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ORIGINAL ARTICLE

Inhibition of P-glycoprotein and multidrug resistance protein 1 by dietary phytochemicals

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Abstract
Purpose For the development of a safe and effective dual inhibitor of anticancer drug efflux transporters P-glycoprotein and multidrug resistance protein 1 (MRP1) to conquer multidrug resistance, we investigated the effects of dietary phytochemicals on the functions of P-glycoprotein and MRP1.
Methods The effects of dietary phytochemicals on the functions of P-glycoprotein and MRP1 were investigated using P-glycoprotein-overexpressing human carcinoma KB-C2 cells and human MRP1 gene-transfected KB/MRP cells. The effects of natural compounds found in dietary supplements, herbs, and foods such as sesame, ginkgo, soybean, and licorice were evaluated.
Results The accumulation of daunorubicin, a fluorescent substrate of P-glycoprotein, increased in the presence of sesamin, ginkgolic acid, matairesinol, glycyrrhetinic acid, glabridin, and phyllodulcin in KB-C2 cells. Glycyrrhetinic acid and matairesinol also increased the accumulation of calcein, a fluorescent substrate of MRP1, in KB/MRP cells. KB-C2 and KB/MRP cells were sensitized to anticancer drugs by glycyrrhetinic acid, showing that glycyrrhetinic acid reverses multidrug resistance. The verapamil-stimulated P-glycoprotein ATPase activity was inhibited by glycyrrhetinic acid. Glycyrrhetinic acid stimulated the ATPase activity of MRP1.
Conclusion These results suggest that dietary phytochemicals, such as glycyrrhetinic acid found in licorice, have dual inhibitory effects on P-glycoprotein and MRP1 and might become useful to enhance the efficacy of cancer chemotherapy.

Keywords P-glycoprotein · MDR1 · MRP1 · ABC transporter · Multidrug resistance · Dietary phytochemical

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Introduction

A major problem in the treatment of cancer is the occurrence of cellular resistance to cytotoxic drugs. Multidrug resistance is a phenomenon whereby tumors become resistant to chemically unrelated anticancer drugs. Although a number of mechanisms mediate multidrug resistance, the first mediator to be characterized at the molecular level was P-glycoprotein (P-gp, ABCB1) encoded by *MDR1*, also referred to as *ABCB1* [2, 7, 17]. P-gp mediates resistance to various classes of chemotherapeutic agents including vinblastine, vincristine, daunorubicin, colchicine, and paclitaxel, by actively extruding the drugs from the cells to lower the intracellular concentrations.

The molecular structure of P-gp consists of 12 transmembrane domains that form a drug-binding pore and two ATP-binding sites belonging to the ATP-binding cassette (ABC) transporter family. Multidrug resistance protein 1 (MRP1, ABCC1), encoded by the *ABCC1* gene, is a second member of the ABC transporter family. MRP1 consists of 17 transmembrane domains and two ATP-binding sites. P-gp and MRP1 differ in substrate specificity. Although the mechanisms by which substrates are recognized by P-gp and MRP1 have not been fully clarified, P-gp seems to

prefer amphipathic cationic compounds, and MRP1, anionic compounds [2, 7, 17]. Both P-gp and MRP1 act as anticancer drug efflux transporters and cause multidrug resistance. Therefore, P-gp and MRP1 are promising targets for the reversal of multidrug resistance and a better outcome of cancer chemotherapy.

The quest for inhibitors of anticancer drug efflux transporters has uncovered natural constituents including flavonoids such as quercetin and (−)-epigallocatechin gallate as promising candidates [8–10]. We have previously reported the inhibitory effects of dietary chemopreventive phytochemicals on the functions of P-gp using human multidrug-resistant carcinoma KB-C2 cells overexpressing P-gp [13]. In this study, for the development of a safe and effective dual inhibitor of P-gp and MRP1 to conquer multidrug resistance, we investigated the effects of dietary phytochemicals on the functions of P-gp and MRP1.

