Department of Pharmaceutics and Therapeutics[1], Graduate School of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan; Center of Excellence in Innovative Drug Delivery and Nanomedicine[2], Mahidol University, Bangkok, Thailand

P-gp modulating effect of *Azadirachta indica* extract in multidrug-resistant cancer cell lines

M. Kawami[1], Y. Yamada[1], O. Issarachot[2], V. B. Junyaprasert[2], R. Yumoto[1], M. Takano[1,*]

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**Corresponding author: Prof. Mikihisa Takano,Department of Pharmaceutics and Therapeutics, Graduate School of Biomedical & Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan takanom@hiroshima-u.ac.jp*

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The extract of *Azadirachta indica*, commonly known as neem, has found extensive use in traditional medicine for treating various human diseases. In this study, the effect of the 50% ethanol extract of *A. indica* (AI01) on P-glycoprotein (P-gp)-mediated multidrug resistance (MDR) was examined using MDR cell lines, specifically paclitaxel-resistant HepG2 (PR-HepG2) and doxorubicin (DOX)-resistant (DR) colon-26 cells. 96-h treatment of the two cell lines with AI01 (30 µg/mL) showed no effect on the expression of P-gp mRNA (human MDR1 and mouse mdr1b) and protein, while AI01 increased the accumulation of rhodamine 123, a P-gp substrate, in both PR-HepG2 and DR-colon-26 cells. The cytotoxic effects of 48-h treatment with AI01 on the viability of PR-HepG2 and DR-colon-26 cells were not observed. Therefore, 30 µg/mL AI01 may have no cytotoxic and P-gp-inducing effects. Finally, AI01 potentiated the sensitivity of PR-HepG2 and DR-colon-26 cell lines to DOX by 8.6- and 15.3-fold, respectively. These findings suggest that *A. indica* may be a promising source for a new class of P-gp modulators without cytotoxic/P-gp induction effects.

1. Introduction

Multidrug resistance (MDR) to anticancer agents is mediated by multiple mechanisms, including the active efflux of drugs by ATP-binding cassette (ABC) transporters, altered activity of specific enzymes, and the deregulation of apoptosis and so on (Krishna and Mayer 2000). Especially, the role of P-glycoprotein (P-gp), a member of the ABC transporter family, in MDR has been extensively studied (Litman et al. 2001). P-gp recognizes structurally and pharmacologically diverse cancer chemotherapeutic agents, including vinca alkaloids, anthracyclines, etoposide, and paclitaxel (PTX) (Takano et al. 2006). Thus, P-gp has an important role in the intrinsic or acquired resistance of tumor cells.

Thus far, various compounds with P-gp inhibiting effects have been examined for their potential in overcoming the P-gp-mediated MDR of cancer cells (Srivalli and Lakshmi 2012). These agents are known as P-gp modulators or chemosensitizers, and are divided into first, second, and third generation inhibitors. First-generation inhibitors include calcium channel blockers, such as verapamil (VRP), and immunosuppressants, such as cyclosporine A. However, the concentrations required for P-gp inhibition are extremely high; thus, many of these inhibitors failed in clinical trials (Dantzig et al. 2003). Subsequently, modified first-generation inhibitors, such as the cyclosporine A analog PSC833 (valspodar), were developed as second-generation P-gp inhibitors (Van Zuylen et al. 2000). Although these compounds are more potent in inhibiting P-gp and less toxic than first-generation modulators, they also interact with cytochrome P450 (CYP) 3A4, causing them to compete with conventional anticancer P-gp substrate drugs as their metabolism is affected by the same enzyme (Darby et al. 2011). The application of quantitative structure-activity relationships to high-throughput screening technology and combinational chemistry methods have led to the identification of a variety of third generation inhibitors including tariquidar (XR9576; an anthranilamide derivative), which are highly specific for P-gp, do not interact with the CYP3A4 system and require no alterations in the chemotherapy doses. However, these P-gp modulators are currently unavailable for clinical use.

In recent years, studies have been performed to discover a new class of P-gp modulators from natural resources such as herbs and fruits, which are expected to be safe and non-toxic (Srivalli and Lakshmi 2012; Yuan et al. 2008). For example, grape fruits juice and green tea themselves are safe and non-toxic, and their components, tangeretin and (-)-epigallocatechin gallate, have been reported to show a potent P-gp inhibitory effect (Jodoin et al. 2002; Honda et al. 2004). In addition, several non-toxic plant components have also been proposed as usefull MDR modulators, including curcumin (Limtrakul et al. 2007), polyphenols (Michalak and Wesolowska 2012), and flavonoids (Di Pietro et al. 2002). These findings indicate that non-toxic natural resources may be useful to find a new class of P-gp modulators.

