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ORIGINAL ARTICLE

Effect of MDR modulators verapamil and promethazine on gene expression levels of *MDR1* and *MRP1* in doxorubicin-resistant MCF-7 cells

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Abstract
Purpose One of the major problems of cancer chemotherapy is the development of multidrug resistance (MDR) phenotype. Among the numerous mechanisms of MDR, a prominent one is the increased expression of membrane transporter proteins, the action of which leads to decreased intracellular drug concentration and cytotoxicity of drugs. Among them, P-gp and MRP1, encoded by *MDR1* and *MRP1* genes, respectively, have been associated with MDR phenotype. Chemical modulators can be used to reverse MDR. These chemicals can either modulate MDR due to their substrate analogy (such as calcium channel blocker verapamil) or interact with phospholipid membranes (such as antihistaminic drug promethazine). This study focuses on the effect of verapamil and promethazine on the expression levels of *MDR1* and *MRP1* genes and the drug transport activity in doxorubicin-resistant MCF-7 breast carcinoma cell line.
Methods Doxorubicin-resistant MCF-7 (MCF-7/Dox) cells were incubated with either verapamil or promethazine, and total RNA was isolated. Real-time PCR (qPCR) was carried out by using specific primers for *MDR1, MRP1,* and *ß-actin* genes. Intracellular doxorubicin accumulation was also examined by confocal laser scanning microscopy in treated cells.
Results Results demonstrated a significant decrease in both *MDR1* and *MRP1* expression levels after promethazine applications. It has also been shown that treatment of the cells with verapamil results in significant decrease in *MDR1* mRNA levels. Confocal laser scanning microscopy images demonstrated that the intracellular accumulation of doxorubicin was increased after verapamil treatment in MCF-7/Dox cells.
Conclusions The present study gives an idea about the efficiency of verapamil and promethazine on MDR reversal both in gene expression and in transport activity levels.

Keywords MDR reversal · MCF-7 · Promethazine · Verapamil

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Introduction

Most cancer patients respond to initial chemotherapy, but many have disease recurrence. Commonly, patients who are refractory to disease show resistance to multiple anticancer agents of different structure and mode of action due to emergence of drug-resistant tumor cells [4]. The phenomenon is defined as multidrug resistance (MDR), and it is the major cause of the failure of chemotherapy [27]. There are several mechanisms by which cancer cells develop resistance to cytotoxic agents. Mechanisms of drug resistance include decreased intracellular drug levels that could result from increased drug efflux, decreased conversion of drug to an active form, altered amount of target enzyme or receptor, decreased affinity of target enzyme or receptor for drug, enhanced repair of the drug-induced defect, decreased activity of an enzyme required for apoptosis, altered expression of genes for survival, and altered target type [27].

The ATP-binding cassette (ABC) transporter family contains membrane proteins that translocate a variety of

substrates across extra- and intracellular membranes. These ATPase proteins bind ATP and use the energy to drive the transport of various molecules, including anticancer drugs, across cellular membranes. The ABC transporters responsible for the decreased intracellular drug levels in cells conferring resistance to multiple drugs are P-glycoprotein (P-gp; MDR1), multidrug resistance-associated proteins (MRP1, MRP2, MRP3, MRP4, and MRP5), and breast cancer resistance protein (BCRP). P-gp is normally expressed in a limited number of tissues with barrier function and prevents accumulation of toxic substances in the cells [1]. De novo or overexpression of P-gp was detected in various hematological malignancies and solid tumors, which were also correlated to poor clinical outcome of patients. In particular, an estimated 40% of all breast tumors overexpress *MDR1* [7, 26]. Among known P-gp substrates are such anti-cancer drugs as anthracyclines (doxorubicin, daunomycin, epirubicin), epipodophyllotoxins (etoposide), mitomycin C, taxanes (paclitaxel, docetaxel), and *Vinca* alkaloids (vincristine, vinblastine) [24]. MRP1 is a multispecific organic anion transporter, but is also able to transport non-anionic organic drugs. The ability of MRP1 to transport glutathione conjugates as well as its widespread presence in normal tissues indicates that it is a ubiquitous GS-X pump and acts as a cellular detoxifying factor [13]. When overexpressed in tumor tissues, MRP1 decreases the uptake of anthracyclines, antifolates (methotrexate), epipodophyllotoxins, and *Vinca* alkaloids [24].

