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Rhinacanthin-C enhances chemosensitivity of breast cancer cells via the downregulation of P-glycoprotein through inhibition of Akt/NF-kappa B signaling pathway

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ABSTRACT

Introduction: High expression of P-glycoprotein (P-gp) has been linked to multidrug resistance (MDR) and chemotherapeutic failure. Previously, we demonstrated that rhinacanthin-C, a naphthoquinone from *Rhinacanthus nasutus*, was able to enhance the cytotoxicity of doxorubicin against breast cancer MCF-7 cells via direct P-gp inhibition. In this study, we looked at its effect on P-gp downregulation and the mechanism involved in the resistance of MCF-7 cells to doxorubicin.

Methods: Doxorubicin-resistant MCF-7 (MCF-7/DOX) cells were exposed to rhinacanthin-C for 24-48 hours prior to the assessment of their chemosensitivity via MTT assay, P-gp activity via calcein-AM uptake assay, P-gp expression, and signaling via qRT-PCR and western blot analyses.

Results: Pretreatment with 1 μM of rhinacanthin-C for 48 hours significantly enhanced cytotoxicity of doxorubicin, as well as camptothecin and etoposide, to MCF-7/DOX cells. In the rhinacanthin-C-treated cells, reduction of MDR1 mRNA and P-gp levels and increased intracellular calcein were observed. Moreover, phosphorylation of Akt, NF-κB and IκB-α, along with YB-1 expression, significantly decreased after 24-hour treatment with rhinacanthin-C. In contrast, the naphthoquinone had no effect on expression levels of ERK1/2 and phosphorylated ERK1/2 under similar conditions.

Conclusion: Rhinacanthin-C, at a non-cytotoxic concentration (1 μM), could downregulate P-gp expression in MCF-7/DOX cells via the inhibition of the Akt/NF-κB signaling pathway and YB-1 expression. Long-term exposure to this natural naphthoquinone may increase chemosensitivity of cancer cells with MDR phenotype.

Implication for health policy/practice/research/medical education:
The present study revealed the suppressive activity of rhinacanthiin-C on P-gp overexpression, which suggested its potential use as a chemosensitizer in cancer therapy.

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Introduction

Multidrug resistance (MDR) to cytotoxic drugs has been a major cause of chemotherapeutic failure in cancer patients (1,2). The development of MDR in cancer cells can result from high expression of drug efflux transporters such as P-glycoprotein (P-gp), which is encoded by *MDR1* (or *ABCB1*) gene (3). These proteins actively extrude their drug substrates, such as anticancer agents, out of the cells and consequently lessen the therapeutic efficacy. Suppression of P-gp activity might prevent the drug resistance of cancer cells and increase the efficacy of chemotherapy.

Downregulation of P-gp expression in cancer cells can be a target for the reversal of MDR to chemotherapy. High levels of P-gp expression in the doxorubicin-resistant cells with MDR phenotype, including MCF-7/DOX cells, have been linked to hyperactivation of either MAPK/ERK1/2 or PI3K/Akt/NF-κB signaling pathways

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(4-6). Activation of these signaling pathways increases the activities of certain transcriptional factors related to cellular survival and adaptive responses, particularly NF-kB, YB-1, and AP-1, and has been reported to involve *MDR1* transcription (7-9). Several natural compounds, e.g. asiatic acid, cepharanthine, osthole, and procyanidin, have been demonstrated to suppress the MAPK/ERK1/2 or PI3K/Akt pathway, decrease P-gp expression in drug-resistant cancer cells, and increase their sensitivity to cytotoxic agents (10-13).

Rhinacanthin-C is a major naphthoquinone ester found in *Rhinacanthus nasutus* Kurz (family Acanthaceae), a shrub commonly used in Thai traditional medicine to treat skin diseases, hepatitis, and cancer. Rhinacanthin-C has potential medicinal values as anti-cancer and anti-inflammatory agent (14,15). Its pharmacological activities have been related to its inhibitory effect on the Akt/NF-kB or MAPK/ERK1/2 signaling pathways (16,17). Previously, we demonstrated that rhinacanthin-C enhanced doxorubicin cytotoxicity via direct inhibition of P-gp function in breast cancer MCF-7 and doxorubicin-resistant MCF-7 (MCF-7/DOX) cells (18). However, it is of great interest whether the compound can elicit its MDR reversal effect through the reduction of P-gp expression levels. In the present study, we investigated the chemosensitivity enhancing effect of rhinacanthin-C on doxorubicin-resistant MCF-7 cells (MCF-7/DOX) and its molecular mechanisms.