Azadirachta indica (Meliaceae), commonly known as neem, can be found in various countries in Asia, Africa and Australia, as well as the Central and South Americas (Kumar and Navaratnam 2013). Various parts of *A. indica* including the leaves, fruits, flowers, seeds, and bark, have been extensively used in traditional medicine for treating certain human diseases (Patel et al. 2016; Paul et al. 2011). Recent studies have revealed that *A. indica* extract has potent anti-tumor activity. For example, the growth of Ehrlich's carcinorma in Swiss albino mice was inhibited by pre-treatment with aqueous neem extracts, due to the immunostimulatory activity of neem (Haque et al. 2006). In addition, ethanolic extracts from the leaves of *A. indica* induced apoptosis via regulation of apoptosis-associated proteins, such as B-cell lymphoma-2, Bcl-2-like protein 11, and caspases, in prostate cancer cell lines (Kumar et al. 2006) and hamster buccal pouch carcinogenesis model (Subapriya et al. 2005). However, the P-gp-modulating effect of *A. indica* remained unclear.

Our recent studies have focused on the P-gp modulating effect of the extracts and purified compounds from Thai plants, including *Ancistrocladus tectorius* (Kawami et al. 2010), *Curcuma comosa*, and *Kaempferia marginata* (Takano et al. 2014), in addition to *Kaempheria parviflora* evaluated by Patanasethanont et al. (2007a, 2007b). In addition, the effect of these Thai plant extracts on P-gp function has been evaluated using PTX-resistant HepG2 (PR-HepG2) cells, which exhibit P-gp-mediated resistance to PTX

as well as doxorubicin (DOX), another P-gp substrate anticancer drug (Takano et al. 2009). On the other hand, in order to examine the P-gp modulating effect of AI01 in another cell line other than PR-HepG2 cells, we focused on a murine adenocarcinorma cell line, colon-26, which has been used for prior investigations concerning P-gp-mediated MDR (Yoshizawa et al. 2014). Although there may be species differences between human and mouse, Ogawara et al. (2009) have demonstrated that P-gp expression and function were markedly increased by acquisition of resistance of colon-26 cells to DOX, indicating that DOX-resistant (DR) colon-26 cells would be useful tool to evaluate P-gp modulating effect of AI01. In addition, since we have previously demonstrated that colon-26 cells can be utilized to prepare tumor-bearing mice (Yumoto et al. 2012), DR-colon-26 cells would be applicable for *in vivo* studies concerning MDR and its modulation. The present study aimed to identify novel, safe, and effective P-gp modulators; thus, we examined the effect of *A. indica* extract on P-gp function and cell sensitivity to DOX using PR-HepG2 and DR-colon-26 cells.

2. Investigations and results

2.1. Establishment of DR-colon-26 cells

DR-colon-26 cells were established by long-term treatment of colon-26 cells with low concentrations of DOX, as described below. The expression levels of mdr1b mRNA and P-gp protein in DR-colon-26 cells markedly increased compared with that in the wild-type cells (Figs. 1A, B). In addition, P-gp activity, estimated by subtracting the rhodamine 123 (Rho123; a P-gp substrate) uptake in the absence of VRP (a P-gp inhibitor) from that in the presence of VRP, was higher in DR-colon-26 cells than in colon-26 cells at all time points (Fig. 1C), suggesting that the expression and function of P-gp in DR-colon-26 cells were increased by DOX treatment compared with the wild type. Furthermore, we compared the DOX sensitivity of colon-26 and DR-colon-26 cells using 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt (XTT) assay. As presented in Fig. 1D, the cell viability curve for DR-colon-26 cells was markedly shifted to the right, compared with the curve for colon-26 cells. The estimated IC_{50} values for DOX in colon-26 and DR-colon-26 cells were 0.3 and 10.3 μM, respectively. These findings indicate that DR-colon-26 cells conferred P-gp-mediated resistance to DOX.