A number of drugs including calcium channel blockers, anti-arrhythmics, antidepressants, antipsychotics, and many others belong to the class of so-called chemosensitizers or MDR modulators. They are not cytotoxic by themselves but can reverse P-gp-related MDR [23]. Calcium channel blocker verapamil inhibits the transport function of P-gp. One hypothesis suggests that verapamil leads to a futile cycle of ATP hydrolysis, so the energy spend does not get coupled to substrate translocation, whereas according to another hypothesis, verapamil competes with the transport of other drugs [14]. In addition, Schuldes et al. [25] stated that modulation of doxorubicin toxicity by verapamil may involve changes in plasma membrane fluidity. Phenothiazines and related tricyclic compounds belong to one of the oldest classes of MDR modulators in cancer cells, but their mode of molecular mechanism is still unclear [8]. Pajeva et al. [23] suggested a mechanism for MDR reversal mediated by drug–membrane interactions.

In the present study, the effect of verapamil and promethazine on the expression levels of *MDR1* and *MRP1* genes in the doxorubicin-resistant MCF-7 cells and on the intracellular doxorubucin accumulation was investigated.

Materials and methods

Cells

MCF-7 cell line, which is a model cell line for human mammary carcinoma, was used as model cell line. The cell line exhibits some features of differentiated mammary epithelium and was donated by ŞAP Institute, Ankara-Turkey. 1,000 nM doxorubicin (MCF-7/Dox)-resistant MCF-7 cell line was developed from sensitive MCF-7 cells (MCF-7/S) as previously described and shown to express high levels of P-gp [10, 12]. The cells were 160-fold resistant to doxorubicin, [9] as determined by Cell Proliferation Assay (Biological Industries, Kibbutz Beit Haemek, Israel).

Treatments

To MCF-7/Dox cells, 60 μM verapamil (Vp) (Isoptin®, Germany) and 9.6 μM promethazine (Prm) (kindly denoted by Prof. Dr. Jozsef Molnàr, University of Szeged, Faculty of Medicine, Szeged, Hungary) were applied. Verapamil and promethazine treatment concentrations were determined according to IC_{50} values determined previously in our laboratory [11], and half of the IC_{50} values were selected for applications [6].

RNA isolation and quantitative real-time polymerase chain reaction (qPCR)

In order to determine the expression levels of *MDR1* and *MRP1* genes in cells, total RNA was extracted using TRI Reagent (Sigma, St. Louis, MO, USA) after 48 and 72 h of treatments according to the manufacturer's instructions. Absorbance values (260, 280 nm) were measured for RNA quantification by spectrophotometry. RNA intactness was tested by 1% w/v denaturing agarose gel electrophoresis at 70 V in MOPS (3-*N*-morpholinopropanesulfonic acid) buffer. Purity of RNA in each sample was adjusted to OD_{260}/OD_{280} ratio of 1.8–2.0. cDNA synthesis was performed with 5 μg of total RNA, 20 pmol specific primers for *MDR1*, *MRP1* and *β-actin* (Roche, Germany), and 40 units of M-MuLV Reverse Transcriptase according to the manufacturer's instructions (MBI Fermentas, Lithuania).

For qPCR analysis, Rotor-Gene 6000 (Corbett Research) and Light-Cycler-FastStart DNA Master SYBR Green I kit (Roche, Germany) were used according to manufacturer's instructions. In brief, 10 μL reaction mix contained 2.8 μL cDNA, 0.3 μM from each forward and reverse primers and SYBR master mix. Each sample run was performed in triplicates with non-template controls. *β-actin* was used as an internal control for standardizations.

