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Reversal effect of Dioscin on multidrug resistance in human hepatoma HepG2/adriamycin cells

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ABSTRACT

Multidrug resistance is a serious obstacle encountered in cancer treatment. Since drug resistance in human cancer is mainly associated with overexpression of the multidrug resistance gene 1 (MDR1), the promoter of the human MDR1 gene may be a target for multidrug resistance reversion drug screening. In the present study, HEK293T cells were transfected with pGL3 reporter plasmids containing the 2 kb of MDR1 promoter, and the transfected cells were used as models to screen for candidate multidrug resistance inhibitors from over 300 purified naturally occurring compounds extracted from plants and animals. Dioscin was found to have an inhibiting effect on MDR1 promoter activity. The resistant HepG2 cell line (HepG2/adriamycin) was used to validate the activity of multidrug resistance reversal by Dioscin. Results showed that Dioscin could decrease the resistance degree of HepG2/adriamycin cells, and significantly inhibit P-glycoprotein expression, as well as increase the accumulation of adriamycin in HepG2/adriamycin cells as measured by Flow Cytometric analysis. These results suggest that Dioscin is a potent multidrug resistance reversal agent and may be a potential adjunctive agent for tumor chemotherapy.

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1. Introduction

Drug resistance to tumor cells is a major obstacle to the success of cancer chemotherapy. After developing resistance to a single drug or a class of drugs, cancer cells show cross-resistance to other functionally and structurally unrelated drugs. This phenomenon known as multidrug resistance has a profound effect on successful chemotherapy (Gottesman et al., 2002). Although many mechanisms of multidrug resistance in cancer cells have been studied, the most important and thoroughly studied mechanism of multidrug resistance is the efflux mechanism based on the function of P-glycoprotein, an ABC transporter, which is a 170 kD plasma membrane glycoprotein encoded by the human MDR1 gene. This P-glycoprotein acts as a drug efflux pump to extrude a wide range of different chemotherapeutic drugs out of multidrug resistance cancer cells (Ambudkar et al., 2003). P-glycoprotein not only elicits drug resistance at the cellular level, but was also found to alter the pharmacokinetics of various drugs and was associated with poor bioavailability (Glavinas et al., 2004; Varma et al., 2003; Johnson, 2002). Therefore inhibition of P-glycoprotein-mediated drug efflux will lead to resensitization of multidrug

resistance cancer cells to treatment with chemotherapeutic agents, and may allow a successful chemotherapy in patients with multidrug resistance tumors (Fojo and Bates, 2003).

The development of the multidrug resistance phenotype is a major hurdle for successful treatment of cancer, whereby patients with multidrug resistance tumor types are often left with few options but exceptionally high doses (Van Vlerken et al., 2007). Currently, many clinical anticancer drugs such as alkaloids, anthracycline antibiotics, and podophyllotoxins may easily induce multidrug resistance. Therefore, development or discovery of safe and effective multidrug resistance reversal agents is urgently required. Natural products from Chinese traditional medicine are a fertile area in which to look for novel drugs with activity against multidrug resistance. In this study, a screening cell model was established by HEK293T cells, which were transfected with pGL3 reporter plasmids containing the 2 kb of MDR1 promoter. The cell model was used to screen candidate multidrug resistance inhibitors from over 300 purified naturally occurring compounds. One compound which was extracted from the roots of *Polygonatum zanlanscianense*, Dioscin (diosgenin-3-yl α -L-rhamnopyranosyl-(1 \rightarrow 2)-[α -L-rhamnopyranosyl-(1 \rightarrow 4)]- β -D-glucopyranoside), (Fig. 1), was found to be a potent inhibitor of the MDR1 promoter.

Saponins belong to a family of glycoconjugates with a broad range of biological and pharmacological activities, including immunomodulation and anticancer effects (Editorial Committee of Flora of China, 1980). Dioscin, a plant glucoside saponin, was shown to inhibit pancreatic lipase with an IC₅₀ of 20 μ g/ml (Birari and Bhutani, 2007).

