

Phytoestrogens/Flavonoids Reverse Breast Cancer Resistance Protein/ABCG2-Mediated Multidrug Resistance

Yasuo Imai,¹ Satomi Tsukahara,¹ Sakiyo Asada,¹ and Yoshikazu Sugimoto^{1,2}

¹Division of Molecular Biotherapy, Japanese Foundation for Cancer Research, Tokyo, Japan, and ²Department of Chemotherapy, Kyoritsu University of Pharmacy, Tokyo, Japan

ABSTRACT

Breast cancer resistance protein (BCRP), also called ABCG2, confers resistance to anticancer agents such as 7-ethyl-10-hydroxycamptothecin (SN-38), mitoxantrone, and topotecan. We found previously that sulfated estrogens are physiologic substrates of BCRP. Flavonoids with weak estrogenic activities are called phytoestrogens. In this study, we show that phytoestrogens/flavonoids, such as genistein, naringenin, acacetin, and kaempferol, potentiated the cytotoxicity of SN-38 and mitoxantrone in BCRP-transduced K562 (K562/BCRP) cells. Some glycosylated flavonoids, such as naringenin-7-glucoside, also effectively inhibited BCRP. These flavonoids showed marginal effect on the drug sensitivity of K562 cells. Genistein and naringenin reversed neither P-glycoprotein-mediated vincristine resistance nor multidrug resistance-related protein 1-mediated VP-16 resistance. Genistein and naringenin increased cellular accumulation of topotecan in K562/BCRP cells. K562/BCRP cells also accumulated less [³H]genistein than K562 cells. [³H]genistein transport in the basal-to-apical direction was greater in BCRP-transduced LLC-PK1 (LLC/BCRP) cells, which express exogenous BCRP in the apical membrane, than in parental cells. Fumitremorgin C abolished the increased transport of [³H]genistein in LLC/BCRP cells compared with parental cells. TLC analysis revealed that genistein was transported in its native form but not in its metabolized form. These results suggest that genistein is among the natural substrates of BCRP and competitively inhibits BCRP-mediated drug efflux. The results have two important clinical implications: (a) flavonoids and glycosylated flavonoids may be useful in overcoming BCRP-mediated drug resistance in tumor cells; and (b) coadministration of flavonoids with BCRP-substrate antitumor agents may alter the pharmacokinetics and consequently increase the toxicity of specific antitumor agents in cancer patients.

INTRODUCTION

Multidrug-resistance (MDR; Ref. 1) is a phenomenon in which cancer cells display cross-resistance to structurally unrelated drugs (2). During chemotherapy, cancer cells displaying an MDR phenotype gradually appear in the course of repeated chemotherapeutic drug regimens, and patients displaying MDR phenotype eventually become nonresponsive to these treatments. Breast cancer resistance protein (BCRP), also called ABCG2, is a half-transporter with a molecular weight of M_r 70,000 and is a member of the ATP-binding cassette transporters (1, 3, 4). BCRP mediates concurrent resistance to chemotherapeutic agents, such as SN-38 (an active metabolite of CPT-11), mitoxantrone, and topotecan, presumably by pumping these compounds out of the cell and thus decreasing their cytotoxic effects (1, 3–6). We reported previously that estrone and 17 β -estradiol circumvented BCRP-mediated drug resistance (7), and we have demonstrated recently that BCRP transports sulfated estrogens as physiologic substrates (8). In light of the findings that BCRP interacts with

estrogens and sulfated estrogens, we then screened synthesized estrogen agonists and antagonists for BCRP inhibitors and found that tamoxifen derivatives effectively circumvented BCRP-mediated drug resistance (9). These tamoxifen derivatives showed weaker affinity for estrogen receptors than 17 β -estradiol, which might serve for development of BCRP inhibitors with fewer clinical side effects.

In the present study, we examined the possible effects of phytoestrogens and other flavonoids in BCRP-mediated MDR. The chemical structures of isoflavones resemble those of estrogens, and their weak estrogenic activities have been reported previously (10). Isoflavones constitute a group of flavonoids that are particularly abundant in soybean, and genistein, a member of the isoflavones, revealed stronger BCRP-inhibitory effects than estrone. Naringenin, a member of the flavanones that is contained in grapefruit juice, also showed BCRP-inhibitory effects. In addition, many other flavonoids, especially flavones, were found to strongly reverse BCRP-mediated drug resistance with few growth-inhibitory effects on cells. The BCRP-inhibitory effect of flavonoids might be explained, in part, by competitive inhibition of the BCRP-mediated efflux of anticancer agents because genistein was found to be a natural substrate that is transported by BCRP. The mechanisms by which isoflavones and other flavonoids inhibit drug export by BCRP currently are under investigation.

MATERIALS AND METHODS

Reagents. Flavonoids used in these experiments were purchased from Funakoshi (Tokyo, Japan). Anti-P-glycoprotein monoclonal antibody C219 was purchased from Centocor (Malvern, PA), and anti-MRP1 monoclonal antibody MRPM6 was obtained from Nichirei (Tokyo, Japan). [³H]genistein (5 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO).

Establishment of K562/BCRP, LLC/BCRP, K562/MDR, and KB/MRP Cell Lines. K562 human leukemic cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in 5% CO₂. K562/BCRP cells were established by transduction of K562 cells with HaBCRP retrovirus, bearing human BCRP cDNA, and subsequent selection with 20 ng/ml SN-38 for 5 days. LLC-PK1 cells, epithelial cells of the porcine kidney, were cultured in M199 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. LLC/BCRP cells were established by the transduction of LLC-PK1 cells with HaBCRP retrovirus and subsequent selection with increasing doses of mitoxantrone (2, 4, and 8 nM) for 17 days. The resulting mixed population of drug-resistant cells was used in this study as described previously (8, 11). K562/MDR cells were established by transduction of K562 cells with HaMDR retrovirus containing human MDR1 cDNA, and this was followed by selection using 4 ng/ml vincristine for 7 days (12). KB-3-1 human epidermoid carcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in 5% CO₂. KB/MRP cells were established by introduction of the pJ3U-MRP1 construct bearing human MRP1 cDNA into KB-3-1 cells, followed by selection with increasing concentrations of doxorubicin (13). Expression of BCRP in K562/BCRP and LLC/BCRP cells, expression of P-glycoprotein in K562/MDR, and expression of MRP1 in KB/MRP cells were confirmed by Western blot analysis with the anti-BCRP polyclonal antibody 3488, anti-P-glycoprotein monoclonal antibody C219, and anti-MRP1 monoclonal antibody MRPM6, respectively. The Western blot analysis procedure is described elsewhere (11).

