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Reversal of P-glycoprotein-mediated multidrug resistance by guggulsterone in doxorubicin-resistant human myelogenous leukemia (K562/DOX) cells

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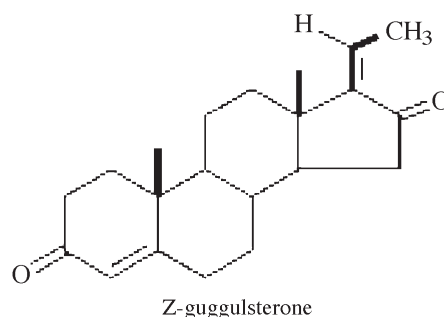
Multidrug resistance (MDR) has been a major problem in cancer chemotherapy. The development of P-glycoprotein inhibitors could be effective to reverse multidrug resistance. The aim of this study was to observe the effects of guggulsterone, the active component of gugulipid, on multidrug resistance in doxorubicin-resistant K562 cells (K562/DOX) and the parental K562 cells. Its cytotoxicity and reversal effects on multidrug resistance were assessed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Apoptosis percentage of cells was obtained from Annexin V/fluorescein isothiocyanate (FITC) and propridium iodide (PI) double staining. The effects of guggulsterone on P-glycoprotein activity were evaluated by measuring rhodamine 123 (Rh123)-associated mean fluorescence intensity and P-glycoprotein expression on the basis of the flow cytometric technology, respectively. The results showed that guggulsterone up to 100 μM had little cytotoxicity against K562/DOX cells. When combined with doxorubicin, it significantly promoted the sensitivity of K562/DOX cells toward doxorubicin through increasing intracellular accumulation of doxorubicin in a dose-dependent manner. Further study demonstrated that the inhibitory effect of guggulsterone on P-glycoprotein activity was the major cause of increased stagnation of doxorubicin inside K562/DOX cells, indicating that guggulsterone may effectively reverse multidrug resistance in K562/DOX cells via inhibiting expression and drug-transport function of P-glycoprotein.

1. Introduction

The exposure of cancer cells to a single hydrophobic cytotoxic agent such as vinka alkaloids, anthracyclines or taxol frequently results in a specific resistance not only to the cytotoxic agent but also to other structurally and mechanically unrelated anticancer drugs. Multidrug resistance is a major obstacle for cancer chemotherapy. It is correlated with the overexpression of P-glycoprotein in the plasma membrane of resistance cells, where P-glycoprotein acts as an ATP-dependent efflux pump by extruding the anticancer drugs and decreasing their intracellular accumulation (Gottesman and Pastan 1993). P-glycoprotein is encoded by MDR1 gene in mammalian cells, and has been implicated in drug resistance in a number of human tumors (Gros et al. 1986; Ling 1997). Therefore, inhibition of P-glycoprotein transporter by pharmacological agents should improve the activity of chemotherapy against human cancer (Ford and Haiz 1990). Numerous agents interfering with the activity of P-glycoprotein have been described; however clinical application has been limited by deleterious toxicities (Dale et al. 1998; Newman et al. 2000; Sparrebom et al. 1999). Therefore, development of safe and effective MDR reversing agents is eagerly required.



Z-guggulsterone

Guggulsterone [4,17(20)-pregnadiene-3,16-dione], which is the active component of gugulipid, is derived from the gum resin of the tree *Commiphora mukul*, and has *cis*-and *trans*-isomers. This gum resin has been used for centuries in Ayurvedic medicine to treat obesity, arthritis, and hyperlipidemia (Sinal and Gonzalez 2002; Urizar and Moore 2003). A previous study has shown that guggulsterone could inhibit P-glycoprotein-mediated transport of daunorubicin and rhodamine 123 (Nabekura et al. 2008). The aim of this study was to investigate the effects of guggulsterone, the active component of gugulipid, on the P-glycoprotein function and P-glycoprotein-mediated MDR in human myelogenous leukemia (K562) cells and K562/DOX cells.

