Combined Effects of Multiple Flavonoids on Breast Cancer Resistance Protein (ABCG2)-Mediated Transport

Shuzhong Zhang,[1] Xinning Yang,[1] and Marilyn E. Morris[1,2]

Received January 9, 2004; accepted March 25, 2004

Purpose. The purpose of this study was to determine the dynamic parameter (EC_{50}) of flavonoids apigenin, biochanin A, chrysin, genistein, kaempferol, hesperetin, naringenin, and silymarin for breast cancer resistance protein (BCRP) inhibition when used alone, and to evaluate their potential interactions (additive, synergistic, or antagonistic) with regards to BCRP inhibition when used in multiple-flavonoid combinations.

Methods. The effects of flavonoids on BCRP-mediated transport were examined by evaluating their effects on mitoxantrone accumulation and cytotoxicity in MCF-7 MX100 cells overexpressing BCRP. The EC_{50} values of these flavonoids for increasing mitoxantrone accumulation were estimated using a Hill equation. The potential interactions among multiple flavonoids with regard to BCRP inhibition were assessed by isobologram and Berenbaum's interaction index methods.

Results. The EC_{50} values of these flavonoids for increasing mitoxantrone accumulation ranged from 0.39 ± 0.13 μM to 33.7 ± 2.78 μM. Quantitative analysis of the combined effects of multiple flavonoids on mitoxantrone accumulation indicated that these flavonoids act additively in inhibiting BCRP when given as 2-, 3-, 5-, or 8-flavonoid combinations with equimolar concentrations of all constituents. The results of the mitoxantrone cytotoxicity studies were consistent with these findings.

Conclusions. The additive effects of multiple flavonoids for BCRP inhibition suggests that prediction of BCRP-mediated food (herbal product)-drug interactions should also take into consideration the presence of multiple flavonoids and provides a rationale for using "flavonoid cocktails" as a potential approach for multidrug resistance reversal in cancer treatment.

KEY WORDS: BCRP; drug interaction; flavonoids; Loewe additivity; MDR reversal.

INTRODUCTION

Multidrug resistance (MDR) is defined as the cross-resistance to a variety of agents with distinct chemical structures or mechanisms of action and is believed to be the primary obstacle to successful cancer chemotherapy. Overexpression of a family of the so-called ABC (ATP binding cassette) transporters, such as P-glycoprotein and MRP1, in the plasma membrane, which actively extrude cytotoxic agents out of the cancer cells and thus prevent efficient cell killing, is a well-recognized mechanism underlying MDR (1). Therefore, pharmacological inhibition of these transporters with transporter inhibitors represents a promising approach for MDR reversal (2). Breast cancer resistance protein (BCRP, MXR, ABCP, ABCG2) is a newly identified ABC transporter (3–5), and overexpression of this transporter has been shown to cause cross-resistance to doxorubicin, topotecan, SN38, mitoxantrone, methotrexate, and flavopiridol, as well as to nucleoside HIV reverse transcriptase inhibitors including zidovudine and lamivudine (3,6–9). Significant BCRP expression has been detected in a number of human tumors (10–13), and possibly associated with clinical MDR (14). Thus, BCRP appears to be another potential molecular target for reversing MDR. Additionally, BCRP is also expressed in a number of normal tissues, such as the canalicular membrane of liver hepatocytes, the apical membrane of intestinal epithelium, the luminal surface of brain capillaries, as well as human placenta (15,16). The mRNA level of BCRP is higher than that of P-glycoprotein, an important ABC efflux transporter in drug disposition, in human intestinal epithelium (17). Therefore, it is likely that BCRP also plays an important function in the disposition of drugs and xenotoxins; in fact, inhibition of BCRP-mediated transport has been shown to alter the pharmacokinetics of BCRP substrates, producing increased bioavailability, increased distribution across the placenta and decreased clearance (18,19).

Flavonoids are the most abundant polyphenols present in the human diet in vegetables, fruits and plant-derived beverages, and are the main components of many herbal products. More than 6500 naturally occurring flavonoids have been described, and the daily intake of total flavonoids in the Western diet was estimated to be 200 mg to 1 g (20). Epidemiological and animal studies have suggested that a high intake of flavonoids may be beneficial for decreasing the risk of cancer (21,22), coronary disease (23), and osteoporosis (24). Due to these perceived beneficial health activities, the lack of toxicity (25,26), as well as the increasing public interest in alternative medicine (27–29), flavonoid-containing dietary supplements and herbal products can now be found in almost any health food store, and consumption of these compounds will most likely increase, posing a serious potential for flavonoid-drug interactions. Therefore, understanding the interactions of flavonoids with molecular entities important in drug disposition, such as drug metabolizing enzymes and transporters, is urgently needed and the information will aid our understanding and prediction of potential food-drug and herbal product-drug interactions.

We reported previously (30) that many naturally occurring flavonoids can inhibit BCRP and produce increased mitoxantrone accumulation and cytotoxicity in BCRP-overexpressing cells, with no or minimal effects in BCRP-negative cells. The most potent flavonoids biochanin A and chrysin produced significant increases in mitoxantrone accumulation at concentrations of 0.5 or 1.0 μM, and in mitoxantrone toxicity at 2.5 μM. Considering these low effective concentrations and the wide consumption of the flavonoid-containing foods, especially the mega dose intake of these compounds present in dietary supplements and herbal products, clinically

[1] Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, State University of New York, Amherst New York 14260, USA.

[2] To whom correspondence should be addressed. (e-mail: memorris@buffalo.edu)

ABBREVIATIONS: ABC, ATP binding cassette; BCRP, breast cancer resistance protein; FTC, fumitremorgin C; MDR, multidrug resistance; MRP1, multidrug resistance-associated protein 1; MX, mitoxantrone; P-gp, P-glycoprotein; SRB, sulforhodamine B.

relevant drug interactions might be anticipated. However, the EC_{50} values of these flavonoids for inhibiting BCRP are not known, and should be characterized in order to better associate these *in vitro* observations with the potential for *in vivo* interactions. In addition, food intake and ingestion of herbal products usually involve the simultaneous consumption of multiple flavonoids, and thus prediction of potential food-drug interactions mediated by BCRP also requires an understanding of the potential interactions, in terms of BCRP inhibition, among these co-ingested flavonoids. It has been reported that the modulation of ABC transporters such as MRP1 (multidrug resistance associated protein 1) by different flavonoids could occur through different interactions with the transporter molecules and that "the flavonoids should be considered individually rather than a class of compounds" (31). Thus, additive, synergistic or antagonistic effects among multiple flavonoids regarding their modulation of ABC transporters could be possible. Therefore, the objective of the present study was to characterize the EC_{50} values of flavonoids for BCRP inhibition and to evaluate the potential interactions (additive, synergistic or antagonistic) among a group of flavonoids regarding their BCRP inhibitory activities. MCF-7 MX100 cells were used as the BCRP-overexpressing cell model and the flavonoids apigenin, biochanin A, chrysin, genistein, kaempferol, hesperetin, naringenin and silymarin were included in the study. All these flavonoids have been shown to possess substantial BCRP inhibition activity (30).

MATERIALS AND METHODS

Materials

Mitoxantrone and the flavonoids apigenin, biochanin A, chrysin, genistein, kaempferol, hesperetin, naringenin, and silymarin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Silymarin refers to collectively silibin (major component), silydianin and silychristin (22), and the molar concentration was calculated based on the molecular weight of silibin. RPMI (Roswell Park Memorial Institute) 1640, fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were purchased from Gibco BRL (Buffalo, NY, USA). Human breast cancer MCF-7 sensitive and MCF-7 MX100 (MCF-7 cells selected with mitoxantrone) cells and fumitremorgin C (FTC) were the kind gifts of Dr. Susan E. Bates (National Cancer Institute, Bethesda, MD, USA). MCF-7 MX100 cells overexpress BCRP with no detectable expression of P-glycoprotein or MRP1, and there is no detectable expression of BCRP, P-glycoprotein, or MRP1 in MCF-7 sensitive cells as demonstrated by Western blot analysis (30). Both MCF-7 sensitive and MCF-7 MX100 cells were also shown to have no detectable MRP2 expression (data not shown) by Western blot analysis.

