

Effects of monensin liposomes on the cytotoxicity, apoptosis and expression of multidrug resistance genes in doxorubicin-resistant human breast tumour (MCF-7/dox) cell-line

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Abstract

We have evaluated the effects of monensin liposomes on drug resistance reversal, induction of apoptosis and expression of multidrug resistance (MDR) genes in a doxorubicin-resistant human breast tumour (MCF-7/dox) cell line. Monensin liposomes were prepared by the pH-gradient method. MCF-7/dox cells were treated with various anticancer drugs (doxorubicin, paclitaxel and etoposide) alone and in combination with monensin liposomes. The cytotoxicity was assessed using the crystal violet dye uptake method. The induction of apoptosis in MCF-7/dox cells was assessed by established techniques such as TUNEL (terminal deoxynucleotidyl transferase-mediated nick end labelling) staining and caspase-3 assay. The effect of monensin liposomes on doxorubicin accumulation in MCF-7/dox cells was monitored by fluorescent microscopy. Finally, the expression of MDR genes (MDR1 and MRP1) in MCF-7/dox cells following the exposure to doxorubicin alone and in combination with monensin liposomes was evaluated by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Our results indicated that monensin liposomes overcame drug resistance in MCF-7/dox cells to doxorubicin, etoposide and paclitaxel by 16.5-, 5.6- and 2.8-times, respectively. The combination of doxorubicin ($2.5 \mu\text{g mL}^{-1}$) with monensin liposomes ($20 \times 10^{-8} \text{M}$) induced apoptosis in approximately 40% cells, whereas doxorubicin ($2.5 \mu\text{g mL}^{-1}$) or monensin liposomes ($20 \times 10^{-8} \text{M}$) alone produced minimal apoptosis (<10%) in MCF-7/dox cells. Fluorescent microscopy revealed that monensin liposomes increased the accumulation of doxorubicin in MCF-7/dox cells. RT-PCR studies demonstrated that the expression of MDR1 and MRP1 was increased by 33 and 57%, respectively, in MCF-7/dox cells following treatment with doxorubicin ($2.5 \mu\text{g mL}^{-1}$) for 72 h as compared with control MCF-7/dox cells. Furthermore, the levels of MDR1 and MRP1 in MCF-7/dox cells exposed to both doxorubicin and monensin liposomes showed a modest decrease as compared with MCF-7/dox cells treated with doxorubicin alone. In conclusion, the delivery of monensin via liposomes provided an opportunity to overcome drug resistance.

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Introduction

Resistance to chemotherapy is an important issue in the management of breast cancer patients. The occurrence of multidrug resistance (MDR) phenotype in patients treated with chemotherapeutic drugs, and sometimes even in their first treatment, limits the therapeutic efficacy. MDR is primarily responsible for the insensitivity of anticancer drugs in metastatic breast cancer (Gottesman 1993). Resistance may be due to pharmacological, cancer multicellular and unicellular mechanisms. One of the cellular mechanisms is the regulation of the ability of the anticancer agent to reach its intracellular target and its intracellular accumulation operated via efflux mechanisms by a group of transporter proteins. Among these proteins, P-glycoprotein (P-gp) represents the one most thoroughly studied, which is implicated in MDR in-vitro and in-vivo (Leonessa & Clarke 2003). P-gp is part of a large family of proteins called the ABC protein superfamily. Other ABC transporter proteins include multidrug resistance-associated proteins (MRP 1–7), lung resistance protein (LRP) and breast cancer resistance protein (MXR/BCRP/ABCP1). Tumours may express several of these transporter proteins (Murren 2002).

Several drugs have been studied to sensitize MDR cells to chemotherapy, which include calcium channel blockers, calmodulin inhibitors, ciclosporins and steroid hormones (Ford & Hait 1990). The mechanisms that have been postulated for MDR modulators include direct binding to P-gp and competitive inhibition of transport of cytotoxins (Safa 1988), and interference with expression and inhibition of P-gp function (Ahmad et al 1994; Muller et al 1995; Li et al 2001).

Monensin, a metabolite of *Streptomyces cinnamomensis*, is a monovalent carboxylic polyether, which regulates Na^+/H^+ exchange across cell membranes (Mollenhauer et al 1990). Monensin has been shown to potentiate the in-vitro cytotoxicity of immunotoxins (Derbyshire et al 1992; van Horssen et al 1999), ribonuclease (Newton et al 2001), and overcome the drug resistance (Ling et al 1993; Wood et al 1996). However, the lipophilicity and short half-life of monensin precludes its use and therefore, a suitable drug delivery system is needed to obtain the desired in-vivo effects. Our laboratory has been involved with the development of delivery systems for monensin such as long-circulating liposomes (Singh et al 1999, 2001; Shaik et al 2001a) and nanoparticles (Shaik et al 2001b). Our studies have shown that the delivery of monensin via long-circulating nanoparticles increases the in-vitro cytotoxicity of anticancer drugs and immunotoxins (Shaik et al 2001b). We have also shown that long-circulating monensin liposomes overcome the doxorubicin resistance in human breast adenocarcinoma (MCF-7/dox) cells (Singh et al 1999). Recently, we reported a modified method for increasing the entrapment of monensin in liposomes using a pH-gradient method (Shaik et al 2001a). Such liposomes have been shown to improve the in-vivo anti-tumour activity of an immunotoxin in severe combined immunodeficient mice (Shaik et al 2003).