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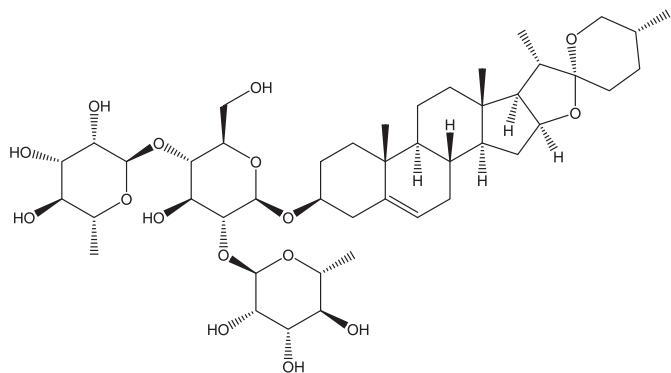


Fig. 1. Chemical structure of Dioscin.

and induce apoptosis in a number of human carcinoma cell lines with an IC_{50} value typically in μM concentrations (Liu et al., 2004; Mi et al., 2002; Wang et al., 2001; Cai et al., 2002). Prosapogenin A of Dioscin had moderate MDR reversal activity on human *mdr1* gene-transfected mouse lymphoma cell line (Ivanova et al., 2009). However, little information has been reported concerning the molecular mechanism on multidrug reversion ability of Dioscin. Accordingly, we further investigate the anti-multidrug resistance activity of Dioscin using the multidrug resistant cell line HepG2/adriamycin. The effects of Dioscin on the expression and function of P-glycoprotein were also tested.

2. Materials and methods

2.1. Cell lines and cell culture

Human embryonic kidney HEK293T cells, human hepatocyte L02 cells, and human liver carcinoma HepG2 cells, were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 10% fetal bovine serum (FBS, TBD, Tianjin, China), 100 U/ml penicillin and 100 $\mu g/ml$ streptomycin (Ameresco, USA), at 37 °C, 5% CO_2 .

2.2. Generation of MDR1 promoter reporter plasmid

Human genomic DNA was prepared from human whole blood by using the phenol/chloroform extraction protocol. To obtain the MDR1 promoter fragments, PCR amplification on human genomic DNA; (forward primer 5'-GGGGTACCCAGTCTCTACG-3', reverse primer 5'-CAAGCTTGTC CGACTGAAGAG-3') was performed in a 50 μl reaction mixture. After an initial denaturation step at 94 °C for 5 min, the PCR reaction was carried out for 30 cycles at 94 °C for 30 s, 60 °C for 50 s, and 72 °C for 2 min, with a final extension of 10 min at 72 °C. The PCR product was cloned into the *KpnI*/*HindIII* site of the pGL3-basic vector.

2.3. Screening for inhibitors of MDR1 transcription

HEK293T cells were plated in a 6-well plate in a concentration of 2.5×10^6 cells in 2 ml of growth medium. After 24 h, cells were transfected with 2 μg of pGL3-MDR1-promoter plasmid by using Calcium Phosphate Cell Transfection kit (Beyotime, China) according to the manufacturer's instructions. 4–6 h after transfection, cells were reseeded in 96-well plates and natural compounds being tested were added to the cells. After 24 h, the cells were harvested with extraction buffer (25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA and 1% Triton X-100). Luciferase activity was measured as described previously (Shoji et al., 2000) using a FLUOstar OPTIMA system. All transfection experiments were repeated at least three times.

2.4. Development and determination of multidrug resistance on HepG2/adriamycin cells

In order to further investigate the effect and the mechanism of Dioscin on anti-MDR, the multidrug resistant cell line (HepG2/adriamycin) was used. HepG2/adriamycin cells were induced by our lab and developed by stepwise increasing concentration of adriamycin (Haizheng, China) on HepG2 cells. MTT assay showed that HepG2/adriamycin cells were resistant not only to adriamycin but also to 5-FU, Vincristine, and Paclitaxel. The IC_{50} of these drugs for HepG2/adriamycin cells was significantly higher than for the HepG2 cells. HepG2/adriamycin cells were about 30-fold more resistant than the parental HepG2 cells to adriamycin (Zheng et al., 2008).