Materials and Methods

Chemicals and reagents

Rhinacanthin-C was obtained from Dr. Pongpun Siripong (National Cancer Institute, Bangkok, Thailand), which had been isolated from the roots of *R. nasutus*, using the purification and identification processes as previously described (Figure 1) (14). Briefly, *R. nasutus* roots were collected from Prachinburi, Thailand. The plant was identified and a voucher specimen (NCIP No. 0129) was kept at the herbarium of the Natural Product Research Section, Research Division, National Cancer Institute, Bangkok, Thailand. The dried roots were ground and extracted with methanol using a Soxhlet apparatus. The concentrated extract was partitioned with n-hexane, chloroform and methanol, respectively. The chloroform extract was chromatographed on a silica gel column eluted with a gradient of chloroform and methanol to provide 5 subfractions (A-E). Purification of subfraction B on a silica gel column eluted with a gradient of n-hexane and chloroform afforded rhinacanthin-C as a yellow-red oil. Its identification was done by comparing its spectroscopic data with literature values. The purity of rhinacanthin-C was at least 94%. Calcein acetoxymethyl (calcein-AM), (S)- (+)-camptothecin, etoposide, Hank's Balanced Salt Solution (HBSS) and verapamil were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and LY294002 were purchased from Merck (Darmstadt, Germany). Doxorubicin hydrochloride (DOX) was purchased from Abcam (Cambridge, UK). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Invitrogen, Life Technologies (Carlsbad, CA, USA). RPMI-1640 medium and L-glutamine were obtained from Gibco Life Technologies (Grand Island, NY, USA). The ImProm-II™ reverse transcription system was from Promega (Madison, WI, USA). The SsoFast™ EvaGreen® Supermix kit was from Bio-Rad (Berkeley, CA, USA). Mouse monoclonal anti-phosphorylated IκB-α (sc-8404), anti-IκB-α (sc-1643), anti-phosphorylated NF-κB p65 (sc-136548), anti-NF-κB p65 (sc-8008), anti-P-gp (sc-55510), and anti-YB-1(sc-101198) antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Mouse monoclonal anti-phosphorylated ERK, anti-ERK, rabbit monoclonal anti-phosphorylated Akt, and anti-Akt antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal anti-GAPDH and horseradish peroxidase-conjugated anti-(Mouse-IgG) antibody were purchased from Calbiochem (San Diego, CA, USA). Oligonucleotide primers were obtained from Integrated DNA Technologies (Singapore Science Park II, Singapore). Super Signal® West Pico Chemiluminescent Substrate was obtained from Pierce Biotechnology Inc. (Rockford, IL, USA).

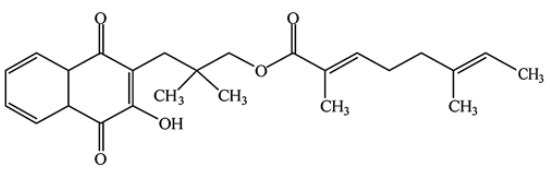

Figure 1. Chemical structure of rhinacanthin-C (RN-C).

Cell culture and treatment

Human breast adenocarcinoma MCF-7 (ATCC® HTB-22™) cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in RPMI-1640 medium containing 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The cells were further developed into the doxorubicin-resistant subline MCF-7/DOX cells by stepwise selection for resistance upon increasing concentrations of doxorubicin (0.01-1.5 μM) (18, 19). MCF-7/DOX cells were maintained in RPMI-1640 complete medium containing 1.5 μM doxorubicin.

One week before the experiments, the medium for MCF-7/DOX cells was replaced by doxorubicin-free RPMI-1640 complete medium. On the day of the experiments, rhinacanthin-C (0.25, 0.5 and 1 μM) or verapamil (60 μM; a positive control) was added to the doxorubicin-free medium for 24 to 48 hours. Then, the cells were washed and harvested for further assessment of their chemosensitivity, P-gp function and expression.