Previous studies showed that chemosensitizers modulated the expression of MDR genes in various drug resistant tumour cells. Herzog et al (1993) showed that P-gp antagonists such as verapamil, nifedipine and ciclosporin increased the MDR1 mRNA levels in drug-resistant colon carcinoma cells, whereas another P-gp antagonist, quinidine had no effect on MDR1 mRNA levels. However, Muller et al (1995) showed that verapamil reduced MDR1 gene expression in two leukaemic multidrug resistant cell lines. Beketic-Oreskovic et al (1995) reported that selection of human MES-SA sarcoma cells with doxorubicin and PSC 833 suppressed the emergence of MDR1 mutants. Nielsen et al (2002) showed that the selection of the wild-type Ehrlich ascites tumour cell line (EHR2), adapted to grow in the presence of daunorubicin and a combination of daunorubicin with chemosensitizers (verapamil and ciclosporin), resulted in a significant decrease in the levels of MDR1a mRNA and P-gp, as compared with cells selected with daunorubicin alone. Li et al (2001) have reported that the treatment of MCF-7/dox cells with nomegestrol showed a time-dependent decrease in the expression of MDR1 mRNA levels with a maximum decrease observed on the third day, followed by a gradual increase to its normal levels by the tenth day. However, so far there has been no report on the effect of monensin on the expression of MDR genes.

Therefore, we have evaluated the utility of long-circulating monensin liposomes prepared using a pH-gradient method (Shaik et al 2001a, 2003) in overcoming doxorubicin resistance. We have studied the role of monensin liposomes on the induction of apoptosis and expression of MDR genes (MDR1 and MRP1 mRNA levels) in MCF-7/dox cells.

Materials and Methods

Materials

[^3H]Monensin (sp. act. 5 Ci mmol^{-1}) was obtained from American Radiolabeled Chemicals Inc. (St Louis, MO). Monensin, D-mannitol, D-(+)-trehalose, dipalmitoylphosphatidylcholine, cholesterol, stearylamine, doxorubicin, etoposide, paclitaxel, ciclosporin and all tissue culture media were obtained from Sigma Chemical Company (St Louis, MO). Distearoyl glycerophospho-ethanolamine-polyethyleneglycol 2000 was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). The human breast tumour cell line MCF-7 (doxorubicin sensitive) was obtained from American Type Culture Collection (Rockville, MD). MCF-7/dox cell line was kindly provided by Dr K.C. Agarwal (Department of Pharmacology, Tulane University School of Medicine, New Orleans, LA). Acridine Orange Stain Dropper solution was obtained from Becton Dickinson and Company (Sparks, MD). DeadEnd Colorimetric Apoptosis Detection System and CaspACE Assay System colorimetric kits were obtained from Promega Corporation (Madison, WI). Indometacin, probenecid and (\pm)-verapamil hydrochloride were purchased from MP Biomedicals, Inc. (Aurora, OH).

Preparation and characterization of long-circulating monensin liposomes

Monensin liposomes were prepared using a pH-gradient method and characterized for their particle size (BI 90 Particle sizer, Brookhaven Instruments, New York, NY) and monensin content (by scintillation counting) essentially as described elsewhere (Shaik et al 2001a).

Reversal of drug resistance in MCF-7/dox cells by monensin liposomes/MDR1 and MRP1 inhibitors

MCF-7/dox cells were seeded at a density of 10 000 per well in 96-well plates and incubated overnight. Subsequently, they were treated with various concentrations of doxorubicin/paclitaxel/etoposide alone and in combination with a fixed non-toxic concentration of monensin liposomes ($20 \times 10^{-8}\text{ M}$). Upon treatment, the cells were incubated for 72 h and the cytotoxicity was assayed by crystal violet dye uptake assay by measuring the absorbance at 540 nm (Shaik et al 2001a, b). For experiments using known MDR1 inhibitors (verapamil and ciclosporin) and MRP1 inhibitors (indometacin and probenecid), the same procedure was followed except that the MDR1/MRP1 inhibitors were employed at their

non-toxic concentrations and used in combination with doxorubicin against MCF-7/dox cells.

Assessment of apoptosis in MCF-7/dox cells by the combination of doxorubicin with monensin liposomes by acridine orange and TUNEL (terminal deoxynucleotidyl transferase-mediated nick end labelling) staining

MCF-7/dox cells were plated into each well of a chamber slide (Nunc Lab-Tek II) at a concentration of 50 000 cells in 1 mL minimum essential medium (MEM) and allowed to grow overnight. Subsequently, cells were treated with doxorubicin ($2.5 \mu\text{g mL}^{-1}$), monensin liposomes ($20 \times 10^{-8} \text{ M}$), or doxorubicin ($2.5 \mu\text{g mL}^{-1}$) with monensin liposomes ($20 \times 10^{-8} \text{ M}$) and incubated for 72 h. The cells were fixed with 0.25% w/v glutaraldehyde and the chamber slide was studied for TUNEL staining using a DeadEnd Colorimetric Apoptosis Detection System kit (Promega) as per the manufacturer's instructions. Finally, the slide was observed for apoptotic cells under an Olympus BX40 microscope equipped with a computer-controlled digital camera (Qimaging, Burnaby, BC, Canada).

