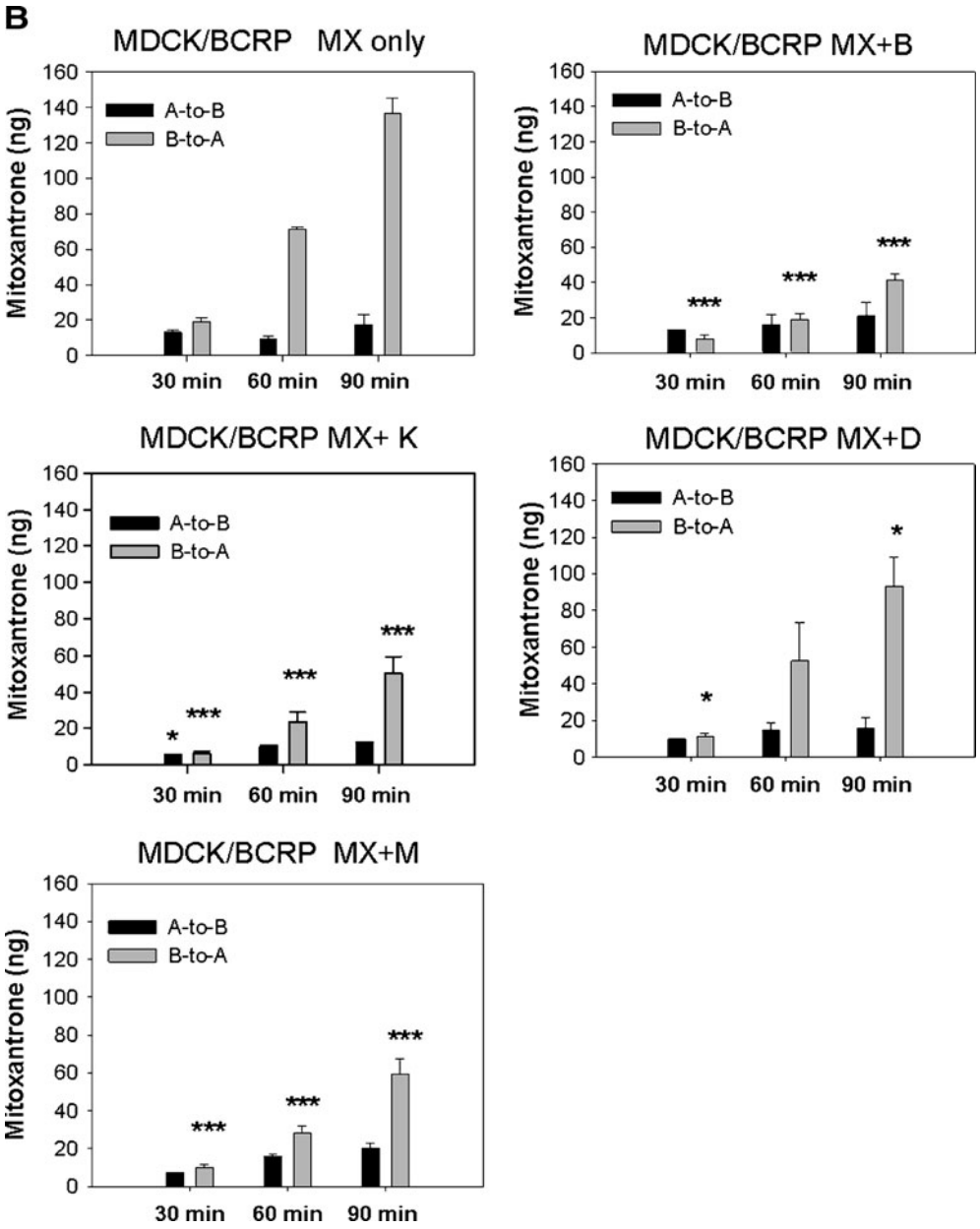


Fig. 5 The effects of the flavonoids biochanin A (B), kaempferide (K), 5,7-dimethoxyflavone (D) and 8-methylflavone (M), on the transport of mitoxantrone (5 μM) in murine Bcrp1- or human BCRP- expressing MDCK cells. Data are presented as mean ± SD, $n = 3$.*, $p < 0.05$; ***, $p < 0.001$ compared with corresponding control group for MDCK/ Bcrp1 or MDCK/ BCRP cells.