Cell viability measurement

Cell viability was determined by MTT assay. The cells were seeded in 96-well plates (5×10^3 cells/well) overnight and then incubated for 48 h with a cytotoxic agent (i.e. camptothecin, doxorubicin and etoposide) at various concentrations. After washing the cells at the end of treatment period, MTT reagent (0.5 mg/mL) was added and incubated for 4 hours. In viable cells, MTT was enzymatically reduced to formazan dye. The formazan crystals were dissolved with DMSO (100 µL) and quantified spectrophotometrically at 570 nm using a microplate reader (Wallac 1420 VICTOR 3, PerkinElmer Inc., MA, USA). Half maximal inhibitory concentration (IC_{50}) values were estimated by linear regression analysis of the concentration-response curve. The ratio between the IC_{50} values of cytotoxic agents obtained from the rhinacanthin-C-treated cells and those from control cells (Reversal Fold, RF) was calculated and used as a measure of chemosensitivity shift.

Determination of P-gp function

P-gp function was assessed by substrate uptake assay, as described previously (18). Briefly, the cells were seeded onto 24-well plates (2.5×10^5 cells/well) overnight and then incubated with calcein-AM (0.4 µM) in the dark for 60 minutes. At the end of the incubation period, cells were washed and lysed with 1% Triton X-100. Fluorescence intensity of calcein was determined at 485/535 nm (excitation/ emission wavelength) and normalized to the protein content in each sample. The amount of protein was determined using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

The expression level of *MDR1* mRNA was determined by qRT-PCR technique. RNA was isolated from the cells using TRIzol® reagent and reversely transcribed to cDNA using the ImProm-II™ reverse transcription system. The qPCR amplification was performed using the SsoFast™ EvaGreen® Supermix kit. The sense (S) and antisense (AS) primers were *MDR1*, S: 5'-CCCATCATTGCAATAGCAGG-3' and AS: 5'-TGTTCAAACTTCTGCTCCTGA-3'; and GAPDH, S: 5'-AAGGTCGGAGTCAACGGATTTGGT-3' and AS: 5'-ATGGCATGGACTGTGGTCATGAGT-3'. The cycling conditions of qPCR were at 95°C for 2 minutes, followed by 40 cycles of 95°C for 5 seconds, 60°C for 5 seconds. The mRNA content was normalized to that of GAPDH (an internal control) (20).

Western blot analysis

Extents of P-gp and signaling proteins were determined by western blot analysis as described previously (18). An equal amount of protein (30 µg) in each sample was separated by 10% or 12% SDS-PAGE and electrically transferred to a PVDF membrane. Then, the membranes were probed with primary antibodies against P-gp, ERK1/2 and p-ERK1/2, Akt and p-Akt, IκB-α and p-IκB-α, NF-κB and p-NF-κB, YB-1 and GAPDH at the dilution of 1:1000, 4°C, overnight. After that, the blots were incubated with HRP-conjugated secondary antibody (1:2000) and developed with the SuperSignal® West Pico chemiluminescent substrate. The signals were captured using a luminescence-image analyzer (ImageQuant™ LAS 4000, GE Healthcare Bio-sciences, Japan). The density of protein bands was quantified by Image J software (NIH, Bethesda, MD, USA) and normalized to that of GAPDH.

Statistical analysis

Data (mean ± SEM) were obtained from 3-4 separated experiments. Statistical difference was assessed by either the Student's *t*-test or one-way ANOVA with subsequent Tukey's post hoc analysis, where appropriate. $P<0.05$ was considered statistically significant.