Materials and methods

Dulbecco's modified Eagle's medium (D-MEM) and fetal bovine serum (FBS) were purchased from Invitrogen Corp. (Carlsbad, CA). Colchicine, doxorubicin, daunorubicin, vinblastine, indomethacin, glycyrrhizin, glycyrrhetinic acid (18β-glycyrhetinic acid, enoxolone), glabridin, phyllodulcin, stevioside, and piperine were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Calcein-AM was provided by Dojindo Laboratories (Kumamoto, Japan). Genistein, daidzein, glycitein, isoliquiritigenin, *N*-ethylmaleimide (NEM), and glutathione (GSH) were purchased from Sigma Chemical (St Louis, MO). Sesamin, matairesinol, and MK-571 were obtained from Cayman Chemical (Ann Arbor, MI). Ginkgolide A, B, and C, (−)-bilobalide, and ginkgolic acid I (15:1) and II (17:1) were acquired from Nagara Science (Gifu, Japan). Liquiritigenin, steviol and isosteviol were purchased from Chroma-Dex Inc. (Santa Ana, CA). Figure 1 shows the chemical structures and sources of several dietary phytochemicals. Human P-gp membranes and luminescent ATP detection kit (Pgp-Glo Assay Kit) were purchased from Promega (Madison, WI), and human MRP1 membranes were from BD Gentest (Woburn, MA). *N*-Ethylmaleimide–glutathione (NEM–GS) was synthesized by incubating equal amount of NEM and GSH solution. All other chemicals used were of the highest purity available. KB-C2 cells were kindly provided by Prof. Shin-ichi Akiyama of Kagoshima University (Kagoshima, Japan), and human MRP1 gene-transfected KB/MRP cells were generated and kindly provided by Prof. Kazumitsu Ueda of Kyoto University (Kyoto, Japan) [14, 18].

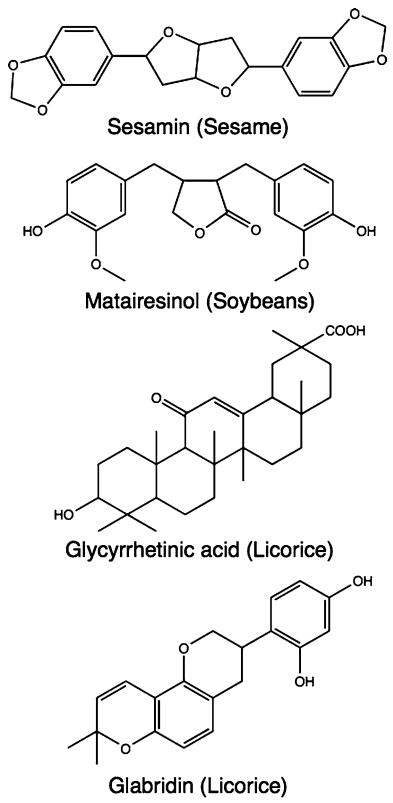

Fig. 1 Chemical structures and sources of dietary phytochemicals

Cell culture

KB-C2 cells were cultured in D-MEM supplemented with 10% FBS and 2 μg/ml of colchicine. KB/MRP cells were established by transfecting KB-3-1 cells with the plasmid pJ3Ω-MRP containing the human MRP1 gene and selection in the presence of increasingly higher concentrations of doxorubicin [18]. KB/MRP cells were cultured in D-MEM supplemented with 10% FBS and 5 ng/ml of doxorubicin. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 and 95% air.