Assessment of apoptosis in MCF-7/dox cells by caspase-3 assay

One million MCF-7/dox cells in 10 mL MEM were plated in a 25 cm² flask and incubated overnight. Subsequently, they were treated with doxorubicin ($2.5 \mu\text{g mL}^{-1}$), monensin liposomes ($20 \times 10^{-8} \text{ M}$), or doxorubicin ($2.5 \mu\text{g mL}^{-1}$) with monensin liposomes ($20 \times 10^{-8} \text{ M}$) for 48 h. Finally, the specific activity of caspase-3 was estimated using CaspACE Assay system, colorimetric (Promega Corporation, Madison, WI) as per the manufacturer's protocol.

Effect of monensin liposomes on fluorescence visualization of doxorubicin in MCF-7/dox cells

One million MCF-7/dox cells were plated in each well of a chamber slide and incubated overnight. The cells were then treated with doxorubicin ($10 \mu\text{g mL}^{-1}$) alone and in combination with monensin liposomes ($20 \times 10^{-8} \text{ M}$) for 30 min. Subsequently, they were washed with cold phosphate-buffered saline (PBS) and examined by fluorescence microscopy (Olympus BX 40). In another set of experiments, the PBS washed cells as described above were incubated in MEM for a further 30 min. Finally, the cells were washed with cold PBS and observed under fluorescent microscope equipped with a 520–550 nm excitation and 580 nm emission filter (Kitazono et al 1999). Studies were performed also using verapamil ($15 \mu\text{g mL}^{-1}$) as a chemosensitizer in place of monensin liposomes, essentially in the same way as described above.

Influence of monensin liposomes on the expression of MDR1/MRP1 in MCF-7/dox cells

MCF-7/dox cells (1×10^6 in 25 cm² flask) were treated with MEM (control), doxorubicin ($2.5 \mu\text{g mL}^{-1}$), monensin

liposomes ($20 \times 10^{-8} \text{ M}$) or $2.5 \mu\text{g mL}^{-1}$ doxorubicin with $20 \times 10^{-8} \text{ M}$ monensin liposomes for 72 h and the total RNA was eluted using the Eppendorf Perfect RNA Mini Kit (Brinkman Instruments, Westbury, NY). Reverse transcription was performed with Moloney-murine leukaemia virus reverse transcriptase (MuLV-RT) (Applied Biosystem, CA) according to the manufacturer's protocol with some modifications. The PCR reaction was performed with MDR1 (sense, 5' CCA TCA TTG CAA TAG CAG G 3'; antisense, 5' GAG CAT ACA TAT GTT CAA ACT T 3'), MRP1 (sense 5' GGA CCT GGA CTT CGT TCT CA 3'; antisense 5' CGT CCA GAC TTC TTC ATC CG 3') and β -actin (sense, 5' GAT CAT GTT TGA GAC CTT C 3'; antisense, 5' GTC AGG CAG CTC GTA G 3') primer pairs and ATAQ DNA polymerase (Applied Biosystem) at 94°C for 2 min, 30 cycles of 94°C, 60°C/52°C for MDR1/MRP1 and 72°C (1 min each), and then 10 min at 72°C before holding at 4°C. The 126 bp for MDR1 and 252 bp for MRP1 PCR products were separated in a 1.5% agarose gel and the band intensities were normalized with respect to β -actin using Scion Image Software (Beta 3b version, Scion Corporation, Frederick, MD).

Statistical analysis

One-way analysis of variance followed by Tukey's multiple comparison test was performed to determine the significance of difference in the caspase-3 activity and expression levels of MDR1 and MRP1 in MCF-7/dox cells subjected to various treatments. Statistical analysis was performed using GraphPad Prism (version 3.0) software (San Diego, CA).

Results

Enhancement of the cytotoxicity of anticancer drugs by monensin liposomes

Table 1 shows the IC₅₀ (inhibitory concentration to produce 50% cell kill) values for the anticancer drugs alone and their combination with monensin liposomes. It was evident that the MCF-7/dox cell line showed 23.3-, 2.9- and 132-fold drug resistance (expressed as the ratio of IC₅₀ in resistant cell line to its parental cell line) to paclitaxel, etoposide and doxorubicin, respectively, as compared with its parental MCF-7 cell line (Table 1). The combination of monensin liposomes ($20 \times 10^{-8} \text{ M}$) with anticancer drugs increased the cell kill resulting in a 2.8-, 5.6- and 16.5-fold increase in the cytotoxicity of paclitaxel, etoposide and doxorubicin, respectively, against MCF-7/dox cells (Table 1). These results were in agreement with our previous study (Singh et al 1999) in which long-circulating monensin liposomes prepared by a conventional method were shown to increase the cytotoxicity of doxorubicin in MCF-7/dox cells. This study differed from the previous one in two aspects: MCF-7/dox cells used in this study had significantly increased resistance to doxorubicin as compared with the previous study (Singh et al 1999), and the monensin liposomes employed in this study were prepared by a pH-gradient method.

