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Modulation of P-glycoprotein function and multidrug resistance in cancer cells by Thai plant extracts

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The effects of ethanol extracts from Thai plants belonging to the families of Annonaceae, Rutaceae, and Zingiberaceae on P-glycoprotein (P-gp) function and multidrug resistance were examined in paclitaxel-resistant HepG2 (PR-HepG2) cells. All the extracts tested, significantly increased the accumulation of [^{3}H]paclitaxel, a P-gp substrate, in the cells. Among nine extracts, Z01 and Z02, extracts from *Curcuma comosa* and *Kaempferia marginata* (Zingiberaceae family), respectively, potently increased the accumulation. In addition, Z01 and Z02 increased the accumulation of other P-gp substrates, rhodamine 123 and doxorubicin, in PR-HepG2 cells in a concentration-dependent manner. Increased accumulation of rhodamine 123 and doxorubicin by Z01 and Z02 was also confirmed by confocal laser scanning microscopy. The effect of Z01 and Z02 pretreatment on the expression of MDR1 mRNA was also examined. The expression of MDR1 mRNA was not affected by the treatment of PR-HepG2 cells with these extracts for 48 hours. Cytotoxicity of paclitaxel was examined by XTT and protein assays in the absence and presence of Z02. Z02 potentiated the cytotoxicity of paclitaxel in PR-HepG2 cells. These results suggest that *Curcuma comosa* and *Kaempferia marginata* belonging to Zingiberaceae are useful sources to search for new P-gp modulator(s) that can be used to overcome multidrug resistance of cancer cells.

1. Introduction

Resistance of cancer cells to chemotherapy is a serious problem of cancer treatment. One of the main mechanisms involved in multidrug resistance of cancer cells is the active efflux of anticancer drugs by ATP-binding cassette (ABC) superfamily of transporters, which reduces the intracellular concentration of anticancer drugs (Gillet et al. 2007). P-Glycoprotein (P-gp; MDR1/ABCB1) is a member of the ABC transporters, and has been well known to confer multidrug resistance to various cancer cells (Baguley 2010). P-gp is about 170 kDa in size, and has 2 homologous parts of approximately equal length, 2 ATP-binding domains, and 12 transmembrane regions in its structure (Takano et al. 2006). P-gp pumps out various structurally and pharmacologically unrelated anticancer drugs as well as other drugs including immunosuppressants (Morjani and Madoulet 2010). Thus, human P-gp plays an important role in intrinsic or acquired resistance of cancer cells.

There are two major strategies to overcome P-gp-mediated multidrug resistance of cancer. One strategy is to co-administer a compound having P-gp inhibitory effect (P-gp modulator) with an anticancer drug, which would increase the intracellular concentration of the anticancer drug and therefore its pharmacological action (Szakacs et al. 2006). Another strategy is to employ anticancer drugs of which cellular transport and anticancer activity are not affected by P-gp (Ozben 2006).

In recent years, a great effort have been made in the discovery of a new class of P-gp modulators from natural plants (Bansal et al. 2009; Zhou et al. 2007). We also have been studying the extracts and purified compounds from Thai plants for this purpose (Patanasethanont et al. 2007a, 2007b). Among various extracts we have tested so far, some extracts from Thai plants belonging to the families of Annonaceae, Rutaceae, and Zingiberaceae showed P-gp inhibitory effect and/or anticancer effect (Kawami et al. 2010; Yumoto et al. 2013). In addition, some of these extracts potentiated cytotoxicity of anticancer drugs in cultured cancer cells, most likely by inhibiting P-gp efflux activity and increasing intracellular accumulation of anticancer drugs in cancer cells (Kawami et al. 2010). For example, MM80, an ethanol extract from *Micromelum minutum* (a Thai plant belonging to the family of Rutaceae), enhanced intracellular accumulation of paclitaxel, a P-gp substrate, and potentiated cytotoxicity of paclitaxel in paclitaxel-resistant HepG2 (PR-HepG2) cells. PR-HepG2 is a cell line which we have established by long term treatment of wild-type HepG2 cells with the low concentration of paclitaxel (Takano et al. 2009). In PR-HepG2 cells, enhanced expression and function of P-gp was found to be a predominant mechanism of the resistance.

