Inhibitory effects of terpenoids on multidrug resistance-associated protein
2- and breast cancer resistance protein-mediated transport

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Running title

Inhibition of MRP2- and BCRP-mediated transport by terpenoids

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Abbreviations

P-gp, P-glycoprotein; MRP2, multidrug resistance-associated protein 2; BCRP, breast cancer resistance protein; E217βG, estradiol 17-β-D-glucuronide; MRP2(–) vesicles, membrane vesicles from vector-transfected Sf9 cells; MRP2(+) vesicles, membrane vesicles from MRP2-overexpressing Sf9 cells; BCRP(–) vesicles, membrane vesicles from vector-transfected LLC-PK1 cells; BCRP(+) vesicles, membrane vesicles from BCRP-overexpressing LLC-PK1 cells.
Abstract

The possibility of interactions between natural products/supplements and conventional prescription medicines is one of the most important issues in pharmacotherapeutic safety. Recently, we reported that some terpenoids such as (R)-(+)-citronellal and glycyrrhetic acid, which are present in herbal medicines, can act as inhibitors of P-glycoprotein (MDR1/ABCB1). In the present study, the effects of seven terpenoids on multidrug resistance-associated protein 2 (MRP2/ABCC2) and breast cancer resistance protein (BCRP/ABCG2)-mediated transport were investigated in vitro. Membrane vesicles were prepared from MRP2 cDNA transfected Sf9 cells derived from pupal ovarian tissue of Spodoptera frugiperda, a fall armyworm, and BCRP cDNA transfected LLC-PK1 cells derived from porcine kidney. MRP2 or BCRP-mediated efflux transport was measured as ATP-dependent accumulation of [3H]estradiol 17-β-D-glucuronide (E217βG) into membrane vesicles collected by a rapid filtration technique. The effects of (R)-(+)-citronellal, (S)-(–)-β-citronellol, α-terpinene, terpinolene, (–)-β-pinene, abietic acid and glycyrrhetic acid on the intravesicular accumulation of [3H]E217βG were examined. Large decreases in the [3H]E217βG accumulation into vesicles from MRP2-expressing Sf9 cells were observed in the presence of glycyrrhetic acid and abietic acid, and their IC50 values were about 20 µM and 51 µM, respectively. [3H]E217βG accumulation into vesicles from BCRP-overexpressing LLC-PK1 cells was suppressed by only glycyrrhetic acid, with an IC50 value of about 39 µM. Other terpenoids used in this study did not alter the ATP-dependent accumulation of [3H]E217βG. These findings suggest that glycyrrhetic acid and abietic acid can potently inhibit MRP2- or BCRP-mediated membrane transport and may interact with their substrates in pharmacokinetic processes.
Introduction

The ATP-binding cassette (ABC) transporter superfamily plays important roles in drug absorption and disposition. ABC transporters were originally implicated in multidrug resistance in tumor cells (Sarkadi et al., 2006). Further research has demonstrated that these transporters are distributed throughout many normal tissues of the body. For instance, P-glycoprotein (P-gp/ABCB1), multidrug resistance-associated protein 2 (MRP2/ABCC2), and breast cancer resistance protein (BCRP/ABCG2) exist on the apical membrane of intestinal epithelia and function as a defense system against xenobiotics (Benet et al., 1996; Borst et al., 2000; Maliepaard et al., 2001; Leslie et al., 2005). P-gp, MRP2, and BCRP have been reported to have well-defined roles in the transport of clinically relevant drugs and to mediate cellular resistance to these drugs (Leslie et al., 2005; Litman et al., 2001). These ABC transporters can transport diverse substrates to the outside of cellular membranes using the hydrolytic energy of ATP as a driving force (Litman et al., 2001).

Recently, the possibility of drug interactions has been increasing due to multiple and complex medications, and significant revisions of safety profiles in the product information have been frequently undertaken in clinical practice (Yoshida et al., 2006 c). Moreover, herbal medicine and diet supplements made from natural products are widely used in patients treated with conventional prescription medicines. Recent advancements in biopharmaceutical research have revealed physiological and pharmacological aspects of transporters, however, drug interactions with other drugs, endogenous substrates, and food ingredients have not been fully clarified.

