Original Article

The Influence of Phenothiazine Derivatives on Doxorubicin Treatment in Sensitive and Resistant Human Breast Adenocarcinoma Cells

(phenothiazine derivatives / doxorubicin / breast adenocarcinoma / multidrug resistance)

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Abstract. Breast cancer is commonly treated by various combinations of surgery, radiation therapy, chemotherapy and hormone therapy. Most cancers either are increasingly resistant to any initial treatment or acquire resistance to a broad spectrum of anticancer drugs over time. Combination of more than one drug or combination with multidrug resistance (MDR) modifiers will possibly support the efficiency of the applied therapy. Understanding the MDR mechanisms in malignancies is crucial for developing novel strategies for treatment. The main goal of our study was to determine the cytostatic effect of doxorubicin in combination with phenothiazine derivatives (PD; promazine and triflupromazine) in doxorubicin-sensitive (MCF-7/WT) and -resistant (MCF-7/DOX) human breast adenocarcinoma cell lines. We determined cytotoxicity of the investigated compounds (MTT assay) after 24 and 48 h. The effect of phenothiazine derivatives was evaluated and doxorubicin localization was performed using confocal microscopy. The mode of the cell death was examined by the comet assay. We also determined the expression of P-glycoprotein (P-gp), which is a membrane-associated protein responsible for the multidrug resistance.

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Abbreviations: ABC – ATP-binding cassette, ATP – adenosine-5'-triphosphate, DOX – doxorubicin, MDR – multidrug resistance, MRP – MDR-related proteins, MTT – 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PD – phenothiazine derivatives, P-gp – P-glycoprotein.

Introduction

Chemotherapy is applied in all stages of breast cancer. In early stages, as well as in all advanced stages, its application depends on the risk and hormone receptor status. The efficiency of chemotherapy in cancer treatment is decreased by the resistance to cytostatics called multidrug resistance (MDR). Resistance to chemotherapy is a common clinical problem in patients with all types of cancer. The medicine targets of malignant cells often show a cross-refractoriness to a variety of drugs that have different structures and functions (Hait, 1990; Bartosz, 1997; Lenart, 2005).

Multidrug resistance is a consequence of complex molecular events, which consolidated in malignant cells during evolution. MDR is still one of the reasons of the adversity in anticancer treatment. The active cytostatics transport out of the cells is also responsible for MDR occurrence. The main role in this process is played by proteins called multidrug transporters (Michalak, 2002). The expression of adenosine-5'-triphosphate (ATP)-binding cassette (ABC) proteins is increased in the membranes of resistant cells. Irregular, increased expression of transporter proteins is a factor involved in cancer resistance to cytostatics. The ABC protein family includes membrane proteins. They are constructed of several or dozen domains built in the cell membrane. Their common feature is a specific domain responsible for binding and hydrolysis of ATP (Bartosz, 1997).

The energy from ATP decomposition is exploited by these proteins for transport of different substances. Transporters in normal cells play important physiological functions involved in inner and outer transport and participate in detoxification processes. The main transporters of MDR are P-glycoprotein (MDR1) and MDR-related proteins (MRP). They possess the ability to pump out a wide spectrum of compounds differing in structure. The mechanism of action of these MDR trans-

porters is still under investigation. Scientists look for compounds with inhibitory properties of MDR (MDR modulators) to apply them in combination with other methods of treatment. MDR modulators can act directly as transport protein inhibitors and indirectly by influencing the biophysical properties of the lipid membrane phase, where MDR transporters are inserted. Multidrug resistance can be modulated by many structurally unassociated chemical substances, e.g. phenothiazine derivatives (Bartosz, 1997; Michalak, 2002; Dean, 2005; Lenart, 2005).