To further search for the safe and effective P-gp modulators, we have collected some ethanol extracts of Thai plants belonging to the families of Annonaceae, Rutaceae, and Zingiberaceae. By focusing on these Thai plants, the probability to find expected extracts may be enhanced. In the present study, the effects of

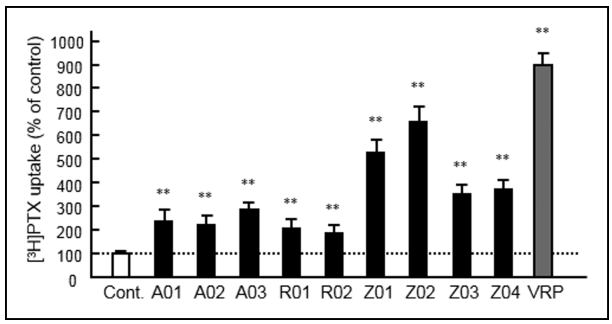

Fig. 1: Effect of various ethanol extracts of Thai plants on the uptake of [^{3}H]PTX in PR-HepG2 cells. PR-HepG2 cells were incubated with 15 nM [^{3}H]PTX without (control, open column) or with each ethanol extract (100 μg/ml, black column). For comparison, the effect of 100 μM VRP (gray column) on [^{3}H]PTX uptake was also examined. Each value represents the mean ± S.E. of three determinations. $^{**}P < 0.01$, significantly different from control.

these Thai plant extracts on the cellular uptake of P-gp substrates were examined in PR-HepG2 cells. In addition, the effects of the extracts on cytotoxicity of paclitaxel and MDR1 expression were also examined.

2. Investigations, results and discussion

2.1. Effect of various Thai plant extracts on [^{3}H]paclitaxel uptake in PR-HepG2 cells

PR-HepG2 is a cell line with an enhanced expression and function of P-gp compared with wild-type HepG2 cells, which confers paclitaxel-resistance to the cells (Takano et al. 2009). As shown in Fig. 1, verapamil, a potent inhibitor of P-gp, markedly increased the cellular uptake of [^{3}H]paclitaxel (about 9-fold), by inhibiting P-gp-mediated efflux of the drug. As we expected, all the extracts at the fixed concentration of 100 μg/ml significantly increased [^{3}H]paclitaxel uptake, though the potencies differed among the extracts (about 2- to 7-fold). Thus, many, though it may not be all, Thai plants belonging to the families of Annonaceae, Rutaceae, and Zingiberaceae may contain compounds having inhibitory effects on P-gp. Among nine extracts, Z01 (an extract from *Curcuma comosa*) and Z02 (an extract from *Kaempferia marginata*) belonging to Zingiberaceae showed potent enhancing effects on [^{3}H]paclitaxel uptake.

2.2. Effect of Z01 and Z02 on [^{3}H]paclitaxel uptake in PR-HepG2 cells

The effect of Z01 and Z02 on [^{3}H]paclitaxel uptake in PR-HepG2 cells was examined at the extract concentration range of 1 to 100 μg/ml (Fig. 2). Z01 showed a biphasic effect on [^{3}H]paclitaxel uptake; it decreased the uptake at low concentrations (1 - 10 μg/ml), while it increased the uptake at higher concentrations (30 - 100 μg/ml). This result may indicate that both P-gp stimulatory and inhibitory compounds are contained in Z01 extract. In fact, some natural compounds are reported to have P-gp stimulatory effects. Critchfield et al. (1994) reported that flavonoids such as galangin, kaempferol, and quercetin inhibited adriamycin accumulation in HCT-15 colon cells. They suggested that the decreased accumulation of adriamycin would be due to the stimulation of P-gp-mediated efflux of the drug by these flavonoids. On the other hand, Z02 increased [^{3}H]paclitaxel uptake in a concentration-dependent manner at this concentration range, and its effect was stronger than that of Z01.