Previously, we investigated possible interactions between Japanese traditional herbal medicines and conventional medicines (Kawakami et al., 2002), and reported that an extract of Zanthoxyli Fructus and some terpenoids can inhibit P-gp-mediated efflux transport in vitro and in vivo (Yoshida et al., 2005; Yoshida et al., 2006 b). In the present study, the inhibitory effects of seven terpenoids (Fig. 1) that which can be P-gp inhibitors (Yoshida et al., 2006 b) on MRP2- and BCRP-mediated transport were investigated using membrane vesicles.
Methods

Materials

[^3]H estradiol 17-β-D-glucuronide (E217βG, 55 Ci/mmol) was purchased from Perkin Elmer Life Science Products (Boston, MA). ATP, AMP, creatine phosphate and creatine phosphokinase were obtained from Sigma-Aldrich Co. (St. Louis, MO). SF9 cells were maintained as a suspension culture at 37°C with serum-free Excel 420 (Nichirei Corp., Tokyo, Japan) supplemented with an antibiotic-antimycotic mixture (Life Technologies, Tokyo, Japan). (R)-(+) -citronellal, (S)-(−)-β-citronellol and α-terpinene were purchased from Sigma-Aldrich Co. (St. Louis, MO). Terpinolene, (−)-β-pinene and abietic acid were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Glycyrrhetic acid was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other reagents are commercially available and analytical grade.

Transport studies with membrane vesicles

Membrane vesicles were prepared from MRP2-overexpressing SF9 cells and SF9 cells infected with a baculovirus encoding the green fluorescent protein according to the method described previously (Ito et al., 2001) and from BCRP-overexpressing and vector-transfected LLC-PK1 cells (Takada et al., 2005). The membrane vesicles were frozen in liquid nitrogen and then transferred to a freezer (−80°C) until use. Protein concentration was determined by the Lowry method.

First, the effects of (R)-(+) -citronellal, (S)-(−)-β-citronellol, α-terpinene, terpinolene, (−)-β-pinene, abietic acid, and glycyrrhetic acid on the intravesicular transport of [^3]H E217βG for 1 min were screened. The terpenoid concentration for this screening was set at 100 µM. After screening, time profiles (up to 2 min) and the concentration dependency of abietic acid and glycyrrhetic acid on the inhibition of MRP2- or BCRP-mediated transport were determined, and their IC50 values were evaluated.

Accumulation of [^3]H E217βG (100 nM) into vesicles were examines using a rapid filtration technique (Suzuki et al, 2003). Radioactivity retained on the filter (HAWP; Millipore Corp., Bedford, MA) was determined using a liquid scintillation counter (TRI-CARB 2100TR, Packard Inst. Co., Meriden, CT).

Statistical analysis

All data are presented the mean with SEM of three experiments. Significant differences between the control and inhibitor data were determined by Bonferroni’s multiple t-test. The IC50 and its 95% confidence interval were calculated by probit analysis. P values of <0.05 were considered to be statistically significant.
Results

Effects of terpenoids on MRP2-mediated transport of [3H]E217βG

[3H]E217βG accumulation into membrane vesicles from vector-transfected (MRP2(–) vesicles) and MRP2-overexpressing Sf9 cells (MRP2(+) vesicles) is shown in Fig. 2. In MRP2(–) vesicles, there was no significant difference between [3H]E217βG accumulation in the presence of ATP and that in the absence of ATP. Then, intravesicular accumulation of [3H]E217βG was compared between MRP2(–) and MRP2(+) vesicles to confirm MRP2 functioning. In the absence of ATP (open column), there was no significant difference between [3H]E217βG accumulation into MRP2(–) and MRP2(+) vesicles. In the control group of MRP2(–) vesicles, there was no significant difference between [3H]E217βG accumulation in the presence of ATP (closed column) and that in the absence of ATP. In the control group of MRP2(+) vesicles, [3H]E217βG accumulation in the presence of ATP was markedly higher than that in the absence of ATP, suggesting ATP-dependent [3H]E217βG transport by means of MRP2.

This ATP-dependent accumulation of [3H]E217βG was also examined in the presence of each of the seven terpenoids and compared with that in the control group (Fig. 2). The [3H]E217βG accumulation decreased significantly in the presence of 100 µM abietic acid or glycyrrhetic acid, but not in the presence of (R)-(+)citronellal, (S)-(−)-β-citronellol, α-terpinene, terpinolene, or (−)-β-pinene.