Results

P-gp levels in MCF-7/DOX cells

The doxorubicin-resistant MCF-7 (MCF-7/DOX) cells were developed after long-term continuous exposure of MCF-7 cells to doxorubicin. As shown in Figure 2A, the MCF-7/DOX cells were approximately 62-fold more resistant to doxorubicin cytotoxicity than its parental MCF-7 cells. The IC_{50} values of doxorubicin in MCF-7/DOX cells and MCF-7 cells were 155.85 ± 1.04 µM and 2.52 ± 1.55 µM, respectively. Since doxorubicin is a known P-gp substrate, we further assessed the function and expression of P-gp in the doxorubicin-resistant MCF-7/DOX cells compared to parental doxorubicin-sensitive MCF-7 cells. The activity of P-gp as an efflux pump was demonstrated by using calcein-AM, a specific P-gp substrate, in a substrate uptake assay. As shown in Figure 2B, the accumulation of calcein in the resistant MCF-7/DOX cells was approximately 6.32-fold less than that in the sensitive parental cells. These findings correlated well with higher P-gp expression levels observed in the resistant cells than in MCF-7 cells (Figure 2C, D). Hence, a higher degree of doxorubicin resistance of MCF-7/DOX cells could be attributed to their higher P-gp expression levels.

MDR reversal effect of rhinacanthin-C in MCF-7/DOX cells

The MDR reversal effect of rhinacanthin-C was demonstrated after MCF-7/DOX cells were exposed to non-cytotoxic concentrations of this compound for 48 h. Our results showed that the rhinacanthin-C-treated MCF-7/DOX cells were more sensitive to camptothecin, doxorubicin, and etoposide than the untreated MCF-7/DOX cells, and this effect of rhinacanthin-C was concentration-dependent. After exposure to 1 µM rhinacanthin-C, the IC_{50} values of these cytotoxic drugs in

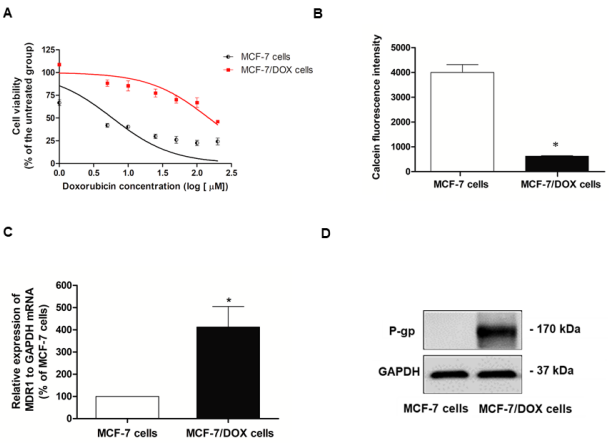

Figure 2. Chemosensitivity, basal activity, and expression of P-gp in MCF-7 and Doxorubicin-resistant MCF-7 (MCF-7/DOX) cells. (A) Cytotoxicity of doxorubicin (DOX) after 48-h exposure. (B) Basal activity of P-glycoprotein (P-gp). The bar graphs represent intracellular accumulation of calcein. (C) Basal expression of *MDR1* mRNA. The bar graphs represent extents of *MDR1* mRNA in relative to those of GADPH and are expressed as the percentage of MCF-7 cells. (D) Immunoblots of P-gp and GAPDH (an internal control). *$P < 0.05$ compared with the MCF-7 group (n=4).

the resistant cells significantly decreased by approximately 1.97 to 2.6-fold (Table 1). Interestingly, rhinacanthin-C (1 μM) could elicit a greater MDR reversal effect than the positive control verapamil (60 μM).

Effects of rhinacanthin-C on MDR1 mRNA and P-gp levels

Camptothecin, doxorubicin, and etoposide are known P-gp substrates. We determined whether, after 48-hour exposure, rhinacanthin-C could reverse the resistance to these cytotoxic compounds via the reduction of P-gp expression in MCF-7/DOX cells. As shown in Figure 3A-C, rhinacanthin-C could reduce the levels of *MDR1* mRNA and P-gp expression in a concentration-dependent manner. Extents of *MDR1* mRNA and P-gp in the MCF-7/DOX cells after exposure to 1 μM rhinacanthin-C for 48 hours were significantly decreased by 84.8-fold and 35.5-fold, respectively, compared to untreated control. These findings correlated well with decreased P-gp activity in the treated cells, as measured by substrate accumulation assay (Figure 3D). When the concentrations of rhinacanthin-C were increased to 1 μM, calcein content in the treated cells increased significantly (3.4-fold compared to untreated group) in concentration-dependent manner. Our results indicated that the rhinacanthin-C-treated cells had lower P-gp activity than the untreated cells. This suppressive effect on P-gp function was also greater than that of the positive control verapamil, a known suppressive agent for P-gp expression.