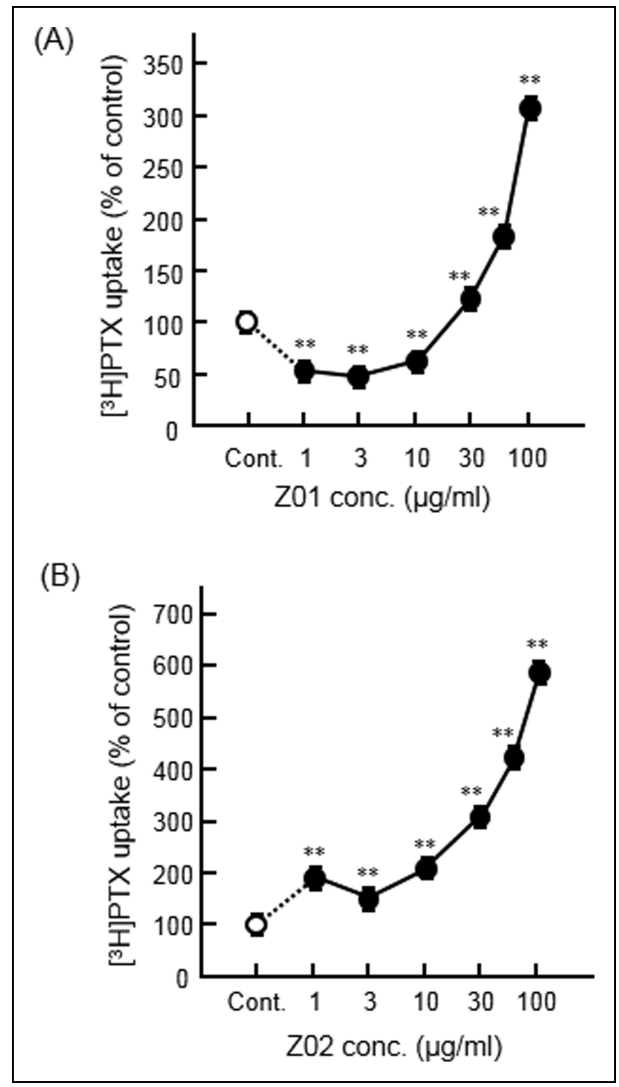

Fig. 2: Effect of Z01 (A) and Z02 (B) on the uptake of [^{3}H]PTX in PR-HepG2 cells. PR-HepG2 cells were incubated with 15 nM [^{3}H]PTX without (control, open circle) or with various concentrations of Z01 (A) and Z02 (B) (solid circle). Each value represents the mean ± S.E. of three determinations. $^{**}P < 0.01$, significantly different from each control.

2.3. Effect of Z01 and Z02 on rhodamine 123 and doxorubicin uptake in PR-HepG2 cells

The effects of Z01 and Z02 on other P-gp substrates, rhodamine 123 and doxorubicin, were examined. As shown in Fig. 3, both Z01 and Z02 increased rhodamine 123 uptake in PR-HepG2 cells in a concentration-dependent manner. In addition, Z01 and Z02 also increased doxorubicin uptake in a concentration-dependent manner (Fig. 4). However, the stimulation effects of these extracts on the uptake of rhodamine 123 and doxorubicin were not as potent as those on [^{3}H]paclitaxel uptake. We have previously observed similar findings; KP018 (an extract from *Ellipeiopsis cherrevensis*) and AT80 (an extract from *Ancistrocladus tectorius*) showed more potent stimulatory effect on the uptake of paclitaxel than that of rhodamine 123 in PR-HepG2 cells (Kawami et al. 2010). This may be due to the differences in substrate concentrations, affinity of each substrate for P-gp, and/or transporters involved in their cellular uptake and efflux. Rhodamine 123 and doxorubicin, but not paclitaxel, are known to be substrates of an efflux transporter BCRP (breast cancer resistance protein; ABCG2) (Sharom 2008), which is expressed in PR-HepG2 cells (unpublished observation).

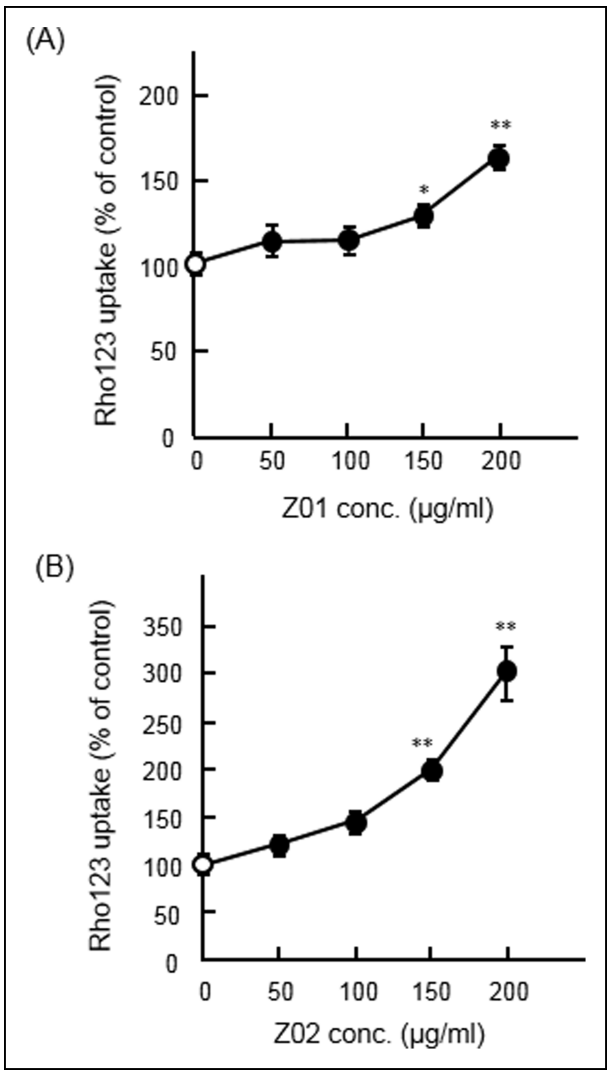

Fig. 3: Effect of Z01 (A) and Z02 (B) on the uptake of Rho123 in PR-HepG2 cells. PR-HepG2 cells were incubated with 50 μM Rho123 without (control, open circle) or with various concentrations of Z01 (A) and Z02 (B) (solid circle). Each value represents the mean ± S.E. of three determinations. $*P < 0.05$, $**P < 0.01$, significantly different from each control.