Measurement of the cellular accumulation of daunorubicin in KB-C2 cells

The accumulation of daunorubicin, a fluorescence substrate of P-gp, was measured and the effects of dietary phytochemicals were determined as described previously [13]. Briefly, KB-C2 cells, plated at 1×10^5 cells/well in 24-well plates, were incubated with 50 μM daunorubicin in the absence or presence of phytochemicals for 2 h in a CO_2 incubator at 37°C. After the incubation, the medium was removed by aspiration and the cells were washed with ice-cold phosphate-buffered saline (PBS), and lysed with 1% sodium dodecyl sulfate (SDS) in PBS. Fluorescence inten-

sity was measured with a microplate fluorometer (Fluoroskan Ascent, Thermo Fisher Scientific, Waltham, MA). The excitation and emission wavelengths were 485 and 590 nm, respectively. Protein concentrations were measured by the Lowry method using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard. Accumulation ratios were calculated using the accumulation of daunorubicin in cells incubated without phytochemicals as a control.

Measurement of the cellular accumulation of calcein in KB/MRP cells

The accumulation of calcein, a fluorescent substrate of MRP1, in KB/MRP cells was measured as in KB-C2 cells [13, 20]. Cells were incubated with 1 μM calcein-AM, an acetoxymethyl ester form of calcein, in the absence or presence of 50 μM dietary phytochemicals for 2 h at 37°C. After the incubation, the cells were washed with ice-cold PBS, and lysed with 1% Triton-X100 in PBS. The fluorescence intensity of the calcein converted from calcein-AM in cells was measured. The excitation and emission wavelengths were 485 and 538 nm, respectively.

Determination of resistance to vinblastine or doxorubicin

The resistance of KB-C2 cells to vinblastine and of KB/MRP cells to doxorubicin were determined by a calorimetric assay using a new water-soluble tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5(2,4-disulfophenyl)-2*H* tetrazolium monosodium salt (WST-8), (Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan), performed in 96-well plates as described previously [13, 19]. First, 5×10^3 cells in 180 μl of culture medium were inoculated into each well. After 24 h in a CO_2 incubator at 37°C, various concentrations of vinblastine or doxorubicin (20 μl) in the absence or presence of phytochemicals were added and the plates were incubated for 3 days (KB-C2 cells) or 2 days (KB/MRP cells). Thereafter, the medium was removed by aspiration, and 90 μl of fresh culture medium and 10 μl of Cell Counting Kit-8 solution (5 mM WST-8) were added to each well. The plates were incubated for a further 4 h, and the absorbance at 450 nm was measured using a microplate reader (model 550, Bio-Rad Laboratories, Hercules, CA).

Determination of ATPase activity

The ATPase activities of P-gp or MRP1 were determined using P-gp or MRP1 membranes from human P-gp or MRP1 gene containing baculovirus infected insect High Five cells (BD Gentest, Woburn, MA) and luminescent ATP detection kit (Pgp-Glo Assay Kit, Promega, Madison, WI) according to the manufacturers' recommendation [11, 15]. Briefly, 0.5 mg/mL P-gp or MRP1 membranes and 5 mM MgATP were incubated in the absence or presence of 100 μM (for P-gp) or 400 μM (for MRP1) sodium orthovanadate at 37°C for 40 min, and the remaining ATP were detected as a luciferase-generated luminescent signal. Basal P-gp or MRP1 ATPase activities were determined as the difference between the ATP hydrolysis in the presence or absence of vanadate. Verapamil-stimulated P-gp ATPase activity was measured in the presence of 200 μM verapamil. NEM-GS-stimulated MRP1 ATPase activity was measured in the presence of 5 mM NEM-GS and 3 mM GSH.

Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SE). Statistical differences were determined with a one-way analysis of variance (ANOVA) followed by Dunnett's test. P values less than 0.01 were considered significant.