HepG2/adriamycin cells were revived and maintained the multidrug resistance at 1 $\mu g/ml$ adriamycin for one week. Reverse transcription-PCR (RT-PCR) was used to re-verify the MDR1 mRNA levels of HepG2 and HepG2/adriamycin cells. Total RNA was extracted from cells by using Trizol Reagent (Invitrogen, USA) according to the manufacturer's protocol. The concentration and purity of the extracted RNA were measured by the optical densities at 260 and 280 nm. Reverse transcription (RT) was carried out by a PrimeScript™ RT Reagent kit (TaKaRa, Japan), and the following primers were used to amplify the target genes: MDR1: 5'-CCCATCATGTGAATAGCAGG-3', 5'-AGT CCTCGTCTTCAAACCTG-3'; β -actin: 5'-TCGTGCGTGACATTAAGGAG-3', 5'-ATGCCAGGT ACATGGTGGT-3'. PCR was performed for 35 cycles, each cycle comprised denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 45 s, before a final extension at 72 °C for 10 min. The amplified PCR products were separated on 1% agarose gels by electrophoresis, and PCR fragments were visualized by ethidium bromide staining and were quantified by Tanon GIS-2000 gel image processing.

2.5. Cell proliferation analysis

The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT (Sigma, USA) assay was performed to quantify the effect of Dioscin on cell growth and viability. L02 and HepG2 cells were plated (1×10^4 cells/well) in 100 μl of growth medium in 96-well plates and allowed to grow for 24 h. The cells were treated with 0, 0.1, 1, 10, 100 $\mu g/ml$ Dioscin in the presence of 3% serum medium. Following 44 h of treatment, 20 μl of 5 mg/ml MTT (Sigma Chemical Co) in phosphate-buffered saline was added to each well for an additional 4 h of incubation. The blue MTT formazan precipitate was dissolved in 100 μl of dimethylsulfoxide and agitated for 10 min. Absorbance in each well was read at 570 nm by an automated microplate reader (Bio-Rad, CA, USA).

Since we require concentrations of reversal multidrug agents which are neither inhibitory nor toxic, the inhibition rate of different concentration of Dioscin on these two cell lines was evaluated and IC_{10} values were calculated by SPSS method and data are shown as the mean \pm standard deviation of three independent experiments. Dioscin concentrations of 2 $\mu g/ml$ (lowered IC_{10}) were used to study the reversal of multidrug resistance.

2.6. Analysis of multidrug resistance reversal activity

HepG2/adriamycin cells were plated (1×10^4 cells/well) in 100 μl of growth medium in 96-well plates and allowed to grow for 24 h. Serial dilutions of adriamycin with or without 2 $\mu g/ml$ Dioscin were added to the cells. Since the color of adriamycin at 100 $\mu g/ml$ affects the absorbance results, we selected a concentration of 30 $\mu g/ml$ adriamycin as the highest concentration. By using the same MTT assay as above, the inhibition ratio of each adriamycin concentration with or without Dioscin was calculated by SPSS method. The increase in sensitivity to adriamycin was expressed as a gain of sensitivity (Keller et al., 1992).

2.7. Protein extraction and western blotting assay

HepG2 and HepG2/adriamycin cells were plated in a 6-well plate in a concentration of 5×10^5 cells in 2 ml of growth medium. After 24 h, 2 $\mu\text{g}/\text{ml}$ Dioscin was added to the designated HepG2/adriamycin cells for another 24 h. Cells were harvested and rinsed twice with PBS. To preserve intact surface glycoproteins, cells were not harvested with trypsin. After three washes with ice-cold PBS, the cells were scraped and incubated with cell lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 4 $\mu\text{g}/\text{ml}$ leupeptin, and 1 $\mu\text{g}/\text{ml}$ aprotinin) for 30 min with occasional rocking followed by centrifugation at 21,000 g, for 15 min at 4 °C. Supernatants were mixed with one-quarter volume of 4 \times SDS sample buffer, boiled for 5 min, and then separated through a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to polyvinylidene difluoride membrane, and blocked with 5% nonfat dry milk in TBST buffer (20 mM Tris-HCl pH 7.6, 150 mM NaCl and 0.02% Tween-20) for 1 h, at room temperature. The membrane was immunoblotted with rabbit anti-human P-glycoprotein polyclonal antibody (1:200) (Santa Cruz, USA) in 1% milk/TBST. To assure equivalent protein loading, the membranes were simultaneously incubated with glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (1:2000) (Kangcheng, China) at 4 °C, overnight. Membranes were washed three times, incubated with HRP-conjugated secondary antibodies for 1 h at room temperature, and washed extensively before detection. The membranes were subsequently developed using ECL reagent (Beyotime, China) and exposed to film according to the manufacturer's protocol.

