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Inhibitory Effects of Herbal Extracts on Breast Cancer Resistance Protein (BCRP) and Structure-Inhibitory Potency Relationship of Isoflavonoids

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Summary: The inhibition of intestinal breast cancer resistance protein (BCRP), which restricts the absorption of xenobiotics, may increase the systemic availability of its substrates. The aim of this study was to evaluate the inhibitory effects of herbal extracts and their constituents on BCRP-mediated transport. The inhibitory effects of 9 herbal extracts and 23 isoflavonoids, including soybean-derived isoflavones, on BCRP-mediated methotrexate (MTX) transport were evaluated using BCRP-expressing membrane vesicles. The structure-inhibitory potency relationship was investigated by multiple factor analysis. Extracts of soybean, Gymnema sylvestre, black cohosh and passion flower and rutin strongly inhibited BCRP-mediated transport of MTX at 1 mg/ml, while inhibition by chlorella, milk thistle and Siberian ginseng extracts was weak. Among the 23 isoflavonoids examined, all of which inhibited BCRP-mediated transport, coumestrol showed the most potent inhibition (IC50 = 63 nM). The inhibitory potencies of 6 isoflavonoid glucosides were 10- to 100-fold lower than those of the corresponding aglycones. The addition of a 5-hydroxyl or 6-methoxyl moiety tended to potentiate the inhibition. The inhibitory potency of daidzein was decreased 100-fold by 7-glucuronidation, but was virtually unaffected by 4'-sulfation. Thus, some herbal and dietary supplements and isoflavonoids may increase the systemic availability of BCRP substrates when concomitantly given orally.

Keywords: breast cancer resistance protein (BCRP); ATP-binding cassette transporter G2 (ABCG2); dietary supplement; drug interactions; isoflavonoids; structure-activity relationship

Introduction

ATP-binding cassette (ABC) transporters such as P-glycoprotein and multidrug resistance-associated protein 2 restrict intestinal drug absorption,1) and the inhibition of ABC transporters has been shown to increase intestinal absorption of their substrates.2) Breast cancer resistance protein (BCRP, encoded by ABCG2), a member of the ABC transporter family,3–5) is an efflux transporter expressed in intestinal epithelial cells, as well as bile canaliculus, kidney, blood-brain barrier and placenta.6,7) BCRP transports various drugs, including methotrexate (MTX),8) topoisomerase I inhibitors,9) flavopiridol,10) statins11,12) and proton pump inhibitors.13,14) In vivo murine studies revealed that intestinal Bcrp restricts the absorption of orally administered drugs, such as topotecan,15) irinotecan,16) nitrofurantoin,17) GV196771, N-methyl-D-aspartate receptor antagonist18) and sulfasalazine.19) Recent studies showed that the genetic polymorphism of ABCG2 (c.421C > A) affects the absorption of BCRP substrate drugs.20–22) These results imply that BCRP plays an important role in restricting the intestinal absorption of various drugs.

Various phytochemicals, such as flavonoids, chalcones, terpenoids, isothiocyanates and nonprenylated rotenoids, are known to inhibit BCRP.23–29) Among them, flavonoids...
have been extensively investigated in relation to the
relationship between inhibitory potency and chemical
structure by using BCRP-overexpressing cells.\textsuperscript{30-32} In
contrast, little is known about isoflavonoids other than
the isoflavonoids, daidzein and coumestrol, which were reported
to be substrates of human BCRP and mouse Bcrp.\textsuperscript{23,24,33}

In rats, the plasma concentration of nitrofurantoin, a
BCRP substrate, was increased by the concomitant oral administration of chrysiryn, a structural analog of flavone,\textsuperscript{34} suggesting that some BCRP inhibitors derived from herbs may increase the systemic availability of BCRP substrate drugs. Some plant-derived health foods and herbal supplements contain these compounds at high levels,\textsuperscript{35} whereas the amounts of these compounds taken from foods are usually limited. In the United States, herbal supplements are used widely, often concomitantly with drugs (18.4% of all prescription users\textsuperscript{36}).

The purpose of the present study was to clarify the in-
hibitory potencies of various herbal extracts and their
consituents on BCRP in order to estimate the likelihood of drug-herbal supplement interactions. The relationship between the chemical structure of isoflavonoids and inhibitory potency towards BCRP was also examined by
means of multiple factor analysis.

