Psoralen reverses the P-glycoprotein-mediated multidrug resistance in human breast cancer MCF-7/ADR cells

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Abstract. The resistance of cancer to chemotherapeutic agents is a major obstacle during chemotherapy. Clinical multidrug resistance (MDR) is commonly mediated by membrane drug efflux pumps, including ATP-binding cassette subfamily B member 1, also termed P-glycoprotein (P-gp). P-gp is a membrane transporter encoded by the MDR1 gene. The current study aimed to investigate the impact of psoralen on the expression and function of P-gp. The 10% inhibitory concentration (IC10) of psoralen, and its capacity to reduce MDR in adriamycin (ADR)-resistant MCF-7/ADR cells were determined using MTT assay. The ability of psoralen to modulate the transport activity of P-gp in MCF-7/ADR cells was evaluated by measuring the accumulation and efflux of rhodamine 123 (Rh 123) and adriamycin with flow cytometry. The present study evaluated the mRNA level of MDR1 in MCF-7 and MCF-7/ADR cells treated with psoralen using reverse transcription-quantitative polymerase chain reaction. The protein expression level of P-gp was examined by western blot analysis. The current study demonstrated that the IC10 of psoralen in MCF-7/ADR cells was 8 μg/ml. At 8 μg/ml, psoralen reduced MDR and the sensitivity of the MCF-7/ADR cells to ADR compared with untreated cells. Additionally, psoralen significantly increased the intracellular accumulation of ADR and Rh 123. However, the IC10 of psoralen did not affect the protein expression levels of P-gp or mRNA levels of MDR1 (P>0.05). Psoralen reduces MDR by inhibiting the efflux function of P-gp, which may be important for increasing the efficiency of chemotherapy and improving the clinical protocols aiming to reverse P-gp-mediated MDR.

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Introduction

Breast cancer is a common malignancy that affects the health of women worldwide (1). Currently, breast cancer treatment typically requires surgery and adjuvant chemotherapy or radiotherapy. Among various treatment strategies, chemotherapy has remained one of the most effective tools for the treatment of breast cancer (2-4). However, the phenomenon of multidrug resistance (MDR) severely limits the efficacy of chemotherapy. MDR is the predominant cause of the failure of chemotherapeutic treatment, therefore, it is important to investigate methods that may reverse MDR during chemotherapy of breast cancer (5,6). Overexpression of ATP-binding cassette (ABC) family proteins and elevation of the apoptotic threshold contribute to drug resistance (7-9). P-glycoprotein (P-gp; also termed ABC subfamily B member 1, ABCB1), multidrug resistance proteins (MRPs; also termed ABCCs), and breast cancer resistance protein (BCRP; also termed ABCG2) are commonly overexpressed in chemoresistant cells (10).

The human ABCB1 gene, also termed multidrug resistance 1 (MDR1), encodes P-gp. Overexpression of this gene is considered to be one of the major obstacles to successful cancer chemotherapy (11). The MDR1 phenotype is commonly observed to produce resistance to chemotherapy in breast cancer (12). The overexpression of P-gp has also been observed in the tumor tissue of 40-50% of patients with cancer (13). Inhibition of P-gp-mediated drug efflux may increase the sensitivity of tumor cells to chemotherapeutics, and enhance the success of chemotherapy for cases of multidrug-resistant cancer (14,15). Thus, inhibiting the function of P-gp and MRPs, or enhancing the efficacy of apoptosis induced by chemotherapeutics, has become important during the investigation of novel breast cancer treatments (16). Although several agents have been demonstrated to effectively reverse P-gp-mediated MDR, no drugs have been successfully developed for clinical use. The observation that numerous plant-derived dietary compounds modulate P-gp transport has led to interest in the possible use of natural compounds, which exhibit fewer side effects than traditional chemotherapy, for cancer treatment (17-19).