Phenothiazine derivatives are known as psychotropic medicines. Many studies demonstrated the possible application of PD as modulators of multidrug resistance. Phenothiazine derivatives are tricyclic, amphiphilic chemical compounds applied as antidepressants (Roberts, 1984). Pharmacological features of phenothiazine derivatives (PD) depend in particular on the type of phenothiazine ring substituent. This substituting group undergoes the interactions between the lipid polar heads and thus influences the thermal phase behaviour of the lipid (Barbieri, 2003). The aim of the current study was to determine whether efficient phenothiazine derivatives are able to modulate the multidrug resistance in doxorubicin-sensitive and -resistant cells after chemotherapy.

Material and Methods

Cell culture

The studies were performed in human doxorubicin-sensitive (MCF-7/WT) and -resistant (MCF-7/DOX) breast adenocarcinoma cell lines. Both cell lines were a kind gift from the Department of Tumor Biology, Comprehensive Cancer Center, Maria Sklodowska-Curie Memorial Institute (Gliwice, Poland). Cells were grown in DMEM (Sigma, Poznań, Poland) containing 10% foetal bovine serum (Biowhittaker, Lonza, Walkersville, MD) and supplemented with antibiotics. For the experiments, the cells were removed by trypsinization and washed with PBS. The cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2.

Doxorubicin and PD treatment

The cells were treated with doxorubicin (Sigma, Poland) without phenothiazine derivatives (PD, Sigma-Aldrich) and doxorubicin (10 mg/ml) with PD (10 and 50 μM) during 24 h and 48 h. The control cells were treated separately with PD and doxorubicin.

MTT assay

The 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) test was used for the assessment of cell viability. The cells were seeded into 96-well microculture plates (Nunc, NunclonTM Surface, Biokom, Janki, Poland) at the concentration 5×10^3 cells/well. Then 4 h incubation with doxorubicin only and doxorubicin in combination with phenothiazine derivatives was performed. After that time the cell culture medium was changed and the cells were incubated for 24 and 48 h at 37 °C and 5% CO_2. The MTT assay was then performed according to the manufacturer's protocol. The absorbance was determined using a multiwell scanning spectrophotometer at 570 nm (Labsystem Multiscan MS type 352, Helsinki, Finland). Mitochondrial function was expressed as a percentage of viable cells under treatment relative to untreated control cells; the outstanding results were not taken into account.

Localization of doxorubicin and phenothiazine derivatives

From the culture dishes microcultures were trypsinized and transferred to cover glasses. The cells were incubated with doxorubicin (10 mg/ml) or phenothiazine derivatives (10 μM) for 4 h. Then, after washing in PBS, cells were fixed in 4% formalin buffer and washed in PBS. After that the cells were placed on glass slides (SuperFrost®, Bionovo, Legnica, Poland) with fluorescence mounting medium (Dako, Glostrup, Denmark) and then examined under a confocal scanning laser microscope (Carl Zeiss GmbH, Jena, Germany).

Immunofluorescence - P-glycoprotein expression in breast adenocarcinoma cells

The cells were plated in growth medium into cover glasses (Thermo Scientific, Braunschweig, Germany) and grown for two days. Then the cells were fixed in 4% formalin buffer and washed in PBS. The antibody against P-gp (concentration 1 : 50, MDR-1, Santa Cruz Biotechnology, Inc., Heidelberg, Germany) was labelled with FITC (concentration 1 : 60, Sigma, excitation wavelength: 470 ± 20 nm and emission wavelength: 525 ± 20 nm). Fluorescence was monitored using a confocal scanning laser microscope (Carl Zeiss GmbH, Jena, Germany).