Materials and Methods

Materials: Membrane vesicles isolated from mammalian cells expressing wild-type BCRP (SB-BCRP-M-VT) were purchased from SOLVO Biotechnologies (Budapest, Hungary). MTX hydrate and fumitremorgin C (FTC) were purchased from Sigma-Aldrich (St Louis, MO, USA). MTX disodium salt [\(3',5',7'-\text{H}(N)\)] (740 GBq/mmol, ethanol:water = 4:6) was purchased from Moravek Biochemicals, Inc. (Brea, CA, USA).

Daidzein, genistein and glycitein (each \(>98.0\%\) pure) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Genistin and daidzin (each \(>98.0\%\) pure) were purchased from LC Laboratories (Woburn, MA, USA). Glycitin (\(\geq 98\%\)) was purchased from Nagara Science (Gifu, Japan). Formononetin (91.0%), ononin (99.9%), sissotrin (99.9%), sophoricoside, 4',7-dimethoxy-3-hydroxyisoflavone (genistein-4',7-dimethyl ether, \(\geq 96.0\%\)) and 5-methyl-7-methoxyisoflavone were purchased from ChromaDex (CA, USA). Daidzein-7-glucuronide potassium salt, daidzein-4'-sulfate and 3',4',7-trihydroxyisoflavone (98.0%) were purchased from Toronto Research Chemicals (Ontario, Canada). Biochanin A, 4',7-dimethoxyisoflavone, prunetin (\(\geq 98.0\%\)) and coumestrol (\(\geq 97.0\%\)) were purchased from Sigma-Aldrich (St Louis, MO, USA). 4',6,7-Trihydroxyisoflavone and 4',6,7-trimethoxyisoflavone were purchased from Extrasynthese (Genay, France). Ipriflavone (\(\geq 97.0\%\)) was purchased from Wako Pure Chemical Ind-

dustries. Coumestrin was a kind gift from Dr. Satoshi Morimoto (Department of Medico-Pharmaceutical Sciences, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan). All other chemicals were commercial products of reagent grade.

**Herbal extracts:** The nine herbal extracts (extracts of soybean, barley, chlorella, milk thistle, Gymnema syl
vestre, black cohosh, passion flower and Siberian ginseng, and rutin, which are industrially added to various end products) were kind gifts from Tokiwa Phytochemical Co (Tokyo, Japan). The putative active ingredients of each herb were enriched in the extracts, the extraction methods of which are briefly shown below.

An extract from barley flour of Hordeum vulgare was purified, mixed with cycloexdrin and dried. Chlorella pyrenoidosa was extracted with water and the extract was purified, mixed with cycloexdrin and dried (the value of OD260 was 4,150). Soybean extract was made from Glycine max (mainly its hypocotyl), concentrated, purified and dried; the product contained 30.4% isoflavones as glucosides (product name: Isomax-30). An extract of passion flower was made from whole Passiflora incarnata L. and dried. An extract of Siberian ginseng was obtained by extraction with hydrous ethanol from rhizomes of Eleutherococcus senticosus and dried. Rutin was extracted from buds of Sophora japonica L. and dried; its purity was 96.2%. Milk thistle extract from seeds of Silybum maria
num was purified and dried; the product contained 31.7% silybin. An extract of black cohosh was obtained by extraction with hydrous ethanol from roots of Cimicifuga racemosa Nutt. and dried. An extract of Gymnema sylvestre R.Br. leaves was obtained by extraction with dilute alcohol and dried; the product contained 10.5% gymnemic acid as nonhydrate.