Effects of rhinacanthin-C on the PI3K/Akt/ NF-κB signaling pathway and YB-1 expression

To determine whether the PI3K/Akt/NF-κB signaling pathway was involved in rhinacanthin-C-mediated *MDR1*/P-gp downregulation, we measured the expression levels of Akt and NF-κB, as well as their phosphorylated forms, in MCF-7/DOX cells after 24-hour treatment. Compared with the untreated group, the levels of phosphorylated Akt significantly decreased in the cells treated with 0.5 and 1 μM rhinacanthin-C (Figure 4A). The naphthoquinone could also inhibit the activity of NF-κB, which is the well-known downstream effector of Akt signaling cascade. As shown in Figure 4B, 24-hour treatment with rhinacanthin-C at 0.5 and 1 μM significantly reduced phosphorylation of NF-κB and IκB-α. These effects on the activities of Akt/NF-κB signaling pathway were concentration-dependent.

Furthermore, we studied the effect of 24-hour treatment with rhinacanthin-C on YB-1 promoter in MCF-7/DOX cells. YB-1 is another downstream transcription factor in the PI3K/Akt signaling pathway, which regulates chemosensitivity and P-gp expression in cancer cells (7,10). Compared with untreated group, 1 μM rhinacanthin-C could significantly reduce YB-1 expression level (Figure 4C).

We also confirmed that the suppression of PI3K/Akt signaling pathway led to the inhibition of NF-κB and YB-1 activities in MCF-7/DOX cells. Treatment of these

Table 1. Chemosensitivity shift of MCF-7/DOX cells after 48-h exposure to rhinacanthin-C or verapamil[a]

Groups	Camptothecin		Etoposide		Doxorubicin	
	IC_{50} (μM)	Reversal fold[b]	IC_{50} (μM)	Reversal fold[b]	IC_{50} (μM)	Reversal fold[b]
Untreated	0.99 ± 1.06		893.40 ± 1.87		155.84 ± 1.04	
Rhinacanthin-C 0.25 μM	0.81 ± 1.54	1.22	737.66 ± 2.55	1.21	102.77 ± 1.51*	1.51
Rhinacanthin-C 0.5 μM	0.92 ± 0.29	1.07	727.68 ± 1.85	1.22	89.79 ± 2.57*	1.73
Rhinacanthin-C 1.0 μM	0.47 ± 0.37*	2.10	452.31 ± 0.65*	1.97	59.61 ± 1.10*	2.61
Verapamil 60 μM	0.60 ± 0.98*	1.65	801.99 ± 2.76	1.11	81.59 ± 0.40*	1.91

Data are expressed as means ± S.E.M. (n = 4). *$P < 0.05$ compared with the untreated group.

[a] The cells were treated with either rhinacanthin–C or verapamil for 48 h prior to determination of cell viability in the presence of a cytotoxic agent (i.e. camptothecin, doxorubicin, and etoposide) at various concentrations.

[b] Reversal fold (RF) was calculated from the ratio between the IC50 value of each cytotoxic agent obtained from the rhinacanthin-C-treated (or verapamil-treated) cells and those from untreated cells.