Neutral comet assay

For the detection of DNA fragmentation associated with apoptosis, the neutral comet assay was used. The cells were harvested in 6-well plates (Nunc), and then 4 h incubation with drug combinations was performed. After that time cells were detached by trypsinization and washed two times with DMEM. The cells were then counted in Bürker counting chamber. The cells, at a concentration of 1×10^5/ml, were mixed with low-temperature-melting agarose (Sigma) at a ratio of 1 : 10 (v/v) and spread on a slide. The slides were submerged in precooled lysis solution (2.5 M NaCl, 100 mM EDTA, pH 10, 10 mM Tris base, and 1% Triton X-100) at 4 °C for 60 min. After lysis and rinsing, the slides were equilibrated in TBE solution (40 mM Tris/boric acid, 2 mM EDTA, pH 8.3), electrophoresed at 1.0 V/cm^2 for 20 min, and then silver staining was performed. For visual (microscope counting by two independent researchers) scoring the comet pattern, 100–200 nuclei on each slide were counted and assigned to a category from 0 to 4,

depending on the relative staining intensity of DNA in the tail (0 = no DNA in tail; 4 ≥ 75 % of DNA in tail).

Results

Figure 1 presents the effect of doxorubicin, phenothiazine derivatives and their combination on two cell lines determined by the MTT assay. As was expected, MCF-7/DOX cells were more resistant to doxorubicin incubation. The strongest cytotoxic effect on both cell lines was displayed by promazine and trifluprom azine at 50 μM concentration after 48 h and in combination with DOX. Very interesting is also the toxicity of promazine derivatives. However, in combination with DOX, MCF-7/WT were more susceptible to this therapy.

When the multidrug-resistant and -sensitive cells used in this study were incubated with 10 mg/ml DOX, we observed that the MCF-7/WT cell line demonstrated higher accumulation of doxorubicin than cells from the corresponding MDR sub-line MCF-7/DOX. In Fig. 2 A and B we can observe cells with interphase nuclei, where DNA is located as hetero- and euchromatin. Doxorubicin accumulated mainly in the nuclear envelope, where heterochromatin is found in high concentrations. In case of the resistant cell line, DOX was accumulated on the cell surface (outer cell membrane). DOX was also detected accumulated in the cell membrane and cytoplasm, as presented in Fig. 2 A and B.

Immunofluorescence of P-glycoprotein was also performed and demonstrated in Fig. 3 A and B. We can no-