The feasibility of using traditional Chinese medicine to combat MDR has received increasing attention and extensive research (20). Psoralen is the main active ingredient extracted

from the natural products of *Psoralea corylifolia*, and is widely used as an anti-neoplastic agent in the treatment of leukemia and other types of cancer. Previous studies have demonstrated psoralen to be a potent inhibitor of cutaneous T-cell lymphoma (21-23), and cytotoxic to MEC-1 mucoepidermoid carcinoma cells *in vitro* (24). Furthermore, intraperitoneal administration of psoralen can inhibit the growth of ascitic tumors in mice (25). However, the effects and mechanisms of psoralen on MDR remain unclear. Therefore, in the present study, the effect of psoralen on MDR in breast cancer cells was investigated. An adriamycin (ADR)-resistant human breast cancer cell line (MCF-7/ADR) was used to determine whether psoralen may reverse MDR by modulating the function of P-gp.

Materials and methods

Reagents. Psoralen was obtained from Baoji Herbest Bio-Tech Co., Ltd. (Baoji, China). Cell culture reagents were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Adriamycin (ADR) was obtained from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). Mouse monoclonal P-gp (ab3083) and β-actin (ab49900) antibodies were purchased from Abcam (Cambridge, MA, USA). Peroxidase-conjugated Affinipure goat anti-mouse IgG (SA00001-1) was obtained from ProteinTech Group, Inc. (Chicago, IL, USA). A RevertAid First Strand cDNA Synthesis kit (#K1621) and Power SYBR Green PCR Master Mix (#4367659) were purchased from Thermo Fisher Scientific, Inc. TRIzol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT) and Rhodamine 123 (Rh 123) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. MCF-7 human breast cancer cells and ADR-resistant (MCF-7/ADR) cells were obtained from the Laboratory of Type Culture Collection, Binzhou University of Medicine (Binzhou, China). The MCF-7 cell line was cultured in RPMI-1640. The MCF-7/ADR cell line was cultured in the same medium containing 1 μg/ml ADR to maintain the MDR phenotype. All media were supplemented with 10% Gibco fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) (all from Thermo Fisher Scientific, Inc.), and all cells were incubated at 37˚C in a 5% CO_2 humidified atmosphere.

Cell viability assay. Inhibition of cell proliferation by psoralen was determined using the MTT assay. The MCF-7/ADR cells were seeded at $1x10^4$ cells/well in 96-well flat-bottomed culture plates with 100 μl RPMI-1640 for 8 h. RPMI-1640 medium alone was used as a blank control. The medium was then removed and replaced by fresh medium containing a range of psoralen concentrations (2, 4, 6, 8, 12, 16 and 20 μg/ml) and cells were incubated for 48 h. Following addition of 20 μl MTT solution (5 mg/ml), incubation was continued for 4 h at 37˚C. The supernatant was then removed and 150 μl dimethyl sulfoxide was added to each well and incubated for 10 min, with agitation to dissolve the purple formazan crystals. The absorbance at 490 nm was measured using an automatic microplate reader (ELx800; BioTek Instruments, Inc., Winooski, VT, USA). Values are presented as the mean ± standard deviation (SD) from three independent experiments. The IC10 value was defined as the concentration resulting in 90% cell survival. The IC10 value was calculated using the SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). The IC10 concentration was used as the experimental concentration in the proceeding experiments.

MDR reversal activity. The effect of psoralen (4, 8 and 12 μg/ml) on MDR in MCF-7 and MCF-7/ADR cells treated with ADR was measured using MTT assay. The concentration of drug that inhibited 50% of cells (IC50) was calculated, and was subsequently used to calculated the MDR reversal fold. The reversal fold value was calculated by dividing the IC50 value of the MDR cells (MCF-7/ADR) by the value of the treated cells (MCF-7/ADR + psoralen).