Cell Culture

MCF-7 sensitive and MX100 cells were cultured in 75-cm^2 flasks with RPMI 1640 culture media supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO_2/95% air. The culture media also contained 100 U/ml penicillin and 100 μg/ml streptomycin; the MCF-7 MX100 cell culture media also included 100 nM of mitoxantrone.

Mitoxantrone Accumulation Studies

The accumulation studies were performed using flow cytometric analysis as reported (32) with some modification. Briefly, cells grown in 75-cm^2 flasks with about 90% confluence were trypsinized and washed with FBS-free RPMI 1640 and resuspended in this medium with a cell density of about 10^6 cells/ml. The accumulation of mitoxantrone was performed by incubating 1 ml of cells with various concentrations of the test compounds or the vehicle (0.1% DMSO) at 37°C for 15 min, followed by addition of 3 μM of mitoxantrone. FTC (10 μM), a specific BCRP inhibitor (33,34), was used as a positive control. After incubation for another 30 min, the accumulation was stopped by adding 3 ml of ice-cold phosphate-buffered saline (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 (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with a standard argon laser for 488-nm excitation, and 670-nm bandpass filter was used to detect mitoxantrone fluorescence. Preliminary studies demonstrated that the fluorescence of all the tested flavonoids at this setting is negligible. The accumulation of mitoxantrone was expressed as percent of the control (in the presence of the vehicle, 0.1% DMSO).

Mitoxantrone Cytotoxicity Studies

Cytotoxicity studies were performed in 96-well plates, with cells (5×10^3) seeded in each well. After cell attachment (24 h incubation), the culture medium in each well was replaced with fresh medium containing 0–1000 μM mitoxantrone as well as the specified concentrations of flavonoids, flavonoid combinations, or the vehicle (0.1% DMSO). FTC (10 μM) was used as a positive control. After a 24-h incubation, the drug-containing medium was aspirated 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 a sulforhodamine B (SRB) assay (35). The absorbance value at 570 nm (OD_{570}) from the SRB assay indicates the cell number in each well of the 96-well plates. In these cells, all the tested flavonoids at a 10 μM concentration produced no cytotoxicity in the absence of mitoxantrone; a concentration of 50 μM also produced no or little cytotoxicity (less than 15% growth inhibition).

Data Analysis

Calculation of EC_{50}, EC_{30}, and EC_{70} in Accumulation Studies

The EC_{50} values of flavonoids for increasing mitoxantrone accumulation in MCF-7 MX100 cells were obtained by fitting the fraction of maximal increase (F) by equation (1), using WinNonlin (Pharsight, Mountain View, CA, USA):

$$F = \frac{C^{\gamma}}{EC_{50}^{\gamma} + C^{\gamma}} \quad (1)$$

F was 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 ($A_{ftc} - A_0$). The concentration of FTC that produced a maximal increase in accumulation was determined in studies examining varying concentrations of FTC. A_{ftc}, A, and A_0 are the mitoxantrone accumulation in the presence of 10 μM FTC, the test compounds, and 0.1% DMSO (control), respectively. C is the concentration of flavonoids or flavonoid combinations. γ is a slope factor. In each experiment, triplicate measurements were obtained for each sample, and three independent experiments were conducted. The EC_{30} and EC_{70} values of flavonoids for increasing mitoxantrone accumulation were obtained by fitting Eq. (2) (36) derived from the Hill equation:

$$EC_x = \left(\frac{E_x}{E_{max} - E_x}\right)^{1/\gamma} \times EC_{50} \quad (2)$$

EC_x is the concentration of the test compounds producing E_x effect. E_{max} is the maximal effect, which, in the current study, is equal to 1. The EC_{50} and γ values were obtained from fitting Eq. (1).

Calculation of Mitoxantrone IC_{50} for Cytotoxicity Studies

Growth inhibition of MCF-7 MX100 cells by mitoxantrone (IC_{50} value) either alone or with the flavonoids (or flavonoid combinations) was obtained by fitting the percent of cell growth (F) by the equation:

$$F = 100 \times \left(1 - \frac{I_{max} \times C^{\gamma}}{IC_{50}^{\gamma} + C^{\gamma}}\right) \quad (3)$$

The observed F values were calculated as 100 times the ratio of the cell growth [$OD_{570} - OD_{570}(1000)$] to the maximum cell growth [$OD_{570}(0) - OD_{570}(1000)$]. $OD_{570}(0)$, $OD_{570}(1000)$ are the absorbance values of cells treated with 0 and 1000 μM of mitoxantrone, respectively. OD_{570} is the absorbance value of cells treated with specified concentrations of mitoxantrone. C is the concentration of mitoxantrone. I_{max} is 1, and γ is a slope factor. In each experiment, quadruplicate measurements were performed for each experiment, and three independent experiments were conducted.

Analysis of the Results of the Flavonoid Combination Studies

Berenbaum's Interaction Index

The interaction index values (I) for each flavonoid combination were calculated by the following equation (37):

$$I = \sum \frac{D_{x,i}}{EC_{x,i}} \quad (4)$$

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" that, when present alone, could also produce x effect. Three independent experiments were conducted, and the mean value and variance and then the 95% confidence interval of I for each flavonoid combination were obtained. 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 (37).

The Isobolographic Analysis

The analysis of the effects of two or three flavonoids as a combination on BCRP-mediated transport was also performed by the traditional graphic analysis—isobolographic analysis, as previously described (37,38). For the binary combinations, the $EC_{x,i}$ (the concentration or dose of constituent drug i that would produce x level of effect when given alone) values are represented by the points on the two axes in a Cartesian plane [designated as points ($EC_{x,1}$, 0) and (0, $EC_{x,2}$), respectively] and the straight line linking these two points (additive line) represents all the doses or dose combinations of the two drugs that will produce the same isoeffect x if the two drugs act additively. However, if the two drugs act synergistically, the dose combination ($D_{x,1}$, $D_{x,2}$) will be located below the additive line (closer to the origin), and if they act antagonistically, the dose combination will be located above the additive line. Similarly, for the tertiary combinations, the $EC_{x,i}$ values are represented by the points located on three axes (($EC_{x,1}$, 0, 0), (0, $EC_{x,2}$, 0) and (0, 0, $EC_{x,3}$), respectively) and the plane connecting these three points represents all the dose combinations that would produce the same isoeffect x if the three drugs act additively. If the dose combination ($D_{x,1}$, $D_{x,2}$, $D_{x,3}$) is located below or above that plane, it indicates that the drugs act synergistically or antagonistically.

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

FTC, a specific BCRP inhibitor (33,34), has been shown to significantly increase the accumulation of mitoxantrone in MCF-7 MX100 cells, but had little effect on mitoxantrone accumulation in BCRP-negative MCF-7/sensitive cells (30). To evaluate the contribution of BCRP in the 30-min mitoxantrone accumulation in MCF-7 MX100 cells and to determine the concentration at which FTC can completely inhibit BCRP in MCF-7 MX100 cells, we examined mitoxantrone accumulation in the presence of various concentrations of FTC (Fig. 1) and the observed values were fitted by the equation

$$A = A_0 + \frac{V_{max} \times C^{\gamma}}{EC_{50}^{\gamma} + C^{\gamma}}, \quad (5)$$

where A_0 is the mitoxantrone accumulation in the absence of FTC, which is 100%; A is the mitoxantrone accumulation in the presence of FTC at concentration of C; EC_{50} is the concentration of FTC for producing 50% of the maximal increase of mitoxantrone accumulation (V_{max}); and γ is a slope factor. The estimated EC_{50} of FTC is 0.215 ± 0.008 μM, and V_{max} is 328 ± 24.3% (n = 3). As can be seen from Fig. 1, FTC at a concentration of 10 μM, which is 46.4-fold greater than its