2.8. Intracellular adriamycin accumulation assay

HepG2 and HepG2/adriamycin cells were plated in a 6-well plate in a concentration of 5×10^5 cells in 2 ml of growth medium. After 24 h, 2 $\mu\text{g}/\text{ml}$ Dioscin was added to the designated HepG2/adriamycin cells for another 24 h. HepG2 and HepG2/adriamycin were incubated with 2 $\mu\text{g}/\text{ml}$ adriamycin (with or without Dioscin) for 1 h at 37 °C. Cells were harvested with trypsin after three washes with ice-cold PBS. The cells were subsequently subjected to flow cytometry (FACS Aria, BD, USA) with excitation measured at 488 nm and emission measured at 575 nm. The HepG2 cell was used as a positive control for maximum adriamycin accumulation.

2.9. Statistical analysis

Statistical analysis was performed using a two-tailed Student's *t*-test, and $P < 0.05$ was considered statistically significant. Data were expressed as mean \pm standard deviation in triplicate, and reproducibility was confirmed in three separate experiments.

3. Results

3.1. Screening of chemosensitizer from natural compounds

A series of natural compounds (named TI1–TI300) from Chinese traditional medicine were screened for their multidrug resistance reversal activity in vitro by our cell lines. The results showed that Dioscin was particularly effective in inhibiting the activity of MDR1 promoter, which showed its potential effect as an anti-MDR agent (Fig. 2). Data from all compounds tested are not shown.

3.2. Determination of multidrug resistance on HepG2/adriamycin cells

Resistant HepG2/adriamycin cells were induced by our lab. MTT assay showed that HepG2/adriamycin cells were resistant not only to adriamycin but also to multiple anticancer drugs. RT-PCR was used to

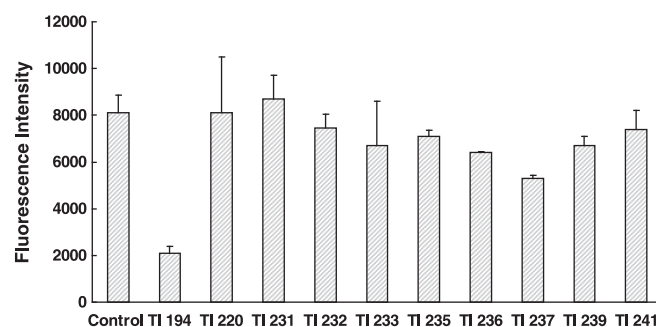


Fig. 2. Screening of chemosensitizers obtained from natural compounds. A series of natural compounds (named TI1–TI300) from Chinese traditional medicine were screened for their multidrug resistance reversal activity in vitro as described in the Materials and methods. Luciferase activity was measured by using a FLUOstar OPTIMA system. TI 194: Dioscin. All experiments were repeated at least three times. * $p < 0.05$ compared with vehicle treated control cells. Partial data are shown.

reverify the multidrug resistance of this cell line. A 157-bp fragment of MDR1 cDNA was detected in HepG2 and HepG2/adriamycin cells. A product of β -actin 303-bp in length was used as an internal control (Fig. 3). MDR1 mRNA levels were significantly increased in the multidrug resistant cells over their control parent cells. Quantities of product in each lane were normalized to β -actin expression.

