Total Saponins from *Paris Forrestii* Reverse Multidrug Resistance of MCF-7/ADM Cells by Suppression of P-gp via ERK Signaling Pathway

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Summary

Our previous study demonstrated that the total saponins from *Paris forristii* (PCT3) had obvious inhibitory effect on the proliferation of Adriamycin-resistant human breast adenocarcinoma cells (MCF-7/ADM), this effect was significantly stronger than that in parental cells (MCF-7). This study was designed to test the reversal effect of PCT3 on MCF-7/ADM cells and to understand its mechanism of action. Results demonstrated that low cytotoxic concentrations of PCT3 (0.3, 1 and 3 µg/ml) reversed resistance of MCF-7/ADM cells to ADM, DDP and 5-FU, with reversal fold of 16.4, 19.5 and 31.7 for ADM, 1.6, 1.4 and 1.4 for DDP, 1.7, 1.8 and 5.6 for 5-FU, respectively. Moreover, PCT3 significantly increased the accumulation of ADM and Rh123 in MCF-7/ADM cells, suggesting that PCT3 may act by affecting the function of drug efflux pump P-gp, which is encoded by MDR1 gene. Both MDR1 gene and P-gp protein expression was downregulated by PCT3 treatment. Further results demonstrated that p38MAPK and ERK pathway was remarkably activated in MCF-7/ADM cells, inhibition of p38 or ERK attenuated P-gp expression. While, only the phosphorylation level of ERK was downregulated by PCT3, indicating that PCT3 sensitized P-gp overexpressed MCF-7/ADM cells to ADM via inhibition of ERK signaling pathway.

**Key words:** Total saponins, *Paris forristii*, MCF-7/ADM, P-gp, ERK signaling pathway
INTRODUCTION

Drug resistance is one of the main reasons for chemotherapeutic failure. Multidrug resistance (MDR) is defined as resistance to one drug accompanied by resistance to other drugs that may have different structures and mechanisms of action.\(^1\) Breast cancer is one of the major malignant tumors that endanger women's health. The main treatments include surgery, radiotherapy, chemotherapy and endocrine therapy. MDR is highly encountered in chemotherapy of breast cancer.

The underlying mechanisms responsible for MDR are complicated and not completely understood. ATP-binding cassette (ABC) transporters are over-expressed in most MDR cancer cells, which act as a pump, pumping a wide range of amphipathic and cationic anticancer drugs out of tumor cells, reducing drug accumulation and resulting in chemotherapeutic failure.\(^2,3\) Research on ABC-transporter-mediated MDR has focused on P-gp.\(^4,5\) Abnormal expression of P-gp is regulated by a variety of factors, including signaling pathways, transcription factors, protein modification and so on. Mitogen-activated protein kinase (MAPK) signaling pathway, including extracellular signal-regulated kinase (ERK), c-Jun NH\(_2\)-terminal kinase (JNK), p38MAPK pathways, is the best characterized signal transduction pathway in cell growth, proliferation, invasion of malignant tumors and resistance to chemotherapeutic drugs. It was reported involving in ABCB1/P-gp-mediated MDR.\(^6\) ABCB1 is a target of the Ras/Raf signaling pathway, suppression of p38, ERK or JNK pathways enhanced sensitivity to chemotherapeutic drugs in multidrug resistant tumor cells.\(^7-10\)

*Paris polyphylla* var. *yunnanensis* (Franch.) Hand.-Mazz. (Melanthiaceae) is a famous traditional Chinese herbal medicine used as antalgic, antipyretic, antibacterial and anti-inflammatory agent.\(^11\) In recent years, it was reported that saponins from *P. polyphylla* var. *yunnanensis* and *P. polyphylla* Sm. exhibit remarkable antitumor activity both *in vitro* and *in vivo*.\(^5,12\) Our previous study showed that total saponins (PCT3) from *P. forrestii* (Takht.) H. Li, a substitute of *P. polyphylla* var. *yunnanensis*, showed stronger cytotoxic activity on a panel of human cancer cells. PCT3
significantly inhibited the proliferation of human breast cancer cells (MCF-7) and Adriamycin (ADM)-resistant human breast adenocarcinoma cells (MCF-7/ADM) with IC\textsubscript{50} values of 10.4 and 5.2 \textmu g/mL. Resistant MCF-7/ADM cells were more sensitive to PCT3, this provoked our interest to explore whether PCT3 could sensitize resistant tumor cells to chemotherapeutic agents. In this study, we focused on reversal activity of PCT3 on MCF-7/ADM cells and its mechanism of action by regulating P-gp and MAPK signaling pathway.