ADR and Rh 123 efflux assays. The intercellular ADR and Rh 123 content in MCF-7/ADR cells treated with psoralen were analyzed by flow cytometry as previously described (26). The cells treated with the IC10 of psoralen were cultured at 37˚C for 3 h. ADR and Rh 123 were added to the cells at a final concentration of 5 μg/ml. The cells were incubated for a further 3 h and 0.5 h, respectively, harvested, washed three times with cold phosphate-buffered saline (PBS), and the fluorescence intensity was measured by flow cytometric analysis on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The mRNA expression of MDR1 was measured by RT-qPCR. MCF-7/ADR cells ($1x10^6$ cells/ml) were seeded in 6-well culture plates and incubated for 8 h to allow attachment. The media was refreshed and psoralen was added to the experimental group at the IC10 concentration (8 μg/ml), and incubation was continued for 48 h. Cells were harvested following treatment, washed twice with cold PBS and collected by scraping. According to the manufacturer's protocols, total RNA was isolated from 6-well plates using TRIzol reagent, and then subjected to RT-qPCR using the RevertAid First Strand cDNA Synthesis kit and Power SYBR Green PCR Master Mix according to the manufacturer's protocol. The RT reactions were performed at 42˚C for 60 min and 70˚C for 60 min. The cDNA was dissolved in 20 μl diethylpyrocarbonate-H_2O and used in the proceeding PCR reactions. In addition, the mRNA levels of β-actin were measured as a reference and used to normalize the mRNA levels of the drug resistance genes. The primer sequences were designed and supplied from Sangon Biotech Co., Ltd. (Shanghai, China) as follows: MDR1, F 5'-CCCATC ATTGCAATAGCAGG-3' and R 5'-GTTCAAACTTCTGCT CCTAG-3'; β-actin, F 5'-TGTCACCAACTGGGACGATA-3' and R 5'-GGGGTGTTGAAGGTCTCAAA-3'. The cDNA (1 μl) was amplified by PCR on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA), at 95˚C for 1 min and 45 sec, followed by 35 cycles of 95˚C for 30 sec and 60˚C for 30 sec, with a final extension at 72˚C for 7 min. The data were analyzed by $2^{-\Delta\Delta Cq}$ (27).

Western blot analysis. The MCF-7/ADR cells treated with psoralen (8 μg/ml), and untreated MCF-7 and MCF-7/ADR

cells were incubated for 48 h. The cell lysates were prepared in radioimmunoprecipitation assay buffer. Protein concentrations were determined by bicinchoninic acid assay using a GeneQuant 1300 spectrophotometer (GE Healthcare Life Sciences, Milwaukee, WI, USA) to measure absorbance at 562 nm. Proteins were separated by 8% SDS-PAGE (80 V for 20 min and 100 V for 70 min) and transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc.). Following blocking with 5% non-fat dry milk, the membranes were incubated with the mouse P-gp (1:400) and β-actin (1:5,000) antibodies overnight at 4˚C. The membranes were washed with Tris-buffered saline with 0.1% Tween 20 (TBS-T) and incubated for 2 h with peroxidase-conjugated anti-mouse secondary antibodies (1:5,000), then washed again with TBS-T. The blots were detected using Clarity Western ECL Blotting Substrate (Bio-Rad Laboratories, Inc.). The relative photographic density was quantified using BandScan software, version 4.0 (Glyko Biomedical, Ltd., Hayward, CA, USA). All experiments were performed in triplicate.

Statistical analysis. Statistical analyses were performed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). Significant differences between the groups were evaluated with t-tests. The values are expressed as the mean ± SD. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inhibitory effects of psoralen on cell proliferation of MCF-7/ADR cells. Prior to the experiments of the current study, no cytotoxic effects of psoralen on MCF-7/ADR cells had been established. Therefore, the cytotoxic effects of psoralen were evaluated prior to use. MTT assays were performed to evaluate the antiproliferative effects of psoralen on MCF-7/ADR cells. The cells were treated with the different concentrations of psoralen (2, 4, 6, 8, 12, 16 and 20 μg/ml) for 48 h. As presented in Fig. 1, the concentration resulting in a 10% growth inhibition (IC10) of psoralen was 8 μg/ml in MCF-7/ADR cells.

