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Ergosterol reverses multidrug resistance in SGC7901/Adr cells

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Multidrug resistance (MDR) is a major obstacle in the chemotherapeutic treatment of tumors. Elevated expression of the P-glycoprotein (P-gp) transporter is associated with MDR and responsible for the resistance of tumor cells against a variety of anticancer drugs. In this study, the reversal effect of ergosterol (Erg) on SGC7901/Adr cells was investigated. At concentrations of 1 μM and 5 μM, Erg could reverse the resistance of SGC7901/Adr to adriamycin up to 4.84 and 3.92 folds, respectively. Mechanistically, Erg could increase the intracellular accumulation of adriamycin and Rh123 in SGC7901/Adr cells through inhibiting the transcription of MDR1 gene and down-regulating the expression of P-gp. In conclusion, Erg could reverse the MDR of SGC7901/Adr cells *via* its influence on P-gp expression and thus be a promising lead compound for future studies.

1. Introduction

As a major obstacle in cancer chemotherapy, MDR is a phenomenon that cancer cells could develop the resistance to a wide variety of structurally and pharmacologically unrelated anticancer drugs. One mechanism of MDR is associated with the up-regulation of P-glycoprotein (P-gp) expression, which belongs to a member of the ATP-Binding Cassette (ABC) super family of membrane transporters encoded by the MDR1 gene (Maliepaard et al.1999; Krishna and Mayer 2000). The ABC transporters constitute a large family of membrane proteins, which transport a variety of compounds through the membrane against a concentration gradient at the cost of ATP hydrolysis. Substrates of the ABC transporters include phospholipids, ions, steroids, polysaccharides, organic anions, lipids, bile acids, xenobiotics, and peptides for antigen presentation (Choudhuri and Klaassen 2006; Leslie et al. 2005). P-gp can lead to MDR and expel many kinds of anti-cancer agents out of cells (Sikic et al. 1997), such as adriamycin, vinblastine, teniposide, paclitaxel and mitomycin C. In order to regain sensitivity of MDR tumor cells to chemotherapeutics, researchers have focused on the drugs that can inhibit P-gp activity. Many compounds have been found to enhance the intracellular accumulation of anticancer drugs *via* inhibiting P-gp, such as cyclosporine (Theis et al. 2000), valspodar, biricodar (VX-710) (Fojo and Bates 2003) and so on. Nevertheless, these compounds failed to achieve clinical success mainly due to their toxicity or side effects. Therefore, the development of more potent reversal agents with low toxicity is the high priority of research on MDR. Ergosterol (Erg, Fig. 1), the major sterol present in lower eukaryotes, is an evolutionary precursor of cholesterol (Dupont et al. 2011). The fluorescent ergosterol analog dehydroergosterol (DHE) has been used to monitor the initial steps of sterol uptake. The studies showed that absorption of sterol molecules to plasma membrane was associated with two ABC pumps – Aus1p and Pdr11p. Deficiency in Aus1p or Pdr11p can inhibit exogenetic Erg absorption (Kohut et al. 2011). Mukhopadhyay et al. (2004) have demonstrated that reduction of either of membrane ergosterol or sphingolipids could alter the location of Aus1p and Pdr11p and result in increased susceptibilities of *C. albicans* cells to the drugs. This phenomenon suggests that Erg interacts with the ABC family. Erg is an important constituent of fungal cell membranes. Its function is similar to that of cholesterol in mammal cell membrane. Fenyvesi et al. (2008) have suggested that P-gp mediates the ATP-dependent re-localization of cholesterol from the cytosolic to the exoplasmic leaflet of the plasma membrane and is involved in stabilizing the cholesterol-rich microdomains, rafts, and caveolae (Garrigues et al. 2002). The activity of the transporter is highly sensitive to the presence of cholesterol, and changes in cholesterol level can affect drug resistance of cells and the activity of P-gp(Shrivastava and Chattopadhyay 2007). Based on these results, we hypothesized that Erg might affect the MDR of cells. In this study, the reverse effects of Erg on SGC7901/Adr cells and its relevant mechanisms were investigated.