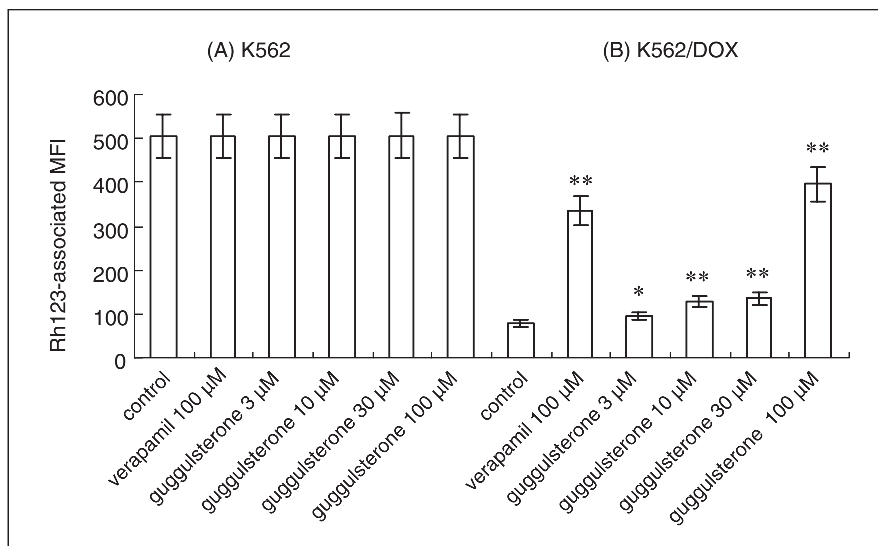


Fig. 1: Effect of guggulsterone on Rh123 accumulation in K562 and K562/DOX cells. Cells were incubated in the presence or absence of guggulsterone (3, 10, 30,100 μM) with medium containing 5 μM Rh123 at 37 °C for 1 h.Rh123-associated mean fluorescence intensity (MFI) was evaluated by flow cytometry. Data were each expressed as a mean ± S.E.M. (n = 3).
*P < 0.05,
**P < 0.01, compared with untreated K562/DOX cells

2. Investigations and results

2.1. Cytotoxicity assay

The effect of guggulsterone on K562/DOX cell growth was determined with MTT assay. The viability of the cells was evaluated as described below. Up to a concentration of 200 μM guggulsterone had no significant inhibitory effects on the growth of K562/DOX cells (data not shown). To minimize the effect of guggulsterone itself on the resistant cell growth, we chose lower concentrations of guggulsterone (3, 10, 30,100 μM) in the reversal experiments.

We used MTT assay to determine the cytotoxicity of a combination of doxorubicin with different concentrations of guggulsterone. As shown in the Table, guggulsterone could significantly increase doxorubicin toxicity in K562/DOX cells in a concentration-dependent manner. The RF of 100 μM guggulsterone (14.39) was comparable to that of 100 μM verapamil (12.55). However, no such activity was found in K562 cells. These finding indicated that guggulsterone enhanced the potency of doxorubicin against K562/DOX cells, whereas, had little effects on K562 cells, supporting the notion that guggulsterone could reverse P-glycoprotein-mediated resistance of K562/DOX cells.

2.2. Effect on intracellular Rh123 accumulation and efflux inhibition

Rh123 acts as a good substrate for MDR-associated P-glycoprotein, and agents that block P-glycoprotein have been found to increase the retention of Rh123 in MDR cells (Yoshimura et al. 1990). Therefore, we further investigated the effect of guggulsterone on the P-glycoprotein activity by detecting accumulation of Rh123 in cancer cells. As shown in Fig. 1, K562/DOX cells in the absence of guggulsterone exhibited a significant decrease of Rh123 compared to K562 cells, while a notable increase was seen in K562/DOX cells in the presence of guggulsterone (3, 10, 30,100 μM). The short time (60 min) treatment with guggulsterone induced the increase of Rh123 in K562/DOX cells, suggesting that guggulsterone had the ability to inhibit the drug-transport activity of P-glycoprotein.

The role of guggulsterone to block outward transporting function of P-glycoprotein was also determined in the Rh123 efflux experiment. Figure 2 demonstrates that without guggulsterone, a rapid decrease of intracellular Rh123 level was observed in K562/DOX cells after incubation in Rh123-free medium for 90 min. Guggulsterone (100 μM) could significantly inhibit the efflux of Rh123 from K562/DOX cells. In the presence of guggulsterone, Rh123 efflux was suppressed in K562/DOX cells, suggesting that P-glycoprotein's active outward transport was inhibited.