**Uptake experiments using BCRP-overexpressing membrane vesicles:** MTX uptake of BCRP-expressing membrane vesicles was measured using a rapid filtration technique, following the protocol (version 1.3) provided by the manufacturer. Briefly, BCRP-expressing membrane vesicles were quickly thawed at 37°C. Substrate uptake was initiated by adding a preincubated substrate buffer (final concentration: 3-(\(N\)-morpholino)propanesulfonic acid (MOPS)-Tris 39.8 mM, KCl 55.7 mM, MgCl\(_2\) 32.7 mM and [\(3\text{H}\)]MTX 18.5 kBq, pH 7.0) with 26.7 mM ATP or AMP to 10 \(\mu\)l (containing 50 \(\mu\)g protein) of preincubated membrane vesicles solution (total volume: 75 \(\mu\)l, the ethanol contained in the radioisotope solution amounted to less than 0.3% (v/v)). To evaluate the concentration dependency of BCRP-mediated transport, the uptake study was run with various final concentrations of MTX, with ATP or AMP. The mixture was incubated for 2 min at 37°C, then 1 ml of ice-cold washing buffer (0.04 M MOPS-Tris, 0.07 M KCl, pH 7.0) was added to stop the uptake reaction. Vesicle-associated [\(3\text{H}\)]MTX in the stopped reaction mixture was
separated from free [3H]MTX by rapid filtration through a GF/C filter (Whatman, Maidstone, UK). Filters were further washed twice with 5 ml of ice-cold washing buffer and assayed for radioactivity in 4 ml of liquid scintillation cocktail (Clear-sol I; Nacalai Tesque, Kyoto, Japan). The amount of MTX taken up into vesicles was calculated from the radioactivity. ATP-dependent MTX transport was assessed by subtracting the uptake in the presence of AMP from that in the presence of ATP. The ATP-dependent uptake into control vesicles (SB-M-CTRL; SOLVO Biotechnology) was also measured using the same protocol.

To evaluate the effect of the osmolality of extravesicular buffer, uptake of MTX into the vesicles was evaluated after addition of mannitol to the substrate buffer to give 500 or 1,000 mOsmol final osmolality. The incubation time for uptake of MTX was 20 min in this experiment.

**Inhibition studies with herbal extracts:** To evaluate the inhibitory effects of nine herbal extracts, MTX uptake into the vesicles was measured in the presence of herbal extracts. The herbal extracts were dissolved in dimethyl sulfoxide (DMSO) and water. The herbal extract solutions were filtered through 0.22 μm Millex-GV filters (Millipore Corp., Bedford, MA, USA) and diluted with the substrate buffer to give 1 mg/ml final herbal extract concentration and 0.5% (v/v) final DMSO concentration. To examine the concentration dependency of the inhibitory effect of soybean extract on BCRP-mediated transport, the uptake of MTX into the vesicles was measured in the presence of final concentrations of 1, 10, 100 and 1,000 μg/ml soybean extract. In these experiments, substrate buffer containing 0.5% (v/v) DMSO was used as the control. FTC was used as a positive control BCRP inhibitor. The final concentration of MTX in the substrate buffer was 100 μM and the incubation time for determination of uptake of MTX was 2 min.

**Inhibition studies with isoflavonoids:** To evaluate the inhibitory effects of 23 isoflavonoids, MTX uptake into vesicles was measured in the presence of various concentrations of each isoflavonoid. Isoflavonoids were dissolved in DMSO and diluted with the substrate buffer to give 0.5% (v/v) final DMSO concentration. In this experiment, the substrate buffer containing 0.5% (v/v) DMSO was used as the control. The final concentration of MTX in the substrate buffer was 100 μM and the incubation time for determination of uptake was 2 min.

**Data analysis:** To determine the negative logarithm of half-maximal inhibitory concentration of soybean extract and isoflavonoids (pIC50; logg/ml] for soybean extract and log[mol/l] for isoflavonoids) for the BCRP-mediated uptake of MTX, the following equation was fitted to the data for the ATP-dependent uptake in the presence of soybean extract or isoflavonoids by using a nonlinear least-squares regression analysis program (Mlab, Civilized Software Inc., Bethesda, MD, USA).

\[ \frac{V}{V_0} = \frac{1}{1 + 10^{\log([I]) - pIC_{50}}} \]

where V and V0 are the ATP-dependent transport rates of MTX in the presence or absence of inhibitors, respectively, and [I] is the concentration of inhibitor (soybean extract: g/ml and isoflavonoids: mol/l). The effects of functional moieties on the inhibitory potencies of isoflavonoids for BCRP-mediated transport were analyzed by multiple factor analysis based on Hayashi’s quantification theory type I,37 using all models which contain some of four items of moieties’ position (C5, C6, C7, C4’, Fig. 4), and then the Akaike information criterion (AIC) was assessed for each model to select the best one. In the statistical analysis, the significance of differences between mean values was determined by analysis of variance (ANOVA), followed by Dunnett’s test, and a p value of less than 0.05 was considered statistically significant. The significance of differences between the pIC50 values of aglycones and the corresponding glucosides was determined by paired t-test.