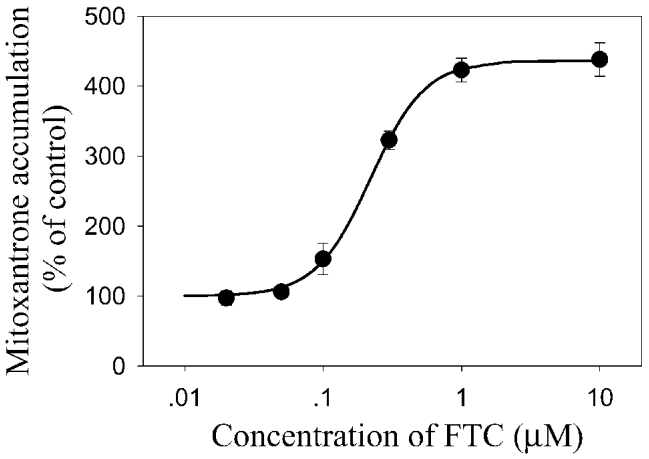

Fig. 1. Concentration-dependent effects of FTC on mitoxantrone accumulation in MCF-7 MX100 cells. The 30-min mitoxantrone accumulation in the presence of various concentrations of FTC in MCF-7 MX100 cells was investigated as described in "Materials and Methods." Data are expressed as mean ± SD, n = 3. The solid line represents the predicted data by fitting the equation:

$$A = A_0 + \frac{V_{max} \times C^{\gamma}}{EC_{50}^{\gamma} + C^{\gamma}}$$

where A_0 is the mitoxantrone accumulation in the absence of FTC, which is 100%; A is the mitoxantrone accumulation in the presence of FTC at concentration of C; EC_{50} is the concentration of FTC for producing 50% of the maximal increase of mitoxantrone accumulation (V_{max}); and γ is a slope factor.

estimated EC_{50} value, produced maximal mitoxantrone accumulation. These results indicate that BCRP in MCF-7 MX100 cells can be completely inhibited by 10 μM FTC, and this is also supported by our previous observation (30) that FTC at a 10 μM concentration completely reversed MCF-7 MX100 resistance to mitoxantrone. Therefore, mitoxantrone accumulation in MCF-7 MX100 cells in the presence of 10 μM FTC represents the maximal mitoxantrone accumulation with complete inhibition of BCRP.

EC_{30}, EC_{50}, and EC_{70} for Flavonoids Administered Alone or in Combinations

In order to predict the potential *in vivo* significance of flavonoid interactions with BCRP-mediated transport and to analyze the potential interactions among multiple flavonoids regarding their BCRP inhibition activities, the EC_{50} values of flavonoids given alone or in combination for increasing mitoxantrone accumulation in MCF-7 MX100 cells were characterized. During the study, the maximal concentrations of these flavonoids were all kept below 100 μM to avoid potential solubility problems, and thus complete inhibition of BCRP or a plateau of response was not achieved by some of these flavonoids. In order to allow the estimation of EC_{50} values by the Hill equation, the effects of flavonoids were thus normalized by the theoretically achievable maximal effect (complete BCRP inhibition) represented by the increase of mitoxantrone accumulation by 10 μM FTC determined in parallel to obtain the F values (fraction of maximal effect, as in equation (1)). The EC_{50} values were obtained by fiting the F values by Eq. (1), and thus truly represent the concentrations at which 50% of BCRP function is inhibited. As shown in Fig. 2A and Table I, the EC_{50} values of the tested flavonoids given alone were apigenin: 1.66 ± 0.55 μM, biochanin A: 1.62 ± 1.02 μM, chrysin: 0.39 ± 0.13 μM, genistein: 14.9 ± 2.69 μM, hesperetin: 12.4 ± 2.21 μM, kaempferol: 6.04 ± 0.09 μM, naringenin: 32.0 ± 3.22 μM, and silymarin: 33.7 ± 2.78 μM (mean ± SD, n = 3 independent triplicate experiments). For flavonoid combinations with equal molar concentrations of the constituents (Fig. 2B and Table I), the EC_{50} values (the value refers to the concentration of each individual flavonoid in a combination) were AB (apigenin + biochanin A): 0.81 ± 0.17 μM, BC (biochanin A + chrysin): 0.32 ± 0.16 μM, ABC (apigenin + biochanin A + chrysin): 0.27 ± 0.01 μM, ABCGK (apigenin + biochanin A + chrysin + genistein + kaempferol): 0.23 ± 0.08 μM, and ABCGKHNS (apigenin + biochanin A + chrysin + genistein + kaempferol + hesperetin + naringenin + silymarin): 0.20 ± 0.10 μM (mean ± SD, n = 3 independent triplicate experiments). In addition, to analyze the interactions among multiple flavonoids at different isoeffect levels, the EC_{30} and EC_{70} values (the concentration at which 30% and 70% of maximal increase in mitoxantrone accumulation was produced, respectively) were also estimated by equation (2) (Table I).

Potential Interaction Among Multiple Flavonoids Regarding BCRP Inhibition

To evaluate potential interactions among multiple flavonoids regarding BCRP inhibition, we used both algebraic (Berenbaum's interaction index) and graphic (isobologram, for binary and tertiary combinations only) analysis methods. As can be seen from Fig. 3, the concentrations of the flavonoids apigenin, biochanin A and chrysin to produce 30%, 50%, and 70% of the maximal increase in mitoxantrone accumulation, when given as combinations AB (apigenin + biochanin A, Fig. 3A), BC (biochanin A + chrysin, Fig. 3B), and ABC (apigenin + biochanin A + chrysin, Fig. 3C) with equal molar concentration for each individual constituent, are all located on or close to the corresponding additive lines (for AB, BC combination) or the corresponding additive planes (ABC combination) except that the dose combination of BC for producing 70% of the maximal increase in mitoxantrone accumulation is located above the additive line. The calculated interaction indices for the above-mentioned binary and tertiary flavonoid combinations ranged from 0.81 ± 0.09 to 1.31 ± 0.19 at either 30%, 50%, or 70% effect levels with their 95% confidence intervals all encompassing 1 (Table II), indicating that the interaction indices of all these combinations at the tested effect levels are not significantly different from 1. Thus, the flavonoids apigenin, biochanin A and chrysin appear to act additively in inhibiting BCRP-mediated transport in combinations AB, BC or ABC. In addition, we also tested the combined effects of multiple flavonoids on BCRP inhibition in combinations consisting of 5 or 8 different flavonoids. As shown in Table II, the interaction indices for these 5-flavonoid and 8-flavonoid combinations are also close to 1 (ranged from 0.85 ± 0.39 to 1.01 ± 0.28) and their 95% confidence intervals include 1, at all the tested effect levels (30%, 50% and 70% of maximal increase in mitoxantrone accumulation) (Table II). Thus, the interaction indices for the 5-flavonoid and 8-flavonoid combinations are not significantly different from 1, indicating that all the constituent flavonoids (apigenin, biochanin A, chrysin, genistein, kaempferol, hesper-