Cell Growth Inhibition Assay. The effects of specific compounds on the sensitivity of cells to SN-38 and mitoxantrone were evaluated by measuring

Received 1/9/04; revised 3/28/04; accepted 4/2/04.

Grant support: Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labor and Welfare, the Hishinomi Cancer Research Fund, and the Virtual Research Institute of Aging of Nippon Boehringer Ingelheim, Japan.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Yoshikazu Sugimoto, Division of Molecular Biotherapy, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 1-37-1 Kami-Kebukuro, Toshima-ku, Tokyo 170-8455, Japan. Fax: 81-3-3918-3716; E-mail: ysugimot@jfc.or.jp.

cell growth inhibition after incubation at 37°C for 5 days in the absence or presence of various concentrations of anticancer drugs in combination with the specific chemicals being examined. Cell numbers were determined using a cell counter (Sysmex, Kobe, Japan). IC₅₀ values (drug dosages that cause 50% inhibition of cell growth) were determined from growth inhibition curves.

Intracellular Topotecan Uptake. The effects of specific compounds on the cellular accumulation of topotecan were determined by flow cytometry. Cells (5×10^5) were incubated with 20 μM topotecan for 30 min at 37°C in the absence or presence of modifying agents, washed in ice-cold PBS, and subjected to fluorescence analysis using FACSCalibur (Becton-Dickinson, San Jose, CA).

Cellular [³H]Genistein Accumulation in K562/BCRP Cells. Either K562 or K562/BCRP cells (2×10^6) were incubated with 30 nM [³H]genistein for 0, 1, 2, or 4 h at 37°C. The cells then were washed with ice-cold PBS, dissolved in 100 μl PBS plus 400 μl Soluene-350 (Packard, Downer's Grove, IL), and mixed with 5 ml ACS II scintillation mixture (Amersham, Piscataway, NJ). Radioactivity levels were measured using a scintillation counter (Beckman, Fullerton, CA).

Transcellular Transport Assay of [³H]Genistein and Silica Gel TLC of Transported Compounds. Details of the experimental procedure are described previously (8). Briefly, exponentially growing LLC-PK1 and/or LLC/BCRP cells were plated on 3-μm pore Transwell 3414 filters (Corning Costar, Cambridge, MA) at a density of 2.4×10^6 cells/well and cultured for 3 days. Culture medium in the upper and lower wells was replaced with 2 ml of serum-free M199 medium 1.5 h before beginning the experiments. When needed, fumitremorgin C was added to the apical and basal side medium at this time (14). The medium in either the upper or lower well then was replaced with 2 ml of medium containing ¹⁴C-labeled inulin and/or ³H-labeled genistein. The cells were incubated at 37°C in 5% CO₂, and 50 μl of the medium from the opposite side were sampled at 1, 2, and 4 h following the addition of radiolabeled compounds. The radioactivity of each sample was measured by liquid scintillation counting and expressed as a percentage fraction of the total radioactivity before incubation. All of the data were presented as mean values with SD of triplicate determinations from three different cultures.

For silica gel TLC, 50 μl of medium in the opposite side of the chamber following incubation were mixed with 100 μl of methanol, spotted, and run on silica gel 60 F₂₅₄ plates (Merck, Darmstadt, Germany) in chloroform/methanol/acetic acid (8:3:1). Separated zones were excised, and their radioactivities were measured using a liquid scintillation counter. The radioactivities were expressed as a percentage fraction of the total radioactivity before incubation. Each point represents a mean value with SD of triplicate determinations.

Statistical Analysis. The two-sided unpaired Student's *t* test was used to evaluate the statistical significance of the differences between the two sets of data. The differences were considered significant when *P* < 0.05.

RESULTS

Characteristics of K562/BCRP, LLC/BCRP, K562/MDR, and KB/MRP Cells. Among the four drug-resistant cell lines used in this study, K562/BCRP cells expressed BCRP but not P-glycoprotein or

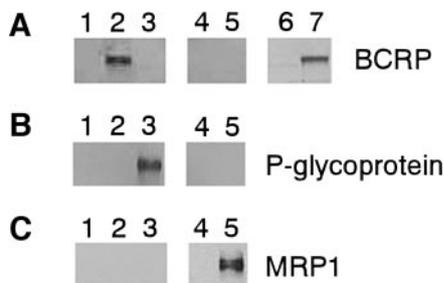


Fig. 1. Analysis of expression levels of breast cancer resistance protein (BCRP), P-glycoprotein, and MRP1 in transfected cells by Western blot analysis. Cell lysates (20 μg/lane) were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. A, BCRP expression. Blots were treated with anti-BCRP polyclonal antibody 3488. B, P-glycoprotein expression. Blots were treated with anti-P-glycoprotein antibody C219. C, MRP1 expression. Blots were treated with anti-MRP1 monoclonal antibody MRPm6. 1, K562 cells. 2, K562/BCRP cells. 3, K562/MDR cells. 4, KB-3-1 cells. 5, KB/MRP cells. 6, LLC-PK1 cells. 7, LLC/BCRP cells.

Table 1 Drug resistances of K562/BCRP, K562/MDR, and KB/MRP cells

Cells were cultured for 5 days with increasing concentrations of anticancer drugs. Cell numbers were measured with a Coulter counter and IC₅₀ values were then determined. The degree of drug resistance is calculated as the IC₅₀ ratio of resistant cells divided by that of the parental cells. The data are represented as mean values ± SD from triplicate determinations.

Parental cell	Resistant cell	Drug	Degree of resistance
K562	K562/BCRP	SN-38	24.8 ± 0.63
K562	K562/BCRP	Mitoxantrone	10.1 ± 0.27
K562	K562/MDR	Vincristine	173 ± 18.7
KB-3-1	KB/MRP	VP-16	10.3 ± 0.60

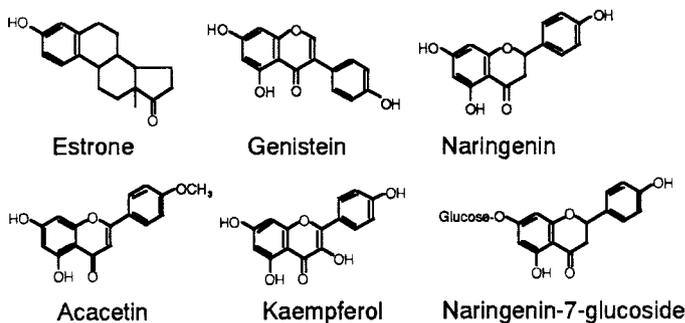


Fig. 2. Chemical structures of estrone and the indicated phytoestrogens/flavonoids.