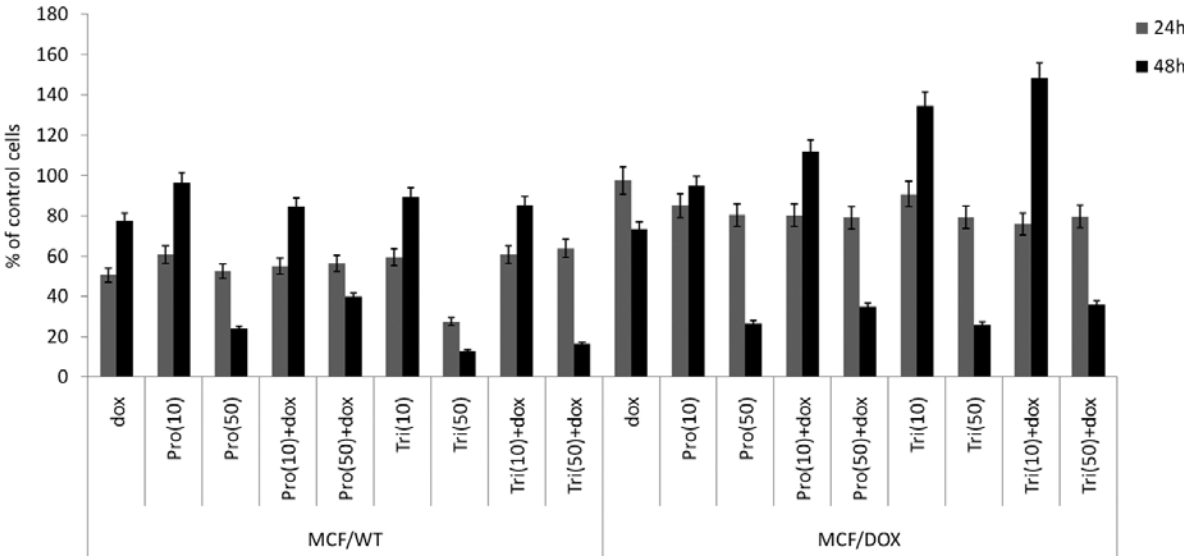

Fig. 1. MTT assay after 24 and 48 h in MCF/WT and MCF/DOX cell lines after 4 h doxorubicin and doxorubicin (10 mg/ml) plus PD (10 and 50 μM) treatment.
Abbreviations: dox – doxorubicin (10 mg/ml); Pro(10) – promazine 10 μM; Pro(50) – promazine 50 μM; Pro(10)+dox – promazine 10 μM in combination with doxorubicin (10 mg/ml); Pro(50)+dox – promazine 50 μM in combination with doxorubicin (10 mg/ml); Tri(10) – triflupromazine 10 μM; Tri(50) – triflupromazine 50 μM; Tri(10)+dox – triflupromazine 10 μM in combination with doxorubicin (10 mg/ml); Tri(50)+dox – triflupromazine 10 μM in combination with doxorubicin (10 mg/ml).

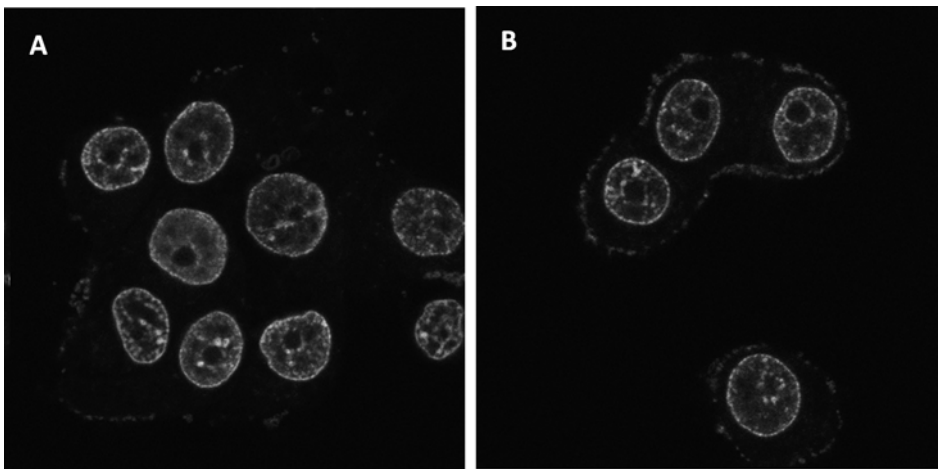

Fig. 2. Doxorubicin localization after 4 h incubation in **A**) MCF-7/WT cells (1000×) and **B**) MCF-7/DOX cells (1000×).

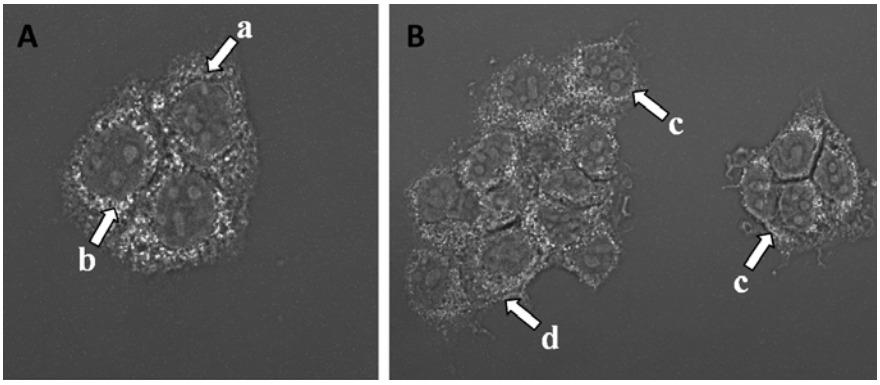

Fig. 3. P-glycoprotein expression in **A**) MCF-7/WT cells (×1000) and **B**) MCF-7/DOX cells (×600). **a** – cytoplasmic distribution; **b** – nuclear envelope; **c** – nuclear membrane; **d** – plasmatic membrane.