The time profiles of [3H]E217βG accumulation into MRP2(+) vesicles were investigated in the presence or absence of ATP and/or abietic acid (Fig. 3A) and in the presence or absence of ATP and/or glycyrrhetic acid (Fig. 3B). [3H]E217βG accumulation in the presence of ATP in the control group increased up to 2 min, and this accumulation was significantly inhibited by abietic acid and glycyrrhetic acid. Therefore, the concentration dependency of abietic acid and glycyrrhetic acid on the inhibition of ATP-dependent accumulation of [3H]E217βG was examined at 1 min. Abietic acid and glycyrrhetic acid inhibited the [3H]E217βG accumulation in a concentration-dependent manner (Fig. 3C and 3D). The apparent IC50 values of abietic acid and glycyrrhetic acid on MRP2-mediated transport were 51.4 µM (95% confidence interval: 45.3-58.7 µM) and 20.1 µM (95% confidence interval: 17.9-24.1 µM), respectively.

Effects of terpenoids on BCRP-mediated transport of [3H]E217βG

[3H]E217βG accumulation into membrane vesicles from vector-transfected (BCRP(–) vesicles) and BCRP-overexpressing LLC-PK1 cells (BCRP(+) vesicles) is shown in Fig. 4. In BCRP(–) vesicles,
there was no significant difference between \([^{3}\text{H}]\text{E217\(^{\beta}\)G}\) accumulation in the presence of ATP and that in the absence of ATP. Then, intravesicular accumulation of \([^{3}\text{H}]\text{E217\(^{\beta}\)G}\) was compared between BCRP(−) and BCRP(+) vesicles to confirm BCRP functioning. In the absence of ATP (open column), there was no significant difference between \([^{3}\text{H}]\text{E217\(^{\beta}\)G}\) accumulation into BCRP(−) and BCRP(+) vesicles. In the control group of BCRP(−) vesicles, there was no significant difference between \([^{3}\text{H}]\text{E217\(^{\beta}\)G}\) accumulation in the presence of ATP (closed column) and that in the absence of ATP. In the control group of BCRP(+) vesicles, intravesicular accumulation of \([^{3}\text{H}]\text{E217\(^{\beta}\)G}\) in the presence of ATP was markedly higher than that in the absence of ATP, suggesting the ATP-dependency of \([^{3}\text{H}]\text{E217\(^{\beta}\)G}\) transport via BCRP.

This ATP-dependent accumulation of \([^{3}\text{H}]\text{E217\(^{\beta}\)G}\) was also examined in the presence of each of seven terpenoids and compared with that in the control group (Fig. 4). \([^{3}\text{H}]\text{E217\(^{\beta}\)G}\) accumulation decreased significantly in the presence of 100 \(\mu\text{M}\) glycyrrhetic acid, but not in the presence of any other terpenoids screened, i.e. (R)-(+-)citronellal, (S)-(+-)\(\beta\)-citronellol, \(\alpha\)-terpinene, terpinolene, (+-)\(\beta\)-pinene, and abietic acid.

The time profiles of \([^{3}\text{H}]\text{E217\(^{\beta}\)G}\) accumulation into BCRP(+) vesicles were investigated in the presence or absence of ATP and/or glycyrrhetic acid (Fig. 5A). \([^{3}\text{H}]\text{E217\(^{\beta}\)G}\) accumulation in the presence of ATP in the control group increased up to 2 min, and this accumulation was inhibited by glycyrrhetic acid to the level of that in the absence of ATP. Therefore, the concentration dependency of glycyrrhetic acid on the inhibition of ATP-dependent accumulation of \([^{3}\text{H}]\text{E217\(^{\beta}\)G}\) was examined at 1 min. Glycyrrhetic acid inhibited \([^{3}\text{H}]\text{E217\(^{\beta}\)G}\) accumulation in a concentration-dependent manner (Fig. 5B), and the apparent IC\(_{50}\) value of glycyrrhetic acid was 39.1 \(\mu\text{M}\) (95% confidence interval: 36.0-42.9 \(\mu\text{M}\)).
Discussion