MATERIALS AND METHODS

Preparation of total saponins (PCT3) from \textit{P. forrestii} Total saponins (PCT3) were extracted from the rhizomes of \textit{P. forrestii} as previously described.\textsuperscript{13, 14} Briefly, the air-dried, powdered \textit{P. forrestii} rhizomes (4.4 kg) were exhaustively extracted with 95\% ethanol at room temperature. The ethanolic extract (550 g) was suspended in water and further partitioned with ethyl acetate and \textit{n}-butanol to yield the ethyl acetate-soluble fraction and the \textit{n}-butanol-soluble fraction (PCT3), respectively. The major chemical constituents of PCT3, including polyphillin D (23.21\%), dioscin (20.79\%), ophiopogonin C' (6.51\%), polyphyllin F (4.04\%), formosanin C (2.79\%), and pennogenin-3-O-\textalpha-L-rhamnopyranosyl-(1→2)-[\textalpha-L-rhamnopyranosyl-(1→4)]-\textbeta-D-glucopyranoside (1.85\%), have been reported.\textsuperscript{14}

Cell lines and culture Human breast carcinoma cell line MCF-7 and the ADM-resistant MCF-7/ADM cell line were provided by the cell bank of School of Pharmaceutical Sciences \& Yunnan Key Laboratory of Pharmacology for Natural Products, Kunming Medical University (Kunming, China). Both lines were cultured in RPMI-1640 medium (Biological Industries; Kibbutz Beit-Haemek, USA) supplemented with 10\% NBS (Biological Industries) and 100 U/mL streptomycin/penicillin (Biological Industries) at 37 °C in a humidified atmosphere containing 5\% CO\textsubscript{2}. MCF-7/ADM cells were additionally cultured with 0.5 \mu M Adriamycin to maintain drug-resistance, which was withdrawn two weeks before the experiment.

Cell proliferation assay MCF-7 and MCF-7/ADM cells were seeded in 96-well
plates (8000 cells/well) and incubated with ADM (Hisun Pfizer, USA), Cisplatin (DDP, Nanjing, China) and 5-Fluorouracil (5-FU, Shanghai, China) at 1, 3, 10, 30 and 100 μg/mL for 48 h. The cytotoxicity of ADM, DDP and 5-FU was measured via MTT assay.\textsuperscript{13,14} The OD value of each well was measured using 570 and 630 nm dual wavelength by Microplate Reader (Plus 384; Molecular Devices, USA). The half inhibitory concentration (IC\textsubscript{50}) was calculated by GWBASIC software. The resistance index (RI) was calculated using the following formula: RI=IC\textsubscript{50} of MCF-7/ADM cells / IC\textsubscript{50} of MCF-7 cells.

8×10\textsuperscript{3} MCF-7/ADM cells were planted in 96-well plate for 24 h, then treated with various concentrations of ADM (1, 5, 25, 125, 250 μg/mL), DDP (1, 3, 10, 30, 100 μg/mL) and 5-FU (0.1, 1, 10, 100, 500 μg/mL) in the presence or absence of PCT3 (0.3, 1, 3 μg/mL) or Verapamil (VER, Mei biological, China, 5 μg/mL) for 48 h. Cytotoxicity of each treatment was analyzed via MTT assay. Reversal fold (RF) of PCT3 was calculated as a ratio of IC\textsubscript{50} for treatment without or with PCT3.

**ADM and Rh123 accumulation assay** Cells were seeded in 6-well plate at a density of 4×10\textsuperscript{5} cells/well and cultured for 48 h. After incubation with various concentrations of PCT3 (5, 15, 45 μg/mL) and ADM 10 μg/mL or Rh123 (Mei biological, China) 10 μg/ml at 37 °C for 3 h, cells were washed with PBS 3 times and lysed in 0.3 mol/L hydrochloric acid (Chuandong Chemical Industry, China) of 50% ethanol solution (Xilong Chemical Industry, China). Then cells were put in the refrigerator overnight. Finally, collected the supernatant and immediately determined the mean fluorescence intensity of intracellular ADM or Rh123, using a multifunctional microplate reader (Molecular Devices, SpectraMax M2, USA) with an excitation wavelength of 470 nm, an emission wavelength of 585 nm for ADM and an excitation wavelength of 485 nm, an emission wavelength of 535 nm for Rh123. VER (Mei biological, China) 10 μg/mL was used as positive control. In addition, intracellular fluorescence of the same conditions was examined by fluorescent microscope (Leica produces, DMI3000B, Germany).