Effect of psoralen on MCF-7/ADR cell resistance to ADR. Effects of psoralen on the MDR were evaluated by an MTT assay. The results demonstrated that psoralen can reduce MDR. The sensitivity of the MCF-7/ADR cells to ADR were 2.0-, 17.5- and 44-fold when the cells were treated with 4, 8 and 12 μg/ml psoralen, respectively. These results indicated that psoralen significantly reduces MDR and increases the cytotoxicity of ADR in MCF-7/ADR cells in a dose-dependent manner.

Psoralen inhibits the transport function of P-gp. The ability of psoralen to inhibit P-gp-mediated transport was analyzed using the P-gp substrates, ADR and Rh 123 (28). Flow cytometric analysis was performed to determine the effect of psoralen on the accumulation and efflux of ADR and Rh 123 (Fig. 2). Compared with cells without psoralen, the fluorescence index of ADR was increased by 1.2-fold in cells treated with 8 μg/ml psoralen (P=0.027). The same doses of psoralen increased the fluorescence index of Rh 123 by 1.6-fold (P=0.022). The results indicate that psoralen increases the accumulation of the ADR and Rh 123 anticancer drugs in MCF-7/ADR cells, which may be associated with an effect on P-gp transport.

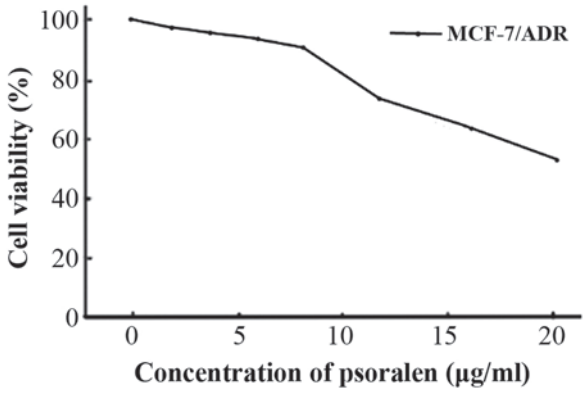

Figure 1. Effects of psoralen on cell viability in ADR-resistant MCF-7/ADR breast cancer cells. Cells were treated with psoralen (2, 4, 6, 8, 12, 16 and 20 μg/ml) for 48 h. ADR, adriamycin.

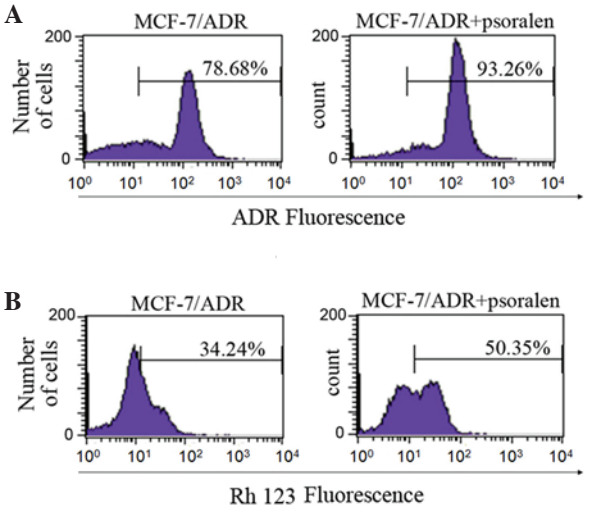

Figure 2. Effects of psoralen on P-gp activity in ADR-resistant MCF-7/ADR breast cancer cells. Cells were treated with psoralen (8 μg/ml) for 48 h. (A) The cellular accumulation of ADR was measured. (B) The P-gp activity was assessed by accumulation of Rh 123. The figures are representative of 3 independent experiments. P-gp, P-glycoprotein; ADR, adriamycin; Rh 123, rhodamine 123.