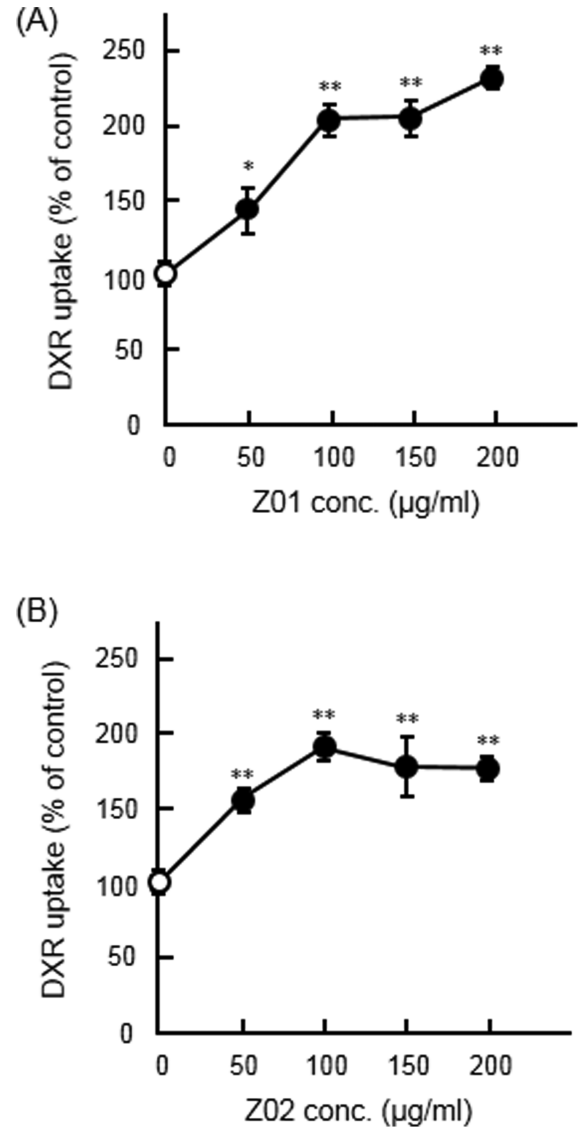

Fig. 4: Effect of Z01 (A) and Z02 (B) on the uptake of DXR in PR-HepG2 cells. PR-HepG2 cells were incubated with 50 μM DXR without (control, open circle) or with various concentrations of Z01 (A) and Z02 (B) (solid circle). Each value represents the mean ± S.E. of three determinations. $*P < 0.05$, $**P < 0.01$, significantly different from each control.

2.4. Confocal laser scanning microscopic study on the effect of Z01 and Z02 on rhodamine 123 and doxorubicin uptake in PR-HepG2 cells

In order to confirm the inhibitory effect of Z01 and Z02 on P-gp function, the effect of Z01 and Z02 on rhodamine 123 and doxorubicin uptake in PR-HepG2 cells was further studied using confocal laser scanning microscopy. Intracellular rhodamine 123 concentration was increased by verapamil, as shown by the strong red color, compared with that in control (Fig. 5A and D). Similarly, the uptake of rhodamine 123 was increased by Z01 and Z02 (Fig. 5B and C). Rhodamine 123 is often used for mitochondrial staining (Johnson et al. 1980). In Fig. 5A-D, rhodamine 123 seemed to distribute to cytoplasm including mitochondria, but not to nuclei. Figure 5E-H showed the effect of Z01, Z02, and verapamil on doxorubicin uptake. The uptake of doxorubicin was increased by these Thai plant extracts and verapamil. In contrast to rhodamine 123, doxorubicin taken up by the cells distributed not only to the cytoplasmic compartment but also to the nuclei. The distribution of doxorubicin to the nuclei should be related to its pharmacological effect. Doxorubicin is known to induce cytotoxic effects against cancer cells mainly by intercalating between base pairs of DNA helix, leading to the inhibition of topoisomerase II and DNA replication (Gewirtz 1999).

Based on these uptake studies using various P-gp substrates and different analytical methods, it was clearly shown that Z01 from *Curcuma comosa* and Z02 from *Kaempferia marginata* are potent P-gp inhibitors.