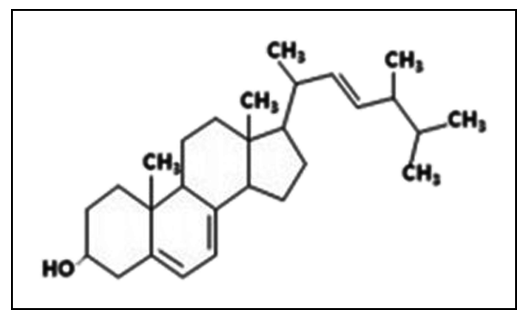

Fig. 1: Structure of Ergosterol

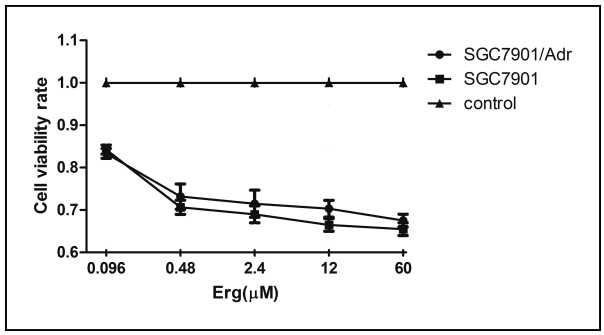

Fig. 2: The growth rate of SGC7901/Adr and SGC7901 cells with Erg. Cells were treated with gradient concentrations of Erg for 48 h and determined by MTT assay. The results were presented as mean ± SD from three independent experiments.*$P < 0.05$ versus control

2. Investigations and results

2.1. Cytotoxicity assay

The MTT assay was used to examine the cytotoxic activity of Erg on tumor cells (Fig. 2). Concentrations below the individual IC_{10} of the drug were used in the study. In this concentration range, Erg showed a weak effect on the cell viability *in vitro*. As shown in the Table, Erg could enhance the cytotoxicity of adriamycin to SGC7901/Adr cells in a dose-dependent manner, but had little effect on drug sensitive SGC7901 cells (Fig. 2). These findings indicated that Erg could enhance the potency of adriamycin against SGC7901/Adr cells, supporting the notion that Erg could reverse multidrug resitance of SGC7901/Adr cells.

2.2. Effect of Erg on intracellular accumulation and efflux of Rh123

We first used intracellular accumulation of Rh123 to determine whether the Erg could affect the transport function of P-gp. Compared with the untreated cells, a significant increase of Rh123 accumulation was observed in SGC7901/Adr cells treated with Erg (Fig. 3A), which indicates a blockage of the P-gp activity by Erg. SGC7901/Adr cells treated with 5 μM Erg exhibited a markedly greater Rh123 accumulation than verapamil. As for the Rh123 efflux, as shown in Fig. 3B, the intracellular Rh123 level was increased in a concentration-dependent manner, which suggested that the outward transporting activity of P-gp was inhibited by this compound.

2.3. Effect of Erg on intracellular accumulation of adriamycin

Adriamycin is a substrate for transport mediated by P-gp, the intracellular adriamycin- associated mean fluorescence intensity (MFI) was thus used to reflect the transport activity of P-gp. As shown in Fig. 4, the accumulation of adriamycin in SGC7901/Adr cells was significantly lowered than that in SGC7901 cells, which is an important cause of adriamycin-resistance in SGC7901/Adr cells. Intracelluar accumulation of adriamycin was markedly increased when used in combination with Erg and the cells showed a concentration-dependent feature.

Table: Effects of Erg on adriamycin cytotoxicity in SGC7901/Adr cells

Treatment	IC_{50}(μM)	RF
Adr	3.53 ± 2.15	1.00
Adr + Ver(10 μM)	$0.53 \pm 0.09^{**}$	6.67
Adr + Erg(1 μM)	$0.90 \pm 0.10^{**}$	3.92
Adr + Erg(5 μM)	$0.73 \pm 0.04^{**}$	4.84

Notes: IC_{50} values of adriamycin were determined in the presence of Erg (1, 5 μM)with various concentrations of adriamycin in SGC7901/Adr cells. After 48 h, the number of viable cells was determined using MTT assay. The resistant fold (RF) values were calculated as follow: RF = IC_{50} of adriamycin alone/IC_{50} of adriamycin + modulator. $^{*}P < 0.05$, $^{**}P < 0.01$ versus adriamycin treatment.