Table 1 Reversal of drug resistance in MCF-7/dox cells by monensin liposomes and MDR inhibitors

Treatment	IC50 value ($\mu\text{g mL}^{-1}$)	Drug resistance reversal*
Paclitaxel	4.2 ± 0.6	0
Etoposide	61.1 ± 10.1	0
Doxorubicin	26.4 ± 7.4	0
Paclitaxel with monensin liposomes ($20 \times 10^{-8} \text{ M}$)	1.5 ± 0.6	2.8
Etoposide with monensin liposomes	10.9 ± 1.0	5.6
Doxorubicin with monensin liposomes	1.6 ± 0.6	16.5
Doxorubicin with ciclosporin	1.0 ± 0.2	26.4
Doxorubicin with verapamil	4.2 ± 0.3	6.3
Doxorubicin with indometacin	9.5 ± 0.5	2.8
Doxorubicin with probenecid	4.1 ± 0.3	6.4

The IC50 values were expressed as mean \pm s.d. of three independent experiments. *Drug resistance reversal = IC50 value for drug alone/IC50 value for drug with drug resistance modifier. For combination experiments using monensin liposomes and MDR inhibitors, the following non-toxic concentrations were used: monensin liposomes $20 \times 10^{-8} \text{ M}$; ciclosporin $6 \mu\text{g mL}^{-1}$; verapamil $15 \mu\text{g mL}^{-1}$; indometacin $40 \mu\text{g mL}^{-1}$; probenecid $150 \mu\text{g mL}^{-1}$. The IC50 values for paclitaxel, etoposide and doxorubicin against doxorubicin sensitive parental MCF-7 cell line were found to be 0.18 ± 0.08 , 20.9 ± 2.4 , $0.2 \pm 0.05 \mu\text{g mL}^{-1}$, respectively. The relative drug resistance to paclitaxel, etoposide and doxorubicin in MCF-7/dox cells was 23.3-, 2.9- and 132-fold, respectively, as compared with MCF-7 cells (expressed as the ratio of IC50 in MCF-7/dox cells to IC50 in MCF-7 cells).

Induction of apoptosis in MCF-7/dox cells

We studied the induction of apoptosis in MCF-7/dox cells by the combination of monensin liposomes ($20 \times 10^{-8} \text{ M}$) with doxorubicin at a concentration much lower than its

IC50 value. Figure 1 shows the TUNEL staining of MCF-7/dox cells treated with MEM (control), monensin liposomes ($20 \times 10^{-8} \text{ M}$), doxorubicin ($2.5 \mu\text{g mL}^{-1}$) and the combination treatment of doxorubicin ($2.5 \mu\text{g mL}^{-1}$) with

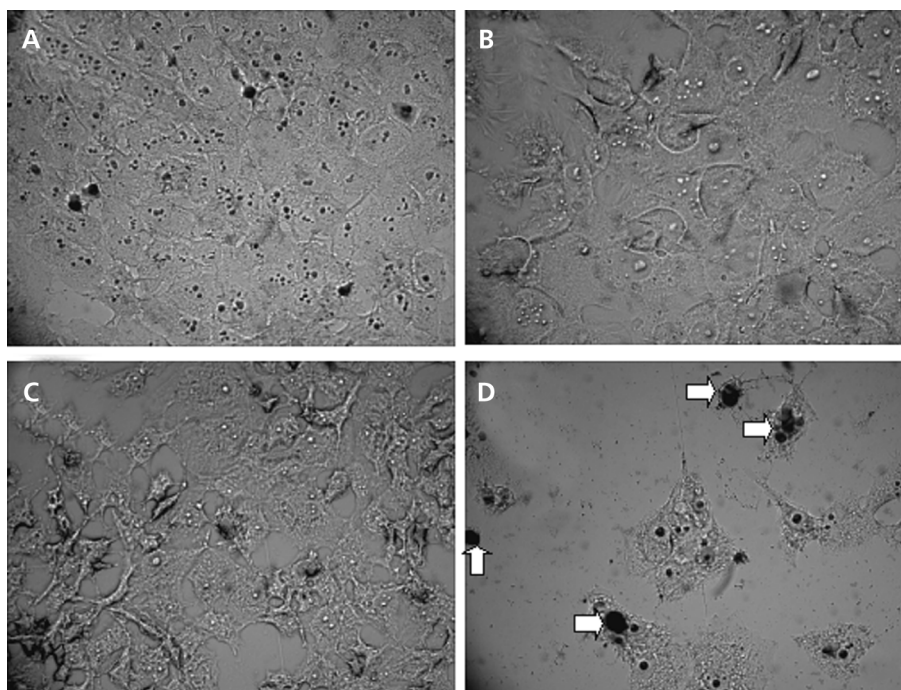


Figure 1 TUNEL staining of MCF-7/dox cells treated with MEM control (A), $20 \times 10^{-8} \text{ M}$ monensin liposomes (B), $2.5 \mu\text{g mL}^{-1}$ doxorubicin (C) and $2.5 \mu\text{g mL}^{-1}$ doxorubicin + $20 \times 10^{-8} \text{ M}$ monensin liposomes (D) for 72 h. Arrows indicate apoptotic cells. The control, monensin liposomes or doxorubicin-treated cells showed negligible apoptosis whereas the cells treated with doxorubicin with monensin liposomes exhibited significant apoptosis. Similar results were observed with acridine orange staining (data not shown). Original magnification: $40\times$.