Fig. 5 (continued)



the plasma by almost seven-fold (i.e. 16.5±5 μM) (36). Since the EC_{50} value of 5,7-dimethoxyflavone for BCRP inhibition was only 1.41±0.26 μM (Table I), it is reasonable to speculate that its MDR-reversing effect, observed *in vitro*, may also occur *in vivo* and, therefore, may have clinical importance.

While the mitoxantrone accumulation and cytotoxicity studies provide us with promising results regarding flavonoids as MDR-reversing agents, we should note that BCRP is present not only in tumor cells, but also in a number of normal tissues. This may cause unwanted pharmacokinetic interactions between mitoxantrone and co-administered flavonoids. Since there is no information about the effect of flavonoids on the transport of mitoxantrone in BCRP-expressing normal cells, mitoxantrone bi-directional transport studies were conducted using MDCK cells that were transfected with human BCRP, murine Bcrp1, or empty vector. Four flavonoids, namely biochanin A, kaempferide, 5,7-dimethoxyflavone, and 8-methylflavone, which exhibited strong MDR-reversing effects in our cytotoxicity study, were selected for the transport study. Consistent with the concentration used in the cytotoxicity study, a 2.5 μM concentration of tested flavonoids, a concentration above the EC_{50} values for BCRP inhibition and that may be achievable *in vivo*, was used. All of the tested flavonoids significantly decreased the B-to-A directed transport of mitoxantrone in both human and murine BCRP-expressing MDCK cell monolayers. However, in contrast to the great

decrease of mitoxantrone B-to-A transport, little to no change in mitoxantrone A-to-B transport was observed in the presence of flavonoids, suggesting that flavonoids have stronger effects on the mitoxantrone B-to-A transport than A-to-B transport. Although the precise mechanism underlying this difference is unknown, this direction-dependent inhibitory effect of flavonoids on mitoxantrone transport across MDCK cell monolayers might occur due to the asymmetry of the apical and basal membrane diffusion of MDCK cells: MDCK cells are canine kidney cells and barrier epithelia, such as that occurring in the urinary bladder and collecting tubule, known to have apical membranes with very low permeability (37), and the diffusion of flavonoids and/or mitoxantrone into or across the membrane may be low. This is important since substrates and inhibitors must reach BCRP binding sites, which are near the membrane-cytoplasm interface.

Our transport data also showed that mitoxantrone was more efficiently transported by murine Bcrp1 than human BCRP, with a transport ratio of 13.1 in murine Bcrp1-expressing MDCK cells and 5.13 in human BCRP-expressing MDCK cells. Similar results have also been reported in several other studies. For example, Merino *et al.* (24) reported highly efficient transport of ciprofloxacin, ofloxacin, and norfloxacin by murine Bcrp1 and only moderate transport of these compounds by human BCRP in MDCK cells. Albendazole sulfoxide and oxfendazole were also found to be transported more efficiently in murine Bcrp1-transduced MDCK cells compared with their transport in human BCRP-transduced cells (38). One of the possible explanations for this transport difference between murine Bcrp1 and human BCRP is likely related to the lower expression of human BCRP than murine Bcrp1 in MDCK cell lines, as indicated by Merino *et al.* (24) However, there also may be differences in the affinity and selectivity for substrates between these two homologues. A study conducted by Enokizono *et al.* (39) reported a higher flux ratio of troglitazone sulfate in human BCRP-expressed cells than that in murine Bcrp1-expressed cells; this difference could not be explained by an expression level difference.