The relative changes in gene expression levels were determined using $2^{-\Delta\Delta C}$T method [15].

Confocal laser scanning microscopy

Cells were trypsinized and pelleted. Trypan blue (Sigma–Aldrich) viable cell count was performed in a Neubauer counting chamber (Bright-line, Hausser Scientic, USA) under light microscopy. Sensitive and doxorubicin-resistant MCF-7 cells were seeded onto autoclaved cover slips as 600,000 cells/slip, and they were allowed to grow overnight. On the following day, cells were washed with PBS for three times and incubated with verapamil or promethazine for 30 min. A control group of non-treated cells was also run. Treated and non-treated cells were incubated with 4 μM doxorubicin for 1 h. After incubation, medium was discarded, cells were fixed with 2% (w/v) paraformaldehyde in PBS, and the coverslips were wet-mounted on microscope slides. Preparations were observed under a Zeiss LSM 510 confocal laser scanning microscope (Jena, Germany) with Plan-Neofluar 40X/1.3 Oil DIC lens. All images were scanned at 1,024 × 1,024 pixels as 12 bit images with pinhole size set to 1 airy unit and with the same laser power and detector sensitivity settings. The excitation and emission wavelengths of doxorubicin were 488 and 530 nm, respectively. For quantification of intracellular drug accumulation, at least 40 cells were randomly picked from cell images and analyzed using Image J I.41 software (USA) as mean doxorubicin fluorescent intensity per pixel.

Statistical analysis

All data are representative of three independent experiments and expressed as mean ± standard error of the means (SEM). They were statistically evaluated by one-way ANOVA test, and the mean difference is significant at the 0.05 level. Further, post hoc Tukey analyses were carried out to find groups whose mean differences were significant.

Results

Expression analysis of MDR1 gene

According to real-time PCR results (Fig. 1), a statistically significant decrease in *MDR1* gene expression was observed in verapamil- and promethazine-treated doxorubicin-resistant cells compared to untreated cells ($P < 0.001$). Expression levels of *MDR1* gene were approximately half of the original cells in 48- and 72-h verapamil-applied cells ($P < 0.001$). Similar decrease was also observed in 48- and 72-h promethazine-applied group ($P < 0.001$).

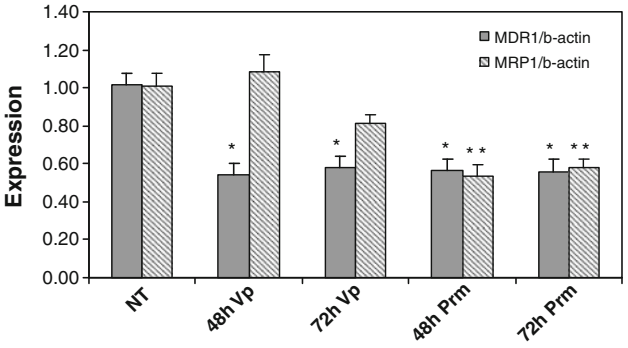

Fig. 1 QPCR expression analysis of *MDR1* and *MRP1* genes in MCF-7/Dox (no treatment; NT) and verapamil- or promethazine-treated MCF-7/Dox cells. * Significant difference in expression level of *MDR1* gene between untreated and treated groups with $P < 0.001$. ** Significant difference in expression level of *MRP1* gene between untreated and treated groups with $P < 0.001$. *Vp* Verapamil, *Prm* Promethazine

Expression analysis of MRP1 gene

According to expression analysis of the *MRP1* gene by qPCR (Fig. 1), there was approximately twofold decrease in the expression level of *MRP1* gene due to 48 and 72 h of promethazine application with respect to untreated cells ($P < 0.001$). On the other hand, neither 48 nor 72 h verapamil application caused a significant change in expression level in doxorubicin-resistant MCF-7 cells although a slight decrease in expression was observed in 72-h verapamil-applied cells.