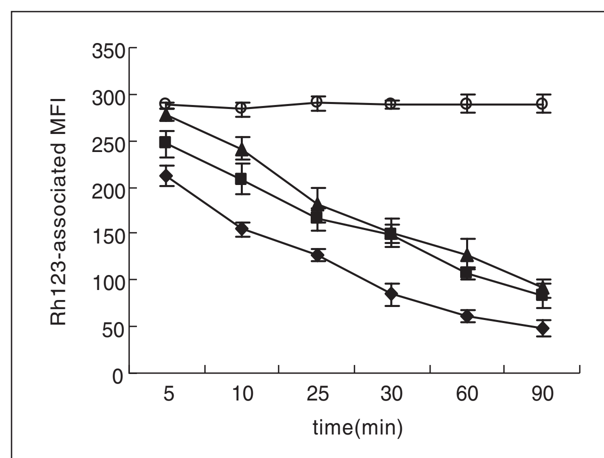


Fig. 2: Effect of guggulsterone on Rh123 efflux in K562/DOX cells. Cells were first incubated with medium containing 5 μM Rh123 at 37 °C for 90 min, washed three times with Rh123-free medium, and then incubated in the presence or absence of 100 μM guggulsterone and 100 μM verapamil at 37 °C for 5, 10, 25, 30, 60, 90 min, respectively. The mean fluorescence intensity (MFI) of retained intracellular Rh123 was then estimated by flow cytometry.
◯ K562 cell control;
▲ K562/DOX cells treated with 100 μM of guggulsterone;
■ K562/DOX cells treated with 100 μM of verapamil;
☐ K562/DOX cells without treatment. Data were each expressed as a mean ± S.E.M. (n = 3)

2.3. Persistence of activity

Figure 3 clearly shows that 100 μM guggulsterone inhibited the efflux of Rh123 from K562/DOX cells and that the inhibitory effect remained even after 100 μM guggulsterone was removed from the medium for 90 min, suggesting that the inhibitory effect

Table: Effect of guggulsterone on doxorubicin cytotoxicity in K562 and K562/DOX cells

Group	IC_{50} (μM)(K562)	IC_{50}(μM))(K562/DOX)	RF(K562/DOX)
DOX	0.19 ± 0.013	8.59 ± 0.17	
DOX + Verapamil 100 μM	0.202 ± 0.005	0.68 ± 0.02[b]	12.55
DOX + Guggulsterone 3 μM	0.198 ± 0.007	6.80 ± 0.13[a]	1.26
DOX + Guggulsterone 10 μM	0.202 ± 0.004	3.26 ± 0.06[b]	2.64
DOX + Guggulsterone 30 μM	0.186 ± 0.004	1.03 ± 0.01[b]	8.37
DOX + Guggulsterone 100 μM	0.190 ± 0.005	0.60 ± 0.05[b]	14.39

Effects of guggulsterone on the sensitivity of K562/DOX and K562 cells toward doxorubicin was examined by MTT method as described in the cytotoxicity assay and multidrug resistance reversal tests. The cells were treated with varying concentrations of doxorubicin in the presence of 3, 10, 30 and 100 μM of guggulsterone. IC50 values for doxorubicin was calculated and the reversal fold (RF) was evaluated. Data were expressed as mean ± S.E.M. (n = 4).
[a] $P < 0.05$,
[b] $P < 0.01$ compared with doxorubicin treatment alone

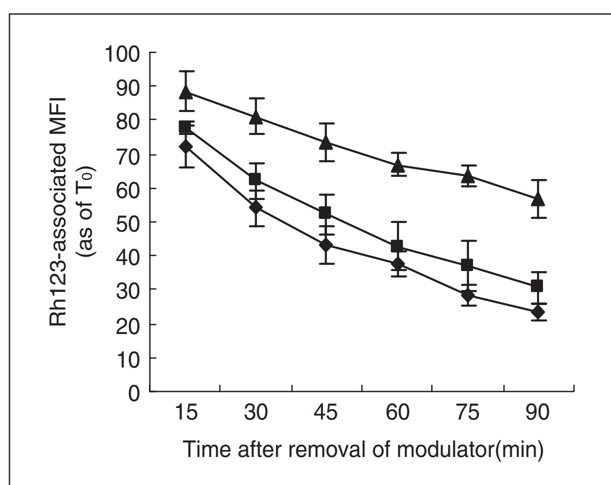


Fig. 3: Persistence of modulatory activity. K562/DOX cells were incubated with medium containing 5 μM Rh123 in the presence or absence of 100 μM guggulsterone and 100 μM verapamil at 37 °C for 90 min, washed three times with rhodamine-free and drug-free medium. At subsequent time points as indicated, the Rh123-associated MFI was measure by flow cytometry.
▲ K562/DOX cells treated with 100 μM of guggulsterone;
■ K562/DOX cells treated with 100 μM of verapamil;
◆ K562/DOX cells without treatment. Data were each expressed as a mean ± S.E.M.
(n = 3)

of the agent on P-glycoprotein function was reversible and the guggulsterone had a longer duration of action than verapamil.