MRP1. LLC/BCRP cells expressed BCRP. K562/MDR cells expressed P-glycoprotein but not BCRP or MRP1, and KB/MRP cells expressed MRP1 but not BCRP or P-glycoprotein (Fig. 1). Expression of BCRP, P-glycoprotein, or MRP1 was not detected in parental K562 and KB-3-1 cells.

K562/BCRP cells showed significantly higher resistance to SN-38 and mitoxantrone than K562 cells (Table 1). LLC/BCRP cells were five to six times more resistant to SN-38 and mitoxantrone than parental LLC-PK1 cells as described previously (8). K562/MDR cells showed significantly higher resistance to vincristine than K562 cells, and KB/MRP cells were significantly more resistant to VP-16 than KB-3-1 cells (Table 1). Protein expression and drug-resistance levels in each resistant cell line were stable for at least 2 months.

Reversal of BCRP-Mediated Drug Resistance by Flavonoids.

Estrone, 17β-estradiol, estrogen agonists, and estrogen antagonists reverse BCRP-mediated drug resistance. In the present study, we examined the potential reversal effects of phytoestrogens/flavonoids because they have been shown to have weak estrogenic activities (10). Structures of representative flavonoids are shown in Fig. 2. We first examined the effects of representative phytoestrogens, genistein and naringenin, on drug resistance in K562/BCRP cells (Fig. 3, A–C). Reversal indexes (ratios of IC₅₀ measurements in the absence of reversing agents divided by levels in the presence of reversing agents) of 3 μM (10 μM) genistein for SN-38 and mitoxantrone were 7.23 ± 0.35 (16.4 ± 0.56) and 6.28 ± 0.51 (11.7 ± 0.40), respectively. In addition, reversal indexes of 3 μM (10 μM) naringenin for SN-38 and mitoxantrone were 5.94 ± 0.26 (15.2 ± 0.92) and 3.42 ± 0.27 (10.6 ± 0.30), respectively. The reversing effects of genistein and naringenin proved to be greater than estrone. Analysis then was extended to other flavonoids, many of which reversed BCRP-mediated SN-38 resistance at a fixed concentration of 3 μM (Fig. 4). The flavones acacetin, apigenin, chrysin, diosmetin, and luteolin and the flavonols kaempferide and kaempferol displayed strong reversal effects (Fig. 3, D and E, and Fig. 4). Reversal indexes of 1 μM (3 μM) acacetin for SN-38 and mitoxantrone were 15.2 ± 1.10 (21.4 ± 0.34) and 9.89 ± 0.27 (9.71 ± 0.81), respectively. Reversal indexes of 1 μM (3 μM) kaempferol for SN-38 and

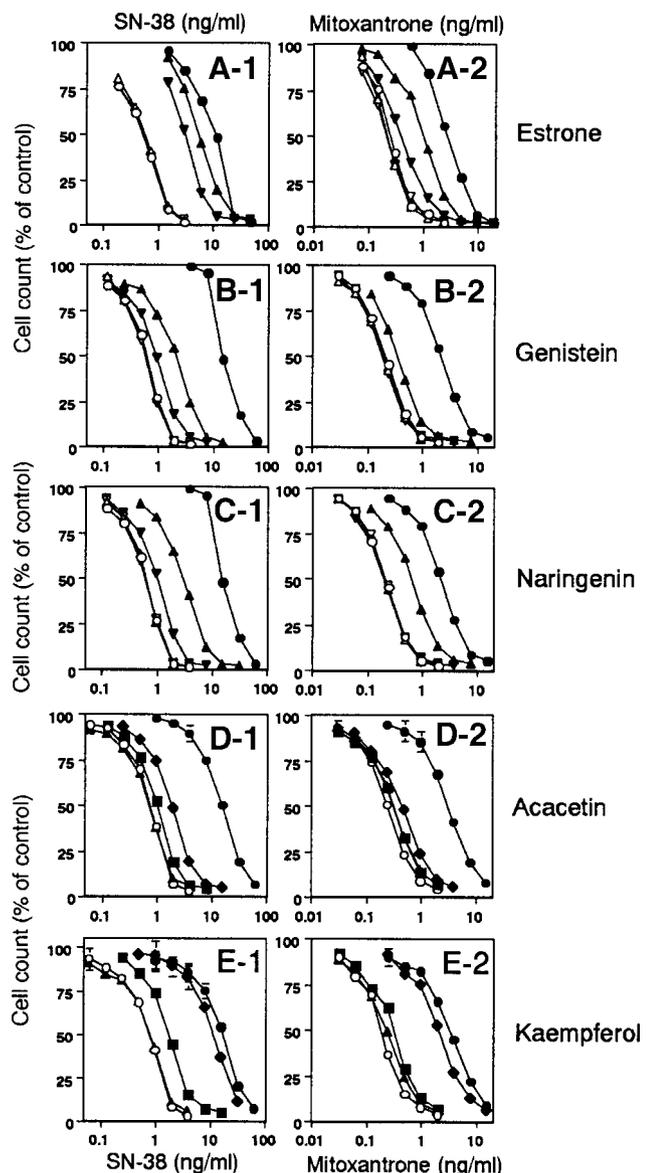


Fig. 3. Reversal effects of estrone and phytoestrogens/flavonoids on breast cancer resistance protein (BCRP)-mediated antitumor drug resistance. K562 (open symbols) and K562/BCRP (closed symbols) cells were cultured for 5 days in the absence (circle) or presence of 0.3 μM (lozenge), 1 μM (square), 3 μM (triangle), and 10 μM (inverted triangle) of the specific compounds indicated under increasing concentrations of antitumor drugs. A, estrone. B, genistein. C, naringenin. D, acacetin. E, kaempferol. Antitumor agents are SN-38 (N-1) and mitoxantrone (N-2; N, A-E). Data points are measurements of the average \pm SD from triplicate determinations. Cell numbers were determined with a cell counter.

mitoxantrone were 9.96 ± 0.38 (21.5 ± 0.22) and 10.6 ± 0.99 (14.2 ± 0.95), respectively. However, the flavonoids did not show growth-inhibitory effects on K562 cells under these experimental conditions. K562/BCRP cells treated with estrone or flavonoids, such as genistein, naringenin, and acacetin, for 5 days expressed similar amounts of BCRP as compared with control K562/BCRP cells (Fig. 5). This result suggested that flavonoids sensitized K562/BCRP cells to SN-38 and mitoxantrone not by reducing BCRP expression but by inhibiting BCRP function. We then examined the effects of glycosylated flavonoids on the drug-resistance properties of K562/BCRP cells. Although most glycosylated flavonoids had little effect on BCRP-mediated drug resistance, some glycosides, such as naringenin-7-glucoside and luteolin-4'-O-glucoside, displayed moderate reversal activity (Fig. 6). Reversal indexes of 3 μM (10 μM) naringenin-7-

glucoside for SN-38 and mitoxantrone were 5.70 ± 0.16 (14.7 ± 0.53) and 5.17 ± 0.23 (9.44 ± 0.42), respectively.