**Quantitative real-time PCR assay** The mRNA expression of ABC transporters genes was measured by quantitative real-time polymerase chain reaction (QPCR).
Cells were planted in 6-well plates at density of $4\times10^5$ cells/well and were allowed to attach overnight. After incubation with various concentrations of PCT3 1, 3, 10 µg/mL at 37 °C for 12, 24 and 48 h, total RNA was extracted with TRIzol® Reagent (Tiangen Co., China). The first strand cDNA was synthesized from 2 µg of total RNA using AccuRT Genomic DNA Removal Kit (Biological Materials Inc, Canada). Genes expression was quantified by ABI PRISM® 7500 Real-Time PCR System (Applied Biosystems, USA). β-actin was used as the internal control. Primers sequences were shown in the following table:

The thermocycling regime was 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. The mRNA expression level of each group was calculated as the mean value of the $2^{-\Delta\Delta C_t}$ of three independent tests. $\Delta C_t = C_{t_{MDR1}} - C_{t_{\beta-actin}}$; $-\Delta\Delta C_t = -(\Delta C_t - \Delta C_t_{control})$

**Flow cytometry analysis for membranous P-gp expression** Membranous P-gp expression was also determined by flow cytometry. Cells were treated by 1, 3 and 10 µg/mL of PCT3 and cultivated for 12, 24 and 48 h. VER (10 µg/mL) was used as positive control. MCF-7 and MCF-7/ADM cells were collected and counted. Cells were rinsed and dissolved with ice-cold PBS containing 10% FBS and incubate with 100 µL of the monoclonal anti- (human P-gp) antibody 17F9 (0.5 µg/10^6 cells, BD Pharmingen™, USA) at 4 °C for 20 min in dark. After incubation, cells were washed and resuspended in PBS, incubated with 100 µL FITC-conjugated affinipure goat anti-mouse IgG (H+L) (1:500 dilution, BD Pharmingen™, USA) at 4 °C for 20 min in dark. Purified Mouse IgG2bκ (0.5 µg/10^6 cells, BD Pharmingen™, USA) was used as isotope control. After incubation, cells were washed and resuspended in chilled PBS containing 10% FBS and were analyzed by flow cytometry BD Accuri C6 (USA). All the experiments were repeated three times. Data were analyzed by the Flowjo software.

**Western blot** MCF-7 and MCF-7/ADM cells were seeded in 6-well plates at a density of $4\times10^5$ cells/well. MCF-7 cells were used as a control, MCF-7/ADM cells were incubated with 3 µg/mL PCT3 and 1 µg/mL ADM alone or in combination for 48 h. Harvesting after trypsinisation, cells were treated with 1 × RIPA lysis buffer (50
mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA and protease inhibitors) (Meilunbio, China) to extract the total proteins. An aliquot of proteins from the total cell lysates 20 μg/lane was separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE, Meilunbio, China), wet-transferred to PVDF membrane (Millipore, USA) and blotted with primary antibodies specific for anti-p38 (1:5000 dilution), anti-phospho-p38(T180) (1:1000 dilution), anti-ERK1/2 (1:1000 dilution), anti-JNK1/2/3 (1:1000 dilution) (Abcam, Cambridge, UK); Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (1:2500 dilution), Phospho-SAPK/JNK (Thr183/Tyr185) (1:2500 dilution) (Cell Signaling Technology, Danvers, MA, USA); P-glycoprotein (1:10000 dilution) (Proteintech Group, Inc, Rosemont, USA); anti-GAPDH (1:50000 dilution) (Santa Cruz Biotechnology, USA) was used as a loading control. HRP-conjugated secondary antibody (Proteintech Group, Inc, Rosemont, USA) was added to the preparation. Bands were visualized with the Clarity™ Western ECL Substrate (Bio-rad Laboratories, Inc, USA) and captured by FluorChem E Imaging System (Proteinsimple, USA). The relative level of a protein was quantified by densitometry using ImageJ software, quantity of GAPDH was used as the internal standard.