2.4. Effect on intracellular doxorubicin accumulation assay

Doxorubicin is also a good substance for MDR-associated P-glycoprotein, and agents that inhibit P-glycoprotein function have been found to increase accumulation of doxorubicin in drug-resistance K562/DOX cells. So the ability of guggulsterone to inhibit the P-glycoprotein function was evaluated by determining the intracellular doxorubicin-associated mean fluorescence intensity (MFI) in K562/DOX cells. As shown in Fig. 4, the doxorubicin-associated MFI was increased in the presence of guggulsterone in a concentration-dependent manner, which could also explain of guggulsterone on doxorubicin cytotoxicity in K562/DOX cells.

2.5. Effect on doxorubicin-induced apoptosis

The regulation of guggulsterone on the cytotoxicity of anticancer drug toward K562/DOX cells was also evaluated by quantification of apoptotic cells. As shown in Fig. 5, 100 μM of guggulsterone treatment resulted in 3.01% of apoptosis of K562/DOX cells, and the percentage of apoptosis was 5.21% when exposed to 10 μM of doxorubicin. When doxorubicin was combined with 10, 30 and 100 μM of guggulsterone, the mean apoptotic population of K562/DOX cells was increased by 2.53, 3.17 and 3.93 times, respectively, compared with 10 μM of doxorubicin treatment alone. Guggulsterone raised the doxorubicin-induced apoptosis percentage in a dose-dependent manner in K562/DOX cells.

2.6. Effect on P-glycoprotein expression in K562/DOX

To determine guggulsterone's MDR modulation is due to alteration of P-glycoprotein protein levels, K562/DOX cells were treated with various concentration of guggulsterone (10, 30, 100 μM) for 24 h. As shown in Fig. 6, guggulsterone could inhibit the expression of P- glycoprotein in plasma membrane from K562/DOX cells. After 24 h incubation with 10, 30 and 100 μM of guggulsterone, the expression level of P-glycoprotein was decreased by 16.41 %, 40.87 %, and 52.63 %, respectively in comparison to untreated K562/DOX cells. The result indicated that guggulsterone downregulated expression of P-glycoprotein in a dose-dependent manner.

3. Discussion

In the present report, we investigated the efficacy of guggulsterone as a potent reversal agent to overcoming multidrug resistance of K562/DOX cells. The results showed that guggulsterone alone ranging from 3 to 100 μM did not display a significant antiproliferative effect on K562/DOX cells, while the compound at these concentrations enhanced the cytotoxicity of doxorubicin toward K562/DOX cells through accumulating doxorubicin inside cells. Moreover, the increased sensitivity of K562/DOX cells to doxorubicin was also confirmed by the enhanced doxorubicin -induced apoptosis in the presence of guggulsterone. Rh123 is a special substrate for P-glycoprotein. The uptake of Rh123 is resulted from passive inward diffusion (Lehnert et al. 1996; Krishna and Mayer 2000), while the efflux is known to be P-glycoprotein-dependent. Rh123 has been used extensively as an indicator of P-glycoprotein activity in drug-resistant cell lines with P-glycoprotein over-expression (Green et al. 2001; Galski et al. 2006). Herein, Rh123 was used to assess the modulating ability of guggulsterone in drug-transport function of P-glycoprotein. As shown in Fig. 1, the treatment of guggulsterone at 3, 10, 30 and 100 μM resulted in a remarkable increase of the fluorescent intensity from Rh123 in K562/DOX cells, indicating that guggulsterone elevated accumulation of doxorubicin in K562/DOX cells by suppressing the drug-transport activity of P-glycoprotein. The accumulation level of Rh123was hardly varied in K562 cells no matter