Additional studies showed that the reversal of MDR by genistein and naringenin was specific to BCRP because they did not show any reversal effects on either P-glycoprotein-mediated vincristine resistance or MRP1-mediated VP-16 resistance (Fig. 7).

Intracellular Topotecan Uptake and Cellular [³H]Genistein Accumulation in K562/BCRP Cells. To address whether reversal of BCRP-mediated drug resistance by flavonoids might be associated with the inhibition of BCRP-mediated drug efflux, the cellular accumulation of topotecan was evaluated in the absence or presence of specific flavonoids by flow cytometric analysis. Intracellular accumulation of topotecan increased in the presence of genistein or naringenin in a dose-dependent manner in K562/BCRP cells (Fig. 8), whereas these levels were not altered in K562 cells (data not shown). The results indicate that these flavonoids reverse anticancer drug

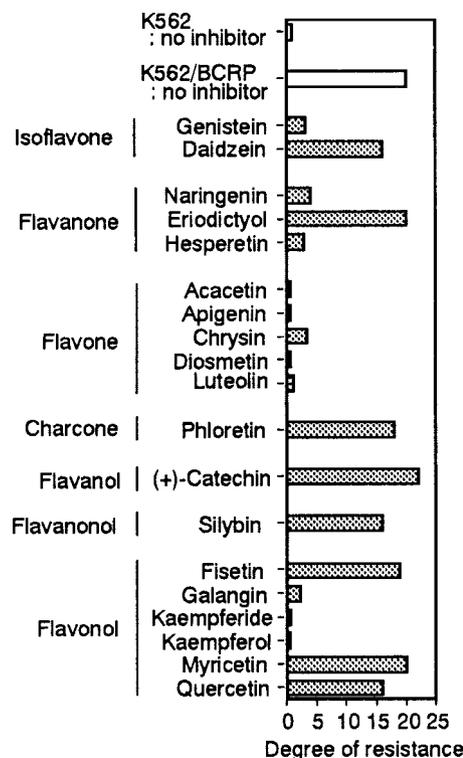


Fig. 4. Inhibitory effects of phytoestrogens/flavonoids on breast cancer resistance protein (BCRP)-mediated SN-38 resistance. K562 and K562/BCRP cells were cultured for 5 days in the absence or presence of 3 μM compound with increasing concentrations of SN-38. Cell numbers were determined using a cell counter, and IC_{50} values then were measured. Open bar, no inhibitor. Dotted bar, treatment with flavonoids. The degree of resistance is the ratio of IC_{50} values of the cells to that of K562 cells under the indicated experimental conditions.

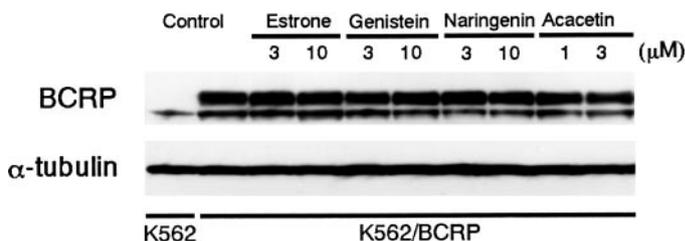


Fig. 5. Western blot analyses of breast cancer resistance protein (BCRP) expression in K562/BCRP cells treated with estrone or flavonoids for 5 days. K562 and K562/BCRP cells were incubated for 5 days in the absence or presence of indicated concentrations of compounds. Cell lysates (20 μg /lane) were used for quantitative analyses of BCRP expression. Expression of α -tubulin was presented as an internal control.