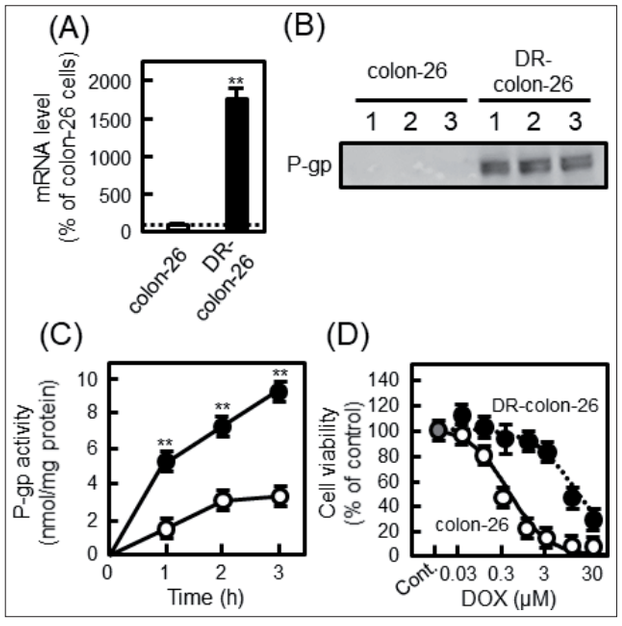

Fig. 1: Establishment of DR-colon-26 cells. (A) The expression of mouse mdr1b mRNA in colon-26 (open column) and DR-colon-26 (closed column) cells was measured by real-time PCR analysis. Relative levels of each target gene mRNA were determined following normalization to GAPDH mRNA. Each value represents the mean±S.E. (n=3). The dotted line indicates the control level in colon-26 cells. (B) Western blot analysis of P-gp proteins was performed with crude membranes from colon-26 and DR-colon-26 cells. (C) Colon-26 (open circles) and DR-colon-26 (closed circles) cells were incubated with Rho123 (25 μM) in the absence or presence of VRP (100 μM) for 1, 2, and 3 h. P-gp activity was estimated by subtracting the Rho123 uptake in the absence of VRP from the uptake in the presence of VRP. Each value represents the mean±S.E. (n=3). $^{**}p<0.01$, significantly different from the value in colon-26 cells. (D) Colon-26 (open circles and solid line) and DR-colon-26 (closed circles and dotted line) cells were incubated with various concentrations of DOX for 48 h. The gray circle in each cell line indicates the % of the value without DOX treatment. Cell viability was estimated using an XTT assay following the treatment, and the fitting-curve was calculated using the Hill equation. Each value represents the mean±S.E. (n=3).

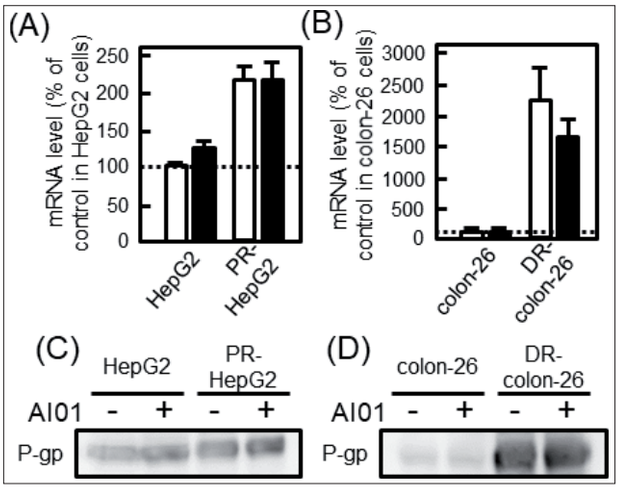

Fig. 2: Effect of AI01 on P-gp expression in wild type and MDR cell lines. The mRNA expression of human MDR1 (A) and mouse mdr1b (B) were measured by real-time PCR analysis in HepG2, PR-HepG2, colon-26 and DR-colon-26 cells treated without (control; open columns) or with AI01 (30 μg/mL; closed columns) for 96 h. Relative levels of each target gene mRNA were determined following normalization to GAPDH mRNA. Each value represents the mean±S.E. (n=3). The dotted lines indicate the control levels in HepG2 and colon-26 cells. (C, D) Western blot analysis of P-gp protein was performed using crude membranes from each cell line treated without (control) or with AI01 (30 μg/mL) for 96 h.

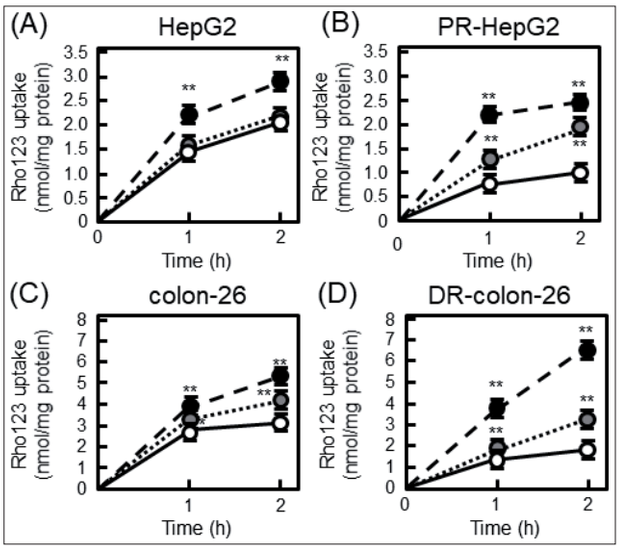

Fig. 3: Effect of AI01 on Rho123 uptake in HepG2, PR-HepG2, colon-26, and DR-colon-26 cells. HepG2 (A), PR-HepG2 (B), colon-26 (C), and DR-colon-26 (D) cells were incubated with Rho123 (50 μM) in the absence (control; open circles) or presence of AI01 (30 μg/mL; gray circles, 100 μg/mL; closed circles) for 1 and 2 h. Each value represents the mean±S.E. (n=3). $^{*}p<0.05$, $^{**}p<0.01$, significantly different from each control.