Doxorubicin accumulation

Confocal laser scanning microscopy images (Fig. 2) of doxorubicin-applied sensitive cells demonstrated high doxorubicin accumulation inside the cells with most of the drug concentrated in the nucleus. On the other hand, doxorubicin accumulation was considerably lower in doxorubicin-resistant cells, and most of doxorubicin accumulated at the cell periphery. Intracellular fluorescence intensity of doxorubicin (Fig. 3) was sixfold higher in sensitive cells when compared to resistant cells ($P < 0.001$). Decreased intracellular fluorescence intensity and increased fluorescence intensity on cell membrane (Fig. 2) indicate doxorubicin efflux from cells. Intracellular doxorubicin accumulation significantly increased (fourfold, $P < 0.001$) in resistant cells treated with verapamil prior to doxorubicin when compared to untreated MCF-7/Dox cells (Figs. 2, 3). Intracellular doxorubicin accumulation in promethazine-treated MCF-7/Dox cells was also significantly increased (1.6-fold, $P < 0.001$) at a lower level when compared to verapamil.

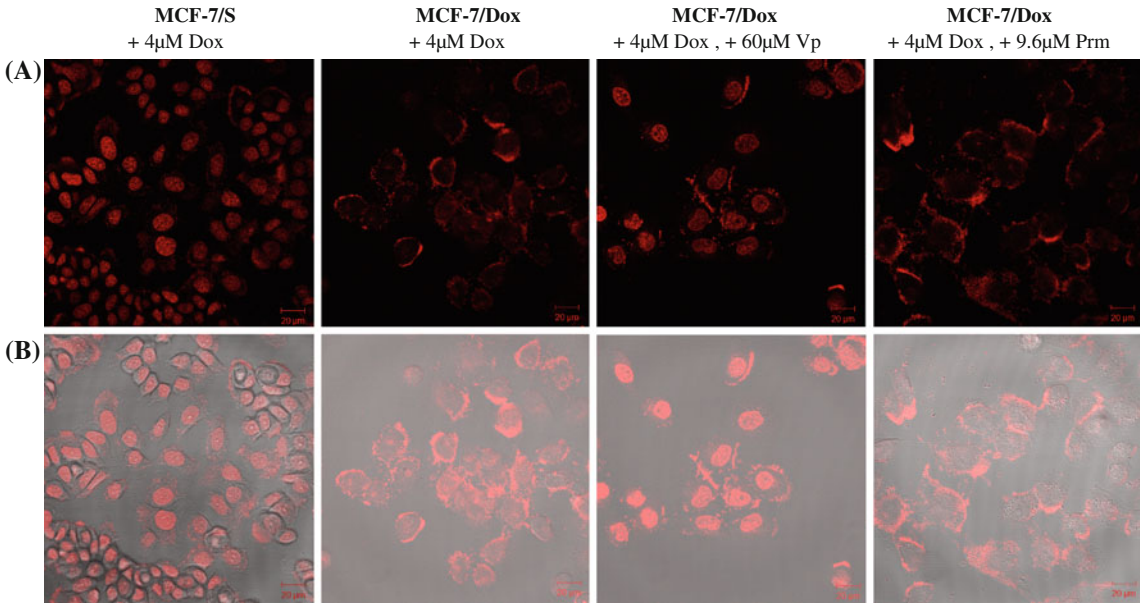

Fig. 2 Representative micrographs for MCF-7 and MCF-7/Dox cells treated with doxorubicin, doxorubicin and verapamil (Vp), and doxorubicin and promethazine (Prm); **a** fluorescence images, **b** superimposed fluorescence and transmission images with Plan-Neofluar 40X/1.3 Oil DIC objective