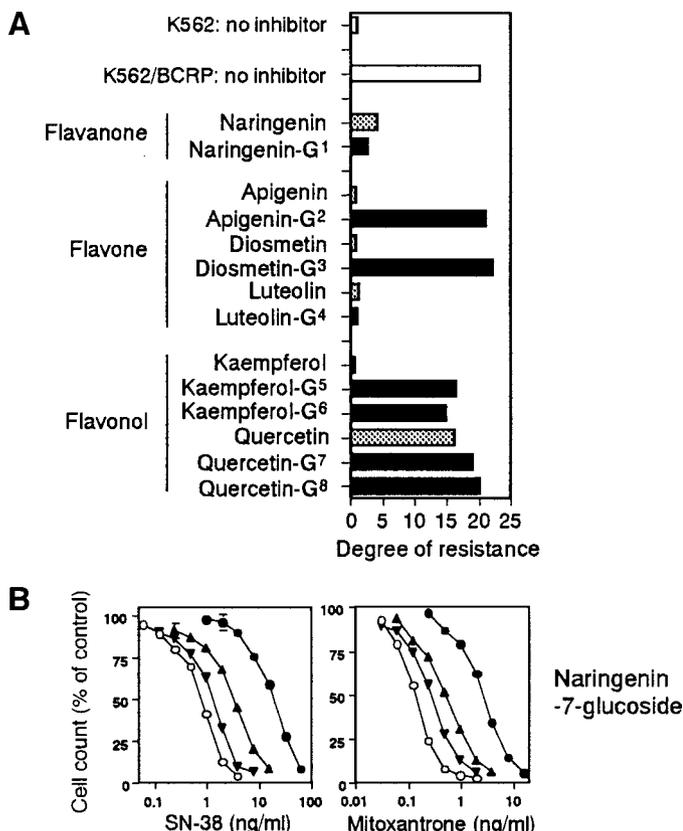


Fig. 6. Inhibitory effects of glycosylated flavonoids on breast cancer resistance protein (BCRP)-mediated drug resistance. *A*, inhibitory effects of glycosylated flavonoids on BCRP-mediated SN-38 resistance. K562 and K562/BCRP cells were cultured for 5 days in the absence or presence of 3 μM flavonoid compounds with increasing concentrations of SN-38. Cell numbers were measured with a cell counter, and IC_{50} values then were determined. The degree of resistance is the ratio of IC_{50} values of the cells to that of K562 cells under the indicated experimental conditions. Open bar, no inhibitor. Dotted bar, treatment with flavonoids. Solid bar, treatment with glycosylated flavonoids. *Naringenin-G¹*, naringenin-7-glucoside; *Apigenin-G²*, apigenin-7-neohesperidoside (rhoifolin); *Diosmetin-G³*, diosmetin-7- β -rutinoside (Diosmin); *Luteolin-G⁴*, luteolin-4'-*O*-glucoside; *Kaempferol-G⁵*, kaempferol-3-glucoside; *Kaempferol-G⁶*, kaempferol-7-*O*-neohesperidoside; *Quercetin-G⁷*, quercetin-3-arabinoglucoside (peltatoside); and *Quercetin-G⁸*, quercetin-3-rutinoside (Rutin). *B*, reversal effects of naringenin-7-glucoside on BCRP-mediated drug resistance. K562 (open symbol) and K562/BCRP (closed symbol) cells were cultured for 5 days in the absence (circle) or presence of 3 μM (triangle) and 10 μM (inverted triangle) naringenin-7-glucoside with increasing concentrations of antitumor drugs. Data points are the average \pm SD from triplicate determinations. Cell numbers were determined by a cell counter.

resistance by increasing the cellular levels of anticancer drugs in BCRP-expressing cells.

To examine whether flavonoids themselves are transported by BCRP, the intracellular accumulation of [^3H]genistein in K562 and K562/BCRP cells also was examined. K562/BCRP cells accumulated a significantly smaller amount of [^3H]genistein than K562 cells, suggesting that there is BCRP-mediated efflux of genistein out of the cells (Fig. 9).

Transcellular Transport of [^3H]Genistein. BCRP-mediated transport of genistein was examined by transcellular transport assays using LLC/BCRP cells, which express BCRP in the apical membrane (8). The paracellular fluxes monitored by [^{14}C]inulin appearance in the other side of the growth chambers were <1% of the total radioactivity/h. Basal-to-apical transport (secretion) of [^3H]mitoxantrone, a BCRP substrate, was greater in LLC/BCRP cells than that in LLC-PK1 cells (8). In the present study, secretion of [^3H]genistein in LLC/BCRP cells also proved to be greater than that in LLC-PK1 cells, whereas apical-to-basal transport (reabsorption) of [^3H]genistein in LLC/BCRP cells was reduced compared with LLC-PK1 cells (Fig.

10). However, in the presence of 3 μM fumitremorgin C, secretion and reabsorption of [^3H]genistein were at similar levels between LLC-PK1 and LLC/BCRP cells.

Our previous study demonstrated that [^3H]estrone was converted to [^3H]estrone sulfate in LLC-PK1 cells and that the latter was exported by BCRP (8). Therefore, transported radioactivity over the apical membrane of the cells was analyzed by silica gel TLC. [^3H]genistein

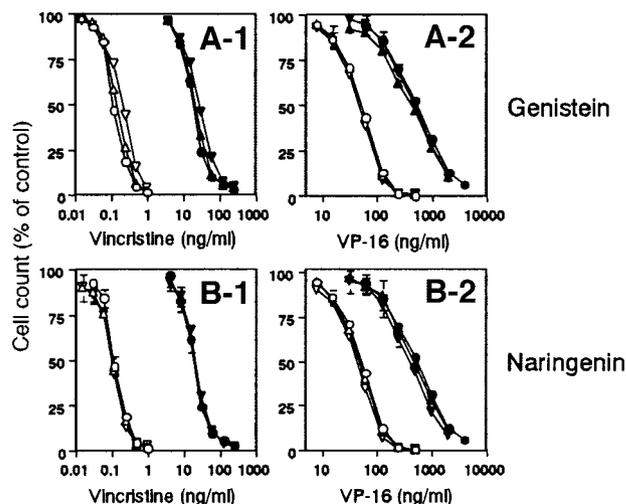


Fig. 7. Reversal effects of genistein and naringenin on either P-glycoprotein- or MRP1-mediated antitumor drug resistance. Parental cells (open symbol) and transfected cells (closed symbol) were cultured for 5 days in the absence (circle) or presence of 3 μM (triangle) or 10 μM (inverted triangle) compounds with increasing concentrations of the antitumor agents. Cell numbers were determined using a cell counter. *A-1*, effect of genistein on the vincristine sensitivity of K562 and K562/MDR cells. *A-2*, effect of genistein on the VP-16 sensitivity of KB-3-1 and KB/MDR cells. *B-1*, effect of naringenin on the vincristine sensitivity of K562 and K562/MDR cells. *B-2*, effect of naringenin on the VP-16 sensitivity of KB-3-1 and KB/MDR cells. Data points are the average \pm SD from triplicate determinations.

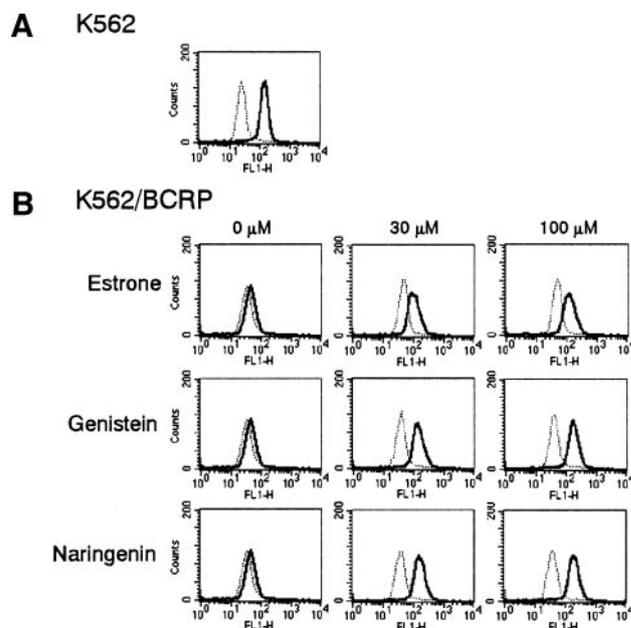


Fig. 8. Effects of estrone and flavonoids on the cellular uptake of topotecan in K562/BCRP cells. Cells were incubated with (bold line) or without (dotted line) 20 μM topotecan in the absence or presence of the indicated compound. Uptake of topotecan was measured using fluorescence-activated cell sorter. A fluorescence peak shift to the right was observed in K562 cells. In K562/BCRP cells, a fluorescence peak shift was not observed in the absence of estrone or flavonoids, but a fluorescence peak shift to the right indicating cellular uptake of topotecan occurred in a dose-dependent manner of the compounds.

migrated at $R_f = 0.8$ (segment 11). TLC of transported radioactivity after 4-h incubation showed that almost all of the [^3H]genistein was transported in its native form and migrated at $R_f = 0.8$ (segment 11) under these experimental conditions. Secretion of [^3H]genistein was significantly greater in LLC/BCRP cells than that in LLC-PK1 cells (Fig. 11), indicating that genistein itself is directly transported by BCRP. In a separate experiment, when [^3H]genistein was incubated with LLC-PK1 cells for 24 h, >80% of the radioactivity shifted to $R_f = 0.12\text{--}0.21$ (segment 3–4) and approximately 10% shifted to $R_f = 0.38\text{--}0.47$ (segment 6–7), in which metabolites of genistein were expected to exist (data not shown). However, only small amounts of such metabolites were found in the 4-h transport assay (Fig. 11).