**Results**

**BCRP-mediated transport of MTX into the vesicles:** The amount of MTX taken up by the vesicles markedly decreased as the osmolarity of the extravesicular medium increased (6.85 μl/mg protein/20 min at 380 mOsmol, 6.54 μl/mg protein/20 min at 500 mOsmol and 4.55 μl/mg protein/20 min at 1,000 mOsmol). This result indicates that MTX was actually transported into the intravesicular space. The uptake of MTX into BCRP-expressing membrane vesicles increased linearly for at least 3 min (data not shown), so initial uptake rates were determined at 2 min in the uptake and inhibition studies. The ATP-dependent transport of MTX increased linearly with concentration from 100 μM to 1 mM, and appeared to be saturated at concentrations above 1 mM (Fig. 1), although uptake studies at MTX concentrations higher than 5 mM were not experimentally feasible due to limited solubility of the drug. ATP-dependent uptake was not found in the experiment using control membrane vesicles (in the presence of ATP 0.34 ± 0.01 μl/mg protein at 1 min vs AMP 0.46 ± 0.01 μl/mg protein at 1 min) and also in the experiment with BCRP-expressed membrane vesicles in the presence of 5 μM FTC, the specific inhibitor of BCRP (Fig. 2). From these results, we examined the ATP-dependent uptake of MTX into BCRP-expressing membrane vesicles as an index of BCRP-mediated transport. The Km value of BCRP-mediated transport of MTX was estimated to be 5.78 ± 0.84 mM. This value was similar to previously reported Km values of MTX in vesicular uptake studies at pH 7.4 (5.7 mM,38 5.21 mM39).

**Inhibitory effects of herbal extracts on BCRP-mediated transport of MTX:** The extracts of soybe-
Effects of Herbal Extracts and Isoflavonoids on BCRP

Fig. 1. Concentration-dependent uptake of methotrexate (MTX) into BCRP-expressing membrane vesicles
The uptake of MTX into human BCRP-expressing mammalian cell membrane vesicles was measured at 37°C at 2 min in the presence of ATP (closed circles) or AMP (open circles). Each point represents the mean ± SEM of three experiments. The dashed line indicates \( V_0 \times [C] \), where \( V_0 \) is the mean of vesicle uptake rate of MTX in the presence of AMP, and \([C]\) is the concentration of MTX (mmol/l). The solid line indicates the best fit to the equation: Vesicle uptake = \( V_{\text{max}} \times [C]/(K_m + [C]) + V_0 \times [C] \), where \( K_m \) is the Michaelis-Menten parameter of MTX, and \( V_{\text{max}} \) is the maximum ATP-dependent vesicular uptake of MTX.

Fig. 2. The effect of various herbal extracts on the ATP-dependent transport of methotrexate (MTX) into BCRP-expressing vesicles
The uptake of MTX in the presence of (A) 1,000 mg/ml herbal extracts or (B) 1, 10, 100 or 1,000 mg/ml soybean extract was measured. BCRP membrane vesicles were incubated with 100 mM MTX at 37°C for 2 min in the presence of ATP (closed bars) or AMP (open bars). Each point represents the mean ± SEM of three experiments. The significance of differences from the respective control (ATP or AMP) was determined by ANOVA followed by Dunnett’s test (\( **p < 0.01; ***p < 0.001 \)).

Soybean extract inhibited BCRP-mediated transport of MTX in a concentration-dependent manner (Fig. 2B) with an IC\(_{50}\) value of 16.5 μg/ml.

Inhibitory effects of isoflavonoids on BCRP-mediated transport of MTX: All 23 isoflavonoids studied, including soybean isoflavones such as daidzein and genistein, inhibited BCRP-mediated transport of MTX in a concentration-dependent manner (Fig. 3). The IC\(_{50}\) values for these isoflavonoids are shown in Table 1.

The influence of the functional moieties of isoflavonoids on the inhibitory potency (IC\(_{50}\) value) for BCRP-mediated transport was quantitatively analyzed by multiple factor analysis (Fig. 4). The model consisting of two items as factors (C5, C7 position, Fig. 4) best described the IC\(_{50}\) values. The presence of a hydroxyl moiety at the C5 position potentiated the inhibitory effects (category scores of moieties: OH 0.275 vs H − 0.206 at the C5 position), while 7-O-glucosidation decreased the IC\(_{50}\) values (category scores of moieties: O-glucoside − 0.653 vs OH 0.363, OCH\(_3\) 0.363 at the C7 position).