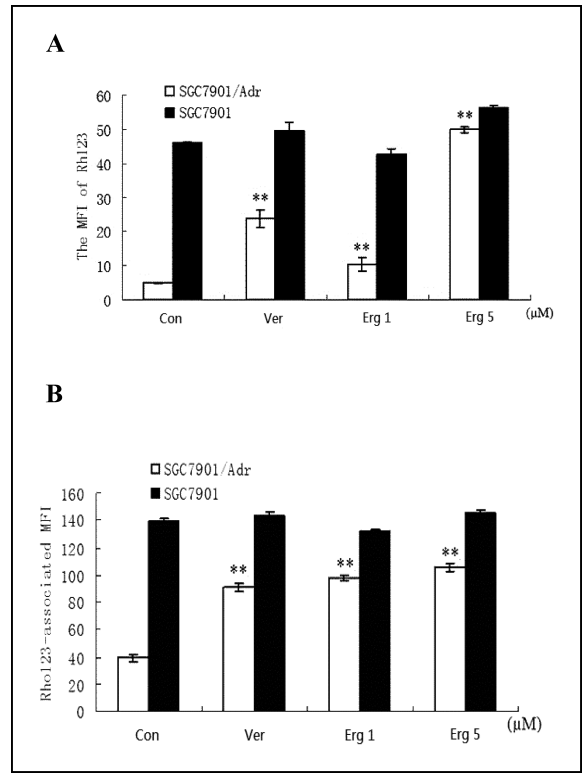

Fig. 3: Effects of Erg on Rh123 accumulation (A) and efflux (B) in SGC7901 and SGC7901/Adr cells. (A) Cells were pretreated with Erg (0, 1, 5 μM) or verapamil (10 μM) for 1 h and then exposed to 5 μM Rh123 for another 1 h. (B) Cells were incubated with 5 μM Rh123 for 1 h, and then incubated in the presence of Erg (0, 1, 5 μM) or verapamil (10 μM) for 1 h. Rh123-associated MFI was evaluated by flow cytometry. Data were expressed as mean ± SD of three independent experiments. *$P < 0.05$,**$P < 0.01$ versus control

2.4. Regulation of Erg on MDR1 gene expression

The MDR1 gene expression of SGC7901 and SGC7901/Adr cells was assessed using semi-quantitative RT-PCR, and analyzed by calculating the relative densitometric value of MDR1 gene expression/β-actin expression. Consistent with our expectation, there was no apparent MDR1 expression in SGC7901

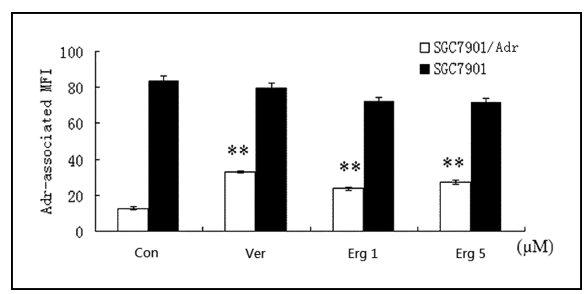

Fig. 4: Effects of Erg on adriamycin accumulation in SGC7901 and SGC7901/Adr cells. Cells were treated with different concentrations of Erg(0, 1, 5 μM) or verapamil (10 μM) combined with adriamycin (0.2 μg/mL) for 48 h and adriamycin-associated MFI was evaluated by flow cytometry. Data were expressed as mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$ versus control

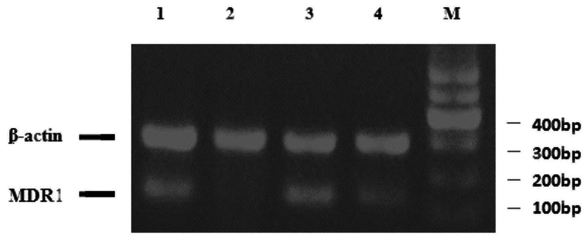

Fig. 5: RT-PCR analysis of MDR1 mRNA expression.The sizes of the specific RT-PCR products were 157 bp for MDR1 and 305 bp for β-actin.1: untreated SGC7901/Adr cells; 2: untreated SGC7901 cells; 3: SGC7901/Adr cells treated with 1 μM Erg; 4: SGC7901/Adr cells treated with 5 μM Erg; M:marker (2000 bp)

cells, but obvious expression in SGC7901/ADR cells (Fig. 5). Compared with the untreated SGC7901/Adr cells, the MDR1 gene expression level in Erg treated (1, 5 μM for 48 h) SGC7901/Adr cells were decreased 36.56% *vs* 57.63%.