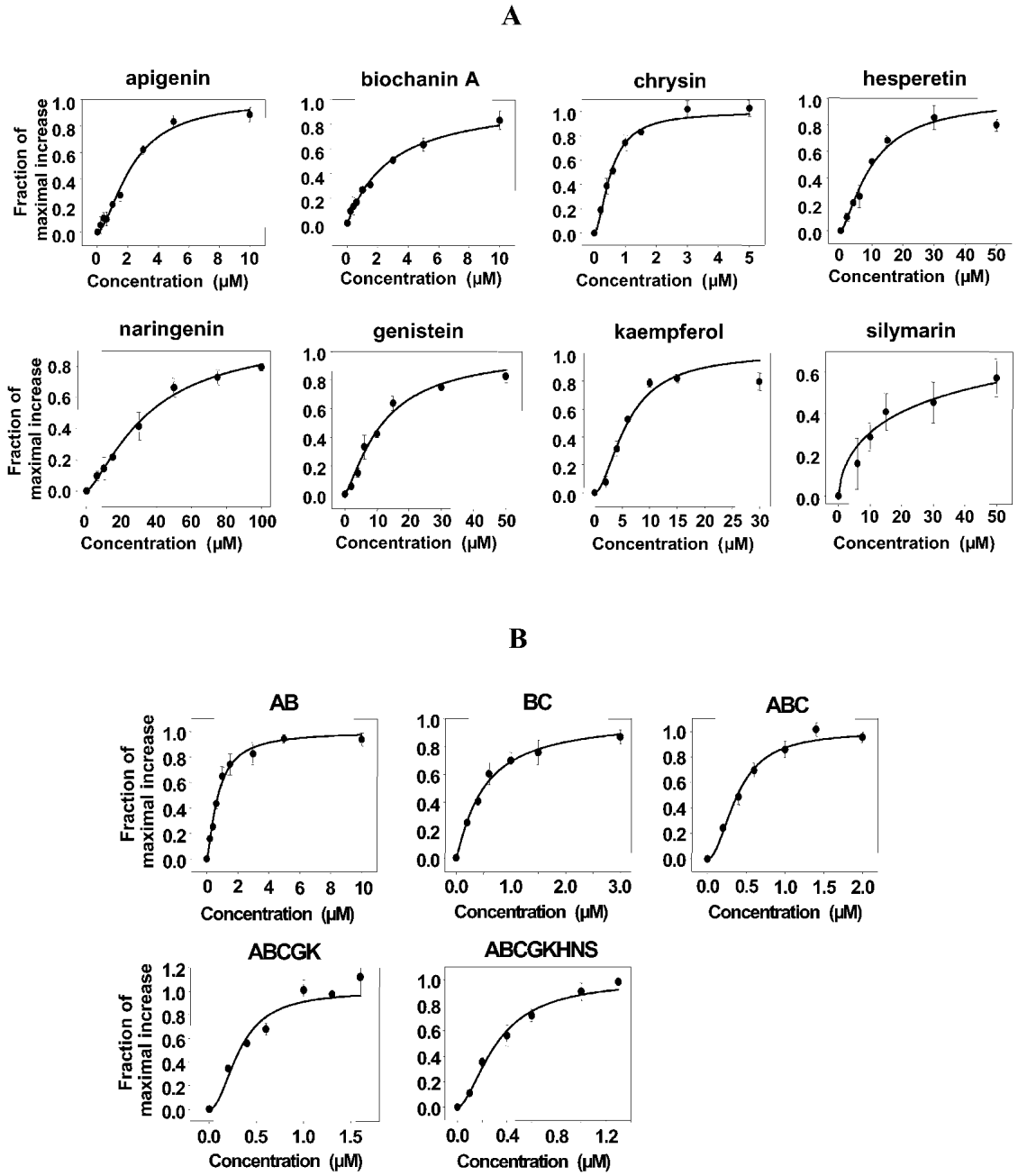

Fig. 2. Concentration-dependent effects of flavonoids or flavonoid combinations on mitoxantrone accumulation in MCF-7 MX100 cells The 30-min accumulation of mitoxantrone in MCF-7 MX100 cells in the presence of individual flavonoids (A), flavonoid combinations (B), or the vehicle (0.1% DMSO) was determined as described in "Materials and Methods." The effects of flavonoids were then expressed as fraction of the maximal increase (F). Data are presented as mean ± SD from a typical triplicate experiment. Three independent experiments were performed. The solid lines represent the predicted value by fitting the F with Eq. (1) as described in "Material and Methods." AB: the combination of apigenin and biochanin A; BC: the combination of biochanin A and chrysin; ABC: the combination of apigenin, biochanin A and chrysin; ABCGK: the combination of apigenin, biochanin A, chrysin, genistein, and kaempferol; ABCGKHNS: the combination of apigenin, biochanin A, chrysin, genistein, kaempferol, hesperetin, naringenin, and silymarin. Equal molar concentrations of all the constituent flavonoids were used in all the flavonoid combinations.

etin, naringenin and silymarin) in these larger combinations also act additively in inhibiting BCRP-mediated transport.

Combined Effects of Multiple Flavonoids on Mitoxantrone Cytotoxicity

The potential interactions among multiple flavonoids with regards to BCRP inhibition were also examined in mitoxantrone cytotoxicity studies. The IC_{50} values of mitoxantrone for inhibiting MCF-7 MX100 cell growth in the presence of flavonoid combinations BC (biochanin A + chrysin), ABC (apigenin + biochanin A + chrysin) and ABCGK (apigenin + biochanin A + chrysin + genistein + kaempferol) were determined and listed in Table III. For ease of comparison, the mitoxantrone IC_{50} values in the same cell line in the pres-

Table I. The EC_{30}, EC_{50}, and EC_{70} Values of Flavonoids or Flavonoid Combinations for Increasing Mitoxantrone Accumulation in MCF-7 MX100 Cells

Flavonoids	EC_{30} (μM)	EC_{50} (μM)	EC_{70} (μM)
Apigenin	0.97 ± 0.38	1.66 ± 0.55	2.86 ± 0.80
Biochanin A	0.70 ± 0.47	1.62 ± 1.02	3.72 ± 2.24
Chrysin	0.24 ± 0.07	0.39 ± 0.13	0.61 ± 0.23
Genistein	8.91 ± 2.35	14.9 ± 2.69	25.0 ± 2.58
Hesperetin	7.12 ± 1.39	12.4 ± 2.21	21.8 ± 3.59
Kaempferol	3.79 ± 0.33	6.04 ± 0.09	9.67 ± 0.65
Naringenin	17.5 ± 2.36	32.0 ± 3.22	59.1 ± 10.5
Silymarin	10.6 ± 1.01	33.7 ± 2.78	109 ± 28.0
AB	0.39 ± 0.04	0.81 ± 0.17	1.69 ± 0.55
BC	0.15 ± 0.08	0.32 ± 0.16	0.69 ± 0.32
ABC	0.16 ± 0.08	0.27 ± 0.01	0.48 ± 0.09
ABCGK	0.14 ± 0.07	0.23 ± 0.08	0.40 ± 0.10
ABCGKHNS	0.13 ± 0.06	0.20 ± 0.10	0.34 ± 0.19

The EC_{50}, EC_{30}, and EC_{70} values for each flavonoid were calculated from each triplicate experiment as described in "Materials and Methods." The data are expressed as mean ± SD from three independent experiments. For flavonoid combinations, the indicated EC_{30}, EC_{50}, and EC_{70} values are the concentration of each individual flavonoid in a particular combination that produced 30%, 50%, and 70% of the maximal effect, respectively, and thus are actually D_{30}, D_{50}, D_{70} values for the individual flavonoid in that particular combination, as equal molar concentrations of individual flavonoids were used in all the combinations. AB: the combination of apigenin and biochanin A; BC: the combination of biochanin A and chrysin; ABC: the combination of apigenin, biochanin A and chrysin; ABCGK: the combination of apigenin, biochanin A, chrysin, genistein and kaempferol; ABCGKHNS: the combination of apigenin, biochanin A, chrysin, genistein, kaempferol, hesperetin, naringenin and silymarin.