The IC\(_{50}\) values of glucosides, including the C4′-O-glucoside sophoricoside, were about 1.0 smaller than those of the corresponding aglycones (Table 1). The decrease of IC\(_{50}\) values due to 7-O-glucosidation was statistically significant (\( p < 0.001 \), paired t-test, Fig. 5), whereas methylation at the C6 position significantly increased the IC\(_{50}\) values by almost 0.3 (glycitein IC\(_{50}\) value 5.99 vs daidzin 5.62, glycitin 4.76 vs daidzin 4.54, 4′,6,7-trimethoxyisoflavone 6.31 vs 4′,7-dimethoxyisoflavone 5.96; \( p < 0.05 \), paired t-test; Table 1).

Discussion

Among the nine herbal extracts examined, soybean extract most strongly inhibited the BCRP-mediated transport of MTX in a concentration-dependent manner (Fig. 2B). This soybean extract contains 30.4% isoflavones as glucosides, and can be consumed in amounts of up to...
Fig. 3. Concentration-dependent inhibition by isoflavonoids of the transport of methotrexate (MTX) into BCRP-expressing vesicles

BCRP membrane vesicles were incubated with 100 μM MTX and the indicated concentrations of isoflavonoids at 37°C for 2 min in the presence of ATP or AMP. ATP-dependent transport was assessed by subtracting the uptake in the presence of AMP from that in the presence of ATP. Each point represents the mean ± SEM of three or four experiments. Each line indicates the best fit to the equation given in Materials and Methods.

160 mg/day (30 mg/day as soy isoflavone aglycones, 48 mg/day as isoflavone glucosides) as a dietary supplement in Japan.40) The upper limit for daily intake of soybean isoflavonoids in Japan is 75 mg/day as aglycones, which is similar to the levels in France and Italy (1.6 mg/kg body weight/day as total isoflavonoids including glucosides,41) 80 mg/day as aglycones42). Assuming administration with a glass of water (250 ml), which volume is derived from typical clinical study protocols,43) the intestinal concentration of soybean extract ([I]2) is estimated to be 632 μg/ml. The US Food and Drug Administration research group proposed a criterion: P-glycoprotein inhibitor drugs that exhibit an [I]2/IC50 > 10 should be evaluated in vivo to determine whether there is clinically relevant P-glycoprotein inhibition with digoxin, a P-glycoprotein substrate with a narrow therapeutic index, for the possibility of drug interaction.43) Given the IC50 value of soybean extract (16.5 μg/ml), the [I]2/IC50 value is 38.3, which exceeds 10, the cutoff value of the criterion. Although it is necessary to consider that the criterion is not for BCRP but for P-glycoprotein, the concomitant administration of soybean extract with a BCRP substrate drug may therefore increase the absorption of the drug. Some of the examined extracts other than soybean may also strongly inhibit intestinal BCRP-mediated transport. However, the intestinal concentrations of these extracts after the recommended amounts have been taken are estimated to be lower than the concentration examined (1 mg/ml). Therefore, additional studies are required to examine the inhibitory profiles of these extracts and to estimate the likelihood of drug-herbal extract interactions in the clinical setting.

Soybean-derived isoflavonoids, such as daidzein, daidzin, genistein, genistin, glycitein, glycitin and ononin,44) all inhibited BCRP-mediated transport (Fig. 3). Based on the assumption that the isoflavone compositions of soybean extract are identical to those of raw soybean,45) we calculated the concentrations of daidzein, genistein, daidzin, genistin and glycitin in 16.5 μg/ml soybean extract solution and compared them with the corresponding IC50 values. The estimated concentrations of genistein and genistin were comparable to the respective IC50 values (0.15 vs 0.24 μM, 2.1 vs 4.3 μM), while those of daidzein, daidzin and glycitin were more than 10-fold smaller than the respective IC50 values (0.08 vs 2.4 μM, 1.6 vs 29 μM, and 0.20 vs 16 μM). Therefore, genistein and genistin are likely to be primarily responsible for the inhibitory effect of soybean extract.