2.5. Effect of Z01 and Z02 on the expression of MDR1 mRNA in PR-HepG2 cells

The expression of MDR1 gene encoding P-gp is known to be induced by various P-gp inhibitors and natural compounds such as ritonavir and St. John's wort (Tian et al. 2005; Zastre et al. 2009; Alvares et al. 2010; Abuznait et al. 2011). Induction of P-gp in cancer cells would counteract the following chemotherapy. Therefore, MDR1 mRNA expression in PR-HepG2 cells was examined, after pretreatment of the cells with 30 μg/ml of Z01 and Z02 for 48 h. As shown in Fig. 6, real-time PCR analysis indicated that the expression of MDR1 mRNA was not affected

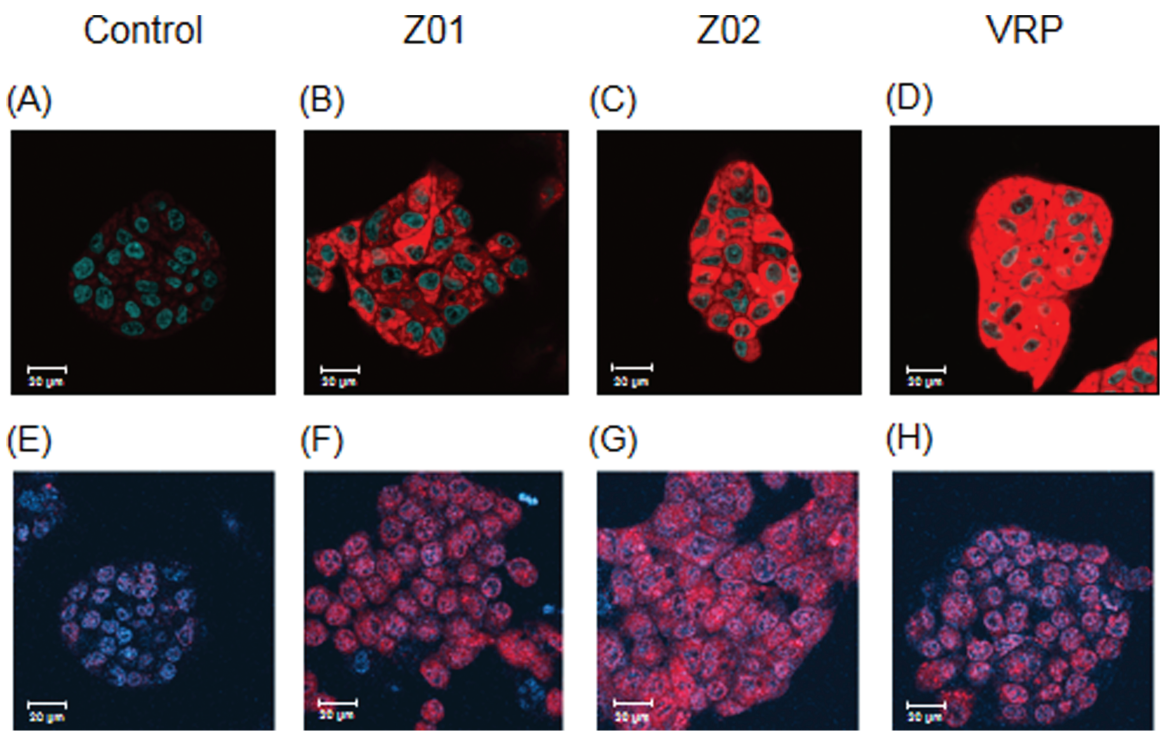

Fig. 5: Effect of Z01 and Z02 on the uptake of Rho123 (A∼D) and DXR (E∼H) in PR-HepG2 cells determined by confocal laser scanning microscopy. PR-HepG2 cells were incubated with 50 μM Rho123 and DXR without (control, A, E) or with 200 μg/ml Z01 (B, F) and Z02 (C, G). For comparison, the effect of 100 μM VRP on Rho123 (D) and DXR (H) uptake was also examined.

by Z01 and Z02. Such characteristics would be desirable for P-gp modulators.

2.6. Effect of Z02 on the viability of PR-HepG2 cells and on the sensitivity of the cells to paclitaxel

Cytotoxicity is an important issue for the development of P-gp modulators, because many candidate compounds are still unavailable in clinical pharmacotherapy due to their toxicities (Verweij et al. 1991; Friedenberg et al. 2006). Figure 7A shows the cytotoxicity of Z02 on PR-HepG2 cells, which was estimated by XTT assay. At 30 μg/ml, no significant cytotoxicity of Z02 was observed, while [^{3}H]paclitaxel uptake was enhanced about 3-fold by 30 μg/ml of Z02 (Fig. 2B). Therefore, 30 μg/ml of Z02 was used to examine its effect on the sensitivity of the cells to paclitaxel. The cytotoxicity was estimated by XTT assay and protein assay. In both assay methods, Z02 potentiated the sensitivity of PR-HepG2 cells to paclitaxel, as shown by the shift of the dose-response curve to the left (Fig. 7B and C). Therefore, Z02 would have a substantial P-gp modulating effect at a concentration which does not show apparent cytotoxicity. Z01 also potentiated the sensitivity of PR-HepG2 cells to paclitaxel in XTT assay, but its effect was weaker than that of Z02 (data not shown), in accordance with the difference in their P-gp inhibitory potencies (Fig. 2).

