Short Communication

Inhibitory Effects of Terpenoids on Multidrug Resistance-Associated Protein 2- and Breast Cancer Resistance Protein-Mediated Transport

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ABSTRACT:

The possibility of interactions between natural products/supplements and conventional prescription medicines is one of the most important issues in pharmacotherapy. 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-overexpressing 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-o-glucuronide (E217G) 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]E217G were examined. Large decreases in the [3H]E217G accumulation into vesicles from MRP2-overexpressing Sf9 cells were observed in the presence of glycyrrhetic acid and abietic acid, and their IC50 values were about 20 and 51 μM, respectively. [3H]E217G 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]E217G. These findings suggest that glycyrrhetic acid and abietic acid can potentially inhibit MRP2- or BCRP-mediated membrane transport and may interact with their substrates in pharmacokinetic processes.

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 shown that these transporters are distributed throughout many normal tissues of the body. For example, 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., 1999; 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 (Litman et al., 2001; Leslie et al., 2005). 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 because of multiple and complex medications, and significant revisions of safety profiles in the product information have been frequently undertaken in clinical practice (Yoshida et al., 2006c). 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, 2006b). In the present study, the inhibitory effects of seven terpenoids (Fig. 1) that can be P-gp inhibitors (Yoshida et al., 2006a) on MRP2- and BCRP-mediated transport were investigated using membrane vesicles.

Materials and Methods

Materials. [3H] Estradiol 17-β-o-glucuronide (E217G, 55 Ci/mmol) was purchased from PerkinElmer, Inc. (Waltham, MA). ATP, AMP, creatine phosphate, and creatine phosphokinase were obtained from Sigma-Aldrich Corp. (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 Inc., Paisley, UK). (R)-(+)citronellal, (S)-(−)β-citronellol, and α-terpinene were purchased from Sigma-Aldrich Corp. 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 the other reagents are commercially available and of analytical grade.

ABBREVIATIONS: ABC, ATP-binding cassette; P-gp, P-glycoprotein; MRP2, multidrug resistance-associated protein 2; BCRP, breast cancer resistance protein; E217G, estradiol 17-β-o-glucuronide.
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. 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 [3H]E217G 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 dependence 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 [3H]E217G (100 nM) into vesicles was examined 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; PerkinElmer, Inc.).

Statistical Analysis. All the data presented are the mean with S.E.M. of three experiments. Significant differences between the control and inhibitor data were determined by Bonferroni multiple t test. The IC50 and its 95% confidence interval were calculated by probit analysis. A p value of <0.05 was considered to be statistically significant.

Results

Effects of Terpenoids on MRP2-Mediated Transport of [3H]E217G. [3H]E217G 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]E217G accumulation in the presence of ATP and that in the absence of ATP. Then, intravesicular accumulation of [3H]E217G 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]E217G accumulation into MRP2(−) and MRP2(+) vesicles. In the control group of MRP2(−) vesicles, there was no significant difference between [3H]E217G accumulation in the presence of ATP (closed column) and that in the absence of ATP. In the control group of MRP2(+) vesicles, [3H]E217G accumulation in the presence of ATP was markedly higher than that in the absence of ATP, suggesting ATP-dependent [3H]E217G transport by means of MRP2.

This ATP-dependent accumulation of [3H]E217G was also examined in the presence of each of the seven terpenoids and compared with that in the control group (Fig. 2). The [3H]E217G 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]E217G 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]E217G 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 dependence of abietic acid and glycyrrhetic acid on the inhibition of ATP-dependent accumulation of [3H]E217G was examined at 1 min. Abietic acid and glycyrrhetic acid inhibited the [3H]E217G accumulation in a concentration-dependent manner (Fig. 3, C and D). 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 \[^{3}H\]E217βG. \[^{3}H\]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 \[^{3}H\]E217βG accumulation in the presence of ATP and that in the absence of ATP. Then, intravesicular accumulation of \[^{3}H\]E217βG was compared between BCRP(−) and BCRP(+) vesicles to confirm BCRP function.
In the absence of ATP (open column), there was no significant difference between \(^{[3}H\)E217\(^{\beta}G\) accumulation into BCRP(−) and BCRP(+) vesicles. In the control group of BCRP(−) vesicles, there was no significant difference between \(^{[3}H\)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 \(^{[3}H\)E217\(^{\beta}G\) in the presence of ATP was markedly higher than that in the absence of ATP, suggesting the ATP dependence of \(^{[3}H\)E217\(^{\beta}G\) transport via BCRP.