Results

Effects of phytochemicals on daunorubicin accumulation in KB-C2 cells

Figure 2a shows the accumulation of daunorubicin in KB-C2 cells in the presence of 50 μM dietary phytochemicals. Sesamin, ginkgolic acid I and II, matairesinol, glycyrrhetinic acid, glabridin, and phyllodulcin increased the cellular accumulation of daunorubicin, which indicates that these phytochemicals inhibit the P-gp-mediated efflux of daunorubicin. In contrast, ginkgolide A, B, and C, bilobalide, genistein, daidzein, glycitein, glycyrrhizin, liquiritigenin, isoliquiritigenin, stevioside, steviol, and isosteviol had no effect on the cellular accumulation of daunorubicin. Figure 2b shows the accumulation ratio of daunorubicin in KB-C2 cells in the presence of various concentrations of phytochemicals. Sesamin, ginkgolic acid I and II, matairesinol, glycyrrhetinic acid, glabridin, and phyllodulcin increased the cellular accumulation of daunorubicin in a concentration-dependent manner.

Effects of phytochemicals on calcein accumulation in KB/MRP cells

Figure 3a shows the accumulation of calcein in KB/MRP cells in the presence of 50 μM dietary phytochemicals. Matairesinol and glycyrrhetinic acid increased the cellular accumulation of calcein, which indicates that these compounds inhibit the MRP1-mediated efflux of calcein. In contrast, piperine, sesamin, glabridin and phyllodulcin,

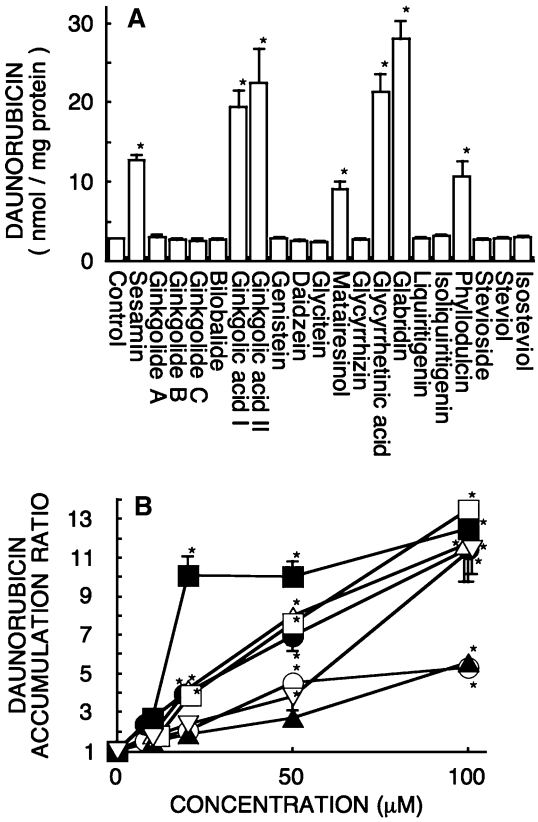

Fig. 2 Effects of 50 μM phytochemicals (**a**) or various concentrations of phytochemicals (**b**) on daunorubicin accumulation in KB-C2 cells. Sesamin (*open circle*), ginkgolic acid I (*filled circle*), ginkgolic acid II (*open triangle*), matairesinol (*filled triangle*), glycyrrhetinic acid (*open square*), glabridin (*filled square*), phyllodulcin (*open nabla*). Mean ± SE of six experiments. * $P < 0.01$, significantly different from control