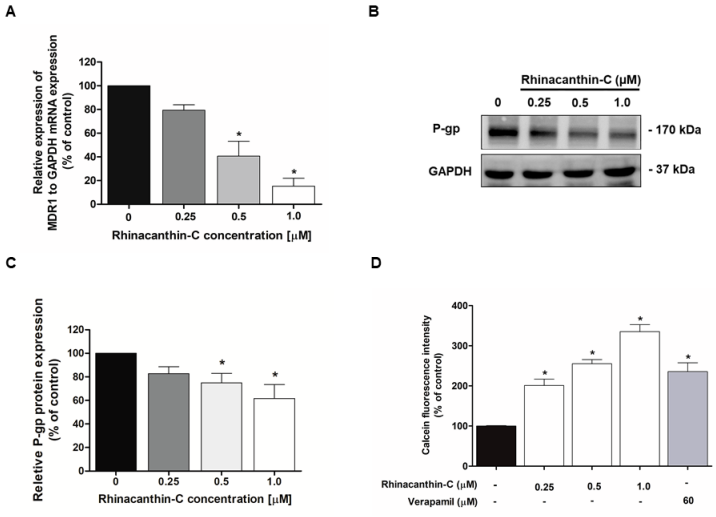

Figure 3. Expression of *MDR1* mRNA and P-glycoprotein (P-gp) protein and its activity in Doxorubicin-resistant MCF-7 (MCF-7/DOX) cells after 48-h treatment with rhinacanthin-C at various concentrations. (A) *MDR1* mRNA, (B) immunoblots of P-gp and GAPDH (an internal control), (C) their densitometrical analysis, and (D) intracellular accumulation of calcein. **P*<0.05 compared with the control (untreated group) (n = 4).

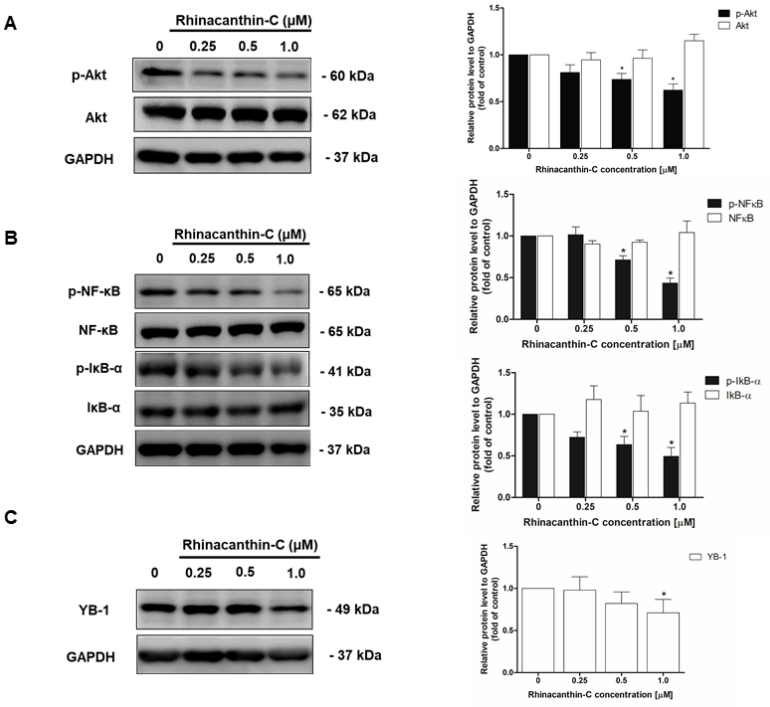

Figure 4. Immunoblots and their densitometrical analysis of (A) Akt and its phosphorylated form, (B) NF-κB, IκB-α and their phosphorylated forms, (C) YB-1 in Doxorubicin-resistant MCF-7 (MCF-7/DOX) cells after 24-h treatment with rhinacanthin-C at various concentrations. **P* < 0.05 compared with the control (untreated group) (n=4).

cells with a known Akt inhibitor, LY294002 (10 μM), for 1 hour significantly decreased phosphorylated Akt (p-Akt), phosphorylated NF-κB (p-NF-κB) and YB-1 protein levels (Figure 5A). Subsequent incubation of LY294002-treated cells with rhinacanthin-C (1 μM) for another 24 h had no additional effects on the expression levels of p-Akt, p-NF-κB, and YB-1 (Figure 5A), suggesting that the suppressive effect of rhinacanthin-C on NF-κB and YB-1 is associated with the inhibition of PI3K/Akt pathway.

Effects of rhinacanthin-C on the MAPKs/ERK1/2 pathway

The MAPKs/ERK1/2 pathway is linked to survival mechanisms and drug resistance in MDR cells (21). We investigated whether 24-hour treatment with

rhinacanthin-C interfered with the MAPK/ERK1/2 pathway in MCF-7/DOX cells. As shown in Figure 5B, rhinacanthin-C (up to 1 μM) did not affect expression levels of either ERK1/2 or its phosphorylated form (p-ERK1/2), compared with the untreated group. This finding suggested that the MDR reversal effects of non-cytotoxic concentrations (up to 1 μM) of rhinacanthin-C in MCF-7/DOX cells did not involve MAPK/ERK1/2 pathway.