3.3. MTT cytotoxicity assay

To further investigate the effect of Dioscin on multidrug resistance, human normal liver cell line L02 was used to determine the non-toxic

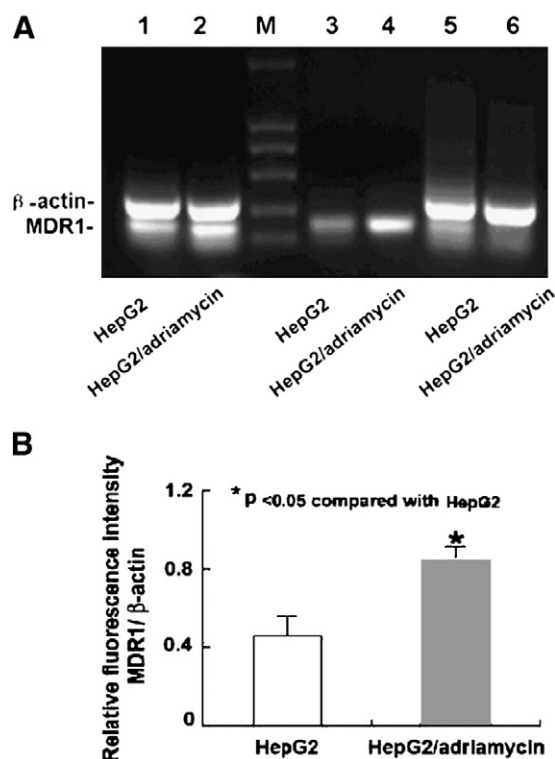


Fig. 3. RT-PCR analysis of MDR1 mRNA expression in HepG2 and HepG2/adriamycin cells. A. Reverse transcription (RT) was carried out by a PrimeScript™ RT reagent kit. The MDR1 PCR product (157-bp) and β -actin PCR product (303-bp) were electrophoresed on 1% agarose gels. Two products were electrophoresed together in lanes 1 and 2; two products were electrophoresed separately in lanes 3–6. M: Marker DL2000. Measurements were performed at least three times. B. The values of relative fluorescence intensity (MDR1/ β -actin) in HepG2 and HepG2/adriamycin cells are shown by representative histograms. * $P < 0.05$ compared with HepG2 cells.

dose of Dioscin. The cytotoxic effects of Dioscin on L02 and HepG2 cells were measured after a 48 h treatment (Table 1). The IC_{10} of Dioscin in L02 and HepG2 cells was 11.50, and 2.07 $\mu\text{g/ml}$ respectively. Consequently a concentration of 2 $\mu\text{g/ml}$ Dioscin was used to further study the reversal effect of multidrug resistance.

3.4. Reversal of multidrug resistance in vitro by Dioscin

To investigate the effect of Dioscin on the sensitivity of cells to a chemotherapeutic agent, cells were incubated with 2 $\mu\text{g/ml}$ Dioscin and a full range of concentrations of adriamycin. Results (Fig. 4) showed that 2 $\mu\text{g/ml}$ Dioscin increased the sensitivity of HepG2/adriamycin to adriamycin. These results suggest that Dioscin was very effective at reversing multidrug resistance in vitro. However, no such activity was found in HepG2 cells (data not shown).

3.5. The effect of Dioscin on P-glycoprotein expression

To assess the effect of Dioscin on P-glycoprotein expression, Western blot analysis was performed (Fig. 5). A high level of P-glycoprotein expression was detected in HepG2/adriamycin cells. However, when HepG2/adriamycin cells were treated with Dioscin, the P-glycoprotein level was significantly decreased.

3.6. The effect of Dioscin on intracellular adriamycin accumulation

Accumulation of adriamycin was measured by flow cytometry (Fig. 6). The fluorescence intensity is shown by representative histograms of HepG2, HepG2/adriamycin, and HepG2/adriamycin cells pre-treated with 2 $\mu\text{g/ml}$ Dioscin for 24 h. The mean fluorescence intensity values of histograms showed that the fluorescence intensity of adriamycin in HepG2 cells was 39.7% higher than HepG2/adriamycin cells, and Dioscin treatment can enhance intracellular adriamycin accumulation in HepG2/adriamycin cells. The fluorescence intensity of adriamycin in 2 $\mu\text{g/ml}$ Dioscin-treated HepG2/adriamycin cells increased 23.7% compared to the untreated HepG2/adriamycin cells.