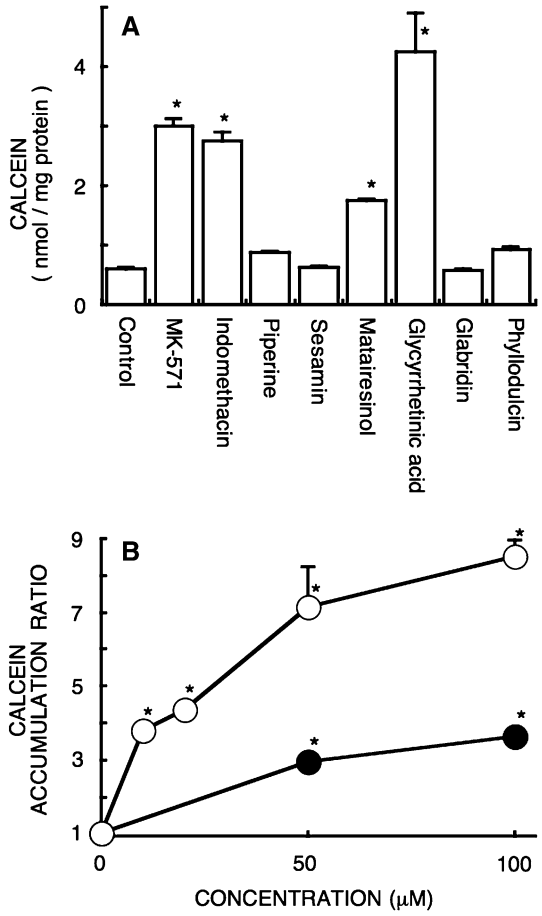

Fig. 3 Effects of 50 μM phytochemicals (**a**) or various concentrations of phytochemicals (**b**) on calcein accumulation in KB/MRP cells. Glycyrrhetinic acid (*open circle*), matairesinol (*filled circle*). Mean ± SE of six experiments. * $P < 0.01$, significantly different from control

phytochemicals that inhibit P-gp function [3], had no effects on the cellular accumulation of calcein in KB/MRP cells. Figure 3b shows the accumulation ratio of calcein in KB/MRP cells in the presence of various concentrations of phytochemicals. Matairesinol and glycyrrhetinic acid increased the cellular accumulation of calcein in a concentration-dependent manner.

Effects of phytochemicals on the cytotoxicity of vinblastine or doxorubicin

Among the dietary phytochemicals tested, glycyrrhetinic acid had the strongest inhibitory effect on the functions of P-gp and MRP1. To confirm these effects and to investigate the potential of using phytochemicals as chemosensitizing agents, glycyrrhetinic acid was tested for effects on the cytotoxicity of vinblastine or doxorubicin. Figure 4 shows cell growth inhibition curves of vinblastine in the absence or presence of glycyrrhetinic acid in KB-C2 cells. Figure 5 shows cell growth inhibition curves of doxorubicin in the absence or presence of glycyrrhetinic acid in KB/MRP cells. In the presence of glycyrrhetinic acid, KB-C2 and KB/MRP cells were more susceptible to the cytotoxicity of vinblastine, a P-gp substrate, or doxorubicin, an MRP1 substrate, as compared with vinblastine or doxorubicin alone, indicating the inhibition of P-gp or MRP1-mediated efflux from cells and an increase in intracellular concentrations of anticancer agents in response to glycyrrhetinic acid.

Effects of phytochemicals on ATPase activities of P-gp and MRP1

Figure 6 shows the basal and verapamil-stimulated P-gp ATPase activity in the absence or presence of glycyrrhetinic acid. Verapamil, a known substrate of P-gp, stimulated ATPase activity of P-gp. Glycyrrhetinic acid alone had no effect on the basal P-gp ATPase activity. However, glycyrrhetinic acid markedly inhibited the ATP hydrolysis by verapamil-stimulated P-gp ATPase. Figure 7 shows the basal and NEM-GS-stimulated MRP1 ATPase activity in the absence or presence of glycyrrhetinic acid. NEM-GS,