Discussion

P-gp-mediated MDR is associated with the loss of chemotherapeutic efficacy in various types of cancer such as breast cancer, ovarian cancer, and leukemia (1,4). Numerous researches in the last decade have focused on the search for novel MDR reversing agents with the ability to suppress P-gp activities. Two classical approaches in the reduction of P-gp-mediated drug extrusion are direct inhibition of P-gp activity and suppression of *MDR1* expression levels in cancer cells (22). Phytochemicals such as cepharanthine, procyanidin, and rhinacanthin-C have been evaluated for their potential MDR reversal effects (18,23,24). Several of these natural compounds are ingredients of herbal medicine or health products with a long history of use.

Rhinacanthin-C is a major bioactive constituent of *Rhinacanthus nasutus*, a medicinal plant native to Thailand as well as South and Southeast Asia countries (14,25). Previously, we demonstrated that at its non-cytotoxic concentrations, rhinacanthin-C could be a promising chemosensitizer through its direct inhibition of P-gp function (18). In the present study, we revealed for the first time that rhinacanthin-C could increase cancer cell sensitivity to cytotoxic anti-cancer drugs via the downregulation of P-gp expression. In this study, doxorubicin-resistant MCF-7 cells (MCF-7/DOX) with MDR1 phenotype, similar to those reported in literatures (19,26), were developed after long term exposure of MCF-7 cells to doxorubicin. These doxorubicin-resistant cells exhibited higher levels of P-gp mRNA, expressed protein and activity. Our MTT assay showed that 48-h exposure to rhinacanthin-C at concentrations lower than 1 μM did not induce cytotoxicity in MCF-7/DOX cells. However, the compound could enhance the cytotoxicity of doxorubicin and other "P-gp substrate" anti-cancer drugs such as camptothecin and etoposide. This observed MDR reversal effect can be strongly correlated to the suppression of P-gp function in the cells as a result of rhinacanthin-C exposure. It should be noted that in this study, the direct inhibitory effect of rhinacanthin-C against P-gp activity was excluded, since the compound was washed out prior to calcein-AM uptake assay. However, a significant reduction of *MDR1* expression at both mRNA and protein levels in the rhinacanthin-C-treated MCF-7/DOX cells was indicative of the loss of P-gp function in these cancer cells. These findings showed that rhinacanthin-C could downregulate P-gp expression at transcription levels and, thus, could reverse P-gp-mediated MDR.

To further investigate the mechanisms by which rhinacanthin-C suppressed *MDR1* expression, we primarily focused on the PI3K/Akt and MAPKs signaling pathways. These two signaling pathways have been linked to the regulation of *MDR1* gene expression in various types of cancer cells, such as breast and ovarian cancer