2.5. Regulation of Erg on P-gp expression

To further confirm whether P-gp expression was down-regulated by Erg, the expression levels of P-gp in SGC7901 cells and Erg treated SGC7901/Adr cells were analyzed by flow cytometry. SGC7901 cells showed virtually fluorescent intensity labeled by anti-P-glycoprotein monoclonal antibody and used as the blank. Compared with SGC7901 cells, SGC7901/Adr cells exhibited a strong fluorescent area. After 48 h incubation with Erg (1, 5 μM),the expression level of P-gp was decreased by 20.4% and 32.2% in SGC7901/Adr cells, by respectively, compared with untreated SGC7901/Adr cells (Fig. 6).

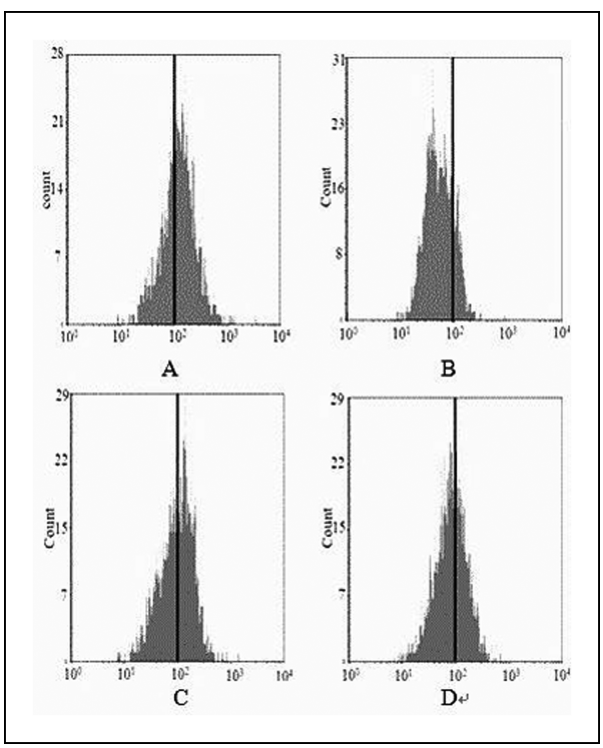

Fig. 6: P-gp expression in SGC7901 and SGC7901/Adr cells. Cells were incubated with various concentrations of Erg for 48 h and P-gp was determined by flow cytometry. (A) untreated SGC7901/Adr cells; (B) untreated SGC7901 cells; (C) SGC7901/Adr cells treated with 1 μM Erg; (D) SGC7901/Adr cells treated with 5 μM Erg

3. Discussion

MDR is one of the major causes for chemotherapeutic treatment failure in cancer patients, which is often associated with up-regulated expression of P-gp (Fojo and Bates 2003). P-gp belongs to the family of ABC transporters and utilizes the energy of ATP hydrolysis (Gottesman and Pastan 1993; Bacso et al. 2004), which is the most well known of the transmembrane efflux transporters and first surfaced in the 1970 s as the reason for multidrug resistance in cancer cells.

The strategies for combating MDR that targeted to P-gp are divided into three types: Firstly, inhibitors that could decrease the P-gp "pump" activities by competitive or noncompetitive with chemotherapeutics. They appear to interact with the same binding site(s) as drugs, and compete with them for transport. Many modulators (*e.g.* verapamil, cyclosporin A, trans-flupenthixol) are transported by the protein (Liu et al. 2010). The way in which modulators exert their action at the molecular level is still not well understood. Secondly, with the development of genetic engineering techniques, antisense RNA or ribozyme technology was used to inhibit the transcription of MDR1. Antisense RNA prevents the translation of the mRNA through specifically combining with the target mRNA, therefore leading to inhibition of P-gp expression (Chin et al. 1989). Thirdly, many modulators inhibit the activity of P-gp through regulating the signaling pathway relevant to P-gp expression. Studies confirmed that pharmacologic inhibition of JNK activity or dominant-negative suppression of c-Jun remarkably abolished the ability of JNK to down-regulate P-gp (Zhou et al. 2006). Inhibitor PD098059 of ERK1/2 MAPK and inhibitor LY294002 of PI-3K are both able to significantly increase the sensitivity of tumor cells to chemotherapy (Ding et al. 2006).

In our study, adriamycin and Rh123 were employed to study P-gp transport function since they were P-gp substrates with an autofluorescence capacity (Ponce de León and Barrera-Rodríguez 2005). Erg could concentration-dependently enhance the accumulation of intracellular adriamycin and Rh123 (Fig. 2 and Fig. 3). In the efflux assay, the Erg could inhibit the efflux of Rh123 from treated SGC7901/Adr cells. The further mechanistic study suggests that Erg suppressed the drug-transport activity of P-gp by down-regulation of MDR1 and P-gp expression. The interaction between Erg and the related signaling pathway should be further studied.