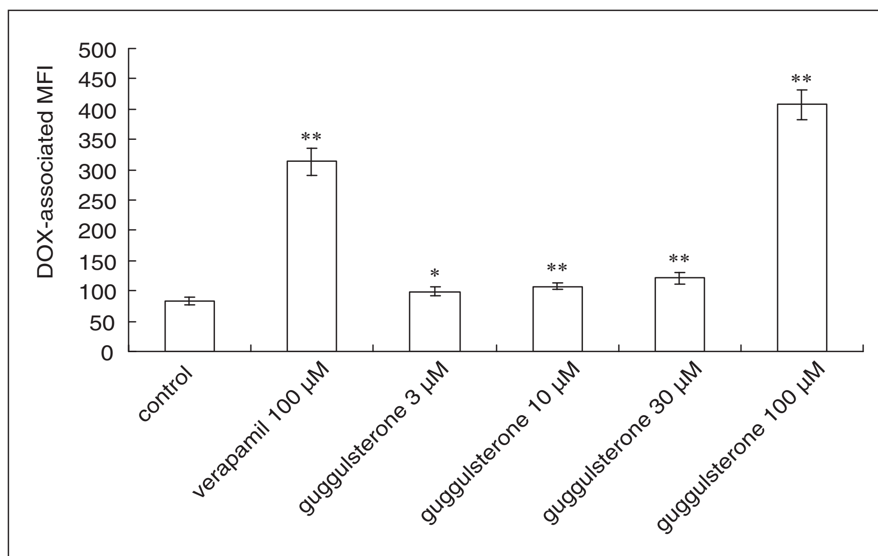


Fig. 4: Effect of guggulsterone on doxorubicin accumulation in K562/DOX cells. Cells were incubated in the presence or absence of guggulsterone (3, 10, 30,100 μM) with medium containing 10 μM doxorubicin at 37 °C for 1 h. Doxorubicin-associated mean fluorescence intensity (MFI) was evaluated by flow cytometry. Data were each expressed as a mean ± S.E.M. (n = 3).
*$P < 0.05$,
**$P < 0.01$, compared with untreated K562/DOX cells

with or without guggulsterone treatment, supporting the notion that guggulsterone enhanced doxorubicin-induced cytotoxicity toward K562/DOX was attributed to its inhibition activity to P-glycoprotein.

In a previous study, Tomohiro examined the activity of the compound in reversing P-gp-overexpressing human carcinoma KB-C2 cells. Guggulsterone might enhance the accumulation of doxorubicin or rhodamine 123 via inhibiting the ATPase activities of P-glycoprotein (Nabekura et al. 2008). The result is quite similar to the effects which block the function of P-glycoprotein or inhibit the expression of P-glycoprotein. On the one hand, guggulsterone inhibited drug-transport function of P-glycoprotein; this conclusion was confirmed by the fact that short time incubation (60-90 min) of K652/DOX cells with guggulsterone caused an increase of intracellular Rh123 and an inhibition of its efflux (Fig. 1 and Fig. 2). On the other hand, the reversal effect of guggulsterone on multidrug resistance of K562/DOX cells may be actualized by downregulating P-glycoprotein (Fig. 6).

Inhibiting the activity of P-glycoprotein is an ideal means to overcome tumor multidrug resistance. Many natural compounds, such as curcuminoid and curcumin, have been reported to inhibit the P-glycoprotein activity through blocking its drug-transport function or down-regulating its expression (Anuchapreeda et al. 2002; Limtrakul et al. 2004). In this study, we added a new member to the class of compounds with P-glycoprotein inhibition activities to the multidrug-resistant cancer cells. Guggulsterone appears to be similar to verapamil in the mechanism to reverse P-glycoprotein-mediated drug resistance. Therefore, the compound is of dual functions of inhibiting P-glycoprotein's drug-pump and expression.

In conclusion, guggulsterone has potent effects in reversing P-glycoprotein-mediated multidrug resistance. This suggests that guggulsterone may be a potential candidate for reversing drug resistance in cancer chemotherapy.

4. Experimental

4.1. Cell culture

The human chronic myeloid leukemia cell line K562, and its multidrug-resistant counterpart K562/DOX, were obtained from the Department of Pharmacology, the Institute of Hematology of Chinese Academy of Medical Sciences (Tianjin, China). K562/DOX cells were maintained in a complete RPMI-1640 medium containing 1 μM doxorubicin (DOX, Wanle, Shenzhen, China) at 37 °C in a humidified atmosphere of 5% CO2. The cells were cultured for two weeks in drug-free medium prior to their use in the experiments.

4.2. Drug

Z-Guggulsterone was purchased from Steraloids (Newport, RI) and its structure was identified as reported previously (Agrawal et al. 2004). The compound was dissolved in dimethylsulphoxide (DMSO) as a stock solution of 100 mM and added to the extracellular solutions to obtain a desired concentration. The final concentration of DMSO was <0.1%, which did not affect the test.