RESULTS

**MCF-7/ADM cells are multidrug resistant with MDR1 and P-gp overexpression phenotype** In order to determine whether MCF-7/ADM cells are multidrug resistant, MTT assay was performed to detect cytotoxic activity of ADM, DDP and 5-Fu on both MCF-7 and MCF-7/ADM cells. Results showed that the inhibitory activity of all drugs on MCF-7 cells were stronger than that on MCF-7/ADM cells. IC₅₀ values of ADM, DDP, 5-FU on MCF-7 cells were 0.46, 2.28 and 10.30 μg/mL, however, those on MCF-7/ADM cells were 140.04, 4.55 and 78.57 μg/mL (Table 2). The resistance index (RI) to ADM, DDP and 5-FU was 304.43, 2.00 and 7.63, respectively. This indicated that MCF-7/ADM cells were multidrug-resistant, which were highly resistant to ADM, moderately resistant to 5-Fu and slightly resistant to DDP.
P-gp overexpression is a characteristic phenotype of MDR cells. Therefore, we measured MDR1 mRNA and P-gp expression by QPCR and flow cytometry. Results demonstrated that MDR1 mRNA was 4862.93 fold higher in MCF-7/ADM cells compared with MCF-7 cells (Fig. 1A). MCF-7 cells showed a positive P-gp expression of 2.40%, while that was 91.43% in MCF-7/ADM cells (Fig. 1B), indicating a great increase of P-gp expression in MDR cells.

**PCT3 reverses drug resistance of MCF-7/ADM cells**  Our previous results exhibited that PCT3 inhibited proliferation of MCF-7/ADM cells with IC_{20} value of 3.14 µg/mL. 0.3, 1 and 3 µg/mL were considered as low cytotoxic concentrations. To investigate whether these low cytotoxic concentrations of PCT3 can improve the sensibility of MCF-7/ADM cells to chemotherapeutic agents, 0.3, 1 and 3 µg/mL of PCT3 were utilized in combination with ADM, DDP and 5-FU. Verapamil (5 µg/mL), which is a competitive P-gp inhibitor, was used as positive control. PCT3 significantly sensitized MCF-7/ADM cells to ADM, DDP and 5-FU in a concentration-dependent manner, indicating a reversal effect of multidrug resistance, the reversal fold to ADM was 16.37, 19.50 and 31.71, to DDP was 1.64, 1.42 and 1.39, and to 5-FU was 1.74, 1.83 and 5.60, respectively (Table 3). Reversal effect to ADM and DDP was equivalent to VER, while that to 5-FU was higher than that to VER.

**PCT3 increases ADM and Rh123 accumulation in MCF-7/ADM cells**  As shown above, PCT3 re-sensitized MCF-7/ADM cells to chemotherapeutic drugs, that may be a result of an increase of drug accumulation in resistant cells. We further examined the effects of PCT3 on ADM and Rh123 accumulation in MCF-7/ADM cells and their parental cells by multifunctional microplate reader and fluorescent microscope. In MCF-7 cells, after 3 h co-culture with ADM and RH123, the intracellular fluorescence intensity of ADM and Rh123 was 259.38 and 514.59, while that in MCF-7/ADM cells was 21.17 and 59.44, indicating a great decrease of ADM and Rh123 accumulation in resistant cells. However, when treated by 5, 15 and 45 µg/mL of PCT3 and 5 µg/mL of VER, much more ADM was accumulated in MCF-7/ADM cells in a dose-dependent manner than the control (Fig. 2A). Intracellular fluorescent intensity was increased to 21.62, 36.97 and 113.95 for PCT3
treatment, and to 66.07 for VER treatment (Fig. 2A). Effect of PCT3 at 45 μg/mL was stronger than positive control VER. We also tested the effect of PCT3 on accumulation of Rh123, which is a specific P-gp substrate. The fluorescent value of Rh123 was also enhanced from 59.44 to 125.34, 154.78 and 169.83 for PCT3 treatment, and to 129.53 for VER treatment (Fig. 2B). However, the accumulation of both ADM and Rh123 in MCF-7/ADM cells was still lower than that in MCF-7 cells.

Images taken by fluorescent microscope showed that PCT3 and VER treatment recovered red (indicating ADM accumulation, Fig. 2C) and green (indicating Rh123 accumulation, Fig. 2D) fluorescence in MCF-7/ADM cells. Both experimental results suggested that PCT3 downregulated efflux of exogenous agents, which may contribute to its reversal activity.