Erg is an important plant sterols and rich in *Ganoderma lucidum* (Yuan et al. 2006), which is a necessary constituent of cell membrane. Our study indicated that Erg had enhanced the cytotoxicity of adriamycin on SGC7901/Adr cells with low toxicity on SGC7901/Adr cells at concentrations below 5 μM. Erg is the predecessor of vitamin D2 and used to produce steroid hormone drugs. Our research showed the efficacy of Erg as a potent reversal agent to overcome multidrug resistance of SGC7901/Adr cells. Since Erg itself has other physiological activity, such as anti-inflammatory anti-tumor anti-aging etc (Kuo et al. 2011; Wu et al. 2007; Weng 2010), so it should also be promising as an adjuvant drug in tumor therapy.

In conclusion, Erg could effectively reverse the MDR of SGC7901/Adr cells, and thus way be a candidate for MDR reversing agents in cancer chemotherapy. Further work is needed to study the interactions between Erg and cell membranes and to evaluate *in vivo* efficacy of Erg on reversing MDR of tumor cells.

4. Experimental

4.1. Materials

Erg (purity > 99% by HPLC) was provided by Jiulong Pharmaceutical Co. Ltd. (Xi'an, China). Rhodamine123 (Rh123), 3-(4,5-dimethylthiazol-

2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and verapamil (Ver) were purchased from Sigma Chemical; Adriamycin was obtained from Pfizer Italia S.r.I, RPMI1640 medium were purchased from GIBCO-BRL; Trizol reagent was purchased from TaKaRa; cDNA Synthesis Kit was purchased from Thermo fisher (USA); Rabbit anti-human monoclonal antibody against P-gp and Fluorescein Isothiocyanate Conjugated (FITC) Goat anti-Rabbit lgG were purchased from Beijing BIOSS; Primer pairs and probe were purchased from Sangon Co. Ltd..

4.2. Cell lines and cell culture

Drug sensitive human gastric carcinoma cell line SGC7901 and its adriamycin-resistant counterpart SGC7901/Adr cells were obtained from The Fourth Military Medical University (China). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum at 37 °C, humidity 95% and 5% CO_2. SGC7901/Adr cells were maintained in the medium containing 0.7 μg/mL adriamycin and cultured in drug-free medium for 15 days before experiments.

4.3. Determination of cytotoxicity and multidrug resistance reversal fold (RF)

The *in vitro* cytotoxicity of the drugs was determined using the MTT assay. The cells were harvested in the exponential growth phase, 200 μL aliquots of cells were seeded into 96-well plates at 5×10^4 cells/mL for SGC7901 and SGC7901/Adr cells per well. The cells were incubated for 24 h at 37 °C before the addition of modulators. After pre-incubation, the cells were treated with various concentrations of Erg (0, 0.096, 0.48, 2.4, 12, 60 μM) for 48 h. Then 20 μL of freshly prepared MTT (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. 200 μl of medium was then carefully removed, and 150 μL of dimethyl sulfoxide (DMSO) were added and agitated for 15 min to dissolve the formazan crystals. The absorbance was measured on a Bio-Rad Model/550 microplate reader (Hercules, CA, USA) at a wavelength of 570 nm. IC_{10} (IC_{10} is defined as the concentration required inhibiting cell growth by 10%) and IC_{50} (IC_{50} is defined as the concentration required inhibiting cell growth by 50%) values were calculated by SPSS 17.0, the concentration ranges of Erg were less than IC_{10} in the following experiments to avoid drug toxicity.

The reversal folds were measured by the following method: SGC7901 and SGC7901/Adr were seeded into 96-well plates and treated with various concentrations of adriamycin (0, 0.16, 0.8, 4, 20, 100 μg/mL) combined with different concentrations of Erg (0, 1, 5 μM) for 48 h. The inhibitory rate of adriamycin to cell growth was obtained through IC_{50} values. The reversal fold (RF) values, as potency of reversal, were calculated from fitting the data to RF = IC_{50} of adriamycin alone/IC_{50} of adriamycin in combination with Erg. Triplicate experiments with triplicate samples were performed. Control medium included equivalent amount of DMSO (as solvent control), verapamil (10 μM) was used as the positive control drug.