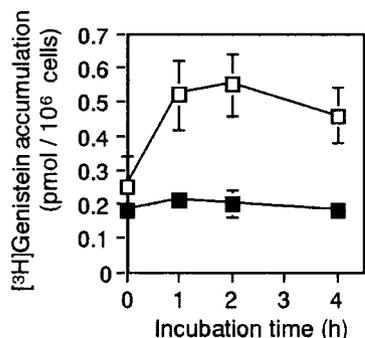


Fig. 9. Intracellular accumulation of [^3H]genistein in K562/BCRP cells. Cells (2×10^6) were incubated with 30 nM [^3H]genistein for 0, 1, 2, or 4 h at 37°C. After washing, the cells were dissolved in 100 μl PBS and 400 μl Soluene-350 and mixed with 5 ml ACS II scintillation mixture. Radioactivity was measured using a scintillation counter. The data are mean \pm SD from triplicate determinations.

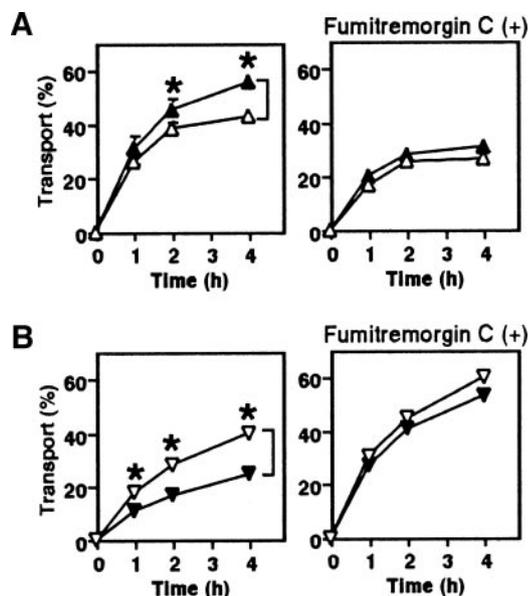


Fig. 10. Transcellular transport of [^3H]genistein by breast cancer resistance protein (BCRP). Cells (2.4×10^6 /well) were plated on 3- μm pore filters and cultured for 3 days. The apical and the basal sides of the medium were replaced with 2 ml serum-free medium 1.5 h before beginning the experiment. When required, 3 μM fumitremorgin C was added to the apical and basal side medium at this time. [^3H]genistein (30 nM) was added to either the apical or basal side medium. After 1, 2, and 4 h, the percentage of radioactivity that appeared in the opposite side was measured. *A*, basal-to-apical transport of [^3H]genistein. *B*, apical-to-basal transport of [^3H]genistein. Open triangles, basal-to-apical transport in LLC-PK1; closed triangles, basal-to-apical transport in LLC/BCRP; inverted open triangles, apical-to-basal transport in LLC-PK1; and inverted closed triangles, apical-to-basal transport in LLC/BCRP. The data, expressed as a percent fraction of the total radioactivity, are mean \pm SD of triplicate determinations from three different cultures. When a vertical bar is not shown, the SD is within the symbol. * $P < 0.05$. The data are representative of two independent experiments.

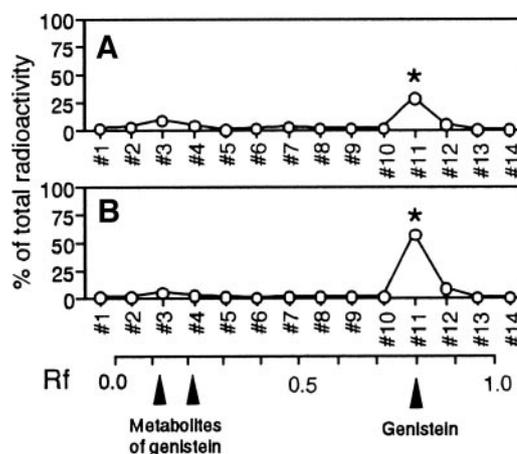


Fig. 11. Silica gel TLC of secreted [^3H]genistein and its corresponding metabolites. The experimental procedure was the same as that of the transepithelial transport assay until sample collection. Fifty μl of medium in the apical side after 4-h incubation were mixed with 100 μl of methanol, spotted, and run on silica gel plates in chloroform/methanol/acetic acid (8:3:1). Separated zones then were excised, and their radioactivities were measured. The radioactivity measurements are expressed as a percentage fraction of the total radioactivity before incubation. Data points are mean \pm SD of triplicate determinations from three different cultures. *A*, TLC analysis of secreted [^3H]genistein compounds for 4 h in LLC-PK1 cells. *B*, TLC analysis of secreted [^3H]genistein compounds for 4 h in LLC/BCRP cells. Numbers 1–14 indicate segments in the silica gel TLC plate. [^3H]genistein is fractionated in segment 11, and metabolites of [^3H]genistein are found in segments 3 and 4. The data are means of triplicate determinations from three different cultures. The SD is within the symbol. * $P < 0.05$ between LLC-PK1 and LLC/BCRP cells.

DISCUSSION

Estrone and 17 β -estradiol are the first endogenous compounds that were shown to exert strong BCRP-reversing activity (7). Synthesized estrogen agonists and antagonists also showed strong reversing activity of BCRP-mediated drug resistance (9). Therefore, we extended our studies to natural estrogenic compounds in the search for BCRP inhibitors.