**PCT3 decreases MDR1 mRNA and surface expression of P-gp in MCF-7/ADM cells** To investigate whether reversal effect of PCT3 is depended on regulation of MDR1 mRNA and P-gp protein expression, MCF-7/ADM cells were treated by 1, 3 and 10 μg/mL of PCT3 at 12, 24 and 48 h, and analyzed by qPCR and flow cytometry. After 12 h of treatment, MDR1 expression was elevated, while was decreased after 24 h of treatment, and after 48 h of treatment, 3 and 10 μg/mL of PCT3 significantly decreased MDR1 expression (Fig. 3A).

Flow cytometry analysis showed that in MCF-7/ADM cells, surface P-gp expression was 91.43% (Fig. 3B). MCF-7/ADM cells treated by 1 and 3 μg/mL of PCT3 for 12, 24 and 48 h, P-gp expression was downregulated to 85.03%, 82.40%, 81.77% and 83.57%, 83.87%, 83.97%, respectively (Fig. 3B). 10 μg/mL of PCT3 strongly inhibited the expression of P-gp protein to 74.87%, 64.70% and 54.07%, respectively, in a time-dependent manner. When treated by 10 μg/mL VER for 12 h, 24h and 48h, P-gp expression was decreased to 77.00%, 72.10% and 73.83%, respectively. These results indicated that PCT3 attenuated expression of MDR1 mRNA and P-gp protein in MCF-7/ADM cells.

**PCT3 suppresses P-gp expression via interfering MAPK signaling pathway** It was demonstrated that ADM in lymphocytoma B is able to induce the expression of P-gp to generate drug resistance by activating MAPK pathway. We therefore
studied the expression and activation of MAPK pathway proteins as well as P-gp in the sensitive and resistant breast cancer cells by western blotting. Results showed that phosphorylated level of ERK and p38 was much higher in MCF-7/ADM cells than its parental counterparts, while, no difference was detected for JNK activation (Fig. 4A). P-gp expression was also remarkable in the resistant cells, indicating a correlation of P-gp up regulation with ERK and p38 signaling pathway activation (Fig. 4A). This was further demonstrated by using ERK and p38 signaling inhibitor: U0126 and SB203580, treatment with U0126 and SB203580 significantly decreased not only the phosphorylated level of ERK and p38, but also P-gp expression in MCF-7/ADM cells (Fig. 4B, D). Although JNK activation was not elevated in MCF-7/ADM cells, P-gp expression was also suppressed by its inhibitor, SP600125 (Fig. 4C). These data suggested that activation of ERK and p38 was involved in the ADM-resistant phenotype of breast cancer cells.

In order to investigate reversal mechanism of PCT3 on MCF-7/ADM cells, low cytotoxic concentration of PCT3 (3 µg/mL) was used in combination with ADM. After 48h of treatment, P-gp expression and Phosphorylated level of ERK was significantly decreased by PCT3 and PCT3 combined with ADM (Fig. 5A, B). However, no obvious changes of JNK and p38 activation were observed in both treatments (Fig. 5A, B). These data indicated that suppression of P-gp expression by PCT3 may be mediated by inhibition of ERK pathway.

DISCUSSION

Our previous study demonstrated that total saponins from *P. forrestii* (PCT3) strongly inhibited proliferation of a panel of human cancer cells and adriamycin resistant MCF-7/ADM cells. In this study, we explore to know whether PCT3 could reverse resistant MCF-7/ADM cells to chemotherapeutic agents and its possible mechanism of action.

Firstly, MCF-7/ADM cells was proved to be multi-resistant, not only to ADM (RI=300), but also to 5-FU (RI=7.63) and DDP (RI=2.00). Low cytotoxic
concentrations of PCT3 showed remarkable reversal effect on MCF-7/ADM cells, with maximum reversal fold of 31.7 to ADM, 5.6 to 5-Fu and 1.6 to DDP. These results indicated that PCT3 efficiently reversed the multidrug resistance of MCF-7/ADM cells at low cytotoxic concentrations.