monensin liposomes (20×10^{-8} M). There was no significant apoptosis ($<10\%$) in the control, doxorubicin and monensin liposome-treated cells (Figure 1). On the other hand, the combination treatment produced apoptosis in 40% cells. The specific caspase-3 activity (pmol pNA liberated h^{-1} ($\mu\text{g protein}^{-1}$)) in control, doxorubicin ($2.5 \mu\text{g mL}^{-1}$), monensin liposomes (20×10^{-8} M) and the combination of doxorubicin ($2.5 \mu\text{g mL}^{-1}$) with monensin liposomes (20×10^{-8} M) treated cells was found to be 4.9 ± 0.7 , 9.9 ± 1.2 , 3.9 ± 1.2 and 20.6 ± 4.0 , respectively. Thus, there was a 2.1-fold increase in the caspase-3 activity in the cells treated with the combination of doxorubicin with monensin liposomes as compared with doxorubicin-only treated cells. The caspase-3 activity in the cells treated with doxorubicin with monensin liposomes was significantly different ($P < 0.05$) as compared with control, doxorubicin and monensin liposomes treated cells. There was no significant difference in the caspase-3 activity among control, doxorubicin and monensin liposomes treated cells.

Enhanced cellular accumulation of doxorubicin in MCF-7/dox cells by monensin liposomes

We exploited the fluorescent properties of doxorubicin to visually demonstrate the effect of monensin liposomes on doxorubicin accumulation in MCF-7/dox cells, as demonstrated by Kitazono et al (1999). The cells treated with doxorubicin and monensin liposomes (Figure 2B) had considerably higher fluorescence as compared with

doxorubicin-alone treated cells (Figure 2A). We observed an increase in the doxorubicin fluorescence in MCF-7/dox cells treated with doxorubicin and verapamil (Figure 2C). After the initial doxorubicin uptake study and further incubation of the cells for 30 min in the fresh medium (without doxorubicin), we could still observe the fluorescence (Figure 2D) in cells initially treated with doxorubicin and monensin liposomes (during the initial loading time). However, we could not detect any fluorescence in the doxorubicin-only treated MCF-7/dox cells after 30 min of incubation in drug-free medium. These observations suggested that monensin liposomes increased the cellular accumulation of doxorubicin from MCF-7/dox cells. We had shown previously that the combination of doxorubicin with monensin liposomes increased the doxorubicin uptake by more than threefold in a doxorubicin-resistant human promyelocytic leukaemia, HL-60/dox cell-line, as compared with doxorubicin-treated cells at 30-min period during uptake studies. Furthermore, at post 30-min period in the efflux study, there was a threefold decrease in the doxorubicin efflux from cells treated with monensin liposomes and doxorubicin as compared with doxorubicin alone (Singh et al 1999).

Effect of monensin liposomes on MDR1 and MRP1 mRNA expression in MCF-7/dox cells

To study the effect of monensin liposomes on genes related to the MDR expression, we studied the mRNA expression levels of MDR1 and MRP1 in MCF-7/dox