It is interesting to note that, even in MDCK mock cells, we still can see that mitoxantrone was transported more efficiently in the B-to-A than in the A-to-B direction, which is in line with the data obtained by other groups (40,41). For example, in a study conducted by Pan and Elmquist (40), at a concentration of 20 μM, the Papp of mitoxantrone in the B-to-A direction (3.32×10^{-6}) was higher than that in the A-to-B direction (1.85×10^{-6}) in wild-type MDCK cells, with an efflux ratio of 1.78. Xiao *et al.* (41) also reported that the transport of mitoxantrone, at a concentration of 50 μM, was more efficient in the B-to-A direction than in the A-to-B direction in wild-type MDCK cells, with an efflux ratio of 2.3. One possible explanation for this phenomenon is that P-gp might be involved in this directed transport of mitoxantrone in MDCK/mock cells since mitoxantrone is not only a substrate of BCRP but also P-gp. To the best of our knowledge, the endogenous expression of P-gp in the wild-type MDCK cells has been confirmed using Western blot analysis by three independent research groups (37,42,43), either in total cell lysates or in membrane fractions. However, other than P-gp expression, the asymmetry of apical and basal membrane diffusion of mitoxantrone, as mentioned before, might also contribute to this phenomenon.

In conclusion, multiple flavonoid combinations act additively and exhibit strong BCRP inhibition, resulting in increased mitoxantrone accumulation in breast cancer cells. The flavonoid EC_{50} values ranged from 0.031 to 0.455 μM when they were given in 2-, 3-, 4-, 7-, 9-, or 10-flavonoid combinations. Kaempferide, biochanin A, 5,7-dimethoxyflavone, and 8-methylflavone, at both 2.5 μM and 5 μM concentrations, greatly improved the cytotoxicity of mitoxantrone in BCRP-overexpressing breast cancer cells, indicating their potential as chemosensitizing agents. In addition, the B-to-A-directed transport of mitoxantrone in murine Bcrp1- and human BCRP-expressing MDCK cells in the presence of 2.5 μM of kaempferide, biochanin A, 5,7-dimethoxyflavone, and 8-methylflavone was also significantly decreased. These results indicate that, while these flavonoids demonstrate MDR-reversing effects, they may also influence the disposition of mitoxantrone and cause unwanted pharmacokinetic interactions. To further confirm the results that were observed *in vitro*, studies involving the effects of flavonoids on the pharmacokinetics and pharmacodynamics of mitoxantrone *in vivo* are currently ongoing in our laboratory.

ACKNOWLEDGEMENT

This study was supported in part by a grant from the Susan G. Komen Breast Cancer Foundation (BCTR0601385).

REFERENCES

1. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. Nat Rev Drug Discov. 2006;5:219–34.
2. Perez-Tomas R. Multidrug resistance: retrospect and prospects in anti-cancer drug treatment. Curr Med Chem. 2006;13:1859–76.
3. Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK *et al.* A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci USA. 1998;95:15665–70.
4. Diestra JE, Scheffer GL, Catala I, Maliepaard M, Schellens JH, Scheper RJ *et al.* Frequent expression of the multi-drug resistance-associated protein BCRP/MXR/ABCP/ABCG2 in human tumours detected by the BXP-21 monoclonal antibody in paraffin-embedded material. J Pathol. 2002;198:213–9.