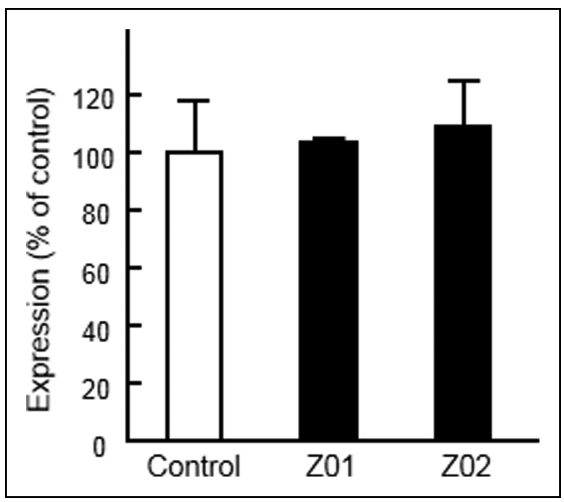

Fig. 6: Effect of Z01 and Z02 on the expression of MDR1 mRNA in PR-HepG2 cells. PR-HepG2 cells were incubated without (control, open column) or with 30 μg/ml Z01 and Z02 (black column) for 48 h. The expression of MDR1 mRNA was estimated by real-time PCR after treatment. Each value represents the mean ± S.E. of three determinations.

Thai plant extracts employed in this study are crude extracts prepared with 50% ethanol, and therefore would contain a lot of compounds. So far, some compounds contained in the plants we employed have been reported. Nakamura et al. (2008) reported that compounds such as 4-hydroxybenzaldehyde and (+)-rhododendrol are contained in *Curcuma comosa*, and Thongnest et al. (2005) reported that compounds such as sandaracopimaradine are contained in *Kaempferia marginata*. It should be interesting to study the effects of these purified compounds on P-gp function in PR-HepG2 cells, though there are many other constituents that are not yet identified.

In conclusion, Thai plant extracts such as Z02 from *Kaempferia marginata* showed P-gp inhibitory effects, and Z02 potentiated the sensitivity of paclitaxel-resistant cancer cells to paclitaxel. Therefore, Thai plants belonging to the families of Annonaceae, Rutaceae, and Zingiberaceae, especially *Kaempferia marginata*, may be promising sources to search for new classes of P-gp modulators.

3. Experimental

3.1. Chemicals and reagents

Dulbecco's Modified of Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from MP Biomedicals, LLC (Santa Ana, CA, USA).