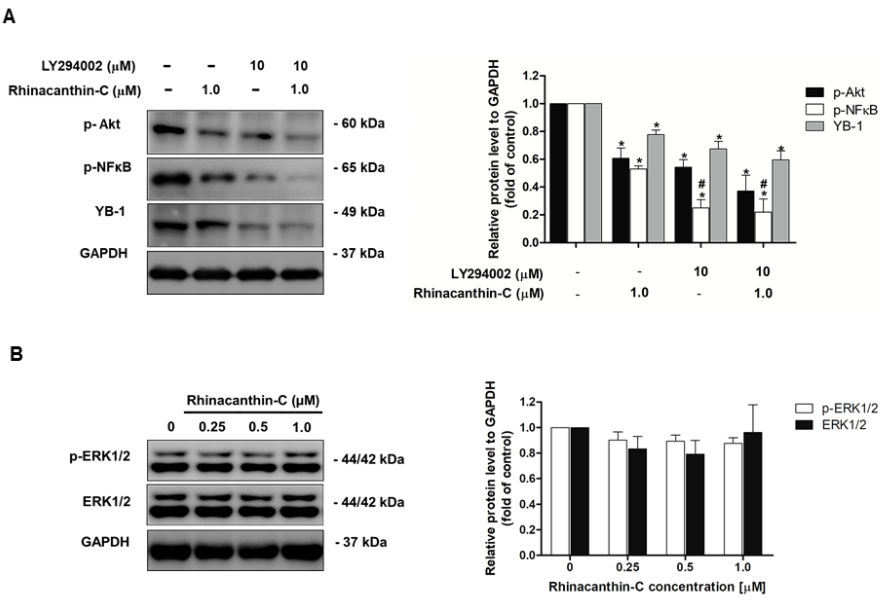

Figure 5. Expression of (A) phosphorylated Akt, phosphorylated NF-κB and YB-1 in the LY294002-treated Doxorubicin-resistant MCF-7 (MCF-7/DOX) cells, and (B) ERK1/2 and phosphorylated ERK1/2 in MCF-7/DOX cells after 24-h exposure to rhinacanthin-C. **P*<0.05 compared with the control (untreated group); #*P*<0.05 compared with rhinacanthin-C-treated group in the absence of LY294002 (n=4).

(5,27). It has been demonstrated that activation of the Akt or MAPK signaling cascades leads to increase activity and nuclear translocation of active transcription factors NF-κB and YB-1 (6,7), which bind to the promoter region of *MDR1* gene in order to initiate the transcription and expression of P-gp (10,22). Moreover, hyperactivation of MAPK/ERK1/2 or Akt/NF-κB pathway, along with transcription factors NF-κB and YB-1, and increased *MDR1* expression were observed in several cancer cells with MDR phenotype resistant to chemotherapeutic agents such as doxorubicin and paclitaxel (26,28,29). Inhibition of the PI3K/Akt or MAPK/ERK1/2 signaling pathway can result in the reduction of P-gp activity and promotion of chemosensitivity of drug-resistant cancer cells to cytotoxic drugs (8,10,30). In this study, we found that 24-hour treatment with rhinacanthin-C could reduce the levels of p-Akt, IκB-α, p-NF-κB, and YB-1 in MCF-7/DOX cells in a concentration-dependent manner, whereas p-ERK1/2 level remained unaffected. Hence, suppression of the PI3K/Akt, but not MAPK/ERK1/2, signaling pathway appeared to be associated with inhibition of NF-κB and YB-1 activities in the resistant MCF-7/DOX cells. It is very likely that rhinacanthin-C downregulated P-gp expression at the transcription level by inhibiting the PI3K/Akt/NF-κB signaling pathway and YB-1 expression. Therefore, the inhibitory effects of rhinacanthin-C on the PI3K/Akt/NF-κB pathway and YB-1 expression were similar to those of specific PI3K/Akt signaling pathway inhibitor LY294002.

Conclusion

Non-cytotoxic concentrations of rhinacanthin-C could downregulate the expression of P-gp at the transcription level in MCF-7/DOX cells, leading to its MDR reversal effect. In addition, rhinacanthin-C-mediated reduction of *MDR1* mRNA may involve inhibition of the PI3K/Akt, but not MAPK/ERK1/2, signaling pathway (Figure 6). The potential clinical application of rhinacanthin-C as a chemosensitizer in anticancer drug regimens should be further explored.

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Authors' contributions

SC: Conceptualization, methodology, data curation, formal analysis, investigation, Original draft preparation. SJ: Conceptualization, data curation, supervision, reviewing and editing, project administration, and funding acquisition.

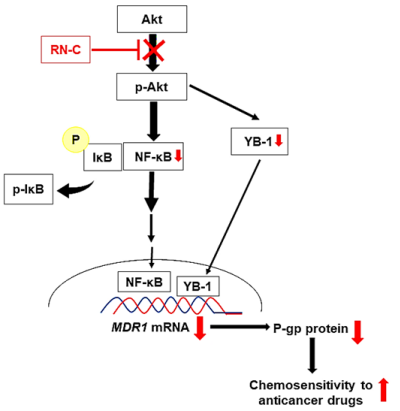

Figure 6. A schematic illustration proposing the mechanism of rhinacanthin-C-mediated downregulation of P-glycoprotein (P-gp) in Doxorubicin-resistant MCF-7 (MCF-7/DOX) cells.

Conflict of interests

Authors declare no conflict of interests.

Ethical considerations

Ethical issues including text plagiarism, misconduct, manipulation or appropriation, data fabrication, falsification, redundant publication as well as duplicate submissions have been carefully observed by authors.

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