4. Discussion

Chemotherapy is an indispensable tool used in cancer treatment. However, the emergence of cancer cells resistance to chemotherapy often hampers treatment efficacy. A major mechanism of resistance is multidrug resistance caused by overexpression of a drug-efflux pump, such as P-glycoprotein (Calatuzzolo et al., 2005). In multidrug resistance cancer cells, the intracellular concentration of drugs is reduced because of the drug-efflux pump. One strategy for reversal of multidrug resistance in cells expressing ABC transporters is combined use of anticancer drugs with modulators (Liscovitch and Lavie, 2002). In order to overcome multidrug resistance, enormous efforts have been made to find an inhibitor of the drug-efflux pump, and various compounds, such as verapamil, cyclosporin, quinidine, tamoxifen, and others have been reported to overcome multidrug resistance in vitro (Sweet et al., 1989; Lavie et al., 1997). To date, most of these compounds have shown less than encouraging results. The aim of this study was to find an effective and safe multidrug resistance reversing agent from Chinese traditional medicine, and to gain insight into its reversal effect and the molecular mechanism of that effect.

Table 1
 IC_{10} of dioscin on L02 and HepG2 cells.

Cells	IC_{10} ($\mu\text{g/ml}$)
L02	11.50 ± 0.48
HepG2	2.07 ± 0.36

Data are mean \pm S.D. of three independent experiments.

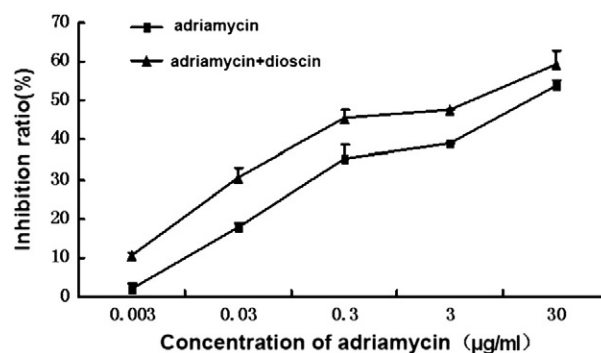


Fig. 4. Reversal of multidrug resistance in vitro by Dioscin. HepG2/adriamycin cells were treated with various concentrations of adriamycin and with 2 $\mu\text{g/ml}$ Dioscin. Cytotoxicity was determined using the MTT assay as described in the Materials and methods. The results represent the mean \pm S.D. of three independent experiments.

However, finding a good anti-multidrug resistance agent quickly and effectively is based on finding a good target and establishing a competent screening model. Screening models include whole animals, organ tissue systems and cellular–molecular level models. Cellular and molecular level screening has been the primary method adopted for drug screening due to its advantages of consumption of less reagents, ability to more explicitly define the mechanism of action and the fact that it can be implemented on a large-scale. For this reason, a cellular–molecular model was chosen for our study. Various tumor cell lines were observed to develop multidrug resistance when the cells were treated with the drugs over a period of time (Gerlach et al., 1986; Beck, 1987). This resistance was due to, apart from other causes, the appearance of P-glycoprotein which exerts efficient pumping action to pump the drugs out of the tumor cells. Recently, there have been reports showing that expression of siRNAs targeting the MDR1 gene is able to reverse the P-glycoprotein mediated multidrug resistance (Shi et al., 2006). Therefore, we established an in-vitro cell model for screening, in which cells were transfected with pGL3 reporter plasmids containing the 2 kb of MDR1 promoter. The cell model was then used to screen candidate multidrug resistance inhibitors from over 300 purified naturally occurring compounds extracted from plants and animals. Ultimately, Dioscin was found to have a significant inhibitory effect on MDR1 promoter activity.