5. Steinbach D, Sell W, Voigt A, Hermann J, Zintl F, Sauerbrey A. BCRP gene expression is associated with a poor response to remission induction therapy in childhood acute myeloid leukemia. Leukemia. 2002;16:1443–7.
6. Hardwick LJ, Velamakanni S, van Veen HW. The emerging pharmacotherapeutic significance of the breast cancer resistance protein (ABCG2). Br J Pharmacol. 2007;151:163–74.
7. Yang CH, Schneider E, Kuo ML, Volk EL, Rocchi E, Chen YC. BCRP/MXR/ABCP expression in topotecan-resistant human breast carcinoma cells. Biochem Pharmacol. 2000;60:831–7.
8. Sharom FJ. ABC multidrug transporters: structure, function and role in chemoresistance. Pharmacogenomics. 2008;9:105–27.
9. Robey RW, Medina-Perez WY, Nishiyama K, Lahusen T, Miyake K, Litman T *et al.* Overexpression of the ATP-binding cassette half-transporter, ABCG2 (Mxr/BCrp/ABCP1), in flavopiridol-resistant human breast cancer cells. Clin Cancer Res. 2001;7:145–52.
10. Staudand F, Pavek P. Breast cancer resistance protein (BCRP/ABCG2). Int J Biochem Cell Biol. 2005;37:720–5.
11. Youngand CW, Raymond V. Clinical assessment of the structure-activity relationship of anthracyclines and related synthetic derivatives. Cancer Treat Rep. 1986;70:51–63.
12. Rentsch KM, Horber DH, Schwendener RA, Wunderli-Allenspach H, Hanseler E. Comparative pharmacokinetic and cytotoxic analysis of three different formulations of mitoxantrone in mice. Br J Cancer. 1997;75:986–92.
13. Ehninger G, Schuler U, Proksch B, Zeller KP, Blanz J. Pharmacokinetics and metabolism of mitoxantrone. A review. Clin Pharmacokinet. 1990;18:365–80.
14. Diah SK, Smitherman PK, Aldridge J, Volk EL, Schneider E, Townsend AJ *et al.* Resistance to mitoxantrone in multidrug-resistant MCF7 breast cancer cells: evaluation of mitoxantrone transport and the role of multidrug resistance protein family proteins. Cancer Res. 2001;61:5461–7.
15. Mao Q, Unadkat JD. Role of the breast cancer resistance protein (ABCG2) in drug transport. AAPS J. 2005;7:E118–33.
16. Consoli U, Van NT, Neamati N, Mahadevia R, Beran M, Zhao S *et al.* Cellular pharmacology of mitoxantrone in p-glycoprotein-positive and-negative human myeloid leukemic cell lines. Leukemia. 1997;11:2066–74.
17. Galli F. Interactions of polyphenolic compounds with drug disposition and metabolism. Curr Drug Metab. 2007;8:830–8.
18. Jang SI, Kim HJ, Hwang KM, Jekal SJ, Pae HO, Choi BM *et al.* Hepatoprotective effect of baicalin, a major flavone from Scutellaria radix, on acetaminophen-induced liver injury in mice. Immunopharmacol Immunotoxicol. 2003;25:585–94.
19. Wen X, Walle T. Methylated flavonoids have greatly improved intestinal absorption and metabolic stability. Drug Metab Dispos. 2006;34:1786–92.
20. Zhang S, Yang X, Morris ME. Flavonoids are inhibitors of breast cancer resistance protein (ABCG2)-mediated transport. Mol Pharmacol. 2004;65:1208–16.
21. Zhang S, Yang X, Coburn RA, Morris ME. Structure activity relationships and quantitative structure activity relationships for the flavonoid-mediated inhibition of breast cancer resistance protein. Biochem Pharmacol. 2005;70:627–39.
22. Leslie EM, Mao Q, Oleschuk CJ, Deeley RG, Cole SP. Modulation of multidrug resistance protein 1 (MRP1/ABCC1) transport and atpase activities by interaction with dietary flavonoids. Mol Pharmacol. 2001;59:1171–80.
23. Zhang S, Yang X, Morris ME. Combined effects of multiple flavonoids on breast cancer resistance protein (ABCG2)-mediated transport. Pharm Res. 2004;21:1263–73.