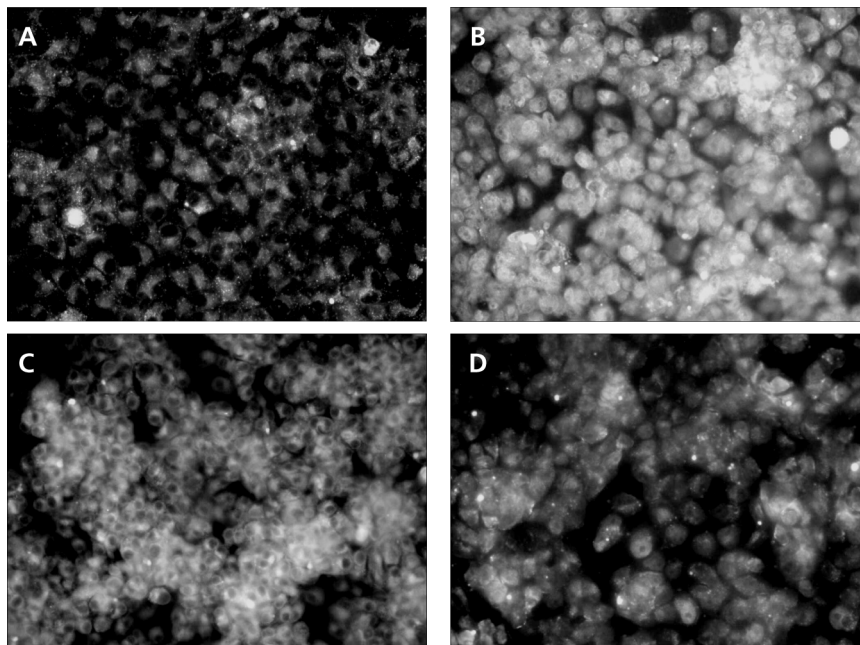


Figure 2 Doxorubicin fluorescence in MCF-7/dox cells exposed to $10 \mu\text{g mL}^{-1}$ doxorubicin (A), $10 \mu\text{g mL}^{-1}$ doxorubicin with 20×10^{-8} M monensin liposomes (B), $10 \mu\text{g mL}^{-1}$ doxorubicin with $15 \mu\text{g mL}^{-1}$ verapamil (C) for 30 min. The cells were washed with cold PBS and observed for doxorubicin fluorescence without fixing the cells. After the initial loading of cells with doxorubicin for 30 min, the cells were washed with cold PBS and again incubated in minimum essential eagle medium for 30 min. We observed no fluorescence in cells treated with doxorubicin only, whereas the cells treated with doxorubicin and monensin liposomes showed considerable fluorescence even after 30 min of efflux in MEM (D). Original magnification: $40\times$.

cells treated with doxorubicin ($2.5 \mu\text{g mL}^{-1}$), monensin liposomes ($20 \times 10^{-8} \text{ M}$) and the combination of doxorubicin ($2.5 \mu\text{g mL}^{-1}$) with monensin liposomes ($20 \times 10^{-8} \text{ M}$) by reverse transcriptase–polymerase chain reaction (RT-PCR). We chose monensin and doxorubicin at these concentrations because doxorubicin or monensin at their studied concentrations were nontoxic to cells and did not induce apoptosis, whereas their combination produced a significant apoptotic response (Figure 1). It was evident from Figure 3 that MCF-7/dox cells expressed both MDR1 and MRP1, whereas their expression was negligible in the parental MCF-7 cells (data not shown). Semi-quantitative analysis of band intensities, followed by normalization to β -actin levels, showed that the exposure of MCF-7/dox cells to doxorubicin increased the expression of MDR1 and MRP1 by 33% (0.62 in control vs 0.83 in doxorubicin-treated cells) and 57% (0.67 in control vs 1.05 in doxorubicin treated cells), respectively. Monensin liposomes by themselves showed a modest decrease of 10 and 24% in the expression of MDR1 and MRP1, respectively (Figure 3). The combination treatment of doxorubicin with monensin liposomes did not decrease the expression of MDR1 or MRP1 below its control levels (Figure 3). However, we observed a statistically significant difference in the expression levels of MDR1 and MRP1 in MCF-7/dox cells treated with the combination of doxorubicin and monensin liposomes as

compared with doxorubicin alone (Figure 3). The relative expression levels of MDR1 and MRP1 in doxorubicin with monensin combination treated cells as compared with the control cells were 110 and 124%, respectively, as compared with 133 (MDR1) and 157% (MRP1) of expression observed with doxorubicin alone. Thus, the combination treatment reduced the doxorubicin-induced elevated expression of MDR1 and MRP1 by 70 and 42%, respectively, in MCF-7/dox cells. These results indicated that monensin liposomes could inhibit the doxorubicin induced elevated expression of MDR genes.

Effect of MDR inhibitors on doxorubicin cytotoxicity in MCF-7/dox cells

To confirm the functional relevance of MDR1 and MRP1, we studied the role of known MDR1 inhibitors (verapamil and ciclosporin) and MRP1 inhibitors (indometacin and probenecid) in overcoming the doxorubicin resistance in MCF-7/dox cells. We employed MDR inhibitors at concentrations which produced cell kill of <15% and, as shown in Table 1, all the known MDR inhibitors were able to enhance the doxorubicin cytotoxicity by 2.8–26.4-fold in MCF-7/dox cells. The results indicated that the expression of both MDR1 and MRP1 contributed to the drug resistance in MCF-7/dox cells and it was possible to sensitize these cells to doxorubicin by both