Isoflavones derived from soybean, such as genistein and daidzein, constitute a subset of flavonoids that have been reported to have weak estrogenic activity (10). In addition, chemical structures of isoflavones resemble those of estrone and 17 β -estradiol (Fig. 2). Naringenin, a flavanone contained in grapefruit juice, also resembles 17 β -estradiol in chemical structure, albeit to a lesser extent than isoflavone (Fig. 2). Flavonoids are remarkably safe nutrients, being the most abundant polyphenolic compounds present in the human diet in fruits, vegetables, and plant-derived beverages such as tea and red wine (15). Some flavonoids also have been reported to interact with ABC transporters, such as P-glycoprotein, MRP1, MRP2, and cystic fibrosis transmembrane conductance regulator (16–21).

Genistein and naringenin displayed stronger interaction with BCRP than estrone (Fig. 3, A–C), and many flavones, such as acacetin, apigenin, chrysin, diosmetin, and luteolin, and some flavonols, such as kaempferide and kaempferol, demonstrated strong reversing activity of BCRP-mediated drug resistances (Fig. 3, D and E, and Fig. 4). Flavanol, flavanonol, and one-half of flavonols tested did not show BCRP-reversing activity (Fig. 4). Two glycosylated flavonoids, naringenin-7-glucoside and luteolin-4'-O-glucoside, did show anti-BCRP activity, whereas six other glycosylated flavonoids did not (Fig. 6). From these results, we speculated that the 3-hydroxyl group of the C ring might be important for BCRP-inhibitory activity, although some exceptions do exist. The flavonoids did not show growth-inhibitory effects in K562 and KB-3-1 cells at the highest concentrations used in our experiments, suggesting that they might be safely used for circumventing BCRP-mediated drug resistance in clinical practice. Glycosylated flavonoids with anti-BCRP activity

also may be useful because of their water solubility. In our preliminary animal experiments, some flavonoid aglycones were insoluble to either water or hydrophilic solvents and therefore would be difficult to administer i.v. Therefore, use of either glycosylated flavonoids or water-soluble derivatives of flavonoids would be an alternative way to develop BCRP inhibitors.

GF120918 was first developed as a P-glycoprotein inhibitor but also was shown to be a BCRP inhibitor (22, 23). TAG-139, which we identified as a tamoxifen-derived BCRP inhibitor, also, like tamoxifen, inhibited P-glycoprotein (9). Genistein was reported to be a substrate/inhibitor of MRP1 because genistein inhibited daunorubicin transport out of cells overexpressing MRP1 at a concentration of 50 μM (20). In the present study, genistein and naringenin effectively inhibited BCRP at a concentration of 3 μM (Fig. 3) but showed little effect on vincristine resistance in K562/MDR cells or on VP-16 resistance in KB/MRP cells even at a concentration of 10 μM (Fig. 7). Therefore, these flavonoids could be specific inhibitors of BCRP.

In previous studies, we showed that estrone inhibits BCRP function but was not transported by BCRP in its native form (7, 8). In contrast, progesterone is known to inhibit the function of P-glycoprotein but is not transported by P-glycoprotein (24). In the case of genistein, K562/BCRP cells accumulated smaller amounts of [^3H]genistein than parental K562 cells (Fig. 9). Secretion of [^3H]genistein from LLC/BCRP cells was greater than from LLC-PK1 cells in transcellular transport assays (Fig. 10). TLC analysis of transported [^3H]genistein suggested that there was increased transport of genistein aglycone in LLC/BCRP cells compared with LLC-PK1 cells (Fig. 11). Intracellular accumulation of [^3H]genistein also was decreased in LLC/BCRP cells compared with levels in parental cells (data not shown). These results suggest that inhibition of BCRP-mediated drug resistance by genistein is caused by the competitive transport of genistein by BCRP. Unlike estrone and 17 β -estradiol, genistein would be transported in its native form but not in either sulfated or glucuronated forms (25, 26).

Another possible mechanism of BCRP inhibition by flavonoids is the interaction with the nucleotide-binding domain of BCRP because some flavonoids, including genistein, have been shown previously to interact with nucleotide-binding domain of P-glycoprotein, which was predicted to suppress ATP-hydrolysis and energy-dependent drug transport (16–18). In the case of BCRP, modulation of ATPase activity by the flavonoids should be investigated further to clarify this possibility.

The data presented here might have clinically important implications because some flavonoids effectively inhibited BCRP-mediated drug resistance at relatively low concentrations. For instance, we showed that 3 μM of genistein effectively circumvented BCRP-mediated drug resistance. Soybean (100 g) contains 100–200 mg isoflavones consisting of genistein, daidzein, glycitein, and their corresponding glycosides. Several groups have investigated the pharmacokinetics of soy isoflavone. A single bolus ingestion of 50 mg genistein in healthy premenopausal women was shown to result in a peak plasma concentration of $1.26 \pm 0.27 \mu\text{M}$ at $9.33 \pm 1.33 \text{ h}$ (25). The rate of unconjugated genistein was only approximately 2–4%. However, in another report of 6-consecutive-day feeding study of 25 g soymilk powder in young adult women, the percentage of plasma aglycone genistein sampled on days 5 and 6 was $26 \pm 7\%$ of total genistein (26). In addition, glucuronide was the main metabolite in that study. Therefore, the concentration of active genistein in cancer tissues may reach sufficient level for BCRP inhibition via oral ingestion because β -glucuronidase activity is elevated in cancer (27). Parenteral administration may validate genistein as BCRP inhibitor. Subcutaneous injection of genistein was shown previously to enable high concentration of active genistein in mice (28).

The findings that glycosylated flavonoids naringenin-7-glucoside

and luteolin-4'-*O*-glucoside effectively inhibited BCRP-mediated drug resistance might prove to be of great importance. These glycosylated flavonoids are well soluble in water. Because deglycosidation by intestinal enzymes precedes glucuronidation via oral routes, the i.v. injection of glycosylated flavonoids would bypass deglycosidation and retain their native structures, which are effective as BCRP inhibitors. In fact, i.v. naringenin-7-glucoside was excreted in urine mostly as its native glucoside form in a rat model (29).

Despite the promising results using flavonoids in the reversal of BCRP-mediated drug resistance, we must bear in mind that that coadministration or intake of flavonoids with BCRP-substrate antitumor agents may result in the alteration of their pharmacokinetics and may increase the toxicity of the antitumor drugs in the recipient patients. In this regard, orally administered GF120918 has been reported to increase oral bioavailability of topotecan (30).

In summary, phytoestrogens/flavonoids reverse BCRP-mediated drug resistance effectively, and these findings may bring direct and immediate clinical benefits via more effective and safer cancer chemotherapy treatments.

ACKNOWLEDGMENTS

We thank Dr. H. Esumi, National Cancer Center Research Institute East, for helpful suggestions and advice. KB/MRP cells were a gift from Dr. K. Ueda, Graduate School of Agriculture, Kyoto University. We also thank Dr. L. Greenberger and Wyeth Ayerst for providing fumitremorgin C.