Psoralen has no marked effect on the expression of P-gp. RT-qPCR and western blotting were performed to measure the effect of psoralen on the expression of P-gp at the mRNA and protein levels, respectively. As demonstrated in Fig. 3, the western blot analysis indicated that the P-gp protein expression levels were markedly increased in MCF-7/ADR cells compared with the parental MCF-7 cells. However, compared with untreated cells, no change in the P-gp protein expression levels in MCF-7/ADR cells were observed following treatment with psoralen (P>0.05).

As determined by RT-qPCR, no significant difference was observed in the MDR1 mRNA expression levels between untreated and psoralen-treated MCF-7/ADR cells (P>0.05; Fig. 4).

In summary, the MDR1 mRNA levels and P-gp protein levels in MCF-7/ADR cells were not changed by psoralen

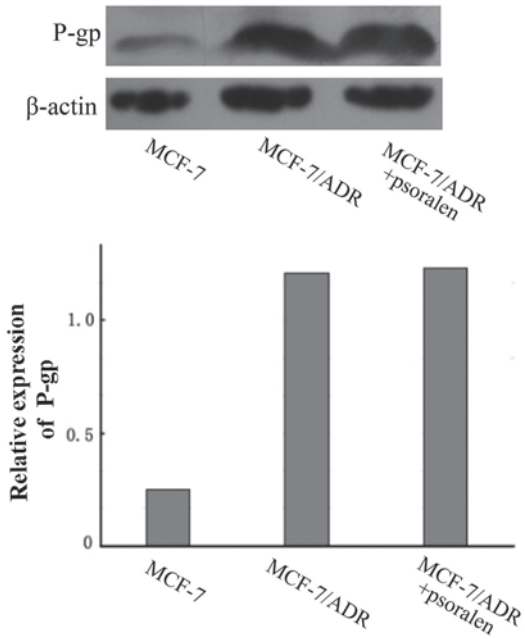

Figure 3. The effect of psoralen on the expression of P-gp. ADR-resistant MCF-7/ADR cells were treated with psoralen (8 μg/ml) for 48 h. P-gp protein levels were detected in MCF-7, MCF-7/ADR cells and psoralen-treated MCF-7/ADR cells by western blot analysis. β-Actin was used as a loading control. P>0.05 MCF-7/ADR + psoralen vs. MCF-7/ADR cells. P-gp, P-glycoprotein; ADR, adriamycin.

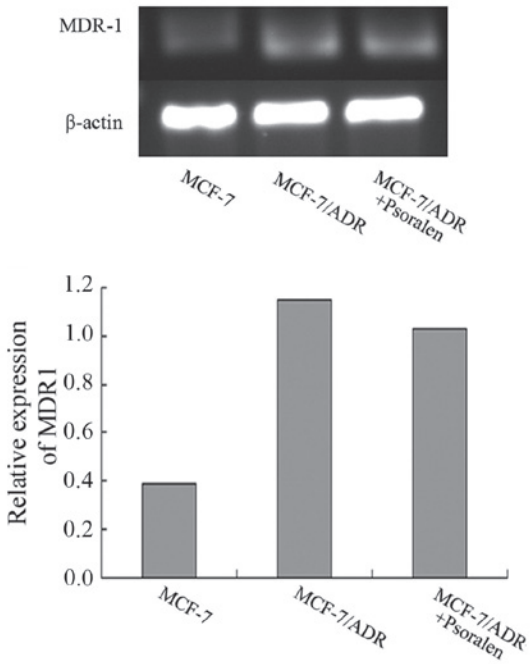

Figure 4. MDR1 mRNA levels in MCF-7/ADR cells. The total RNA from MCF-7, MCF-7/ADR and psoralen-treated (8 μg/ml) MCF-7/ADR cells was reverse-transcribed and amplified using targeted cDNA primers for MDR1 and β-actin. The products of polymerase chain reaction were separated on a 5% agarose gel and quantification of the MDR1 levels was conducted. The data are expressed as the mean ± standard deviation. P>0.05, MCF-7/ADR + psoralen vs. MCF-7/ADR. MDR1, multidrug resistance 1.

treatment. The above results demonstrate that the effect of psoralen on MDR may be associated with inhibition of the P-gp transporter, rather than reducing the expression levels of P-gp mRNA or protein.