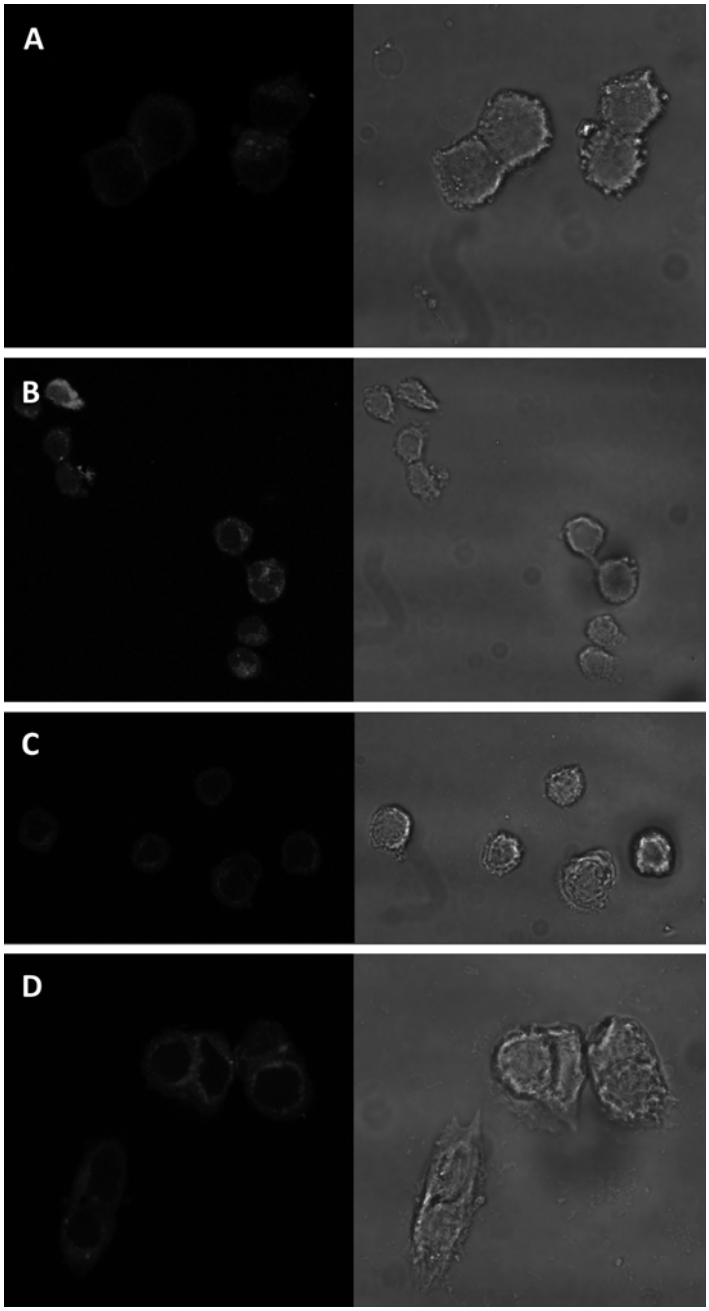

Fig. 4. Intracellular distribution of **A**) promazine in MCF-7/WT cells (1000×); **B**) triflupromazine in MCF-7/WT cells (600×); **C**) promazine in MCF-7/DOX cells (600×); **D**) triflupromazine in MCF-7/DOX cells (1000×).