4.4. Rh123 accumulation and efflux assay

SGC7901 cells and SGC7901/Adr cells were seeded in 6-well plates with 5 mL aliquots at 2.0×10^5 cells/mL per well and cultured for 24 h at 37 °C in an atmosphere containing 5% CO_2. The cells were then treated with Erg (0, 1, 5 μM) or verapamil (10 μM) for 1 h, and cells were incubated with 5 μM Rh123 in culture medium in the dark at 37 °C in 5% CO_2 for another 1 h. Then cells were washed twice with ice-cold PBS. The mean fluorescence intensity (MFI) associated with intracellular Rh123 was examined by flow cytometry (FACS Calibur, USA) at a wave length of 480 nm for excitation and 530 nm for emission.

In the efflux study, cells were firstly incubated with medium containing 5 μM Rh123 at 37 °C for 1 h, washed three times with Rh123-free medium, and then incubated in the presence of Erg (0, 1, 5 μM) or verapamil (10 μM) at 37 °C for 1 h, respectively. Cells were washed twice with ice-cold PBS. The mean fluorescence intensity (MFI) associated with intracellular Rh123 was examined as described above. Data analysis was completed with Cell Quest software.

4.5. Adriamycin accumulation assay

SGC7901 cells and SGC7901/Adr cells were harvested, and 5 mL aliquots of cells were seeded in 6-well plates at 2.0×10^5 cells/mL per well and cultured for 24 h at 37 °C in an atmosphere containing 5% CO_2. Then cells were treated with different concentrations of Erg (0, 1, 5 μM) or verapamil (10 μM) combined with adriamycin (0.2 μg/mL) for 48 h. Then cells were placed in ice-water to cease the reaction followed by harvesting and washing twice with ice-cold PBS (Chen et al. 2004), and the mixture was centrifuged for 3 min at 1,200 rpm. The mean fluorescence intensity (MFI) associated with adriamycin was determined by flow cytometry. Excitation was performed by an argon ion laser operating at 633 nm and the emitted fluorescence was collected through a 660 nm pass filter.

4.6. Determination of MDR1 expression

SGC7901 cells and SGC7901/Adr cells were harvested, and 5 mL aliquots of cells were seeded in 6-well plates at 2.0×10^5cells/mL per well. The cells were treated with different concentrations of Erg (0, 1, 5 μM)for 48 h. Total RNA was extracted using the Trizol reagent of TaKaRa RNAiso^{TM} Plus and and the first strand of cDNA was synthesized by PrimeScript^{TM} RT reagent Kit (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions. RT-PCR was performed as previous reported (Gao et al. 2010). We used the following PCR primers: MDR1, forward 5′-CCCATCATTGCAATAGCAGG-3′ and reverse 5′-GTTCAAACTTCTGCT CCTCA-3′for a 157 bp product; β-actin, forward 5′-CTTCTACAATGAGCTGCGTG-3′ and reverse 5′-TCATGAGGTAGTCGTCAGG-3′ for a 305 bp product. β-Actin was used as the reference gene. The reaction conditions were set as follows: 94 °C for 90 s, 35 cycles at 94 °C for 15 s, 57 °C for 30 s, and 72 °C for 30 s; followed by extension at 72 °C for 10 min. The PCR products were separated on 1.5% agarose gel (Gibco) and visualised with ethidium bromide. Images were fixed with a gel imaging system and the target gene expression level was measured with semi-quantitative analysis by calculating the ratio of density metric value from specific genes expressed in retation to the internal standard (MDR1 gene expression/β-actin gene expression). Triplicate experiments with triplicate samples were performed.

4.7. Determination of P-gp

The P-gp expression levels were measured by flow cytometry (Kim et al. 2007; Xu et al. 2006). SGC7901 and SGC7901/Adr cells were seeded into 6-well plates at a density of 2×10^5cells/mL per well, 5 mL of aliquots cells for each well. The cells were treated with Erg (0, 1, 5 μM) for 48 h, respectively. Then the cells were harvested, washed twice with ice-cold PBS, counted and then labeled with Rabbit anti-human monoclonal antibody against P-gp and Fluorescein Isothiocyanate conjugated (FITC) Goat anti-Rabbit lgG. The fluorescent intensity was analyzed using FACS with isotype as control. Duplicate experiments with triplicate samples were performed.

4.8. Data analysis

The data gathered from this experiment were presented as mean ± SD. Results from three cultures and the significant difference was analyzed by Student's t-test and $P < 0.05$ were considered as statistically significant.

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