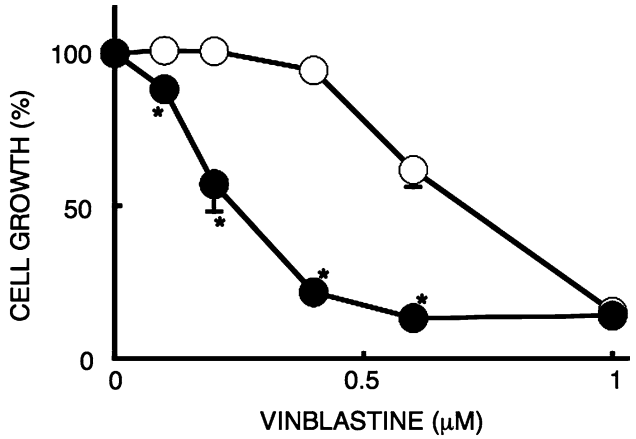

Fig. 4 Effects of glycyrrhetinic acid on the cytotoxicity of vinblastine in KB-C2 cells. Control (*open circle*), 100 μM glycyrrhetinic acid (*filled circle*). Mean ± SE of six experiments. * $P < 0.01$, significantly different from control

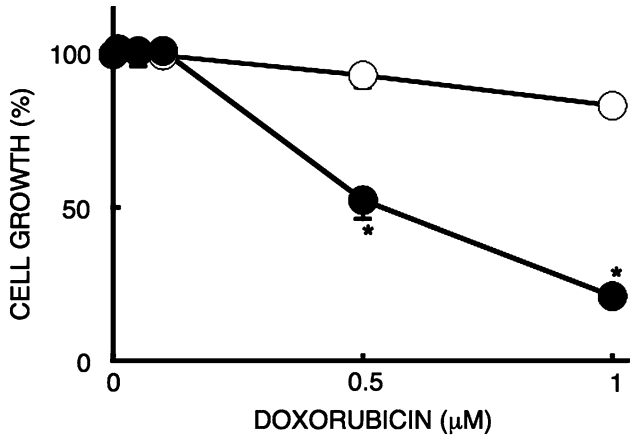

Fig. 5 Effects of glycyrrhetinic acid on the cytotoxicity of doxorubicin in KB/MRP cells. Control (*open circle*), 100 μM glycyrrhetinic acid (*filled circle*). Mean ± SE of six experiments. * $P < 0.01$, significantly different from control

an MRP1 substrate, stimulated ATPase activity of MRP1. In contrast to the results of P-gp, glycyrrhetinic acid alone stimulated the basal MRP1 ATPase activity. Glycyrrhetinic acid further enhanced the NEM-GS-stimulated MRP1 ATPase activity.

Discussion

Cancer chemotherapy is usually a marginal proposition in the sense that the maximum dose tolerated by the patient is often barely sufficient to kill a useful percentage of the cancer cells. Relatively small increases in drug resistance in cancer cells are thus sufficient to render the drug ineffective. ABC (ATP-binding cassette) transporters are expressed at cancer cell membranes and can cause multidrug resistance. Therefore, ABC transporters seem to be good targets for

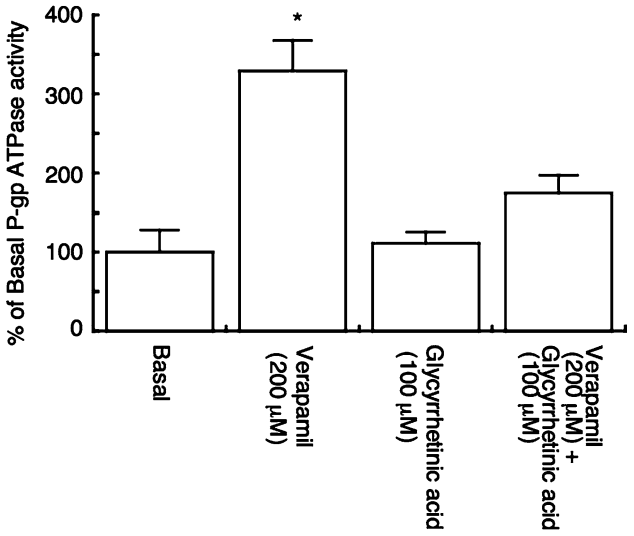

Fig. 6 Effects of glycyrrhetinic acid on P-glycoprotein ATPase activity. Mean + SE of six determinations from three experiments. * $P < 0.01$, significantly different from basal P-gp ATPase activity