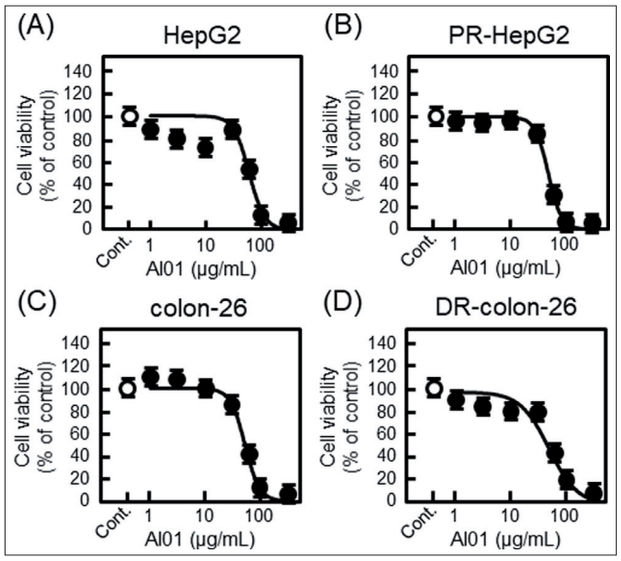

Fig. 4: Effect of AI01 on the viability of HepG2, PR-HepG2, colon-26, and DR-colon-26 cells. HepG2 (A), PR-HepG2 (B), colon-26 (C), and DR-colon-26 (D) cells were incubated with each serum-free culture medium in the absence (control; open circle) or presence of various concentrations of AI01 (closed circles) for 48 h. The cell viability was estimated using an XTT assay following treatment, and the fitting-curve was calculated using the Hill equation. Each value represents the mean±S.E. (n=3).

Table 1: Effect of AI01 on viabilities of HepG2, PR-HepG2, colon-26, and DR-colon-26 cells

Cells	IC_{50} (μg/mL)
HepG2	59.53 ± 5.91
PR-HepG2	56.85 ± 1.64
colon-26	52.16 ± 1.91
DR-colon-26	54.64 ± 4.89

IC_{50} values were calculated by fitting the data in Fig. 4 to Hill's equation.

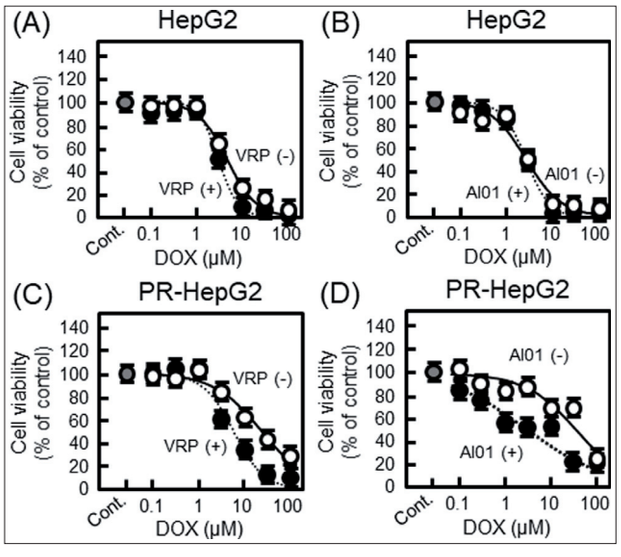

Fig. 5: Effect of AI01 on the sensitivity of HepG2 and PR-HepG2 cells to DOX. HepG2 (A, B) and PR-HepG2 (C, D) cells were incubated with various concentrations of DOX without (open circles and solid lines) or with VRP (10 μM; closed circles and dotted lines) (A, C) and AI01 (30 μg/mL; closed circles and dotted lines) (B, D) for 48 h. The gray circle in each cell line indicates the % of the value without DOX treatment. Cell viability was estimated using an XTT assay following treatment, and the fitting-curve was calculated using the Hill equation. Each value represents the mean±S.E. (n=3).

Table 2: Effect of VRP and AI01 on sensitivities of HepG2, PR-HepG2, colon-26, and DR-colon-26 cells to DOX

Cells	Conditions	DOX IC_{50} (μM)	Ratio
HepG2	Control	4.62 ± 0.20	1.5
	VRP (10 μM)	3.15 ± 0.21	
	Control	2.62 ± 0.28	1.0
	AI01 (30 μg/mL)	2.68 ± 0.16	
PR-HepG2	Control	22.55 ± 2.37	4.0
	VRP (10 μM)	5.60 ± 0.65	
	Control	34.41 ± 7.80	8.5
	AI01 (30 μg/mL)	4.04 ± 1.00	
colon-26	Control	0.38 ± 0.03	2.2
	VRP (10 μM)	0.17 ± 0.01	
	Control	0.51 ± 0.05	4.3
	AI01 (30 μg/mL)	0.12 ± 0.01	
DR-colon-26	Control	4.82 ± 0.32	12.4
	VRP (10 μM)	0.39 ± 0.01	
	Control	10.70 ± 2.71	15.7
	AI01 (30 μg/mL)	0.68 ± 0.15	

IC_{50} values were calculated by fitting the data in Fig. 5, 6 to Hill's equation. Each ratio was calculated by $IC_{50,\ control}/IC_{50,\ VRP\ or\ AI01}$.