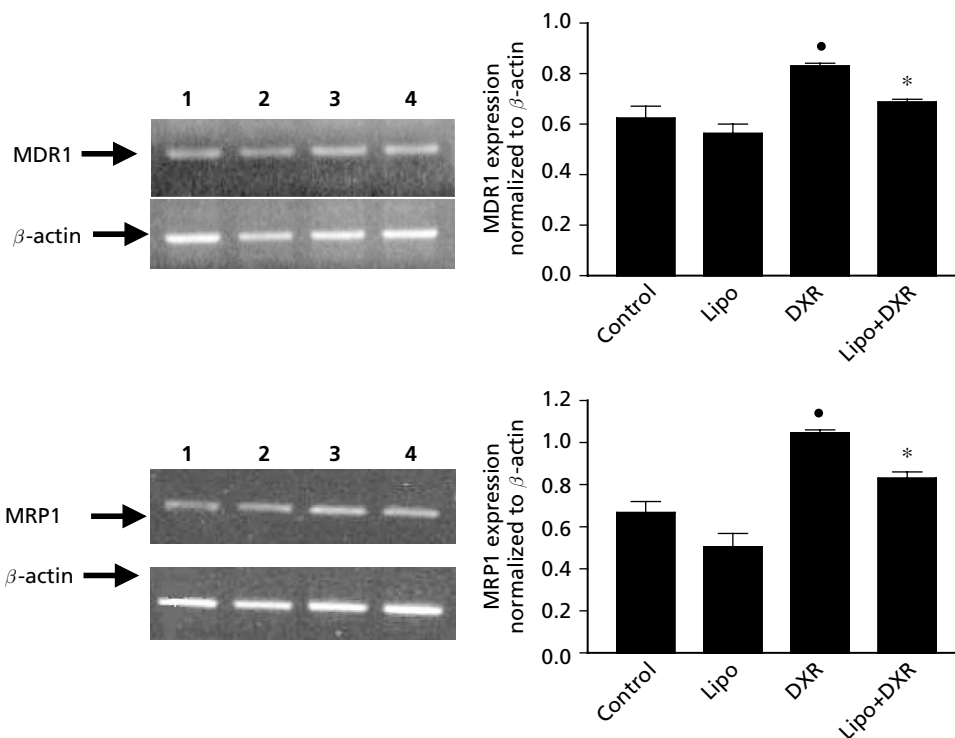


Figure 3 Semi-quantitative RT-PCR analysis of MDR1 and MRP1 m-RNA expression in MCF-7/dox cells. Lane 1, control cells; lane 2, $20 \times 10^{-8} \text{ M}$ monensin liposomes; lane 3, $2.5 \mu\text{g mL}^{-1}$ doxorubicin; lane 4, $2.5 \mu\text{g mL}^{-1}$ doxorubicin + $20 \times 10^{-8} \text{ M}$ monensin liposomes. The bar graphs indicate the relative abundance of MDR1 and MRP1 expression normalized to β -actin. Lipo: monensin liposomes; DXR: doxorubicin; Lipo + DXR: monensin liposomes with doxorubicin combination. • $P < 0.05$, compared with control cells. * $P < 0.05$, compared with doxorubicin-treated cells.

MDR1 and MRP1 inhibitors. However, we observed a greater effect with MDR1 inhibitors as compared with MRP1 inhibitors in the compounds tested.

Discussion

In recent years enormous efforts have been directed to find drug transport-modulating agents to circumvent P-gp and MRP-mediated drug resistance. Compounds such as verapamil, ciclosporin, quinidine, reserpine and other compounds have been shown to overcome MDR *in-vitro*. However, most of these compounds have disappointing results in animal studies because of toxicity considerations. Accordingly, new agents with low toxicity and high reversal activity are being actively evaluated (Li et al 2001; Toppmeyer et al 2002). The carboxylic ionophore monensin has been shown to overcome drug resistance in several tumour cell lines (Sehested et al 1988). The short half-life and lipophilicity of monensin precluded its further use in *in-vivo* studies. Other workers have attempted to synthesize various monensin derivatives (Dosio et al 1996) or monensin conjugates (Colombatti et al 1990). Our laboratory pursued a drug delivery approach for monensin to overcome its shortcomings and developed long-circulating nanoparticulate and liposomal formulations of monensin (Singh et al 1999; Shaik et al 2001a, b). Recently, we demonstrated the potential of long-circulating liposomes in enhancing the *in-vivo* cytotoxicity of anti-Myc9-bR immunotoxin (Shaik et al 2003).

The MCF-7/dox cells employed in our study were made resistant to doxorubicin by growing them in the presence of doxorubicin (600 ng mL^{-1}). The IC_{50} value for doxorubicin against MCF-7/dox cells was $26.5 \mu\text{g mL}^{-1}$, which was 1.5-fold the reported value for a similar type of doxorubicin-resistant MCF-7 cell employed by Li et al (2001). Consistent with the reported findings that cells made resistant to a particular drug also acquire resistance to other classes of drug, we observed that our MCF-7/dox cells exhibited 2.9–23.3-fold resistance to etoposide and paclitaxel, respectively, as compared with the parental MCF-7 cells. As shown in Table 1, monensin liposomes were able to increase the sensitivity of MCF-7/dox cells not only for doxorubicin but also to paclitaxel and etoposide. One advantage of using monensin liposomes was that they worked *in-vitro* at fairly low concentrations ($20 \times 10^{-8} \text{ M}$ or 200 nM) as compared with other reported chemosensitizers, which work in various drug resistant human breast and other tumour cell lines. Chemosensitizers such as nomogestrol, megestrol, droloxifen and verapamil work at $20 \mu\text{M}$ (Li et al 2001), whilst VX-710 works at 0.5–2.5 μM (Germann et al 1997). Another chemosensitizer MS-209 works at 1–10 μM (Naito et al 2002), and PSC 833 works at 1–3 μM (Warmann et al 2002). Previously, we reported the potential of monensin liposomes in overcoming doxorubicin resistance in a human promyelocytic leukaemia cell line, HL-60/dox (Shaik et al 2001c).

Recent studies suggested that the occurrence of MDR in cancer cells may be the result of a decrease in the ability of the cancer cells to initiate apoptosis in response to

cytotoxic agents (Jones et al 2002). Over-expression of P-gp via transfection of MDR1 in MCF-7 cells has been shown to significantly decrease the apoptotic response to doxorubicin as compared with its effect in wild-type MCF-7 cells (Li et al 2003). In this study, we demonstrated that it was possible to induce apoptosis in MCF-7/dox cells by employing doxorubicin at one-tenth of its IC_{50} value in combination with monensin liposomes (Figure 1). Caspase-3 activation is an early event in the apoptosis cascade, whereas its activation triggers PARP cleavage, which is parallel to apoptosis detection by DNA degradation and TUNEL assays (Li et al 2001). Accordingly, caspase-3 estimation was carried out early at 48 h as compared with the TUNEL assay performed at 72 h after drug treatment. Our data supports the theory that monensin liposomes can sensitize the MCF-7/dox cells to doxorubicin and induce apoptosis via caspase-3 activation. However, the molecular mechanisms leading to apoptosis need to be elucidated further.