In this study, the effects of seven terpenoids on MRP2- and BCRP-mediated transport were examined by intravesicular accumulation studies. In our preliminary experiments for the present report, MRP2- or BCRP-mediated transport was attempted to be evaluated by the whole cell based assay such as the intracellular accumulation and transcellular transport studies as shown in our previous reports (Yoshida et al., 2005; Yoshida et al., 2006a; 2006b). However, an active efflux via these transporters was not demonstrated probably because of the high hydrophilicity of the specific substrate used ([3H]E217βG). Therefore, the intravesicular accumulation technique was adopted in this report. In the intravesicular accumulation study, a possibility for the expression of MRP2 in LLC-PK1 cells may be concerned. BCRP-mediated transport was evaluated by the comparison between the vector-transfected (control) and BCRP-overexpressing LLC-PK1 (Takada et al., 2005) to exclude a contribution of endogenous MRP2. Large amount of [3H]E217βG accumulation via MRP2 was observed (about 60 µL/mg protein) in control-MPR2(+) in Fig. 2. Therefore, the inhibitory potency of abietic acid and glycyrrhetic acid on MRP2 can exert a great influence on the net transport of [3H]E217βG. In contrast, [3H]E217βG accumulation via BCRP is practically same level (7 µL/mg protein) with ATP-independent accumulation of [3H]E217βG (open column in Fig. 4) under our experimental conditions. Even if BCRP-mediated transport of [3H]E217βG is fully blocked by glycyrrhetic acid, the change in the net transport will be relatively small. Moreover, IC50 value of glycyrrhetic acid for MRP2 (20 µM) is smaller than that for BCRP (39 µM). These findings suggest that glycyrrhetic acid is a potent inhibitor of MRP2 but less potent inhibitor of BCRP among terpenoids tested in this study.

In this study, MRP2 transport was inhibited by glycyrrhetic acid and abietic acid, while BCRP transport was inhibited by only glycyrrhetic acid. (R)-(+)citronellal, (S)-(−)β-citronellol, α-terpinene, terpinolene, and (−)-β-pinene did not affect these transporters. Previously, we reported that all terpenoids used in this study can inhibit P-gp (Yoshida et al., 2006b). The IC50 values of (R)-(+)citronellal, (S)-(−)β-citronellol, α-terpinene, terpinolene, (−)-β-pinene, abietic acid, and glycyrrhetic acid on the intracellular accumulation of 30 nM [3H]digoxin in P-gp-overexpressing cells were 167, 504, 414, 481, 608, 172, and 80.0 µM, respectively. These terpenoids had no effect on the cellular viability evaluated with a leakage of lactate dehydrogenase (Yoshida et al., 2006b). Based on our findings in a previous study and the
present study; (R)-(+)−citronellal, (S)-(−)−β−citronellol, α−terpinene, terpinolene and (−)−β−pinene inhibit only P−gp, abietic acid inhibits P−gp and MRP2, and glycyrrhetic acid inhibits P−gp, MRP2 and BCRP among the three ABC transporters examined. The IC_{50} values of glycyrrhetic acid for these ABC transporters were lower than that of other terpenoids, suggesting its potent affinity for these transporters. This is the first report, to the best of our knowledge, on glycyrrhetic acid and abietic acid with respect to their inhibitory effects on MRP2 or BCRP transport.

Terpenoids form a large and structurally diverse family of natural products including pharmaceutically important compounds as drug candidates. For instance, anticancer agent paclitaxel is a component diterpenoid isolated from an extract of the bark of the Pacific yew (Taxus brevifolia), and ginsenosides, component triterpenoids of Ginseng Radix, can inhibit P−gp−mediated transport (Walle and Walle, 1998; Wang et al., 2001; Kim et al., 2003). Terpenoids used in this study are componential mono−, di−, and triterpenoids in herbal medicines and natural products. (R)−(+ )−citronellal, (S)−(−)−β−citronellol, α−terpinene, terpinolene and (−)−β−pinene are contained in essential oils and present in Zanthoxyli Fructus and some edible plants (Sakai et al., 1968; Lota et al., 2002; Sun and Petracek, 1999). Abietic acid is a major component of the rosin fraction of turpentine from pines and other conifers (Dewick, 2001). Glycyrrhetic acid is contained in Glycyrrhizae Radix as its major component and has mild anti−inflammatory, antiallergic, and mineral−corticoid activities (Finney and Somers, 1958; Kumagai et al., 1957). Glycyrrhizae Radix is frequently used as an ingredient in traditional herbal prescriptions (e.g. Hochuekkito and Kanzoshashinto) and in the treatment of rheumatoid arthritis, Addison’s disease (chronic adrenocortical insufficiency), and various inflammatory conditions (Yamamura et al., 1992; Kawakami et al., 1993). The extract from Glycyrrhizae Radix is also used in many kinds of foods and for flavoring. Therefore, a concomitant intake of glycyrrhetic acid and other terpenoids with conventional prescription medicines may occur practically.