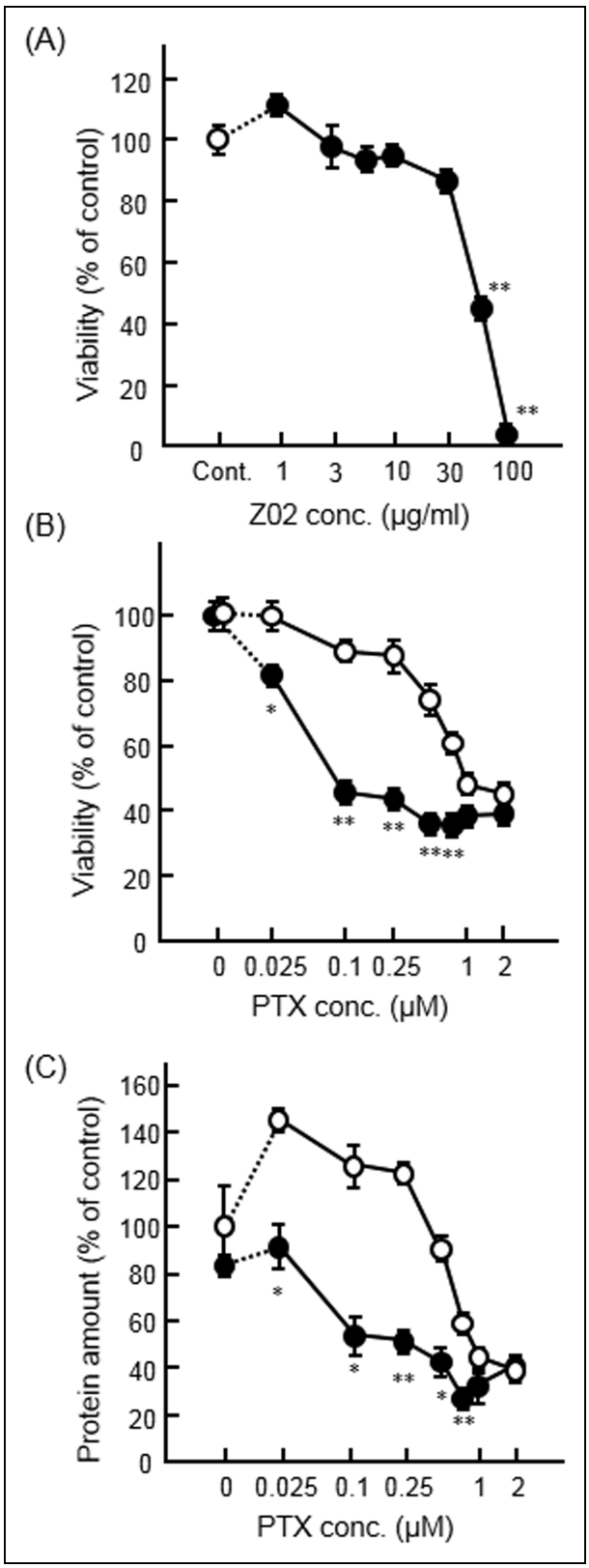

Fig. 7: Effect of Z02 on the viability of PR-HepG2 cells (A) and sensitivity of PR-HepG2 cells to PTX determined by XTT assay (B) and protein assay (C). (A): PR-HepG2 cells were incubated without (control, open circle) or with various concentrations of Z02 (solid circle) for 48 h. The cell viability was estimated by XTT assay after treatment. (B, C): PR-HepG2 cells were incubated with various concentrations of PTX without (open circle) or with 30 μg/ml Z02 (solid circle) for 48 h. The cell viability and protein amount were estimated by XTT assay (B) and Lowry method (C), respectively. Each value represents the mean ± S.E. of three determinations. $*P < 0.05$, $**P < 0.01$, significantly different from each value in the absence of Z02.

Table: Ethanol extracts of Thai plants belonging to Annonaceae, Rutaceae, and Zingiberaceae

No.	Code	Plant	Part	Family
1	A01	*Artabotrys siamensis*	Leaf	
2	A02	*Desmos chinensis*	Leaf	Annonaceae
3	A03	*Melodorum fruticosum*	Leaf	
4	R01	*Citrus hystrix*	Leaf	Rutaceae
5	R02	*Aegle marmelos*	Leaf	
6	Z01	*Curcuma comosa*	Rhizome	
7	Z02	*Kaempferia marginata*	Leaf	Zingiberaceae
8	Z03	*Catymbium peciosum*	Rhizome	
9	Z04	*Amomum* sff. *villosum*	Rhizome	

Trypsin-EDTA and penicillin-streptomycin were purchased from Life Technologies (Carlsbad, CA, USA). [^{3}H]Paclitaxel (PTX, Taxol; 15.0 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA, USA). Unlabeled PTX was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Rhodamine 123 (Rho123) was purchased from Kanto Chemical (Tokyo, Japan). Doxorubicin hydrochloride (DXR), 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt (XTT) and phenazine methosulfate (PMS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Verapamil hydrochloride (VRP) was purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals used for experiments were of the highest purity commercially available.

3.2. Thai plant extracts

In the Table, the information concerning Thai plant extracts examined in this study were summarized. These plants were authenticated and 50% ethanol extracts were prepared by the Center for Research and Development of Herbal Health Products, Khon Kaen University. Voucher specimens of the plant materials have been lodged at the Herbarium, Khon Kaen University. The solvent in the ethanol extract was evaporated under vacuum, and the dried extract was stored at −20 °C until use.

3.3. PR-HepG2 cell culture

The PR-HepG2 cell line was established from wild-type HepG2 cells as described previously (Takano et al. 2009). PR-HepG2 cells were cultured in DMEM medium containing 3 nM PTX, 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 5% CO_2-95% air at 37 °C. The cells were subcultured every 6-7 days using 0.25% trypsin and 1 mM EDTA. The medium was replaced every 2-3 days and the cells were used for the uptake experiments on the sixth day after seeding at a density of 3×10^4 cells/well in a 24-well flat-bottomed plate.