The MDR cell phenotype in tumors is often associated with overexpression of members of the ATP-binding cassette (ABC) transporter family protein, especially ABCB1 (P-gp).\(^5\) P-gp functions as drug efflux pump, which catalyzes the efflux of a variety of drugs and reduce their intracellular concentrations. Elevated expression of P-gp is exhibited in many kinds of resistant cancer cells.\(^{17} \) In our study, drastically elevated expression of MDR1 mRNA and P-gp protein was exhibited in MCF-7/ADM cells compared with its parental cells. This suggested that multidrug resistance of MCF-7/ADM cells is mediated by high expression of P-gp. 12 h treatment of low cytotoxic concentration of PCT3 in MCF-7/ADM cells stimulated a higher expression of MDR1 mRNA, indicating exogenous agent is one of the causes to induce P-gp expression to pump out cytotoxic agents and xenobiotics. However, MDR1 mRNA was finally decreased after 24 and 48 h treatment of PCT3 in a time-dependent manner. This was further confirmed by decreased P-gp expression and an increased accumulation of ADM and Rh123 in MCF-7/ADM cells after PCT3 treatment, which indicated a weaker function of P-gp.

It was demonstrated that inhibitor of ERK kinase reduced endogenous and exogenous expression of P-gp in human colorectal and breast cancer cells and diminished the cellular multidrug resistance.\(^{18} \) In this study, we demonstrated a higher expression of phosphorylated ERK and p38 in MCF-7/ADM cells compared to MCF-7 cells. Inhibition of p38, ERK or JNK by their inhibitors could decrease P-gp expression. As one of the main steroidal saponins detected in PCT3,\(^{13} \) polyphyllin D was proved to sensitize liver and lung cancer cells to chemotherapeutic drugs via inhibition of NF-κB, p38MAPK, ERK or Akt signaling pathways,\(^{19,20} \) while polyphyllin VII could reverse adriamycin-resistant breast cancer cells via P-gp inhibition and apoptosis augmentation.\(^{21} \) In this study, we demonstrated that low
Cytotoxic concentration of PCT3 reversed MCF-7/ADM cells by inhibiting P-gp expression at both transcriptional and post-transcriptional level and this is mediated, at least in part, by inhibiting activation of MAP kinase: ERK, but not p38MAPK and JNK. Collectively, as an effective steroidal saponin fraction of *P. forrestii*, low cytotoxic concentrations of PCT3 showed a reversal activity in P-gp mediated multidrug resistant breast cancer cells. Since it is not difficult to get enough quantity of PCT3, further study need to be performed to explore its reversal activity and mechanism *in vivo*.

**Acknowledgements**

This work was supported by grants from the Joint Application and Basic Research Foundation of Kunming Medical University & Science and Technology Department of Yunnan Province of China (grant no. 2014FB011, 2019FE001(-193)), National Natural Science Foundation of China (grant no. 81960739), International Partnership Program of Chinese Academy of Sciences (grant no. 153631KYSB20160004) and Yunnan Major Biopharmaceutical Project (grant no. 2018ZF002).

**Conflict of interest**

The authors declare no conflict of interest.
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| Gene name | Primer sequence | Product length |
|-----------|----------------|----------------|
| MDR1      | 5’-CCCATCATTGCAATAGCAGG-3’ | 157bp          |
|           | 5’-GTTCAAACTTCTGCTCCTGA-3’ |                |
| β-actin   | 5’-GGGAAATCGTGCGTGACATTAAGG-3’ | 185bp          |
|           | 5’-CAGGAAGGAAGGCTGGAAGAGTG-3’ |                |
Table 2 Cytotoxicity of ADM, DDP and 5-FU on MCF-7 and MCF-7/ADM Cells

| Drug | IC_{50} ±SD (μg/mL) | RI       |
|------|-------------------|----------|
|      | MCF-7             | MCF-7/ADM|          |
| ADM  | 0.46±0.12         | 140.04±15.77*** | 304.43   |
| DDP  | 2.28±0.29         | 4.55±0.56  | 2.00     |
| 5-Fu | 10.30±4.30        | 78.57±7.63*** | 7.63     |