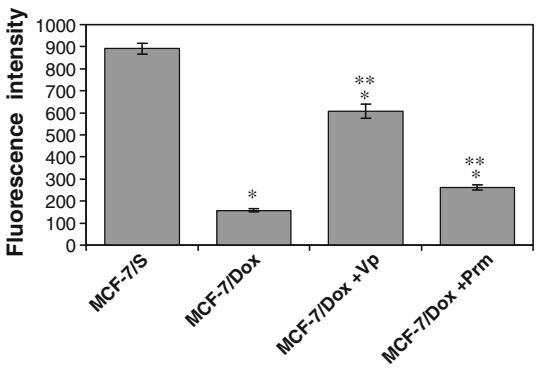

Fig. 3 Doxorubicin accumulation in MCF-7/S and MCF-7/Dox cells. * Significant difference in fluorescence intensity of doxorubicin between MCF-7/S and untreated/treated MCF-7/Dox cells with $P < 0.001$. ** Significant difference in fluorescence intensity of doxorubicin between untreated MCF-7/Dox and verapamil- or promethazine-treated MCF-7/Dox cells with $P < 0.001$

Discussion

In the present study, besides MDR modulation function of verapamil and promethazine at protein activity level, suppression of *MDR1* and *MRP1* at gene expression levels dependent on time of exposure to these reversal agents was also investigated.

QPCR results revealed that verapamil treatment caused significant reduction in *MDR1* gene expression after 48- and 72-h treatment, while there was no significant change in the expression level of *MRP1*. Dose-related downregulation of *MDR1* gene transcription by verapamil exposure for 24 h was first reported by Muller et al. [20], and it was suggested that the decreased transcription rate could be due to the decrease in *MDR1* proximal promoter activity. Molnar et al. [18] also previously described that *MDR1* gene expression level was reduced due to promethazine application in mouse lymphoma cells and suggested that the inhibition of gene expression was at promoter level. There are only few compounds other than verapamil have been reported to downregulate *MDR1* gene expression and potentially reverse multidrug resistance. These compounds are curcumin [2], tryptanthrin [28], estrogen [21], and perospirone [29]. Therefore, the results demonstrating a twofold decrease in the expression of *MDR1* upon exposure to modulators are parallel to literature. To our knowledge, this is the first report demonstrating the suppression effect of promethazine on *MRP1* expression in doxorubicin-resistant MCF-7 cells.

Verapamil is a specific first-generation P-gp efflux pump inhibitor [22]. According to our previous reports [10], it exerted synergic effects in combination with doxorubicin on doxorubicin-resistant MCF-7 cells. The inhibitory effect of verapamil on drug efflux was also demonstrated by flow cytometric measurements in the same study. Promethazine, a phenothiazine derivative, is clinically used as oral antihistamine that mimics the effect of the naturally occurring histamine. It was previously reported that phenothiazines could be used as P-gp suppressor MDR reversal agents [19]. According to confocal laser scanning microscopy, reversal effect of promethazine on doxorubicin efflux was lower when compared to verapamil (Figs. 2, 3). It was also

previously demonstrated by our group that verapamil and promethazine were effective P-gp inhibitors as determined by flow cytometric measurements. However, promethazine had about fivefold lower P-gp modulation activity than that of verapamil in doxorubicin-resistant MCF-7 cells [11]. A study on multidrug resistance reversal in mouse lymphoma and resistant COLO 320 cells has shown that phenothiazine derivative promethazine modulated MDR also [16].

In addition to the interaction of modulators with ABC transporter proteins, their interaction with the lipid bilayers of the plasma membrane is important, and membrane lipids can be regarded as one of the targets for MDR reversing agents [17]. Most of the MDR reversing compounds, such as promethazine, exert an influence on the physical properties of lipid bilayers by altering membrane fluidity and increasing membrane permeability [3, 5]. These alterations in the lipid membrane can modify the functional conformation of P-gp conformation and function.

In conclusion, verapamil as a channel blocker seems to be a better MDR modulator than promethazine in doxorubicin-resistant MCF-7 cells when degree of drug efflux inhibition and *MDR1* suppression effects are considered. Additionally, promethazine can also be suggested as a good resistance modifier with its combined additive effects with doxorubicin [11], its effect on intracellular doxorubicin efflux and gene suppressor effects on *MDR1* and *MRP1*.

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