4.3. Cytotoxicity and multidrug resistance reversal assay

To determine the reversal effect of guggulsterone on resistant tumor cells, the cytotoxicity of guggulsterone toward K562/DOX cells was first measured by MTT method. Briefly, K562/DOX cells (5×10^4 per well) were seeded in 96-well plates. After 24 h incubation, the cells were treated with various concentrations of guggulsterone for 24, 48 and 72 h, respectively. Cell viability was assessed by MTT assay as reported (Hansen et al. 1989). The reversal effect of guggulsterone was further investigated with the same method. Cells seeded into 96-well plates were treated with varying concentrations of doxorubicin in the absence and presence of guggulsterone for 48 h, respectively. IC_{50} values for doxorubicin (concentration resulting in 50% inhibition of cell growth) were calculated from plotted results using untreated cells as 100%. The reversal fold (RF) values, as potency parameter of reversal, were calculated from dividing IC_{50} of doxorubicin alone by IC_{50} of doxorubicin in combination with guggulsterone. Control medium included equivalent amount of DMSO (as solvent control), but the applied dose did not exhibit modulation effects on the cell growth or drug sensitivity in these studies. In all the experiments, verapamil (Wanle, Shenzhen, China) was used as a positive control.

4.4. Rh123 accumulation and efflux assay

Rh123 is a fluorescent dye that could be used as a function assay for P-glycoprotein (Qi et al. 2002, Ji et al. 2005). K562/DOX and K562 cells were seeded into 6-well plates at a density of 1×10^6 per well. Cells were incubated in the presence or absence of guggulsterone (3, 10, 30, 100 μM) with medium containing 5 μM Rh123 at 37 °C for 1 h. Verapamil (100 μM) was used as a positive control. The intracellular mean fluorescence intensity (MFI) associated with Rh123 was measured with FACScan flow cytometry (Becman coulter). Excitation was performed by an argon ion laser operating at 488 nm and the emitted fluorescence was collected through a 530 nm pass filter. Data analysis was performed using Cell Quest software.

In the efflux study, K562/DOX cells were first incubated with medium containing 5 μM Rh123 at 37 °C for 90 min, washed three times with Rh123-free medium, and then incubated in the presence or absence of 100 μM