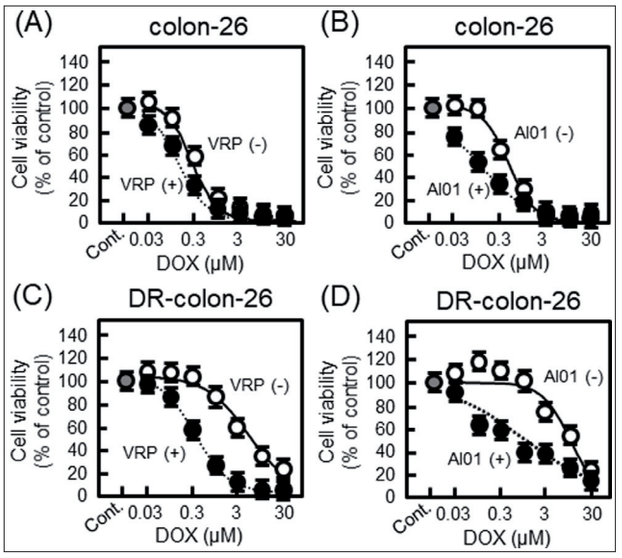

Fig. 6: Effect of AI01 on the sensitivity of colon-26 and DR-colon-26 cells to DOX. Colon-26 (A, B) and DR-colon-26 (C, D) cells were incubated with various concentrations of DOX without (open circles and solid lines) or with VRP (10 μM; closed circles and dotted lines) (A, C) and AI01 (30 μg/mL; closed circles and dotted lines) (B, D) for 48 h. The gray circle in each cell line indicates the % of the value without DOX treatment. Cell viability was estimated using an XTT assay following treatment, and the fitting-curve was calculated using the Hill equation. Each value represents the mean±S.E. (n=3).

2.2. Effects of AI01 on P-gp expression in wild type and MDR cell lines

We further examined whether 30 μg/mL AI01 induces P-gp expression in each wild type cell line, as well as in the MDR cells. Real-time PCR analysis revealed that the mRNA expression of human MDR1 was not affected by long-term (96 h) treatment of HepG2 and PR-HepG2 cells with 30 μg/mL AI01 (Fig. 2A). Similarly, AI01 had no effect on the expression of mouse mdr1b (Fig. 2B) or mdr1a (data not presented) in colon-26 and DR-colon-26 cells. The mRNA expression of MDR1 and mdr1b in MDR cell lines was higher than that in each wild type cells (Figs. 2A, B).

The effect of 30 μg/mL AI01 (96 h) treatment on the protein expression of P-gp in wild type and MDR cell lines was examined

by western blotting. As presented in Figs. 2C and D, the protein levels of P-gp were not altered following AI01 treatment in any of the cell lines. In addition, P-gp expression was higher in multi-drug resistant cell lines than in each wild type cell line, which was comparable to the results obtained for mRNA expression.

2.3. Effect of AI01 on P-gp function in HepG2, PR-HepG2, colon-26, and DR-colon-26 cells

Using HepG2, PR-HepG2, colon-26, and DR-colon-26 cells, the effect of AI01 at concentrations of 30 and 100 µg/mL on the accumulation of Rho123 was examined. As presented in Fig. 3, although the Rho123 uptake in HepG2 cells was not affected by 30 µg/mL AI01, 100 µg/mL AI01 significantly increased Rho123 uptake in HepG2, PR-HepG2, colon-26, and DR-colon-26cells, and P-gp inhibitory effect of AI01 in the MDR cell lines was stronger than that in the wild-types.

2.4. Effect of AI01 on the viability of HepG2, PR-HepG2, colon-26, and DR-colon-26 cells

The effect of 48-h treatment with various concentrations of AI01 on the viability of HepG2, PR-HepG2, colon-26, and DR-colon-26 cells was examined using an XTT assay. As presented in Fig. 4, AI01 decreased the viability of all the cells in a concentration-dependent manner. Calculated IC_{50} values are summarized in Table 1. Conversely, at a concentration of 30 µg/mL, AI01 had no significant effect on the viability of the cells, indicating that a 48-h treatment with 30 µg/mL AI01 would not have a cytotoxic effect on these cell lines. Therefore, the following experiments used 30 µg/mL AI01, which had no cytotoxic effects.

2.5. Efficacy of AI01 in overcoming MDR in HepG2, PR-HepG2, colon-26, and DR-colon-26 cells

We subsequently examined the effect of VRP (10 µM) and AI01 (30 µg/mL) on DOX-induced cytotoxicity in HepG2, PR-HepG2, colon-26, and DR-colon-26 cells. In HepG2 cells, VRP and AI01 had no effect on the sensitivity of the cells to DOX (Figs. 5A, B). On the other hand, both P-gp modulators markedly increased the sensitivity of PR-HepG2 cells to DOX (Figs. 5C, D). Calculated $IC_{50,\ control}/IC_{50,\ VRP}$ and $IC_{50,\ control}/IC_{50,\ AI01}$ were 1.5 and 1.0 in HepG2 cells, and 4.0 and 8.5 in PR-HepG2 cells, respectively (Table 2). In colon-26 and DR-colon-26 cells, AI01 increased DOX sensitivity of the cells (Fig. 6), with $IC_{50,\ control}/IC_{50,\ VRP}$ and $IC_{50,\ control}/IC_{50,\ AI01}$ values of 2.2 and 4.3 in colon-26 cells, and 12.4 and 15.7 in DR-colon-26 cells, respectively (Table 2).