3.4. Uptake of [^{3}H]PTX, Rho123 and DXR by PR-HepG2 cells

Uptake experiments were performed as described previously (Takano et al. 2009). Briefly, after removal of the culture medium, cells were washed twice with phosphate buffered saline (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.4; PBS buffer) supplemented with 5 mM D-glucose (PBS-G buffer) and preincubated for 15 min at 37 °C. Then the substrates of P-gp such as [^{3}H]PTX (15 nM), Rho123 (50 μM), and DXR (50 μM), with or without a Thai plant extract which was dissolved in PBS-G buffer containing 0.5% DMSO, were added and the cells were incubated at 37 °C for 60 min. After incubation, the uptake buffer was aspirated and the cells were rinsed rapidly three times with ice-cold PBS buffer. To estimate the uptake amount of [^{3}H]PTX, the cells were scraped with a rubber policeman into 0.3 ml of 0.1 M NaOH and each well was rinsed again to improve the recovery of the cells. Then, 3 ml of Ultima Gold (PerkinElmer, Waltham, MA, USA) were added and the radioactivity was measured by liquid scintillation counting. Protein concentration was measured by the method of Bradford with bovine serum albumin as a standard (Bradford 1976).

To estimate the uptake amount of Rho123 and DXR, 0.1% Triton X-100 in PBS(-) buffer (PBS buffer without $CaCl_2$ or $MgCl_2$) was added to each well and the cells were scraped with a rubber policeman. The wells were rinsed again to improve the recovery of the cells. The cells were solubilized in 0.1% Triton X-100 in PBS(-) buffer for 30 min at room temperature and centrifuged at 5,600 g for 5 min. The supernatant was used for fluorescence and protein assays. The fluorescence of Rho123 and DXR was measured

using a Hitachi fluorescence spectrophotometer F-2700 (Tokyo, Japan) at Ex 485 nm/Em 546 nm and Ex 500 nm /Em 560 nm, respectively. Protein content was determined by the Lowry method (Lowry et al. 1951).

3.5. Confocal laser scanning microscopy

PR-HepG2 cells were incubated with 50 μM Rho123 or 50 μM DXR, and 1 μM Hochest33342 for nuclei staining at 37 °C for 60 min. After incubation, confocal laser scanning microscopy was performed on an LSM5 PASCAL (Carl Zeiss Microimaging GmbH, Jena, Germany) using a 63 × oil immersion objective lens.

3.6. Cytotoxicity evaluated by XTT assay

XTT assay was performed as described previously (Yumoto et al. 2013). Briefly, PR-HepG2 cells were seeded at a density of 5×10^4 cells/well in a 24-well flat-bottomed plate, and were cultured at 37 °C for 24 h. The medium was replaced with serum-free DMEM containing various concentrations of a Thai plant extract and 0.5% DMSO, and the cells were incubated at 37 °C for 48 h. Then, the cells were washed twice with PBS-G buffer and 0.2 ml of 250 μM XTT in PBS-G buffer containing 10 μM PMS was added to each well. The cells were incubated at 37 °C for 30 min. After transfer of the reaction solution to a microplate well, the amount of orange formazan dye produced was quantitated by measuring absorbance with EnSpire™ Plate Reader (PerkinElmer, Waltham, MA, USA).

3.7. Cytotoxicity evaluated by protein assay

PR-HepG2 cells were seeded and treated with a Thai plant extract as described above. After the treatment, the cells were washed twice with PBS(-) buffer and were lysed in 0.3 ml of 0.1 M NaOH. The lysate was transferred to a 1.5 ml centrifugation tube and incubated at room temperature for 30 min. After centrifugation at 10,000 rpm for 5 min, protein concentration in the supernatant was measured by the method of Lowry with bovine serum albumin as standard.

3.8. Expression of MDR1 mRNA estimated by real-time PCR

PR-HepG2 cells were seeded at a density of 10×10^4 cells/dish in a 35-mm dish, and cultured for 7 days. Total RNA was extracted from the cells with an RNeasy mini kit (Qiagen, Hilden, Germany). The total RNA was reverse transcribed into cDNA by using Rever Tra Dash™ (TOYOBO, Osaka, Japan). Real-time PCR was performed on a Bio-Rad CFX96 (Bio Rad, Hercules, CA, USA) using THUNDERBIRD SYBR qPCR Mix. The reaction mixtures consisted of 2 μl cDNA, 5 μl THUNDERBIRD SYBR qPCR Mix, and primers, in a final volume of 10 μl. The PCR conditions were the following: initial denaturation for one cycle of 30 s at 95 °C, followed by 40 cycles of 10 s at 95 °C (denaturation), 15 s at 60 °C (annealing), and 15 s at 72 °C (extension). After the reaction, a melting curve was obtained to confirm the single product. The primer sequences for MDR1 were sense, 5′-CCCATCATTGCAATAGCAGG-3′, antisense, 5′-TGTTCAAACTTCTGCTCCTGA-3′. The expression level of mRNA was normalized as to that of GAPDH, a housekeeping gene.

3.9. Statistical analysis

The data were expressed as the mean ± S.E.. Statistical analysis was performed by Student's t-test, or by one-way analysis of variance followed by the Tukey's test for multiple comparisons. The level of significance was set at * $P < 0.05$ or at ** $P < 0.01$.

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