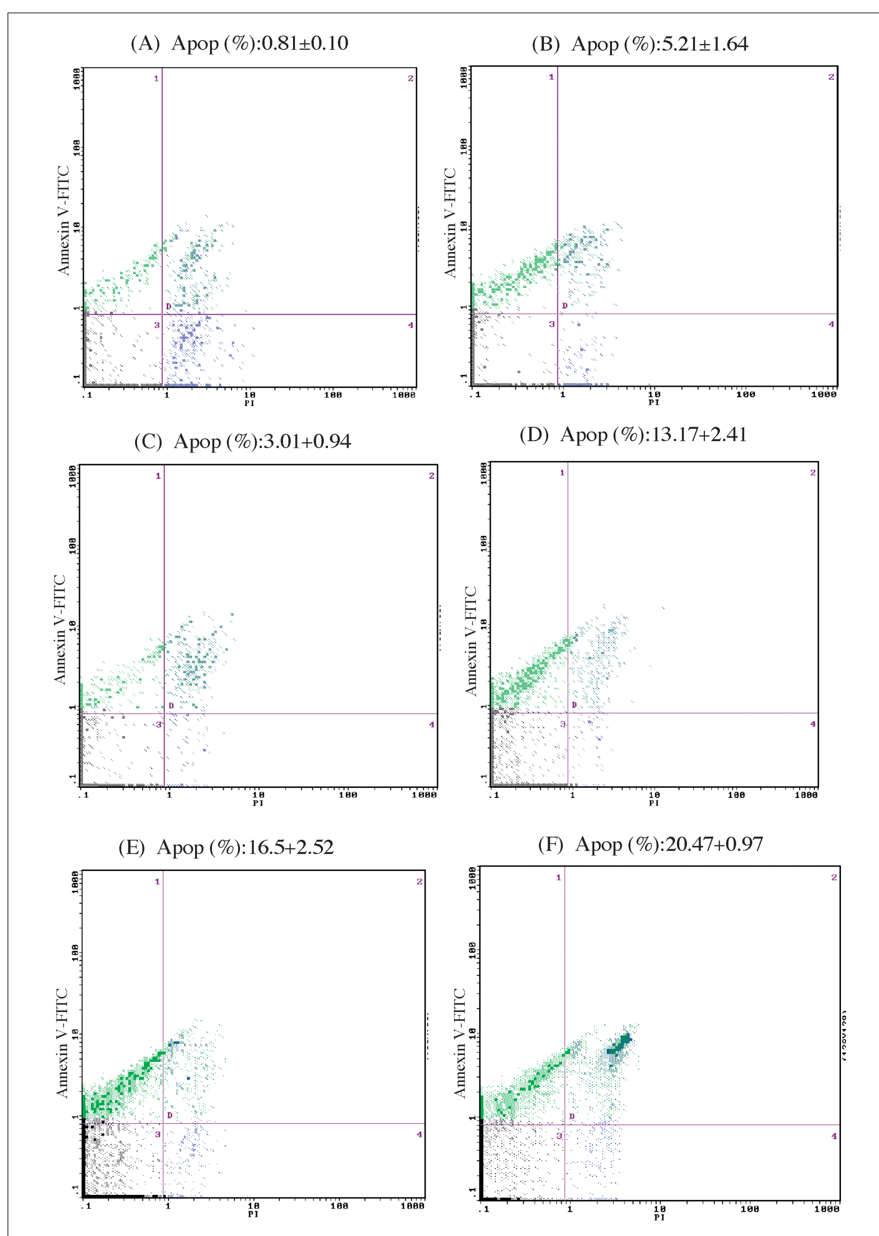


Fig. 5: Flow cytometric analysis of doxorubicin-induced apoptosis of K562/DOX cells. K562/DOX cells were incubated with 10 μM of doxorubicin in the presence of various concentrations of guggulsterone for 48 h.The rate of apoptosis was measured using flow cytometry as described in Materials and methods. A, K562/DOX cells without treatment; B, K562/DOX cells treated with 10 μM doxorubicin; C, K562/DOX cells treated with 100 μM guggulsterone; D-F, K562/DOX cells treated with 10 μM of doxorubicin in the presence of 10,30,100 μM of guggulsterone, respectively

guggulsterone and 100 μM verapamil at 37 °C for 5, 10, 25, 30, 60, 90 min, respectively. The MFI was measured as described for the accumulation assay. Graphs were plotted of cell-associated MFI against time.

4.5. *Persistence of activity*

K562/DOX cells were incubated with medium containing 5 μM Rh123 in the presence or absence of 100 μM guggulsterone and 100 μM verapamil at 37 °C for 90 min, washed three times with rhodamine-free and drug-free medium. At subsequent time points as indicated, the ability of the cells to accumulate Rh123 was assessed. Graphs were plotted of cell-associated MFI against time, where T_0 represents the end of the modulator incubation phase.

4.6. *Intracellular doxorubicin accumulation*

Accumulation of doxorubicin was monitored using a standard procedure by incubating tumor cells with doxorubicin (10 μM) alone or in combination with guggulsterone (3,10,30,100 μM) for 1 h at 37 °C. Then cells were placed in ice-water to cease the reaction followed by harvesting and washing twice with ice-cold PBS. The intracellular mean fluorescence intensity associated with doxorubicin was determined by FACScan flow cytometry. Excitation was performed by an argon ion laser operating at 488 nm and the emitted fluorescence was collected through a 615 nm pass filter. Data analysis was performed using Cell Quest software.

4.7. *Flow cytometric apoptosis assay*

Double staining for Annexin V-FITC/PI (Becton Dickinson, USA) was performed according to Vermes method (Gong et al. 1994). The K562/DOX cells (5×10^5 per well) were seeded into 6-well plates and then treated with 10 μM doxorubicin in the absence or presence of various concentrations of guggulsterone. After 48 h, the cells were harvested and washed twice with ice-cold PBS (0.01 M, pH 7.2). After 5 min of centrifuging at 200 g, Annexin V/FITC and PI double-staining were performed according to manufacturer's instruction. Cell apoptosis was analyzed on a FACScan flow cytometry. Annexin V-positive, PI-negative cells were scored as apoptotic. Double-stained cells were considered either as necrotic or as late apoptotic.