ence of single flavonoid (not flavonoid combination), which were reported previously (30), are also listed in the table (Table III). We have previously demonstrated (30) that the resistance of MCF-7 MX100 cells to mitoxantrone is due to the over-expression of BCRP, and flavonoids can increase mitoxantrone cytotoxicity in MCF-7 MX100 cells with minimal effects in MCF-7/sensitive cells, which express little BCRP. As can be seen from Table III, mitoxantrone cytotoxicity in MCF-7 MX100 cells was also significantly increased in the presence of all the tested flavonoid combinations (BC, ABC and ABCGK), and the potentiation of mitoxantrone cytotoxicity by these flavonoid combinations was flavonoid-concentration dependent, consistent with their BCRP inhibitory activities. Generally, it was found that, the ability of a flavonoid combination to increase mitoxantrone cytotoxicity is greater than that of the less potent constituent flavonoids given alone, and lower than that of the more potent constituent flavonoids given alone. For example, the mitoxantrone IC_{50} values in the presence of flavonoid combination BC at 2.5 μM (1.25 μM biochanin A + 1.25 μM chrysin), 5 μM (2.5 μM biochanin A + 2.5 μM chrysin), and 10 μM (5 μM biochanin A + 5 μM chrysin) concentrations are lower than those in the presence of 2.5 μM biochanin A (BC vs. biochanin A: 62.9 ± 33.6 μM vs 107 ± 17.6 μM, $p > 0.05$), 5 μM biochanin A (BC vs. biochanin A: 9.94 ± 1.75 μM vs. 30.9 ± 5.18 μM, $p < 0.01$), and 10 μM biochanin A (BC vs. biochanin A: 2.84 ± 0.02 μM vs 9.23 ± 2.07 μM, $p < 0.01$), respectively, but the mitoxantrone IC_{50} values in the presence of flavonoid combination BC at 2.5 μM (1.25 μM biochanin A + 1.25 μM chrysin) and 5 μM (2.5 μM biochanin A + 2.5 μM chrysin) are higher than those in the presence of 2.5 μM chrysin (BC vs. chrysin: 62.9 ± 33.6 μM vs. 18.8 ± 0.06 μM, $p > 0.05$) and 5 μM chrysin (BC vs. chrysin: 9.94 ± 1.75 μM vs. 6.25 ± 2.13 μM, $p > 0.05$). Biochanin A is the less potent flavonoid in the combination, and chrysin is the more potent flavonoid in the combination (Tables I and III). An exception to the general trend is that the mitoxantrone IC_{50} value in the presence of flavonoid combination BC at 10 μM (5 μM biochanin A + 5 μM chrysin) is similar to, instead of lower than, that in the presence of 10 μM chrysin (BC vs chrysin: 2.84 ± 0.02 μM vs. 3.35 ± 1.70 μM, $p > 0.05$), probably because the BCRP has already been completely inhibited by both the combination BC and chrysin at this concentration. This is very likely because these mitoxantrone IC_{50} values (BC: 2.84 ± 0.02 μM, chrysin: 3.35 ± 1.70 μM) are similar to or even a little lower than the mitoxantrone IC_{50} value in the MCF-7 sensitive cells (5.30 ± 2.22 μM, $p > 0.05$ for both BC and chrysin). Similar observations were also obtained for the flavonoid combinations ABC and ABCGK (Table III). Another general observation is that the ability of a flavonoid combination to increase mitoxantrone cytotoxicity was greater than that of any single constituent flavonoid when given at the same concentration as the concentration of the single flavonoid present in the combination. For example, the mitoxantrone IC_{50} values in the presence of 5 μM BC (2.5 μM chrysin + 2.5 μM biochanin A) and 5 μM ABC (contains 1.7 μM chrysin) are both lower than the mitoxantrone IC_{50} value in presence of 2.5 μM chrysin alone, the most potent constituent flavonoid in these combinations (5 μM BC vs 2.5 μM chrysin: 9.94 ± 1.75 μM vs. 18.8 ± 0.06 μM, $p < 0.001$; 5 μM ABC vs. 2.5 μM chrysin: 10.9 ± 4.34 μM vs. 18.8 ± 0.06 μM, $p < 0.05$) (Table III).

DISCUSSION

Epidemiology studies have consistently shown that consumption of fruits, vegetables, and plant-derived beverages are strongly associated with the reduced risk of chronic diseases such as heart diseases, cancer and osteoporosis, and a daily consumption of five or more servings of fruits and vegetables was recommended by a National Academy of Sciences Report on Diet and Health in 1989 (39). Among a variety of phytochemicals, the flavonoids abundant in these foods are believed to be key dietary components responsible for these health benefits. As such, hundreds of herbal products containing enriched or even pure flavonoid compounds are marketed as over-the-counter dietary supplements and available in health food stores and pharmacies. Due to the widespread use of flavonoid-containing herbal products in the general population, a careful evaluation of potential drug-flavonoid interactions is urgently needed for the safe consumption of these products. BCRP is an efflux transporter located in the apical membrane of enterocytes, canalicular membrane of hepatocytes, luminal surfaces of the brain capillaries as well as placental syncytiotrophoblasts (15,16), and shown to be an important factor limiting the intestinal absorption, facilitating biliary excretion and preventing placental penetration of xenotoxins or drugs that are BCRP substrates (18,19). Thus, a detailed flavonoid-BCRP interaction study is warranted.

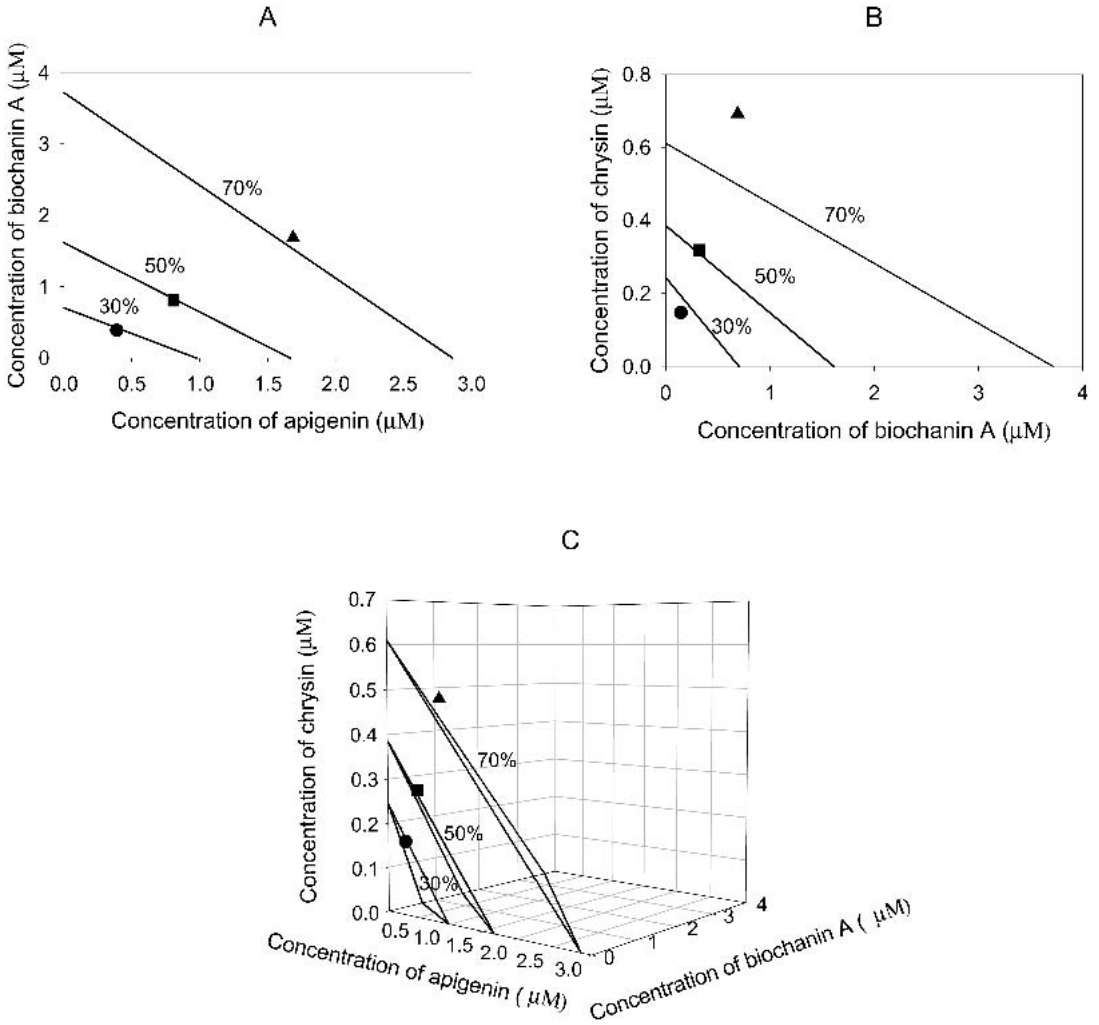

Fig. 3. Isobolographic analysis of the combined effects of flavonoids on mitoxantrone accumulation in MCF-7 MX100 cells The combined effects of flavonoid combinations (A): apigenin + biochanin A (1:1), (B): biochanin A + chrysin (1:1), and (C): apigenin + biochanin A + chrysin (1:1:1) at effect levels of 30% (●), 50% (■), and 70% (▲) of the maximal increase (in the presence of 10 μM FTC) were analyzed by the isobologram method. Data are presented as the mean values of the EC_{30}, EC_{50}, or EC_{70} of apigenin, biochanin A, or chrysin when given alone from three independent triplicate experiments or the mean concentrations of these flavonoids (D_x) in the specified combinations that produced 30%, 50%, or 70% of maximal increase in mitoxantrone accumulation. For the combinations of two flavonoids (A and B), the diagonal straight lines represent the Loewe additivity lines for the corresponding isoeffects designated in the plot. For the combination of three flavonoids (C), the concentrations located on the triangle planes designated for the specified isoeffects indicate Loewe additivism.