3. Discussion

Using PR-HepG2 cells, we identified several Thai plant extracts with the ability to overcome P-gp-mediated MDR in cancer cells. Due to the enhanced expression and function of P-gp in PR-HepG2 cells, they are a useful model for evaluating resistance to P-gp substrate anti-cancer drugs (Takano et al. 2009). Conversely, anti-cancer drugs, including PTX and DOX, are used for the treatment of various types of cancer, thus it is important to use various MDR cell lines to evaluate the P-gp modulating effects of candidate chemosensitizers. As presented in Figs. 1A and B, we could successfully obtain P-gp-over-expressing colon-26 cells by treating the wild type cells with low concentrations of DOX. In addition, the P-gp activity in DR-colon-26 cells was markedly high, when compared with that in colon-26 cells (Fig. 1C). Furthermore, the DOX sensitivity of DR-colon-26 cells was markedly lower than that of colon-26 cells, and the DOX IC_{50} values in colon-26 and DR-colon-26 cells were 0.3 and 10.3 µM, respectively (Fig. 1D). These results indicate that the efflux of DOX due to the overexpression of P-gp in DR-colon-26 cells is primarily responsible for the resistance of the cells to DOX. On the other hand, Suzuyama et al. (2007) demonstrated that the IC_{50} value of VRP to the transport of daunorubicin, a P-gp substrate, in human MDR1-transfected LLC-PK_1 cells was 8.9 µM, while that in mouse mdr1b-transfected cells was 15.0 µM. In addition, Tang-Wai et al. (1995) clarified that DOX sensitivity of human MDR1-transfected Chinese hamster LR73 cells is about 2-fold higher than that of mouse mdr1-transfected LR73 cells. Therefore, although DR-colon-26 cells would be useful to evaluate P-gp modulating effect of the candidates and applicable for *in vivo* studies as described above, attention should be paid to species differences when comparing P-gp modulating effect of a certain compound in human MDR1 and mouse mdr1 expressing cells.

The interactions of various compounds with P-gp have been categorized as inhibitor, substrate, or inducer (Srivalli and Lakshmi 2012). XR9576 is a third-generation P-gp modulator that non-competitively inhibits P-gp efflux mechanisms by binding to an allosteric modulatory site of P-gp (Martin et al. 2000), indicating that XR9576 exhibits only P-gp-inhibitory effects. By contrast, certain herbal medicines and their components have been known to increase P-gp expression and activity (Dan et al. 2010; Wu et al. 2016). If chemosensitizers have a P-gp inducing effect, this may lead to reductions in the pharmacological effects of P-gp substrate anti-cancer drugs. Therefore, we examined whether 30 µg/mL AI01 induces P-gp expression in PR-HepG2 and DR-colon-26 cells; the expression of mRNA (human MDR1 and mouse mdr1b) and P-gp protein was not observed to be affected following 96 h of AI01 treatment (Fig. 2). Considering that AI01 exhibited no P-gp-altering effect, *A. indica* may be a promising source of chemosensitizers with P-gp-inhibitory effect.

Regarding the traditional medicinal uses of *A. indica*, treatable ailments depend on the parts of *A. indica* used. For example, the bark has been used as analgesic and an antipyretic, while the leaf has therapeutic effects against leprosy, skin ulcers, and cancer (Paul et al. 2011). We examined the effects of 50% ethanol extracts from the bark, fruit, and leaf of *A. indica* (200 µg/mL) on Rho123 uptake in PR-HepG2 cells. Notably, the extract from *A. indica* bark AI01 increased Rho123 uptake, while the extracts from other parts of the plant had no effect on P-gp function in PR-HepG2 cells (data not presented). Accordingly, we focused on the bark of *A. indica*, and examined the effect of AI01 on P-gp function in HepG2, PR-HepG2, colon-26, and DR-colon-26 cells. AI01 increased Rho123 uptake in MDR cell lines in a concentration-dependent manner (Fig. 3), although Rho123 uptake in HepG2 cells was not increased by 30 µg/mL AI01. To the best of our knowledge, this is the first study to demonstrate that AI01 has a potent P-gp inhibitory effect in MDR cell lines.

An ideal P-gp modulator is the one that is non-toxic. When HepG2, PR-HepG2, colon-26, and DR-colon-26 cells were treated with various concentrations of AI01 for 48 h, the extract exhibited a concentration-dependent inhibitory effect on the viability of PR-HepG2 and DR-colon-26 cells (Fig. 4). As summarized in Table 1, similar IC_{50} values of AI01 in each cell line were obtained. Considering that there was no difference in IC_{50} values between MDR cell lines and the wild-type cell lines, toxic components in *A. indica* would not be P-gp substrates. Therefore, the constituents having P-gp inhibitory effect in *A. indica* may be considered as non-toxic substances. On the other hand, AI01 at a concentration of 30 µg/mL, which significantly increased Rho123 uptake, had no cytotoxic effect in either cell lines, indicating that 30 µg/mL AI01 could inhibit P-gp without associated cytotoxicity. Manosroi et al. (2014) demonstrated that the methanolic extract of *A. indica* seeds and roots had potent cytotoxic effects in HL60 (leukemia), A549 (lung), AZ521 (stomach), and SK-BR-3 (breast) cancer cell lines, with IC_{50} values of around 10 µg/mL, while the bark extract exhibited no cytotoxic effect in these cells. Moreover, *A. indica* leaves have been known to have potent apoptoic effects on cancer cells (Kumar et al. 2006) as described in the introduction. These findings indicate that the cytotoxic activity of *A. indica* bark may be low compared with other parts of *A. indica*.