***P<0.001, compared to MCF-7 cells. Data are representative of at least three independent experiments.
| Sample       | IC\(_{50}\)±SD (μg/mL) | Reversal fold |
|--------------|--------------------------|---------------|
| **ADM**      |                          |               |
| + 0.3 μg/mL  | 7.05±0.92***             | 16.37         |
| + 1 μg/mL    | 5.92±0.378***            | 19.5          |
| + 3 μg/mL    | 3.64±0.21***             | 31.71         |
| + 5 μg/mL    | 3.26±0.41***             | 35.52         |
| + 0.3 μg/mL  | 3.36±0.21**              | 1.64          |
| + 1 μg/mL    | 3.64±0.21**              | 1.42          |
| + 3 μg/mL    | 3.72±0.09**              | 1.39          |
| + 5 μg/mL    | 3.06±0.13**              | 1.69          |
| + 0.3 μg/mL  | 13.34±1.97               | 1.74          |
| + 1 μg/mL    | 12.66±2.93               | 1.83          |
| + 3 μg/mL    | 4.14±0.08**              | 5.6           |
| + 5 μg/mL    | 12.98±2.66               | 1.79          |

**P<0.01 and ***P<0.001, compared with untreated group. Data are representative of at least three independent experiments.
Fig. 1. Expression of MDR1 gene and P-gp protein in MCF-7 and MCF-7/ADM cells. (A) QPCR analysis of MDR1 gene expression in the two cell lines. The data is representative of at least 3 replicates. (B) Flow cytometry analysis of P-gp expression in MCF-7 and MCF-7/ADM cells. *** $P<0.001$, compared with MCF-7 cells (For Fig. 1A).
Fig. 2. Effect of PCT3 on the accumulation of ADM and Rh123 in MCF-7/ADM cells. (A-B) Accumulation of ADM (10.0 μg/mL) (A) and Rh123 (10.0 μg/mL) (B) in the parental MCF-7 cells and the MCF-7/ADM cells treated by VER (10 μg/mL), as well as PCT3 (5, 15 and 45 μg/mL) for 3 h and detected by a multifunctional microplate reader. (C-D) Results of A and B detected by fluorescent microscope (Bar: 40μm). ###P < 0.001, compared with control of MCF-7 cells. *P < 0.05, **P < 0.01, compared with control of MCF-7/ADM cells (For Fig. 2A and 2B). Data are representative of at least three independent experiments.

(Color figure can be accessed in the online version.)
Fig. 3. Effect of PCT3 on MDR1 and P-gp expression in MCF-7/ADM cells. (A) QPCR analysis of MDR1 gene expression in MCF-7/ADM cells treated with VER (10 μg/mL) and PCT3 (1, 3 and 10 μg/mL) for 12, 24 and 48 h. (B) Flow cytometry analysis of P-gp expression in MCF-7/ADM cells treated with VER (10 μg/mL) and PCT3 (1, 3 and 10 μg/mL) for 12, 24 and 48 h. **P<0.01, ***P<0.001, compared with control group. Data are representative of three independent experiments.
A

MCF-7 MCF-7/ADM

P-ERK
ERK
P-JNK
JNK
P-p38
p38
P-gp
GAPDH

B

MCF-7/ADM

U0126
0 40μmol/L

P-ERK
ERK
P-gp
GAPDH

Control U0126-40μM

Relative protein expression

P-ERK ERK P-gp
Fig. 4. Effect of MAPK signaling pathway inhibitors on P-gp expression. (A) Western blot analysis of the phosphorylated and total proteins of ERK, JNK, p38 and P-gp expression in MCF-7 and MCF-7/ADM cells. GAPDH was used as a loading control. (B-D) MCF-7/ADM cells were treated with MEK (ERK) inhibitor U0126 (40 µmol/L), JNK inhibitor SP600125 (20 µmol/L) and p38 inhibitor SB203580 (80 µmol/L).
µmol/L) for 48 h. Phosphorylated and total protein of ERK, JNK, p38 and P-gp expression was detected by western blot method, GAPDH was used as a loading control. **P<0.01, ***P<0.001, compared with control group. Data are representative of at least three independent experiments.
Fig. 5. Effect of PCT3 on P-gp and MAPK signaling pathway proteins expression in MCF-7/ADM cells. (A) Western blot analysis of P-gp expression and phosphorylated and total protein expression of ERK, JNK, p38 in MCF-7/ADM cells treated with PCT3 (3 µg/mL) and ADM (1 µg/mL) for 48 h. GAPDH was used as a loading control. (B) Expression quantity of P-gp, P-ERK, ERK, P-JNK, JNK, P-p38 and p38. *P<0.05, **P<0.01, ***P<0.001, compared with control group. #P<0.05, ##P<0.01, ###P<0.001, compared with ADM group (For Fig. 5 B). Data are representatives of at least three experiments.