Dioscin, one of the best characterized diosgenyl saponins, has been shown to exhibit potent cytotoxicity toward a number of human cancer cell lines (Wang et al., 2001). However, the effect of Dioscin on

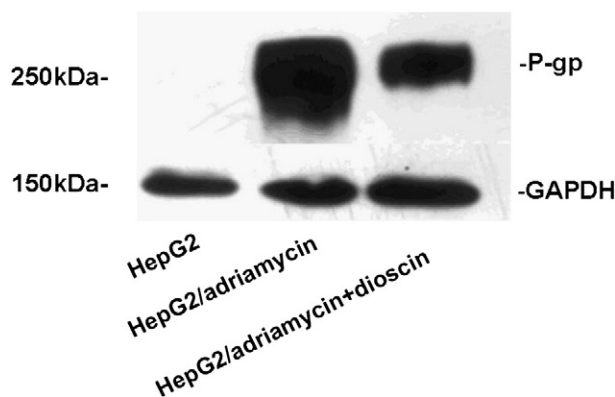


Fig. 5. Effect of Dioscin on P-glycoprotein expression in HepG2/adriamycin cells by western blot analysis. HepG2 and HepG2/adriamycin cells were plated in a 6-well plate. Designated HepG2/adriamycin cells were incubated with 2 $\mu\text{g/ml}$ Dioscin for 24 h. Cell lysates were prepared, and subjected to SDS-PAGE. P-glycoprotein (P-gp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by Western blot using specific antibodies. Lane 1: HepG2 cells, Lane 2: HepG2/adriamycin cells, Lane 3: Dioscin treated HepG2/adriamycin cells.