As AI01 at a concentration of 30 µg/mL exhibited a potent P-gp-inhibitory effects with no associated cytotoxicity or P-gp induction, we examined the effect of AI01 on the sensitivity of HepG2, PR-HepG2, colon-26, and DR-colon-26 cells to DOX (Fig. 5, 6). AI01 markedly potentiated DOX-induced cytotoxicity in MDR cell lines, as illustrated by the decreased DOX IC_{50} values when each cell line was co-treated with AI01. Thus far, we have identified various Thai plant extracts with potent P-gp-inhibitory effects using the PR-HepG2 cell line. Furthermore, the 80% ethanol extract of *Ancistrocladus tectorius* (Kawami et al. 2010) and the 50% ethanol extract from *Curcuma marginata* (Takano et al. 2014) enhanced the

sensitivity of PR-HepG2 cells to PTX by ~3-fold. In this study, AI01 potentiated the DOX sensitivity of PR-HepG2 and DR-colon-26 cells by 8.6- and 15.7-fold, indicating that AI01 may have the highest chemosensitizing effect among the extracts previously investigated.

The specificity of AI01 for P-gp should be considered for evaluating P-gp modulating effect. In the case of HepG2 and PR-HepG2 cells, Rho123 uptake for 2 h was significantly increased by 30 μg/mL AI01 in PR-HepG2 cells (Fig. 3B), but not in HepG2 cells (Fig. 3A). In addition, $IC_{50,\ control}/IC_{50,\ AI01}$ values in HepG2 and PR-HepG2 cells were calculated to be 1.0 and 8.5, respectively (Table 2), indicating that P-gp inhibition by AI01 would contribute to the enhancement of DOX sensitivity. Furthermore, $IC_{50,\ control}/IC_{50,\ VRP}$ values in both cells was calculated to be 1.5 and 4.0, respectively (Table 2). In the case of colon-26 and DR-colon-26 cells, Rho123 uptake increased by AI01 was high in DR-colon-26 cells than colon-26 cells (Figs. 3C, D). Considering $IC_{50,\ control}/IC_{50,\ AI01}$ values of 4.3 in colon-26 cells and 15.7 in DR-colon-26 cells, P-gp inhibitory effect of AI01 may be associated with DOX sensitivity in these cell lines. Furthermore, $IC_{50,\ control}/IC_{50,\ VRP}$ in colon-26 and DR-colon-26 cells were calculated to be 2.2 and 12.4 (Table 2). These results suggest that chemosensitizing effect of AI01 as well as VRP would be due to P-gp suppression in PR-HepG2 and DR-colon-26 cells. However, $IC_{50,\ control}/IC_{50,\ AI01}$ values in MDR cell lines seem to be high compared to $IC_{50,\ control}/IC_{50,\ VRP}$ values, indicating that AI01 may have other effects than P-gp inhibition. For example, it has been shown that meloxicam-induced downregulation of multidrug resistant protein (MRP) 1 and MRP4, members of ABC transporters, increases the accumulation of DOX in A549 cells (Chen et al. 2015), indicating that DOX can be also a substrate for MRPs. Further studies should be needed to clarify the effect of AI01 on MRPs function.

The chemical constituents of *A. indica* are diverse and >300 phytochemicals have been isolated and characterized (Patel et al. 2016). Among them, azadirachtin and nimbolide, limonoids from *A. indica* that have been extensively studied for their anti-cancer properties, exhibited inhibitory effects on the development of 7,12-dimethylbenz(a)anthracene-induced hamster buccal pouch carcinogenesis that were mediated by multiple mechanisms, including the prevention of procarcinogen activation and oxidative DNA damage, the upregulation of antioxidants, and the inhibition of tumor invasion and angiogenesis (Priyadarsini et al. 2009). Conversely, constituents of *A. indica* with P-gp-inhibitory and/or chemosensitizing effects have yet to be reported. Our findings strongly indicate that AI01 includes these compounds, and exhibits potent P-gp-inhibitory effects with no cytotoxicity. To identify the active ingredients, further studies are required.

In conclusion, based on the uptake and cytotoxicity studies using PR-HepG2, DR-colon-26, and their wild-type cells, AI01 was determined to have P-gp-mediated MDR reversing effects. In addition, 30 μg/mL AI01, which exhibited significant P-gp-inhibitory effects in multidrug-resistant cell lines, had no cytotoxic or P-gp-inducing effects in these cell lines.

Although AI01 (*A. indica*) has previously been reported to have various pharmacological effects, this is the first study to demonstrate the potent P-gp-inhibitory and chemosensitizing effects of AI01. These results suggest that the bark of *A. indica* may be a promising source for a novel P-gp modulator that is safe and non-toxic.