Milk thistle extract contains silybin, a weak inhibitor of BCRP.46) The weak inhibitory effect of the milk thistle extract may be largely accounted for by silybin, because the extract contains 31.7% silybin. Passion flower con-
Table 1. Structure of investigated isoflavonoids and pIC_{50} values for BCRP inhibition

<table>
<thead>
<tr>
<th>Compound</th>
<th>C5</th>
<th>C6</th>
<th>C7</th>
<th>C4’</th>
<th>C3’</th>
<th>pIC_{50} (log [M])</th>
<th>S.D.</th>
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<td>-OH</td>
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<td>-OCH_{3}</td>
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<td>0.06</td>
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*The structures of coumestrol and coumestrin are shown below.*

tains chrysin, which inhibits BCRP,24,31) and other flavonoids.46,47) Although the flavonoid compositions of passion flower extract are unknown, the inhibitory effect of passion flower extract may be due to flavonoids, including chrysin. Inhibition of BCRP by rutin seems reasonable, because it is a glycoside of quercetin, which inhibits BCRP.24) With regard to other extracts, the inhibitory constituents responsible for BCRP inhibition remain to be investigated.

Among the 23 isoflavonoids, including soybean-derived isoflavones, coumestrol showed the most potent inhibition (Fig. 3, Table 1). Its pIC_{50} value was comparable to that of Ko143, a typical potent inhibitor of BCRP (7.20 vs 7.89 log[mol/l]48)). A structure-inhibitory potency relationship analysis of 14 isoflavonoids showed that the predicted pIC_{50} values of isoflavonoids were well correlated to the observed pIC_{50} values (Fig. 4).

Among five pairs of isoflavonoids with and without a hydroxyl moiety at the C5 position, four isoflavonoids with a hydroxyl moiety at the C5 position exhibited more potent inhibition than the corresponding isoflavonoids without the hydroxyl group. With regard to the remaining pair, the pIC_{50} value of 4’,7-dimethoxy-5-hydroxyisoflavone was slightly lower than that of 4’,7-dimethoxyisoflavone (5.70 vs 5.96). As a result of multiple factor analysis, the pIC_{50} values of an isoflavonoid with the hydroxyl moiety was expected to be larger than that of the corresponding isoflavonoid without the hydroxyl moiety at C5 (Fig. 4), so that genisteins have higher affinity for BCRP than do daidzeins. Ahmed-Belkacem et al. examined the influence of the C5 hydroxyl moiety in flavonoids and reported that the inhibitory potency of chrysin (5,7-dihydroxyflavone) for mitoxantrone efflux from BCRP-overexpressing cells was greater than that of 7-hydroxyflavone.31) The influence of a hydroxyl moiety at the C5 position on the inhibition of BCRP may be similar in isoflavonoids and flavonoids.

In all three pairs of isoflavonoids with and without a methoxyl moiety at C6, the inhibitory effects of isoflavonoids with the methoxyl moiety were more potent than...
Fig. 4. Predictability of the pIC$_{50}$ values for BCRP inhibition by multiple factor analysis based on quantification theory type I. The observed pIC$_{50}$ value ± S.D. of each isoflavonoid was plotted against the predicted value, calculated by using the best describing equation shown at the bottom of the figure. In the equation, category scores of dummy variables (X$_{ij}$) are shown as their respective factors. The upper table shows the definition of the dummy variable (X$_{ij}$) for each category (j) and item (i). Each point represents the pIC$_{50}$ value of the appropriately numbered compound (1 daidzein, 2 daidzin, 3 formononetin, 4 ononin, 5 4',7-dimethoxyisoflavone, 6 genistein, 7 genistin, 8 biochanin A, 9 sissotrin, 10 4',7-dimethoxy-5-hydroxyisoflavone, 11 prunetin, 12 glycitein, 13 glycitin, 14 4',6,7-trimethoxyisoflavone).

Predicted pIC$_{50}$ value = 5.694±0.206 X$_{1}$+0.275±0.363 X$_{2}$+0.363 X$_{3}$±0.653 X$_{4}$

(r=0.904)

Fig. 5. Comparison of the pIC$_{50}$ values for BCRP inhibition between isoflavonoid aglycone and the corresponding glucoside. Each set represents the combination of an isoflavonoid aglycone and the corresponding glucoside: open circles, daidzein-daidzin; closed circles, formononetin-ononin; open squares, glycitein-glycitin; closed squares, biochanin A-sissotrin; open triangle, genistein-genistin; closed triangle, coumestrol-coumestrin. The significance of differences in mean pIC$_{50}$ values between aglycones and glucosides was determined by use of the paired t-test (**p<0.001).