In the current study, we first evaluated the EC_{50} values for BCRP inhibition (presented as EC_{50} values for increasing mitoxantrone accumulation) for flavonoids apigenin, biochanin A, chrysin, genistein, kaempferol, hesperetin, naringenin and silymarin. These flavonoids are all frequently present in foods or herbal products, and were all shown to possess substantial BCRP inhibition activities in our previous report (30). All these flavonoids (50 μM) were shown to significantly increase mitoxantrone accumulation in MCF-7 MX100 cells (BCRP positive) with little effect in MCF-7 sensitive cells (BCRP negative) (30). Based on their dose-response relationship for increasing mitoxantrone accumulation in MCF-7 MX100 cells, the EC_{50} values of these flavonoids for BCRP inhibition ranged from 0.39 ± 0.13 μM to 33.7 ± 2.78 μM, in the order (more potent to less potent) of chrysin > biochanin A > apigenin > kaempferol > hesperetin > genistein > naringenin > silymarin. This order of potency generally agrees with the order of the ability of these flavonoids to potentiate mitoxantrone cytotoxicity in MCF-7 MX100 cells, which we reported previously (30), with the exception that kaempferol can not increase mitoxantrone cytotoxicity in MCF-7 MX100 cells at concentrations of 5 and 10 μM, but the less potent flavonoids such as hesperetin and genistein (as judged by the EC_{50} values determined in the current study) can significantly increase mitoxantrone cytotoxicity at these concentrations. The reason for this inconsistency is currently unknown, but it might be possible that kaempferol has some cytoprotective effects at these concentrations. Considering these relatively low EC_{50} values, it is reasonable to speculate that clinical inhibition of BCRP by these flavonoids, at least in the intestine, is very likely. For example, the recommended dose for chrysin supplements is 1–4 capsules daily, and each capsule

Table II. The Interaction Index (I) and its 95% Confidence Interval (CI) for the Combined Effects of Flavonoids on Mitoxantrone Accumulation in MCF-7 MX100 Cells

	30% of Maximal effect		50% of Maximal effect		70% of Maximal effect	
Combination	I	CI	I	CI	I	CI
AB	1.12 ± 0.43	[0.05, 2.19]	1.08 ± 0.27	[0.41, 1.75]	1.08 ± 0.10	[0.83, 1.34]
BC	0.81 ± 0.09	[0.57, 1.04]	1.01 ± 0.11	[0.75, 1.27]	1.31 ± 0.19	[0.83, 1.78]
ABC	1.03 ± 0.13	[0.72, 1.35]	1.06 ± 0.03	[0.98, 1.15]	1.14 ± 0.19	[0.66, 1.62]
ABCGK	0.93 ± 0.11	[0.66, 1.20]	0.94 ± 0.11	[0.67, 1.23]	1.01 ± 0.28	[0.30, 1.71]
ABCGKHNS	0.92 ± 0.18	[0.46, 1.36]	0.86 ± 0.28	[0.16, 1.56]	0.85 ± 0.39	[–0.13, 1.83]

The interaction index (I) for each flavonoid combination was calculated from each triplicate experiment as described in "Materials and Methods." The I values are presented as mean ± SD from three independent experiments. The confidence interval of the I values (CI) was calculated based on the three experiments. If the confidence interval of the I value contains 1, it indicates that I is not significantly different from 1, and thus Loewe additivism; if the higher limit of the confidence interval is less than 1, it indicates that I is significantly smaller than 1, and thus Loewe synergism; if the lower limit of the confidence interval is larger than 1, it indicates that I is significantly larger than 1, and thus Loewe antagonism. AB: the combination of apigenin and biochanin A; BC: the combination of biochanin A and chrysin; ABC: the combination of apigenin, biochanin A and chrysin; ABCGK: the combination of apigenin, biochanin A, chrysin, genistein, and kaempferol; ABCGKHNS: the combination of apigenin, biochanin A, chrysin, genistein, kaempferol, hesperetin, naringenin, and silymarin.

contains 500 mg chrysin (http://www.herbsmd.com/shop/xq/asp/pid.1646/qx/productdetail.asp). Based on a 1.65-L intestinal fluid volume (40), the estimated intestinal concentration of chrysin would be 1.18 mM after taking one capsule, which is 3026-fold higher than its EC_{50} value (0.39 ± 0.13 μM). Estimated intestinal concentrations of biochanin A and silymarin after taking a tablet of red clover extracts (26 mg biochanin A) (41) and milk thistle (150–160 mg silymarin) (42) are 55.5 μM and 180 μM, respectively. These concentrations are 34.3- and 5.3-fold higher than their corresponding EC_{50} values (biochanin A: 1.62 ± 1.02 μM; silymarin: 33.7 ± 2.78 μM). Commercially available grapefruit juice contains 750 μM of naringin (a naringenin glycoside) (43), and this concentration (assuming complete release into its aglycone form, naringenin, in the intestine) is 23-fold higher than the EC_{50} of naringenin (32.0 ± 3.22 μM) determined in this investigation. Hesperidin (a glycoside of hesperetin) is the major flavonoid in orange juice and its concentration can be as high as 444 mg/L (727 μM) (44), which (assuming complete release into its aglycone hesperetin) is 58.4-fold higher than the EC_{50} value of hesperetin (12.4 ± 2.21 μM). Therefore, it is reasonable to expect that inhibition of intestinal BCRP resulting in

Table III. The Effects of Flavonoids or Flavonoid Combinations on the Cytotoxicity of Mitoxantrone (IC_{50}) in MCF-7 MX100 Cells

Flavonoid concentration	MCF-7/sensitive	MCF-7 MX100		
		2.5 μM	5 μM	10 μM
Control	†5.30 ± 2.22		†199 ± 19.3	
Apigenin			†219 ± 10.0	†10.5 ± 7.13***
Biochanin A		†107 ± 17.6***	†30.9 ± 5.18***	†9.23 ± 2.07***
Chrysin		†18.8 ± 0.06***	†6.25 ± 2.13***	†3.35 ± 1.70***
Genistein			†148 ± 23.2	†29.3 ± 6.76***
Kaempferol			†196 ± 20.9	†228 ± 13.8
BC		62.9 ± 33.6***	9.94 ± 1.75***	2.84 ± 0.02***
ABC			10.9 ± 4.34***	3.54 ± 0.80***
ABCGK			38.9 ± 1.60***	5.77 ± 2.99***
FTC (10 μM)	†2.30 ± 0.29***		†1.79 ± 1.52***	

Mitoxantrone cytotoxicity (IC_{50}) in MCF-7 MX100 cells overexpressing BCRP in the presence of flavonoid combinations BC, ABC, and ABCGK was determined as described in "Materials and Methods." The IC_{50} values in the presence of individual flavonoids and FTC were reported previously (30) and listed for ease of comparison. For flavonoid combinations, the concentrations of flavonoids indicated referred to the total concentrations of all the constituent flavonoids. Equal molar concentrations of individual flavonoids were used in all the combinations. BC: the combination of biochanin A and chrysin; ABC: the combination of apigenin, biochanin A, and chrysin; ABCGK: the combination of apigenin, biochanin A, chrysin, genistein, and kaempferol.

*** $p < 0.001$ compared with the corresponding control (MCF-7 MX100), ANOVA followed by Dunnett's test.

† Data were from Ref. 30.

increased bioavailability of BCRP substrate drugs may occur following the ingestion of flavonoid-containing foods or dietary supplements.