Discussion

Numerous types of cancer cells eventually develop MDR following treatment with chemotherapeutic drugs (29). MDR is an important issue, as overcoming MDR may enhance chemotherapy results and improve the outcome for patients with cancer (30). One of the mechanisms of MDR is overexpression of P-gp (31), which increases drug efflux and eventually results in the reduced efficacy of chemotherapeutic drugs (32). Extensive research efforts have attempted to reduce MDR using inhibitors of the drug-efflux pump and various other compounds to improve the therapeutic efficiency of chemotherapy (33,34). Several P-gp inhibitors have been investigated for their potential to reduce MDR. However, none have currently been approved for clinical use (35).

A large body of literature now exists indicating that the herbal medicines or natural compounds may be feasible for use as potent chemopreventive drugs (36,37). The goal of the present study was to assess whether psoralen, an active ingredient extracted from *Psoralea corylifolia*, may reverse MDR by modulating the function of P-gp. MTT assays demonstrated that psoralen is a cytotoxic agent, which is consistent with previous results in A549/D16 human lung cancer cells (38), with an IC10 at 8 μg/ml. However, no cell toxicity was detected when psoralen was used at 8 μg/ml as a single agent. This indicates that the concentration of psoralen used in these experiments is clinically achievable.

To assess the potential of psoralen to reduce MDR, its capacity to sensitize MCF-7/ADR cells was measured. The results of the present study demonstrated that 8 μg/ml psoralen significantly reduced the resistance of MCF-7/ADR cells to ADR, whereas it had no effect on the parental MCF-7 cells. The current study demonstrated the potential of psoralen as a P-gp-mediated MDR reversal agent, and this result was further confirmed by the psoralen-induced reduction to ADR resistance in P-gp-overexpressing MCF-7/ADR cells.

P-gp-mediated MDR can be reduced by downregulating P-gp expression or inhibiting P-gp efflux activity. In the experiments of the current study, the P-gp transport function was analyzed by measuring the efflux of P-gp substrates using flow cytometry. This analysis provides a measure of P-gp-mediated transport activity by comparing the intracellular concentrations of ADR and Rh 123 in P-gp overexpressing MCF-7/ADR cells, in the presence and absence of psoralen. These results demonstrated that the intracellular accumulation of ADR and Rh 123 were significantly increased in MCF-7/ADR cells treated with psoralen, compared with untreated cells, however, psoralen had no significant effect on the parental MCF-7 cells. These results are in agreement with the demonstrated effect of psoralen on MDR, indicating that psoralen alters P-gp function and transport to increase the intracellular accumulation of drugs. However, the results of the present study indicated that psoralen did not alter P-gp expression at the mRNA or protein level, suggesting that psoralen does not reduce MDR via inhibition of P-gp expression. Due to the effect of psoralen

on MDR, it may be a promising chemosensitizer for clinical use in combination with other anticancer drugs.

P-gp is a transmembrane drug efflux protein with transmembrane and nucleotide-binding domains. The transport activity of P-gp requires energy from ATP hydrolysis, thus, the ATPase activity of P-gp reflects its transport activity. Substrate recognition and binding occur in the transmembrane domains of P-gp, and are an essential requirement for transportation. Detailed understanding of the mechanisms by which psoralen inhibits P-gp transport function may be important for overcoming MDR. Thus, further research should be undertaken to elucidate these mechanisms, particularly the effect on P-gp ATPase activity.

In conclusion, the current study demonstrated that psoralen significantly reduces P-gp-mediated MDR in human breast cancer MCF-7/ADR cells at pharmacologically achievable concentrations, and affects MDR reversal by inhibiting P-gp function. Based on the results of the present study, psoralen has the potential to be coadministered with chemotherapeutic agents to improve the efficacy of chemotherapy and to reduce P-gp-mediated MDR.

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