Although a previous study has shown that some glucosides of flavonoids seem to inhibit BCRP-mediated transport, the present study is the first to examine quantitatively and systematically the differences in inhibitory potency between glucosides and the corresponding aglycones.

We found that the pIC$_{50}$ values of biochanin A and genistein were comparable (6.62 vs 6.30), whereas Zhang et al. reported that biochanin A was 9 times more potent than genistein in inhibiting BCRP-mediated transport. This discrepancy may be explained by the differences in the experimental systems and substrates used, i.e., Zhang et al. evaluated the increase in the accumulation of mitoxantrone in BCRP-overexpressing cells, while we directly evaluated the inhibition of MTX uptake into inside-out vesicles.

Although daidzein-7-glucuronide, the glucuronic acid conjugate of daidzein, inhibited BCRP-mediated transport, its IC$_{50}$ value was about 100-fold larger than that of daidzein. In an uptake study using membrane vesicles of BCRP-overexpressing cells, the Km value of SN-38 glucuronide was reported to be greater than that of SN-38 (26 vs 4.0 µM). Thus, the results overall indicate that glucuronidation may reduce the affinity of a substrate or inhibitor for BCRP.

The inhibitory potency of daidzein-4'-sulfate was comparable to that of daidzein (pIC$_{50}$ value; 5.41 vs 5.62). Some sulfate conjugates are known to be substrates or inhibitors of BCRP. Estrone-3-sulfate inhibited the BCRP-mediated transport of [3H]estrone-3-sulfate more potent than did estrone (~ 80% vs ~ 30% at 30 µM), and the inhibitory effect of 17β-estradiol-3-sulfate was com-

Table 1

<table>
<thead>
<tr>
<th>category</th>
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<tr>
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<td>C14</td>
<td>X$_{10}$</td>
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X$_{ij}$ = \{ 1 : the case in which item i reacted on categories j 0 : the other case 

Those of the isoflavonoids without it (Table 1), so that glycitins have higher affinity for BCRP than do daidzeins.

7-O-Glucosidation of isoflavonoids reduced the inhibitory potency toward BCRP-mediated transport, and the differences in IC$_{50}$ values between glucosides and the corresponding aglycones were approximately 10- to 100-fold (Table 1). Multiple factor analysis revealed that the pIC$_{50}$ values of 7-O-glucosides can be expected to be approximately 1.0 smaller than those of the corresponding aglycones (Fig. 4). The inhibitory potencies of glucosides tended to be related to those of the corresponding aglycones, although this was not statistically significant (Spearman's rank correlation coefficient = 0.77, p = 0.10). As for compounds other than 7-O-glucosides, the pIC$_{50}$ value of sophoricoside, the 4'-O-glucoside of genistein, was 1.24 smaller than that of genistein.
parable to that of 17β-estradiol.52) These published observations are consistent with the results of the present study.

The mean plasma concentrations of isoflavonoids have been reported to be submicromolar after consumption of soybean products containing abundant isoflavonoids.53) Considering that the plasma free-fractions of isoflavones are relatively low (e.g., < 12% for daidzein54)), calculated unbound concentrations of isoflavonoids were lower than the IC50 values for inhibition of BCRP-mediated transport, whereas epithelial cells of the gastrointestinal tract are exposed to high levels of isoflavonoids. Drug-isoflavonoid interactions on intestinal BCRP were predicted to be induced by taking more than 0.16 mg genistein or 0.042 mg coumestrol ([II]/IC50 > 106). More studies are needed to clarify the possibility of clinical interactions between isoflavonoids and the substrates of BCRP.

In conclusion, some herbal extracts and isoflavonoids strongly inhibited BCRP, and the inhibitory potencies of isoflavonoids were characterized in relation to the functional moieties. Some herbal or dietary supplements containing these extracts or constituents seem likely to increase the systemic availability of BCRP substrates when concomitantly taken orally.

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References


Anxiolytic activity of a phytochemically characterized *Passiflora incarnata* extract is mediated via the GABAergic system. *Planta Med.*, **74**: 1769–1773 (2008).