Recently, herbal medications have been often used to provide the relief from gastrointestinal reactions such as nausea, vomiting, constipation, and diarrhea that are associated with anticancer chemotherapy. The safety evaluation of natural medicines will be required to avoid undesirable interactions with anticancer agents and other conventional medicines. On the other hand, some terpenoids such as glycyrrhetic acid may be useful as reversal agents to overcome P−gp, MRP2 or BCRP−mediated multidrug resistance in anticancer chemotherapy.
In conclusion, we have demonstrated that glycyrrhetic acid can inhibit not only P-gp but also MRP2 and BCRP. Abietic acid can also inhibit P-gp and MRP2. Abietic acid and glycyrrhetic acid may interact with P-gp, MRP2 or BCRP substrates and change their absorption and disposition.
References


Footnotes

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Legends for figures

**Fig. 1.** Molecular structures of terpenoids used in this study.

**Fig. 2.** Effect of terpenoids on MRP2-mediated [3H]E217βG uptake by membrane vesicles. [3H]E217βG (100 nM) accumulation in MRP2(–) and MRP2(+) vesicles was measured in the presence of AMP (open column) or ATP (closed column) at 37°C for 1 min. The concentration of terpenoids were 100 µM. Each value represents the mean ± SEM of three experiments. Significant difference (**p<0.01) compared with the control using Bonferroni’s multiple t-test.

**Fig. 3.** Inhibitory effect of abietic acid and glycyrrhetic acid on MRP2-mediated [3H]E217βG uptake by membrane vesicles. Time profile of [3H]E217βG (100 nM) accumulation in MRP2(+) vesicles was measured in the absence (control, circle) or presence of terpenoids (triangles), 100 µM abietic acid (A) and 100 µM glycyrrhetic acid (B) at 37°C. Open and solid symbols represent the presence of AMP and ATP, respectively. Concentration-dependent inhibition of [3H]E217βG (100 nM) accumulation by abietic acid (C) and glycyrrhetic acid (D) in MRP2(+) vesicles was measured at 37°C for 1 min. Each value represents the mean ± SEM of three experiments. Significant difference (**p<0.01) compared with the control using Bonferroni’s multiple t-test.

**Fig. 4.** Effect of terpenoids on BCRP-mediated [3H]E217βG uptake by membrane vesicles. [3H]E217βG (100 nM) accumulation in BCRP(–) and BCRP(+) vesicles was measured in the presence of AMP (open column) or ATP (closed column) at 37°C for 1 min. The concentration of terpenoids were 100 µM. Each value represents the mean ± SEM of three experiments. Significant difference (**p<0.01) compared with the control using Bonferroni’s multiple t-test.

**Fig. 5.** Inhibitory effect of glycyrrhetic acid on BCRP-mediated [3H]E217βG uptake by membrane vesicles. (A) Time profile of [3H]E217βG (100 nM) accumulation in BCRP(+) vesicles was measured in the absence (control, circle) or presence of glycyrrhetic acid (100 µM, triangle) at 37°C. Open and solid symbols represent the presence of AMP and ATP, respectively. (B) Concentration-dependent inhibition of [3H]E217βG (100 nM) accumulation by glycyrrhetic acid in BCRP(+) vesicles was measured at 37°C for 1 min. Each value represents the mean ± SEM of three experiments. Significant difference (**p<0.01 and *p<0.05) compared with the control using Bonferroni’s multiple t-test.
Fig. 1

(R)-(+)-citronellal

(S)-(−)-β-citronellol

α-terpinene

terpinolene

(−)-β-pinene

abietic acid

glycyrrhetic acid
Fig. 2
Fig. 3
Fig. 4

Graph showing the accumulation of various compounds (µL/mg protein). The y-axis represents accumulation in µL/mg protein, while the x-axis lists different compounds and conditions including control, (S)-(-)-β-citronellol, (R)-(+) -citrone, terpinolene, (-) -β-pinene, abietic acid, and glycyrrhetic acid. The graph includes error bars indicating variability. Significant differences are marked with asterisks (**).
Fig. 5