On the other hand, although the intestinal concentrations of flavonoids may be very high after ingestion of food and herbal products, blood concentrations are likely to be low due to extensive first pass extraction and rapid metabolism (45,46). For example, the peak plasma concentrations of chrysin were only 12–63 nM (3–16 ng/ml) in healthy human volunteers after a oral dose of 400 mg chrysin; most of the dose appeared in feces as chrysin (45). Thus, the systemic inhibition of BCRP by oral dosing of chrysin may be limited and tumor MDR reversal using a single flavonoid would be difficult. To circumvent this problem, a combination of large number of flavonoids, as well as other food-derived active compounds, with a well-defined composition, taking advantage of their additive or synergistic effect, may be an interesting approach. In fact, additivism or synergism is believed to be at least part of the reason why dietary supplements with purified bioactive compound do not appear to have the same health benefits as a diet rich in fruits and vegetables, which contains a complex mixture of hundreds or thousands of phytochemicals (39). To explore the feasibility of this flavonoid "cocktail" approach for BCRP inhibition, the combined effects of multiple flavonoids (additive, synergistic or antagonistic) need to be characterized. The information will also help to better predict potential food (herbal products)-drug interactions since multiple flavonoids are usually coingested upon food intake. In the current study, we investigated the potential interactions among the tested flavonoids with regards to BCRP inhibition when given as a 2-, 3-, 5-, or 8-flavonoid combination using Berenbaum's interaction index method and a traditional isobologram method; both methods are widely-used (34,35). These methods use the Loewe additivity model, which is based on the idea that an agent can not interact with itself, as the reference for "no interaction" (36). For all the above-mentioned combinations, the interaction indices for three isoeffect levels (30%, 50%, and 70% of maximal increase of mitoxantrone accumulation) are all close to 1 (ranged from 0.81 ± 0.09 to 1.31 ± 0.19) and not significantly different from 1, indicating that the tested flavonoids inhibited BCRP in an additive fashion when given as a 2-, 3-, 5-, or even 8-flavonoid combination. This suggestion is also supported by the subsequent mitoxantrone cytotoxicity studies, where the ability of a flavonoid combination to increase mitoxantrone cytotoxicity in MCF-7 MX100 cells was shown to be generally higher than that of the less potent constituents and lower than that of the more potent ones, providing the total concentrations of the flavonoid(s), given alone or in combination, are the same. In addition, the ability of a flavonoid combination to increase mitoxantrone cytotoxicity was shown to be higher than any single constituent alone, given that the concentration of the flavonoid given alone is the same as the concentration of that flavonoid in the combination, indicating that the presence of an additional active flavonoid would provide additional BCRP inhibition activity. Both of these observations are consistent with what we would expect if the flavonoids in the combination act additively to inhibit BCRP. Therefore, qualitatively speaking, these cytotoxicity results are also consistent with the indication that multiple flavonoids may act additively to inhibit BCRP. This finding suggests that prediction of potential food (herbal product)-drug interactions mediated by BCRP may need to consider the additive effects from multiple co-ingested flavonoids. The EC_{50} value (refers to the concentration of each constituent) of flavonoid combination ABCGKHNS was shown to be 0.20 ± 0.10 μM, which is about half of the EC_{50} of the most potent constituent flavonoid chrysin (0.39 ± 0.13 μM). This reduction of EC_{50} value (compared with the EC_{50} of chrysin, the most potent constituent) is not dramatic, because several much less potent flavonoids, such as naringenin and silymarin, were included in the combination. However, based on their additive action, a dramatic reduction of EC_{50} value for BCRP inhibition would be expected if a flavonoid "cocktail" consisting of a large number of flavonoids with potent BCRP inhibitory activities was used. Therefore, the additive inhibition of BCRP by multiple flavonoids also points out that clinical reversal of BCRP-mediated MDR could potentially be accomplished by using a large flavonoid combination without the need of a high dose of any individual flavonoid.

In conclusion, flavonoids apigenin, biochanin A, chrysin, genistein, kaempferol, hesperetin, naringenin, and silymarin are potent BCRP inhibitors. The EC_{50} values of these flavonoids for inhibiting BCRP in MCF-7 MX100 cells ranged from 0.39 ± 0.13 μM to 33.7 ± 2.78 μM. These concentrations are very likely achieved or exceeded in the intestine even after the ingestion of flavonoid-containing foods or dietary supplements, and could potentially increase the bioavailability of drugs that are BCRP substrates. Moreover, these flavonoids, when combined, act additively to inhibit BCRP. Therefore, prediction of potential clinical significance of BCRP-mediated drug-flavonoid interactions should take into consideration the co-presence of multiple flavonoids after food or herbal product ingestion. This additive nature of the combined effects of multiple flavonoids on BCRP-mediated transport also suggests that using a combination of a large number of flavonoids with potent BCRP inhibitory activities may be a potential approach for MDR reversal in cancer treatment.

ACKNOWLEDGMENTS

Financial support for this study was provided by grants from the Susan G. Komen Breast Cancer Foundation and Pfizer Global Research and Development. We would like to acknowledge the discussions and assistance of Dr. Kazuko Sagawa from Pfizer Global Research and Development.

REFERENCES

1. T. Litman, T. E. Druley, W. D. Stein, and S. E. Bates. From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. *Cell. Mol. Life Sci.* **58**:931–959 (2001).
2. G. D. Leonard, T. Fojo, and S. E. Bates. The role of ABC transporters in clinical practice. *Oncologist* **8**:411–424 (2003).
3. L. A. Doyle, W. Yang, L. V. Abruzzo, T. Krogmann, Y. Gao, A. K. Rishi, and D. D. Ross. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* **95**:15665–15670 (1998).
4. K. Miyake, L. Mickley, T. Litman, Z. Zhan, R. Robey, B. Cristensen, M. Brangi, L. Greenberger, M. Dean, T. Fojo, and S. E. Bates. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. *Cancer Res.* **59**:8–13 (1999).
5. R. Allikmets, L. M. Schriml, A. Hutchinson, V. Romano-Spica, and M. Dean. A human placenta-specific ATP-binding cassette

gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. *Cancer Res.* **58**:5337–5339 (1998).
6. M. Maliepaard, M. A. van Gastelen, L. A. de Jong, D. Pluim, R. C. van Waardenburg, M. C. Ruevekamp-Helmers, B. G. Floot, and J. H. Schellens. Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line. *Cancer Res.* **59**:4559–4563 (1999).
7. E. L. Volk, K. M. Farley, Y. Wu, F. Li, R. W. Robey, and E. Schneider. Overexpression of wild-type breast cancer resistance protein mediates methotrexate resistance. *Cancer Res.* **62**: 5035–5040 (2002).
8. R. W. Robey, W. Y. Medina-Perez, K. Nishiyama, T. Lahusen, K. Miyake, T. Litman, A. M. Senderowicz, D. D. Ross, and S. E. Bates. Overexpression of the ATP-binding cassette half-transporter, ABCG2 (Mxr/BCrp/ABCP1), in flavopiridol-resistant human breast cancer cells. *Clin. Cancer Res.* **7**:145–152 (2001).
9. X. Wang, T. Furukawa, T. Nitanda, M. Okamoto, Y. Sugimoto, S. Akiyama, and M. Baba. Breast cancer resistance protein (BCRP/ABCG2) induces cellular resistance to HIV-1 nucleoside reverse transcriptase inhibitors. *Mol. Pharmacol.* **63**:65–72 (2003).
10. D. D. Ross, J. E. Karp, T. T. Chen, and L. A. Doyle. Expression of breast cancer resistance protein in blast cells from patients with acute leukemia. *Blood* **96**:365–368 (2000).
11. A. Kanzaki, M. Toi, K. Nakayama, H. Bando, M. Mutoh, T. Uchida, M. Fukumoto, and Y. Takebayashi. Expression of multidrug resistance-related transporters in human breast carcinoma. *Jpn. J. Cancer Res.* **92**:452–458 (2001).
12. J. M. Sargent, C. J. Williamson, M. Maliepaard, A. W. Elgie, R. J. Scheper, and C. G. Taylor. Breast cancer resistance protein expression and resistance to daunorubicin in blast cells from patients with acute myeloid leukaemia. *Br. J. Haematol.* **115**:257–262 (2001).
13. D. M. van der Kolk, E. Vellenga, G. L. Scheffer, M. Muller, S. E. Bates, R. J. Scheper, and E. G. de Vries. Expression and activity of breast cancer resistance protein (BCRP) in de novo and relapsed acute myeloid leukemia. *Blood* **99**:3763–3770 (2002).
14. D. Steinbach, W. Sell, A. Voigt, J. Hermann, F. Zintl, and A. Sauerbrey. BCRP gene expression is associated with a poor response to remission induction therapy in childhood acute myeloid leukemia. *Leukemia* **16**:1443–1447 (2002).
15. M. Maliepaard, G. L. Scheffer, I. F. Faneyte, M. A. van Gastelen, A. C. Pijnenborg, A. H. Schinkel, M. J. van De Vijver, R. J. Scheper, and J. H. Schellens. Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. *Cancer Res.* **61**:3458–3464 (2001).
16. H. C. Cooray, C. G. Blackmore, L. Maskell, and M. A. Barrand. Localisation of breast cancer resistance protein in microvessel endothelium of human brain. *Neuroreport* **13**:2059–2063 (2002).
17. J. Taipalensuu, H. Tornblom, G. Lindberg, C. Einarsson, F. Sjoqvist, H. Melhus, P. Garberg, B. Sjostrom, B. Lundgren, and P. Artursson. Correlation of gene expression of ten drug efflux proteins of the ATP- binding cassette transporter family in normal human jejunum and in human intestinal epithelial Caco-2 cell monolayers. *J. Pharmacol. Exp. Ther.* **299**:164–170 (2001).
18. J. W. Jonker, J. W. Smit, R. F. Brinkhuis, M. Maliepaard, J. H. Beijnen, J. H. Schellens, and A. H. Schinkel. Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. *J. Natl. Cancer Inst.* **92**:1651–1656 (2000).
19. C. M. Kruijtzer, J. H. Beijnen, H. Rosing, W. W. ten Bokkel Huinink, M. Schot, R. C. Jewell, E. M. Paul, and J. H. Schellens. Increased oral bioavailability of topotecan in combination with the breast cancer resistance protein and P-glycoprotein inhibitor GF120918. *J. Clin. Oncol.* **20**:2943–2950 (2002).
20. H. K. Rooprai, M. Christidou, and G. J. Pilkington. The potential for strategies using micronutrients and heterocyclic drugs to treat invasive gliomas. *Acta Neurochir. (Wien.)* **145**:683–690 (2003).
21. H. P. Lee, L. Gourley, S. W. Duffy, J. Esteve, J. Lee, and N. E. Day. Dietary effects on breast-cancer risk in Singapore. *Lancet* **337**:1197–1200 (1991).
22. H. Kohno, T. Tanaka, K. Kawabata, Y. Hirose, S. Sugie, H. Tsuda, and H. Mori. Silymarin, a naturally occurring polyphenolic antioxidant flavonoid, inhibits azoxymethane-induced colon carcinogenesis in male F344 rats. *Int. J. Cancer* **101**:461–468 (2002).
23. M. G. Hertog, E. J. Feskens, P. C. Hollman, M. B. Katan, and D. Kromhout. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* **342**:1007–1011 (1993).
24. S. M. Potter, J. A. Baum, H. Teng, R. J. Stillman, N. F. Shay, and J. W. Erdman Jr. Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. *Am. J. Clin. Nutr.* **68**:1375S–1379S (1998).
25. E. Middleton Jr., C. Kandaswami, and T. C. Theoharides. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **52**:673–751 (2000).
26. B. H. Havsteen. The biochemistry and medical significance of the flavonoids. *Pharmacol. Ther.* **96**:67–202 (2002).
27. C. B. Powell, S. L. Dibble, J. E. Dall'Era, and I. Cohen. Use of herbs in women diagnosed with ovarian cancer. *Int. J. Gynecol. Cancer* **12**:214–217 (2002).
28. D. M. Eisenberg, R. C. Kessler, M. I. Van Rompay, T. J. Kaptchuk, S. A. Wilkey, S. Appel, and R. B. Davis. Perceptions about complementary therapies relative to conventional therapies among adults who use both: results from a national survey. *Ann. Intern. Med.* **135**:344–351 (2001).
29. H. Ni, C. Simile, and A. M. Hardy. Utilization of complementary and alternative medicine by United States adults: results from the 1999 national health interview survey. *Med. Care* **40**:353–358 (2002).
30. S. Zhang, X. Yang, and M. E. Morris. Flavonoids are inhibitors of breast cancer resistance protein (ABCG2)-mediated transport. *Mol. Pharmacol.* **65**:1208–1216, (2004).
31. E. M. Leslie, Q. Mao, C. J. Oleschuk, R. G. Deeley, and S. P. Cole. Modulation of multidrug resistance protein 1 (MRP1/ABCC1) transport and atpase activities by interaction with dietary flavonoids. *Mol. Pharmacol.* **59**:1171–1180 (2001).
32. H. Minderman, A. Suvannasankha, K. L. O'Loughlin, G. L. Scheffer, R. J. Scheper, R. W. Robey, and M. R. Baer. Flow cytometric analysis of breast cancer resistance protein expression and function. *Cytometry* **48**:59–65 (2002).
33. S. K. Rabindran, H. He, M. Singh, E. Brown, K. I. Collins, T. Annable, and L. M. Greenberger. Reversal of a novel multidrug resistance mechanism in human colon carcinoma cells by fumitremorgin C. *Cancer Res.* **58**:5850–5858 (1998).
34. R. W. Robey, Y. Honjo, A. van de Laar, K. Miyake, J. T. Regis, T. Litman, and S. E. Bates. A functional assay for detection of the mitoxantrone resistance protein, MXR (ABCG2). *Biochim. Biophys. Acta* **1512**:171–182 (2001).
35. P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J. T. Warren, H. Bokesch, S. Kenney, and M. R. Boyd. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* **82**:1107–1112 (1990).
36. W. R. Greco, G. Bravo, and J. C. Parsons. The search for synergy: a critical review from a response surface perspective. *Pharmacol. Rev.* **47**:331–385 (1995).
37. M. C. Berenbaum. Synergy, additivism and antagonism in immunosuppression. A critical review. *Clin. Exp. Immunol.* **28**:1–18 (1977).
38. P. K. Gessner. Isobolographic analysis of interactions: an update on applications and utility. *Toxicology* **105**:161–179 (1995).
39. R. H. Liu. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *Am. J. Clin. Nutr.* **78**:517S–520S (2003).
40. B. Davies and T. Morris. Physiological parameters in laboratory animals and humans. *Pharm. Res.* **10**:1093–1095 (1993).
41. J. B. Howes, D. Sullivan, N. Lai, P. Nestel, S. Pomeroy, L. West, J. A. Eden, and L. G. Howes. The effects of dietary supplementation with isoflavones from red clover on the lipoprotein profiles of post menopausal women with mild to moderate hypercholesterolaemia. *Atherosclerosis* **152**:143–147 (2000).
42. S. C. Piscitelli, E. Formentini, A. H. Burstein, R. Alfaro, and S. Jagannatha. and J. Falloon. Effect of milk thistle on the pharmacokinetics of indinavir in healthy volunteers. *Pharmacotherapy* **22**:551–556 (2002).

43. D. G. Bailey, G. K. Dresser, J. H. Kreeft, C. Munoz, D. J. Freeman, and J. R. Bend. Grapefruit-felodipine interaction: effect of unprocessed fruit and probable active ingredients. *Clin. Pharmacol. Ther.* **68**:468–477 (2000).
44. C. Manach, C. Morand, A. Gil-Izquierdo, C. Bouteloup-Demange, and C. Remesy. Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice. *Eur. J. Clin. Nutr.* **57**:235–242 (2003).
45. T. Walle, Y. Otake, J. A. Brubaker, U. K. Walle, and P. V. Halushka. Disposition and metabolism of the flavonoid chrysin in normal volunteers. *Br. J. Clin. Pharmacol.* **51**:143–146 (2001).
46. K. D. Setchell, N. M. Brown, P. Desai, L. Zimmer-Nechemias, B. E. Wolfe, W. T. Brashear, A. S. Kirschner, A. Cassidy, and J. E. Heubi. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J. Nutr.* **131**:1362S–1375S (2001).