24. Merino G, Alvarez AI, Pulido MM, Molina AJ, Schinkel AH, Prieto JG. Breast cancer resistance protein (BCRP/ABCG2) transports fluoroquinolone antibiotics and affects their oral availability, pharmacokinetics, and milk secretion. Drug Metab Dispos. 2006;34:690–5.
25. Walle T, Otake Y, Brubaker JA, Walle UK, Halushka PV. Disposition and metabolism of the flavonoid chrysin in normal volunteers. Br J Clin Pharmacol. 2001;51:143–6.
26. Manach C, Donovan JL. Pharmacokinetics and metabolism of dietary flavonoids in humans. Free Radic Res. 2004;38:771–85.
27. Weiss J, Rose J, Storch CH, Ketabi-Kiyanvash N, Sauer A, Haefeli WE *et al.* Modulation of human BCRP (ABCG2) activity by anti-HIV drugs. J Antimicrob Chemother. 2007;59:238–45.
28. Sugimoto Y, Tsukahara S, Imai Y, Ueda K, Tsuruo T. Reversal of breast cancer resistance protein-mediated drug resistance by estrogen antagonists and agonists. Mol Cancer Ther. 2003;2:105–12.
29. Ahmed-Belkacem A, Pozza A, Macalou S, Perez-Victoria JM, Boumendjel A, Di Pietro A. Inhibitors of cancer cell multidrug resistance mediated by breast cancer resistance protein (BCRP/ABCG2). Anticancer Drugs. 2006;17:239–43.
30. Su Y, Hu P, Lee SH, Sinko PJ. Using novobiocin as a specific inhibitor of breast cancer resistant protein to assess the role of transporter in the absorption and disposition of topotecan. J Pharm Pharm Sci. 2007;10:519–36.
31. Liu RH. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. Am J Clin Nutr. 2003;78:517S–20.
32. Williamson EM. Synergy and other interactions in phytomedicines. Phytomedicine. 2001;8:401–9.
33. Berenbaum MC. Correlations between methods for measurement of synergy. J Infect Dis. 1980;142:476–80.
34. Imai Y, Tsukahara S, Asada S, Sugimoto Y. Phytoestrogens/flavonoids reverse breast cancer resistance protein/ABCG2-mediated multidrug resistance. Cancer Res. 2004;64:4346–52.
35. Ebert B, Seidel A, Lampen A. Phytochemicals induce breast cancer resistance protein in Caco-2 cells and enhance the transport of benzo [a]pyrene-3-sulfate. Toxicol Sci. 2007;96:227–36.
36. Walle T, Ta N, Kawamori T, Wen X, Tsuji PA, Walle UK. Cancer chemopreventive properties of orally bioavailable flavonoids–methylated *versus* unmethylated flavones. Biochem Pharmacol. 2007;73:1288–96.
37. Ito S, Woodland C, Sarkadi B, Hockmann G, Walker SE, Koren G. Modeling of P-glycoprotein-involved epithelial drug transport in MDCK cells. Am J Physiol. 1999;277:F84–96.
38. Merino G, Jonker JW, Wagenaar E, Pulido MM, Molina AJ, Alvarez AI *et al.* Transport of anthelmintic benzimidazole drugs by breast cancer resistance protein (BCRP/ABCG2). Drug Metab Dispos. 2005;33:614–8.
39. Enokizono J, Kusuhara H, Sugiyama Y. Involvement of breast cancer resistance protein (BCRP/ABCG2) in the biliary excretion and intestinal efflux of troglitazone sulfate, the major metabolite of troglitazone with a cholestatic effect. Drug Metab Dispos. 2007;35:209–14.
40. Pan G, Elmquist WF. Mitoxantrone permeability in MDCKII cells is influenced by active influx transport. Mol Pharm. 2007;4:475–83.
41. Xiao Y, Davidson R, Smith A, Pereira D, Zhao S, Soglia J *et al.* A 96-well efflux assay to identify ABCG2 substrates using a stably transfected MDCK II cell line. Mol Pharm. 2006;3:45–54.
42. Hammerle SP, Rothen-Rutishauser B, Kramer SD, Gunthert M, Wunderli-Allenspach H. P-Glycoprotein in cell cultures: a combined approach to study expression, localisation, and functionality in the confocal microscope. Eur J Pharm Sci. 2000;12:69–77.
43. Raggers RJ, Vogels I, van Meer G. Upregulation of the expression of endogenous Mdr1 P-glycoprotein enhances lipid translocation in MDCK cells transfected with human MRP2. Histochem Cell Biol. 2002;117:181–5.