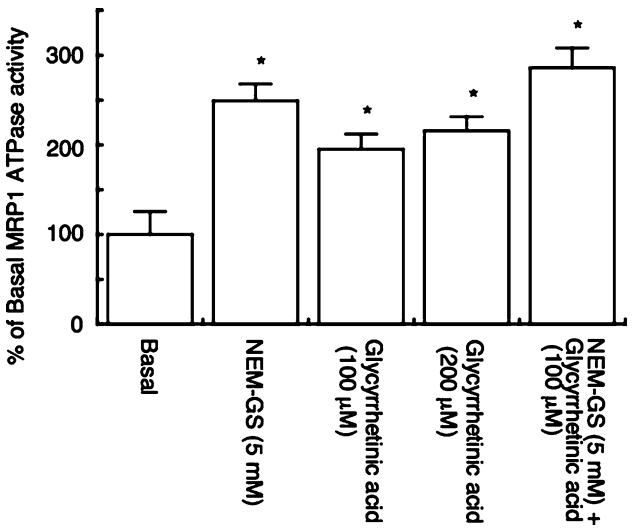

Fig. 7 Effects of glycyrrhetinic acid on MRP1 ATPase activity. Mean + SE of six determinations from three experiments. * $P < 0.01$, significantly different from basal MRP1 ATPase activity

circumventing multidrug resistance. Since the discovery of P-gp, the first of the ABC transporters, in the 1970s, various attempts and clinical trials have been carried out using a P-gp inhibitor such as verapamil or cyclosporine to overcome multidrug resistance. However, due to the side effects or ineffectiveness of these compounds, a successful outcome has not been achieved [17]. In general, natural dietary phytochemicals from foods, herbs, and dietary supplements are thought to be less toxic to the body than medical drugs. Allen et al. [1] reported that P-gp and MRP1 were major determinants of innate drug sensitivity, even when the level of expression was low in drug-naïve tumors. They suggested

that inhibitors of P-gp and MRP1 are useful not only to reverse or prevent acquired drug resistance, but also to sensitize drug-naïve untreated tumors to anticancer drugs. Therefore, dietary phytochemicals that can inhibit P-gp and MRP1 could be promising candidates for chemosensitizing agents. In this study, to develop a safe and effective multidrug reversing agent for better cancer chemotherapy, we screened various dietary phytochemicals for inhibitory effects on both P-gp and MRP1.

The ingredients of sesame, ginkgo, and soybean are now widely used in commercially available dietary supplements. Sesamin, a lignan existing exclusively and abundantly in sesame (*Sesamum indicum*) seeds, is thought to have antioxidative activity and hypocholesteromic effects [5]. Sesamin is a common ingredient of dietary supplements, especially in Japan. Ginkgo (*Ginkgo biloba*) leaf extracts containing ginkgolide A, B, and C, and bilobalide are reported to provide protection against neural and vascular damage [12]. Ginkgo leaf extracts are available as medical drugs in Europe, and as herbal preparations in other countries. Soybean (*Glycine max*) products containing isoflavones such as genistein, daidzein and glycitein, and lignan matairesinol are also well consumed as dietary supplements or foods such as tofu and soy sauce [4]. Licorice (*Glycyrrhiza glabra*) extract containing glycyrrhizin, glycyrrhetinic acid, glabridin, liquiritigenin, and isoliquiritigenin is one of the major components of traditional Chinese medicine [16]. Licorice extracts are also widely used as sweetening agents for foods such as soy sauce and licorice candy. Phyllodulcin from Amacha (*Hydrangea macrophylla* var. *thunbergii*), and stevioside, steviol, and isosteviol from Stevia (*Stevia rebaudiana*) are used as food sweeteners.