REFERENCES

- Doyle LA, Yang W, Abruzzo LV, et al. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci USA* 1998;95:15665–70.
- Gottesman MM, Hrycyna CA, Schoenlein PV, Germann UA, Pastan I. Genetic analysis of the multidrug transporter. *Annu Rev Genet* 1995;29:607–49.
- Allikmets R, Schriml L, Hutchinson A, Romano-Spica V, Dean M. A human placenta-specific ATP-binding cassette gene (*ABCP*) on chromosome 4q22 that is involved in multidrug resistance. *Cancer Res* 1998;58:5337–9.
- Miyake K, Mickley L, Litman T, et al. Molecular cloning of cDNAs which is highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. *Cancer Res* 1999;59:8–13.
- Maliepaard M, van Gastelen MA, de Jong LA, et al. Overexpression of the *BCRP/MXR/ABCP* gene in a topotecan-selected ovarian tumor cell line. *Cancer Res* 1999;59:4559–63.
- Kawabata S, Oka M, Shiozawa K, et al. Breast cancer resistance protein directly confers SN-38 resistance of lung cancer cells. *Biochem Biophys Res Commun* 2001;280:1216–23.
- Imai Y, Tsukahara S, Ishikawa E, Tsuruo T, Sugimoto Y. Estrone and 17 β -estradiol reverse breast cancer resistance protein-mediated multidrug resistance. *Jpn J Cancer Res* 2002;93:231–5.
- Imai Y, Asada S, Tsukahara S, Ishikawa E, Tsuruo T, Sugimoto Y. Breast cancer resistance protein exports sulfated estrogens but not free estrogens. *Mol Pharmacol* 2003;64:610–8.
- Sugimoto Y, Tsukahara S, Imai Y, Sugimoto 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.
- Kuiper GG, Lemmen JG, Carlsson B, et al. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β . *Endocrinology* 1998;139:4252–63.
- Kage K, Tsukahara S, Sugiyama T, et al. Dominant-negative inhibition of breast cancer resistance protein as drug efflux pump through the inhibition of S-S dependent homodimerization. *Int J Cancer* 2002;97:626–30.
- Suzuki M, Sugimoto Y, Tsuruo T. Efficient protection of cells from the genotoxicity of nitrosoureas by the retrovirus-mediated transfer of human O⁶-methylguanine-DNA methyltransferase using bicistronic vectors with human multidrug resistance gene 1. *Mutat Res* 1998;401:133–41.
- Taguchi Y, Yoshida A, Takada Y, Komano T, Ueda K. Anti-cancer drugs and glutathione stimulate vanadate-induced trapping of nucleotide in multidrug resistance-associated protein (MRP). *FEBS Lett* 1997;401:11–4.
- Rabindran SK, Ross DD, Doyle A, Yang W, Greenberger LM. Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. *Cancer Res* 2000;60:47–50.
- Harborne J. Nature, distribution and function of plant flavonoids. In: *Plant flavonoids in biology and medicine: Biochemical pharmacological and structure-activity relationships*. New York: Alan R. Liss, Inc.; 1986. p. 17–28.
- Castro AF, Altenberger GA. Inhibition of drug transport by genistein in multidrug-resistant cells expressing P-glycoprotein. *Biochem Pharmacol* 1997;53:89–93.
- Di Pietro A, Conseil G, Perez-Victoria JM, et al. Modulation by flavonoids of cell multidrug resistance mediated by P-glycoprotein and related ABC transporters. *Cell Mol Life Sci* 2002;59:307–22.

18. Zhang S, Morris ME. Effects of the flavonoids biochanin A, morin, phloretin, and silymarin on P-glycoprotein-mediated transport. *J Pharmacol Exp Ther* 2003;304:1258–67.
19. Walgren RA, Karnaky KJ Jr, Lindenmayer GE, Walle T. Efflux of dietary flavonoid quercetin 4'- β -glucoside across human intestinal Caco-2 cell monolayers by apical multidrug resistance-associated protein-2. *J Pharmacol Exp Ther* 2000;294:830–6.
20. Versantvoort CH, Broxterman HJ, Lankelma J, Feller N, Pinedo HM. Competitive inhibition by genistein and ATP dependence of daunorubicin transport in intact MRP overexpressing human small cell lung cancer cells. *Biochem Pharmacol* 1994;48:1129–36.
21. Singh AK, Schultz BD, Katzenellenbogen JA, Price EM, Bridges RJ, Bradbury NA. Estrogen inhibition of cystic fibrosis transmembrane conductance regulator-mediated chloride secretion. *J Pharmacol Exp Ther* 2000;295:195–204.
22. Hyafil F, Vergely C, Du Vignaud P, Grand-Perret T. In vitro and in vivo reversal of multidrug resistance by GF120918, an acridonecarboxamide derivative. *Cancer Res* 1993;53:4595–602.
23. De Bruin M, Miyake K, Litman T, Robey R, Bates SE. Reversal of resistance by GF120918 in cell lines expressing the ABC half-transporter, MXR. *Cancer Lett* 1999;146:117–26.
24. Ueda K, Okamura N, Hirai M, et al. Human P-glycoprotein transports cortisol, aldosterone, and dexamethasone, but not progesterone. *J Biol Chem* 1992;267:24248–52.
25. Setchell KD, Brown NM, Desai P, et al. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J Nutr* 2001;131:1362–75S.
26. Zhang Y, Hendrich S, Murphy PA. Glucuronides are the main isoflavone metabolites in women. *J Nutr* 2003;133:399–404.
27. de Graaf M, Boven E, Scheeren HW, Haisma HJ, Pinedo HM. Beta-glucuronidase-mediated drug release. *Curr Pharm Des* 2002;8:1391–403.
28. Doerge DR, Twaddle NC, Banks EP, Jefferson WN, Newbold RR. Pharmacokinetic analysis in serum of genistein administered subcutaneously to neonatal mice. *Cancer Lett* 2002;184:21–7.
29. Choudhury R, Chowrimootoo G, Srail K, Debnam E, Rice-Evans CA. Interactions of the flavonoid naringenin in the gastrointestinal tract and the influence of glycosylation. *Biochem Biophys Res Commun* 1999;265:410–5.
30. Kruijtzter CM, Beijnen JH, Rosing H, et al. Increased oral bioavailability of topotecan in combination with the breast cancer resistance protein and P-glycoprotein inhibitor GF120918. *J Clin Oncol* 2002;20:2943–50.