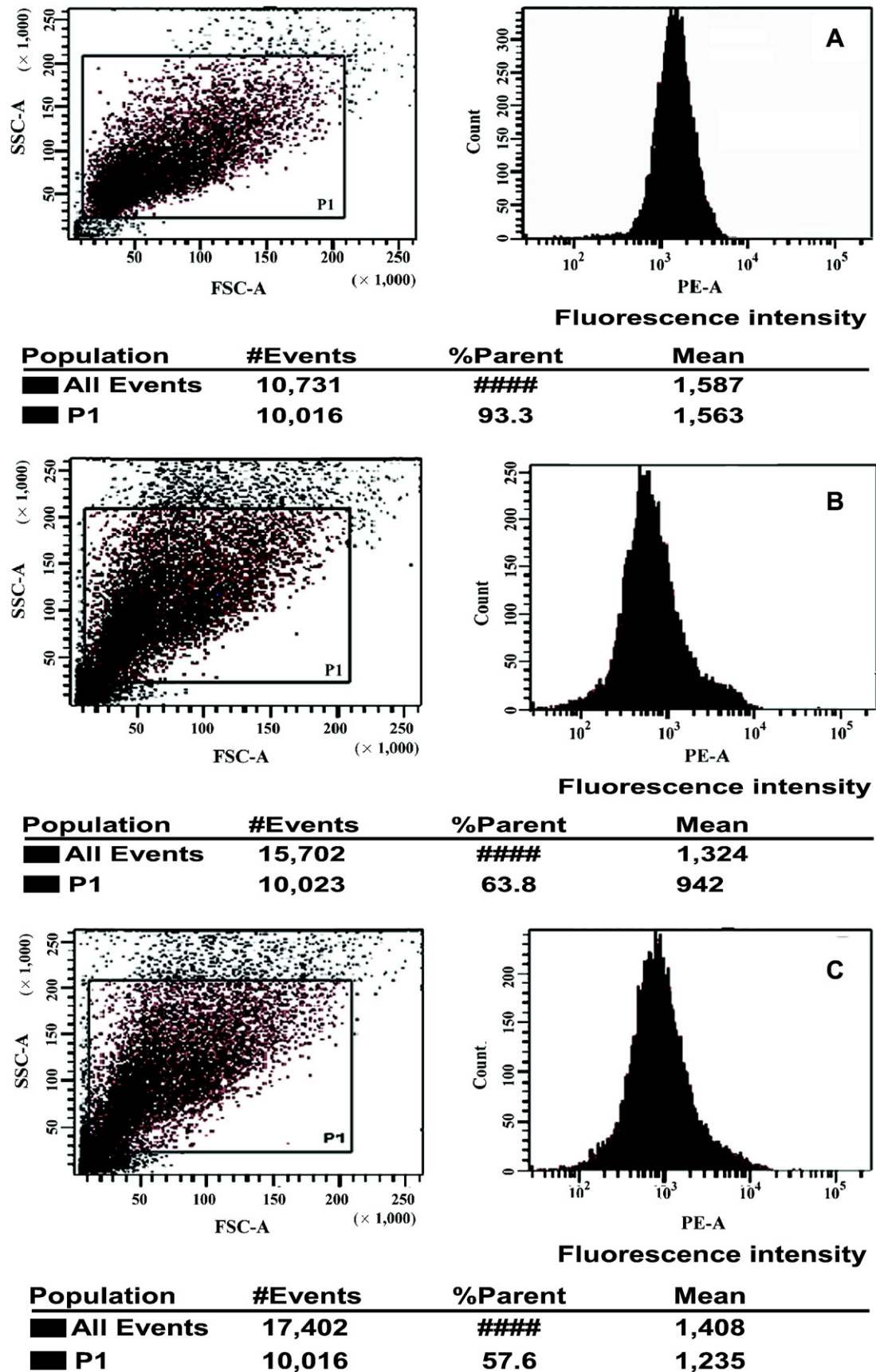


Fig. 6. Effect of Dioscin on adriamycin accumulation. HepG2 and HepG2/adriamycin cells were incubated with 2 µg/ml adriamycin (with or without Dioscin pre-treatment for 24 h) for 1 h at 37 °C. The cells were subjected to flow cytometry with excitation measured at 488 nm and emission measured at 575 nm. The HepG2 cell was used as a positive control for maximum adriamycin accumulation. Representative examples are shown. A: HepG2; B: HepG2/adriamycin; C: HepG2/adriamycin + 2 µg/ml Dioscin.

reversal of multidrug resistance and its molecular mechanism has not been previously reported. In order to further investigate the effects of Dioscin on anti-MDR, a multidrug resistant HepG2/adriamycin cell line was used. HepG2/adriamycin cells were induced by adriamycin, which is a chemotherapeutic agent used for the treatment of many solid and hematologic malignancies (Young et al., 1981). Clinical resistance is often acquired to adriamycin resulting in loss of therapeutic efficacy. By RT-PCR, the MDR1 mRNA level of HepG2/adriamycin is higher than that in parental HepG2 cells. Thus, the HepG2/adriamycin cells were used in subsequent experiments.

Reversal of resistance assays requires Dioscin concentrations which are not inhibitory or toxic by themselves. If more than 90% of the parent cell line appears to be viable after treatment, then the concentration of Dioscin is non-toxic and could be used in reversal experiments. Thus, in the present study, the IC₁₀ values of Dioscin on L02 and HepG2 cells were determined and Dioscin concentrations of 2 µg/ml were used to study the reversal of multidrug resistance. The ability of Dioscin to reverse resistance of HepG2/adriamycin cells to adriamycin is shown in Fig. 4. We can see that Dioscin produced a reversal effect of resistance to adriamycin at a concentration of 2 µg/ml. The results suggest that Dioscin has an effect at reversing multidrug resistance in vitro. To explain the modulating effects of Dioscin in HepG2/adriamycin cells, we analyzed P-glycoprotein expression and function. Additionally, decreased protein expression was found by Western blot analysis. Reduction of the expression of P-glycoprotein may certainly be proposed as one of the mechanisms for certain modulators or agents to reverse multidrug resistance phenotype (Hu et al., 1996). In this study, adriamycin was chosen to study P-glycoprotein function since it is a good P-glycoprotein substrate with an autofluorescence capacity (Ponce de León and Barrera-Rodriguez, 2005). By flow cytometry, the result showed that Dioscin could inhibit intracellular adriamycin efflux and significantly increase the accumulation of adriamycin in HepG2/adriamycin cells.

In conclusion, we have shown here that Dioscin can effectively reverse multidrug resistance, via inhibiting the activity of MDR1 promoter, thus down-regulating MDR1 gene, P-glycoprotein expression and inhibiting the function of P-glycoprotein. Our results suggest that Dioscin is a novel and potent agent for reversing multidrug resistance mediated by P-glycoprotein and may be considered as a promising lead compound for the design of more efficient multidrug resistance chemosensitizers or reversal agents.

Acknowledgments

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