4. Experimental

4.1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from MP Biomedicals, LLC (Santa Ana, CA, USA). RPMI-1640 medium, EDTA and penicillin-streptomycin were purchased from Thermo Fisher Scientific Inc., (Waltham, MA, USA). Rho123 was purchased from Kanto Chemical Co., Inc., (Tokyo, Japan). DOX, XTT, and phenazine methosulfate (PMS) were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). VRP was purchased from Nacalai Tesque, Inc., (Kyoto, Japan). All other chemicals used were of analytical grade.

4.2. Cell culture

Colon-26, a murine colon-adenocarcinoma cell line, and HepG2 cells were provided by RIKEN BioResource Center (Tsukuba, Japan). PR-HepG2 cells were cultured as reported previously (Takano et al. 2009). DR-colon-26 cells were established by treating the wild type cell line with RPMI-1640 medium containing 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and DOX (30, 100 and 300 nM). The concentration of DOX was increased sequentially, and the final concentration for cell maintenance was 300 nM. PR-HepG2 and DR-colon-26 cells were maintained at 37 ºC in a humidified atmosphere containing 5% CO_2.

4.3. A. indica extract

For AI01 preparation, 25 g dried *A. indica* bark was macerated in 250 mL of 50% ethanol for 7 days at room temperature. The evaporated fraction was then dissolved in DMSO at a concentration of 100 mg/mL. The AI01 were stored at -20 ºC until use.

4.4. Uptake of Rho123 in HepG2, PR-HepG2, colon-26, and DR-colon-26 cells

Following removal of the culture media, HepG2, PR-HepG2, colon-26, and DR-colon-26 cells were washed with phosphate-buffered saline (PBS; 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) supplemented with 5 mM D-glucose (PBS-G) and pre-incubated at 37 ºC for 15 min. The cells were then incubated with 50 μM Rho123 in the presence or absence of AI01 (30 and 100 μg/mL) at 37 ºC for 60 and 120 min, and then rinsed three times with ice-cold PBS. The uptake of Rho123 was estimated using a previously reported procedure (Kawami et al. 2010).

4.5. Cytotoxicity evaluated using an XTT assay

An XTT assay was performed as described previously (Kawami et al. 2010). Briefly, after HepG2, PR-HepG2, colon-26, and DR-colon-26 cells were cultured for 24 h, the medium was replaced with serum-free DMEM or RPMI-1640 containing various concentrations (1, 3, 10, 30, 60, 100, and 300 μg/mL) of AI01 (cytotoxic effect of AI01), or various concentrations of DOX with or without 10 μM VRP or 30 μg/mL AI01 (chemosensitizing effect of AI01), and the cells were incubated at 37 ºC for 48 h. Subsequently, 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. After the cells were incubated at 37 ºC for 30 min, the amount of orange formazan dye produced was quantitated by measuring the absorbance at a wavelength of 490 nm with a MultiSkan™ GO Spectrophotometer (Thermo Fisher Scientific, Inc.). Curve fitting to the rate of cell viability and estimation of the IC_{50} values were performed by nonlinear regression analysis, using the Hill equation.

4.6. mRNA expression analyzed by real-time PCR

Total RNA was extracted from HepG2, PR-HepG2, colon-26, and DR-colon-26 cells using a High Pure RNA Isolation Kit (Roche Diagnostics, Laval, QC). The total RNA was reverse transcribed into cDNA using ReverTra Ace (TOYOBO, Osaka, Japan). Real-time PCR was performed with the CFX Connect™ Real-Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the KAPA SYBR® FAST qPCR Kit (NIPPON Genetics, Tokyo, Japan), according to the manufacturer's instructions. The primer sequences were as follows: Human MDR1 sense, 5'-CCCATCATTG-CAATAGCAGG-3' and antisense, 5'-TGTTCAAACTTCTGCTCCTGA-3'; human GAPDH sense, 5'-ACGGGAAGCTTGTCATCAAT-3' and antisense, 5'-TGGACTC-CACGACGTACTCA-3'; mouse mdr1b sense, 5'-ACCAAGCGAGTCCGATACA-3' and antisense, 5'-TCCCGAGGTTTGCTACATTC-3'; mouse GAPDH sense, 5'-CGTG-CCGCCTGGAGAAACCTG-3' and antisense, 5'-AGAGTGGGAGTTGCTGTT-GAAGTCG-3'. The expression levels of the mRNA were normalized to that of GAPDH mRNA, a housekeeping gene.

4.7. Protein expression analyzed by western blotting

Whole cell lysates derived from PR-HepG2 and DR-colon-26 cells were used for western blot analysis, which was performed as described previously (Takano et al. 2016). The primary antibody used in this study was an anti-P-gp mouse monoclonal antibody (C219; 1:50 dilution) (Calbiochem; EMD Millipore, Billerica, MA, USA), and the secondary antibody was a peroxidase-conjugated antibody to mouse IgG (KPL 474-1806; 1:2000 dilution) (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA).

4.8. Statistical analysis

Data are expressed as the mean±standard error of the mean (S.E.). All statistical analyses were performed using Tukey's test for multiple comparisons. The level of significance was set at $*p < 0.05$ or $**p < 0.01$.

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Conflicts of interest: None declared.

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