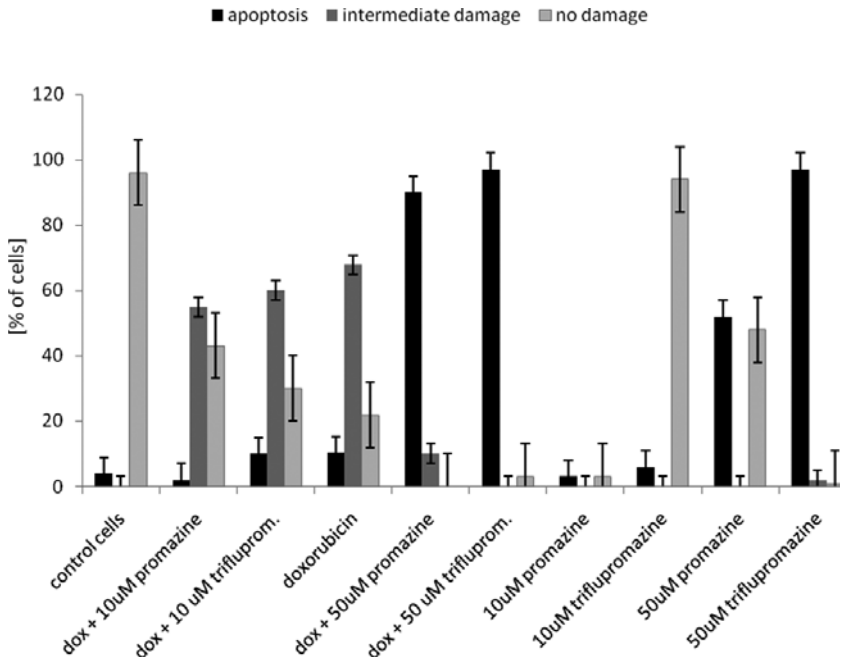

Fig. 5. Neutral comet assay in MCF-7/WT cells was performed after 4 h after doxorubicin (10 mg/ml) treatment and application of doxorubicin (10 mg/ml) with promazine and trifluprom azine in concentrations: 10 and 50 μM. Results expressed as the mean ± SD.

tice that expression of P-gp was irregular and dispersed in the cytoplasm of both cell lines. In MCF-7/WT cells P-gp was mainly located in the nuclear membrane; in MCF-7/DOX in the plasmatic membrane. We did not notice any expression in the nuclei of both cell lines.

In Fig 4. A-D we can observe intracellular distribution of phenothiazine derivatives in both examined cell lines. In both cell lines promazine localized mainly in the cell membranes, nuclear envelope and partially, as we suspect, in lysosomes. In case of trifluprom azine the strongest fluorescence signal was observed in both cell lines mainly in the nuclear membrane.

The intention of the current study was also to determine apoptosis in both cell lines. In Figs. 5 and 6 we can observe results of the neutral comet assay. Our experiments showed that in resistant cells apoptosis was in-