4.8. *Determination of P-glycoprotein level*

The cell-surface P-glycoprotein levels were measured by immunofluorescence flow cytometry. K562/DOX and K562 cells seeded into 12-well plates at a density of 4×10^5 per well were treated with 3, 10, 30 and 100 μM of guggulsterone for 24 h, respectively (Liu et al. 2006). The cells were harvested, washed twice with ice-cold PBS, counted and then labeled

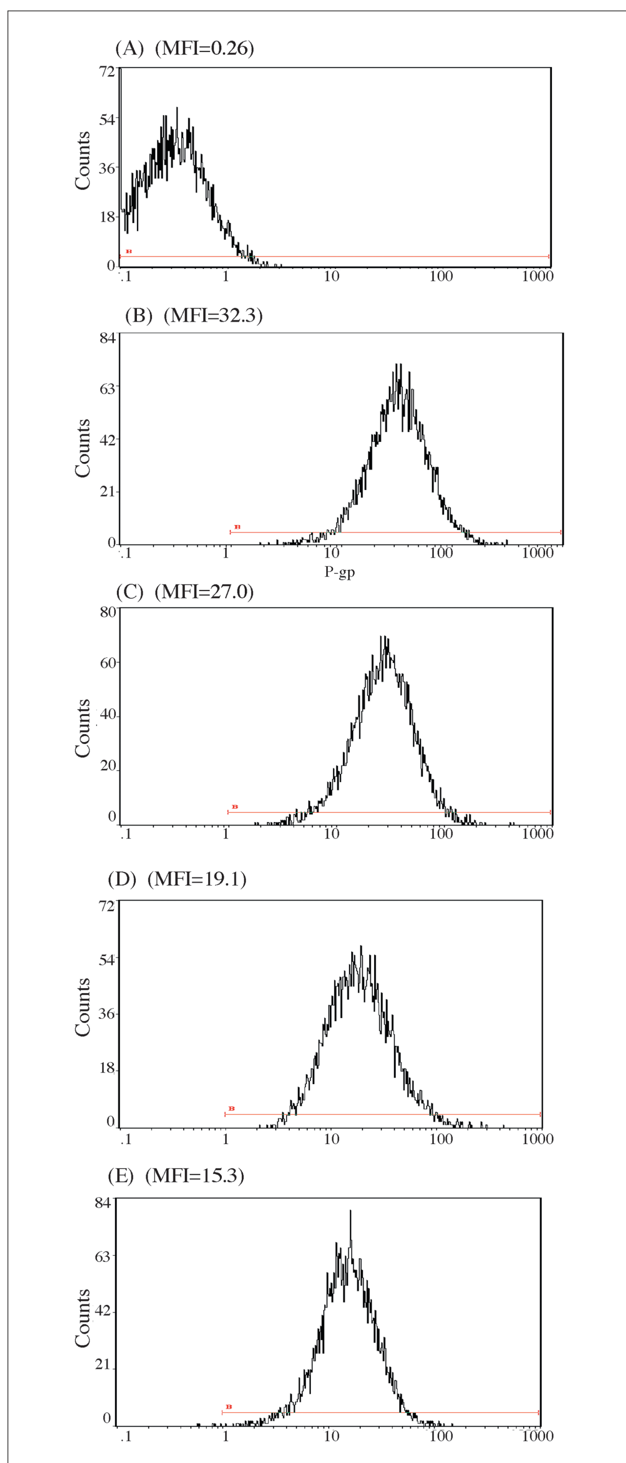


Fig. 6: Effect of guggulsterone on the expression of P-glycoprotein in K562/DOX cells. Cells were incubated with various concentrations of guggulsterone for 24 h and subjected to flow cytometry analysis of P-glycoprotein. P-glycoprotein was determined by flow cytometry using R-PE-conjugated mouse anti-human monoclonal antibody against P-glycoprotein. A, K562 cells without treatment; B, K562/DOX cells without treatment; C–E, K562/DOX cells treated with10, 30,100 μM of guggulsterone

with R-phycoerythrinconjugated mouse anti-human monoclonal antibody against P-glycoprotein according to manufacturer's instruction. The fluorescent intensity was analyzed using FACS Caliber with isotope as control. Excitation was performed by an argon ion laser operating at 488 nm and the emitted fluorescence was collected through a 575 nm pass filter. K562 cells, which do not express P-glycoprotein, were used as a negative control.

4.9. Statistical analysis

Data are each expressed as a mean ± S.E.M. Statistical evaluation was carried out by the Student's unpaired t-test. P values less than 0.05 were considered to indicate a significant difference.

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