KB-C2 is a multidrug-resistant human epidermal carcinoma cell line that over-expresses P-gp, a drug efflux transporter. In the present study, we examined the effects of 20 phytochemicals on P-gp-mediated transport. Sesamin, ginkgolic acid I and II, matairesinol, glycyrrhetinic acid, glabridin, and phyllodulcin increased the accumulation of daunorubicin in KB-C2 cells (Fig. 2a). Since these phytochemicals inhibit the efflux of P-gp substrates, the increase in the amount of substrate accumulated seems to be due to inhibition of the efflux transporter. To investigate the concentration-dependent effects of dietary phytochemicals on P-gp-mediated transport, the effects of seven phytochemicals with strong P-gp-inhibitory activities when tested at 50 μM were evaluated. Sesamin, ginkgolic acid I and II, matairesinol, glycyrrhetinic acid, glabridin, and phyllodulcin demonstrated concentration-dependent inhibition of P-gp (Fig. 2b). Although ginkgolic acids have inhibitory effects on P-gp, it is reported that these compounds have allergenic and toxic effects and governmental regulations have determined that levels of ginkgolic acids must be very low in commercial products [6, 12]. Therefore, we ruled out ginkgolic acid from next evaluation of MRP1's inhibition.

MRP1, the second member of the ABC transporter family, also mediates multidrug resistance in cancer cells. We then tested dietary phytochemicals that inhibit P-gp for effects on MRP1 using KB/MRP cells. Known MRPs inhibitors, MK-571 and indomethacin, increased the accumulation of calcein in KB/MRP cells (Fig. 3a). Dietary phytochemicals, glycyrrhetinic acid and matairesinol, elevated substrate levels through the inhibition of MRP1 (Fig. 3b).

Glycyrrhetinic acid increased the sensitivity to vinblastine of KB-C2 cells (Fig. 4) and to doxorubicin of KB/MRP cells (Fig. 5). This demonstrates that glycyrrhetinic acid can reverse multidrug resistance in cells that express P-gp or MRP1. Overall, these results suggest that glycyrrhetinic acid has a chemosensitizing effect, reversing P-gp and MRP1-mediated multidrug resistance by increasing the intracellular accumulation of anticancer drugs.

It is reported that ATP hydrolysis and substrate transport are tightly coupled, and most compounds that are known to be transported by P-gp stimulate ATPase activity [2]. To explore the inhibitory mechanism of glycyrrhetinic acid on P-gp and MRP1 functions, effects on ATPase activities of P-gp and MRP1 were measured using human P-gp or MRP1 membranes from baculovirus infected insect cells. Glycyrrhetinic acid alone had no effect on the basal P-gp ATPase activity, but inhibited the verapamil-stimulated P-gp ATPase activity (Fig. 6). These results suggest that glycyrrhetinic acid is not a substrate of P-gp, but possibly interacts noncompetitively at ATP hydrolytic site of P-gp. In contrast to the result of P-gp ATPase activity, glycyrrhetinic acid alone stimulated the MRP1 ATPase activity (Fig. 7). In the presence of NEM-GS, the ATP hydrolysis by MRP1 was further stimulated by glycyrrhetinic acid. These results suggest that glycyrrhetinic acid could be a substrate of MRP1, and competitively interact at drug-binding site of MRP1.

In conclusion, we used multidrug resistant KB-C2 and KB/MRP cells to investigate the effects of dietary phytochemicals on the functions of P-gp and MRP1. Natural compounds found in foods and dietary supplements, such as sesamin, matairesinol, glycyrrhetinic acid, and glabridin, have inhibitory effects on the function of P-gp in KB-C2 cells. Glycyrrhetinic acid also showed inhibitory effects on the function of MRP1 in KB/MRP cells. Cytotoxic effects of anticancer agents were sensitized by glycyrhetininc acid in KB-C2 and KB/MRP cells. Glycyrrhetinic acid found in licorice can be considered a promising lead compound for the design of more efficacious and less toxic chemosensitizing agents to enhance the efficacy of cancer chemotherapy.

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