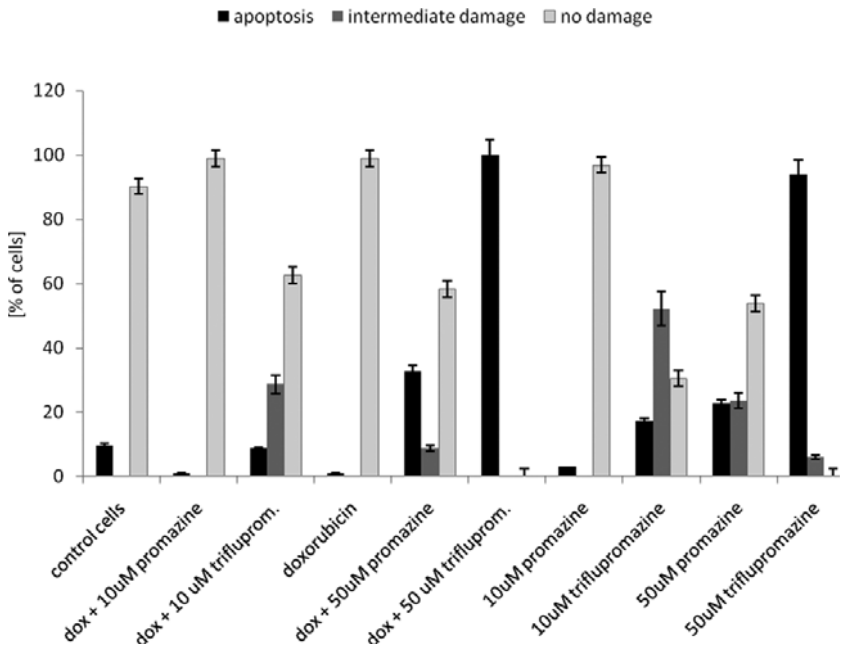

Fig. 6. Neutral comet assay in MCF-7/DOX cells was performed 4 h after doxorubicin (10 mg/ml) treatment and application of doxorubicin (10 mg/ml) with promazine and trifluprom azine in concentrations: 10 and 50 μM. Results expressed as the mean ± SD.

duced by triflupromazine (50 μM) and its combination with DOX. However, in sensitive cells we could observe apoptosis also after treatment with 50 μM promazine and its combination with DOX.

Discussion

The main problem in cancer therapy is the multidrug resistance. It remains imperfectly understood, though in some instances it certainly involves altered membrane transport in tumour cells. Toxicity-lowering export of drugs is mediated by the membrane protein P-glycoprotein, a member of the class of ABC transporters. Phenothiazine derivatives can block DOX-induced mdr-1/P-gp expression with the consequent increase in DOX cellular uptake and cytotoxicity. PD can potentiate the cytotoxic activity of DOX against the growth of MDR cells by an mdr-1/P-gp independent mechanism (Hendrich, 2003). Some authors applied combinations of the antibiotics vancomycin or ampicillin and thioridazine and prochlorperazine at subinhibitory concentrations. They observed that these antibiotics could render vancomycin- or ampicillin-resistant bacteria sensitive to each of the antibiotics (Thanacoody, 2007; Rahbar, 2010). Motohashi et al. (2000, 2003) indicated biological activity of N-acylphenothiazines and their influence on lipid and erythrocyte membrane. Hendriks et al. (2005) also applied verapamil and reserpine as inhibitors of P-glycoprotein-mediated multidrug resistance, but this did not reduce resistance. The results of these authors outline modification of resistance in enterococci induced by phenothiazine derivatives unrelated to P-glycoprotein-mediated multidrug resistance. However, Hendrich et al. (2003) conclude that the molecular mechanisms of action of phenothiazine derivatives may involve interactions with either P-glycoprotein or membrane lipid matrix. Our results suggest that phenothiazine derivatives are highly toxic for both cell lines, with triflupromazine showing higher effects. PD alone and PD with doxorubicin induced a significant decrease in cell viability and apoptosis. Similar results were obtained by Bisi et al. (2008). These authors tested some phenothiazine derivatives and they concluded that the most cytotoxic compounds of the series were able to induce apoptosis in resistant cell lines. They also observed that apoptosis was induced via an atypical pathway of caspase cascade activation, and a synergistic effect in combination with doxorubicin was also found (Bisi et al., 2008). Currently, there are only a few studies related to the research of phenothiazine derivatives. These drugs are used commonly, but their application can be more effective in other fields of medicine. However, phenothiazine derivatives have a wide range of applications. Besides being psychotropic drugs, they can also be useful as MDR modulators or combined with cytostatics. Some researchers used PD as photosensitizers in photodynamic therapy. These compounds revealed phototoxic and allergic skin and eye reactions (Roberts, 1984; Chignell, 1985).

We conclude that PD, especially trifluopromazine, directly interfere with P-glycoprotein and accumulate more intensively in the tested breast cancer cells. Our results confirm that the modifiers have effect on multidrug resistant cells treated with doxorubicin. The usage of DOX as an important anticancer drug in the cancer therapy is usually limited by its severe cardiotoxicity and the development of MDR phenotype. Combination therapy of MDR modulators with chemotherapy should be further investigated and treatment protocols developed.

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