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

The time profiles of \(^{[3}H\)E217\(^{\beta}G\) accumulation into BCRP(+) vesicles were investigated in the presence of absence of ATP and/or glycyrhetic acid (Fig. 5A). \(^{[3}H\)E217\(^{\beta}G\) accumulation in the presence of ATP in the control group increased up to 2 min, and this accumulation was inhibited by glycyrhetic acid to the level of that in the absence of ATP. Therefore, the concentration dependence of glycyrhetic acid on the inhibition of ATP-dependent accumulation of \(^{[3}H\)E217\(^{\beta}G\) was examined at 1 min. Glycyrrhetic acid inhibited \(^{[3}H\)E217\(^{\beta}G\) accumulation in a concentration-dependent manner (Fig. 5B), and the apparent IC\(^{50}\) value of glycyrhetic acid was 39.1 \(\mu\)M (95% confidence interval: 36.0–42.9 \(\mu\)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, 2006a,b). However, an active efflux via these transporters was not shown probably because of the high hydrophilicity of the specific substrate used (\(^{[3}H\)E217\(^{\beta}G\)). 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 \(^{[3}H\)E217\(^{\beta}G\) accumulation via MRP2 was observed (about 60 \(\mu\)l/mg protein) in control-MPR2(+) in Fig. 2. Therefore, the inhibitory potency of glycyrhetic acid on BCRP-mediated \(^{[3}H\)E217\(^{\beta}G\) uptake by membrane vesicles. A, time profile of \(^{[3}H\)E217\(^{\beta}G\) (100 \(nM\)) accumulation in BCRP(+) vesicles was measured in the absence (control, circle) or presence of glycyrhetic acid (100 \(\mu\)M, triangle) at 37°C. Open and solid symbols represent the presence of AMP and ATP, respectively. B, concentration-dependent inhibition of \(^{[3}H\)E217\(^{\beta}G\) (100 \(nM\)) accumulation by glycyrhetic acid in BCRP(+) vesicles was measured at 37°C for 1 min. Each value represents the mean ± S.E.M. of three experiments. Significant difference (**, \(p < 0.01\)) compared with the control using Bonferroni multiple \(t\) test.
abietic acid and glycyrrhetic acid on MRP2 can exert a great influence on the net transport of \[^{3}H\]E217G. In contrast, \[^{3}H\]E217G accumulation via BCRP is practically the same level (7 \(\mu\)g/mg protein) with ATP-independent accumulation of \[^{3}H\]E217G (open column in Fig. 4) under our experimental conditions. Even if BCRP-mediated transport of \[^{3}H\]E217G is fully blocked by glycyrrhetic acid, the change in the net transport will be relatively small. Moreover, IC\(_{50}\) value of glycyrrhetic acid for MRP2 (20 \(\mu\)M) is smaller than that for BCRP (39 \(\mu\)M). 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, whereas BCRP transport was inhibited by only glycyrrhetic acid. \((R)\,\,(-)\)-citronellol, \((S)\,\,(-)\)-\(\beta\)-citronellol, \(\alpha\)-terpinene, terpinoline, and \((-)\)-\(\beta\)-pinene did not affect these transporters. Previously, we reported that all the terpenoids used in this study can inhibit P-gp (Yoshida et al., 2006a). The IC\(_{50}\) values of \((R)\,\,(-)\)-citronellol, \((S)\,\,(-)\)-\(\beta\)-citronellol, \(\alpha\)-terpinene, terpinoline, and \((-)\)-\(\beta\)-pinene, abietic acid, and glycyrrhetic acid on the intracellular accumulation of 30 nM \[^{3}H\]doxorubicin in P-gp–overexpressing cells were 167, 504, 414, 481, 608, 172, and 80.0 \(\mu\)M, respectively. These terpenoids had no effect on the cellular viability evaluated with a leakage of lactate dehydrogenase (Yoshida et al., 2006a). Based on our findings in a previous study and the present study, \((R)\,\,(-)\)\,-citronellol, \((S)\,\,(-)\)-\(\beta\)-citronellol, \(\alpha\)-terpinene, terpinoline, and \((-)\)-\(\beta\)-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 example, anticancer agent paclitaxel is a diterpenoid diterpenoids, and triterpenoids in herbal medicines and natural drug candidates. For example, anticancer agent paclitaxel is a

References


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