We reported (Singh et al 1999) that monensin liposomes increased the doxorubicin uptake and decreased its efflux in HL-60/dox cells. In this study, we have shown morphological evidence for the increased cellular accumulation of doxorubicin in MCF-7/dox cells treated with doxorubicin and monensin liposomes (Figure 2). Previous studies have shown that monensin ($10 \mu\text{M}$) increased the uptake of anthracycline antibiotics in drug resistant cells (Wood et al 1996; Cleary et al 1997). We have consistently observed enhancement of doxorubicin cytotoxicity and altered cellular kinetics in MCF-7/dox cells at a significantly much lower concentration (200 nM) by delivering monensin in the form of liposomes as compared with the concentrations of monensin delivered as a solution (Singh et al 1999; Shaik et al 2001b). Studies by Naito et al (1991) showed that ionophores such as monensin inhibited the binding of vincristine to plasma membrane isolated from resistant K562 cells and reduced the photoaffinity labelling of P-gp by azidopine. Wood et al (1996) proposed that the drug resistance modification by monensin might be due to its binding to P-gp, rather than interfering with vesicular traffic. In the light of recent studies demonstrating the modulation of various MDR genes by chemosensitizing agents (Li et al 2001), we studied the expression of MDR1 and MRP1 genes in MCF-7/dox cells. We observed that MCF-7/dox cells expressed both MDR1 and MRP1 genes by RT-PCR. Significant expression of both MDR1 and MRP1 in MCF-7/dox cells had been reported by Chauvier et al (2002). We found that monensin liposomes alone had a moderate effect, whereas doxorubicin treatment alone had significantly increased the expression of MDR genes. However, the combination of doxorubicin with monensin liposomes showed a decrease in the expression of both MDR1 and MRP1 gene expression as compared with doxorubicin alone (Figure 3). Warmann et al (2002) reported that doxorubicin treatment increased the expression of MDR1 in hepatoblastoma Hep T1 cells. However, their data showed no alteration in MDR1 expression in cells treated with doxorubicin in the presence of chemosensitizers such as verapamil or PSC 833 as compared with doxorubicin

alone. Borrel et al (1994) reported that monensin formed lipophilic neutral complexes with metal ions and such complexes inhibited the P-gp mediated drug efflux, apparently by altering the lipid environment in membranes. Our results suggested that monensin liposomes partially inhibited the doxorubicin induced elevated expression of MDR1 and MRP1 in MCF-7/dox cells.

Thus, the delivery of monensin via liposomes increased the sensitivity and apoptotic response to doxorubicin at a significantly lower concentration. These results were in agreement with our previous studies (Singh et al 1999) and with other reported findings such as enhanced in-vitro therapeutic effect of liposomal delivery of anticancer drugs in resistant cells (Sadava et al 2002; Briz et al 2003). The mechanisms responsible for the increased sensitivity of resistant cells to liposomal anticancer drugs have not been clearly elucidated. It has been reported that liposomes alter the P-gp function by direct interaction and membrane stabilization according to their lipid composition (Poujol et al 1999). Recent studies suggested that P-gp carried out both drug transport and lipid translocation by the same path (Romsicki & Sharom 2001) and phospholipids have been shown to be P-gp substrates (Bosch et al 1997). Liposomes have been thought to interact with cells via intermembrane transfer, adsorption, fusion and endocytosis (Pagano & Weinstein 1978). Based upon these studies, Lo (2000) proposed that liposomal formulations may function as a substrate for MDR1 and/or MDR3 P-gp upon entry into cells via endocytosis or other mechanisms, leaving less P-gp available for pumping out the drug from inside the cells. Alternatively, the liposomal formulations may induce changes in the composition and fluidity of the cell membranes, resulting in the modulation of P-gp activity. These alterations in the cell membrane by liposomes are thought to influence the activity of the other membrane pumps such as MRP, LRP and canalicular multispecific organic anion transporter (cMOAT). The liposomal approach has been extended to various chemosensitizers such as ciclosporin (Lo et al 2001a) and PSC 833 (Lo et al 2001b) and such liposomal formulations have been found to overcome drug resistance in-vitro. Our data indicated that the use of monensin liposomes in combination with doxorubicin in MCF-7/dox cells increased the drug sensitivity as well as its apoptotic response, which may be attributed to the alterations in the drug cellular kinetics. The liposomal delivery of a chemosensitizer such as monensin may alter the activity and or expression of membrane-associated MDR pumps.

In conclusion, monensin liposomes potentiated the in-vitro cytotoxicity of anticancer drugs in MCF-7/dox cells and offered an effective solution to overcome drug resistance in-vitro. However, further in-vivo studies are needed to confirm these in-vitro findings.

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