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Preliminary Studies on Phenothiazine-mediated Reversal of Multidrug Resistance in Mouse Lymphoma and COLO 320 Cells

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Abstract. *The ability of phenothiazine derivatives to inhibit the transport activity of P-glycoprotein in resistant mouse lymphoma and MDR/COLO 320 cells was studied. A rhodamine 123 efflux from the above-mentioned neoplastic cells in the presence of tested compounds was examined by flow cytometry. Two of the phenothiazine derivatives, namely perphenazine and prochlorperazine dimaleate, proved to be effective inhibitors of the rhodamine efflux. Other tested phenothiazine derivatives (promethazine hydrochloride, oxomemazine, methotrimeprazine maleate, trifluoropromazine hydrochloride, trimeprazine) also modulated the intracellular drug accumulation in both resistant cell lines, however, they exerted additional cytotoxic effects. The differences observed between the effects of the test compounds on intracellular drug accumulation could be the outcome of differences in phenothiazine's chemical structure, which is crucial for drug-cell membrane interactions. The results of this study provide information about a new group of compounds that offer promise in multidrug resistance reversal in tumor cells.*

Neoplastic cells exposed to chemotherapeutic agents respond by the activation of defense mechanisms, that result in the development and progression of resistance to a wide range of anticancer drugs. These multidrug resistance (MDR) mechanisms include: decreased uptake, increased detoxification (phase I and II enzymes), altered protein targets, or increased efflux. One of the most widespread mechanisms of MDR is associated with the expression of the 170-kDa P-glycoprotein (P-gp) (MDR1), which belongs to the ATP-binding cassette type transport proteins (1, 3, 5). The presence of P-gp was demonstrated in normal cells, such as capillary endothelium in the brain, colon, kidney and liver, where P-gp is involved in physiological functions. The efflux pump activity of P-gp in human brain capillary endothelium, which is responsible for the blood-brain barrier, was less sensitive to a phenothiazine promethazine than the MDR in cancer cells (10). The main role of P-gp is the transport of toxic compounds into bile or urine, as well as to select compounds translocated to the cerebrospinal fluid (14). Moreover, the *MDR1* gene is overexpressed in some tissues such as adrenal cortical cells, biliary hepatocytes, CD 34 bone marrow cells, type T4 peripheral lymphocytes or renal tubules. In these tissues, P-gp is in charge of transporting steroids, as well as forming the blood-brain barrier (1, 14). With regard to transformed cells, the expression of P-gp is also detectable in some drug-untreated tumor cells originating from adrenal-, kidney-, liver- or colorectal carcinomas, and certain types of lymphomas or neuroblastoma, or carcinoids. Other tumors, that stem from the breast or ovary, are able to overexpress P-gp if they are exposed to anticancer drugs (1). Consequently, when P-gp is overexpressed, the tumors grow and progress rapidly. Needless to say, P-gp is able to pump cytotoxic compounds out of the cell, therefore the intracellular drug concentration decreases to the point that tumor cells can survive.

Because the clinical significance of P-gp is of considerable importance to the outcome of patients with drug-resistant tumors, the development and selection of P-gp modulators or inhibitors have been extensively studied. Among numerous compounds proposed to block P-gp, only a few, which are non toxic on their own, have entered clinical use.

Phenothiazines, apart from their wide biological activity (13), have been described as effective MDR modifiers. During the last few years, a lot of new phenothiazine derivatives have been synthesized and their activity has been extensively studied (7-9, 12, 13, 15).

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The purpose of this study was to test the anti-multidrug resistance activity of seven phenothiazine derivatives. Their ability to inhibit P-gp-dependent transport activity in a mouse lymphoma cell line overexpressing the *MDR1* gene and in the MDR/COLO 320 cell line was studied by flow cytometry, using the standard functional assay with rhodamine 123 as a fluorescent substrate analog.

Materials and Methods

Tested compounds. Perphenazine (Bracco, Italy), promethazine hydrochloride (EGYTE, Hungary), oxomemazine (Rhone, Poulence, France), methotrimeprazine maleate (EGYTE), trifluoropromazine hydrochloride (Squibb, England), trimeprazine (Rhone) and prochlorperazine dimaleate (Farmitalia, Italy) were purchased from the companies indicated. The test compounds, prometazine and prochlorperazine dimaleate, were dissolved in dimethylsulfoxide (Serva, Feinbiochemica, Heidelberg, Germany). The remaining test compounds were dissolved in water. The chemical structures of the compounds are presented in Figure 1.

Cell cultures. The COLO 320 cell line was propagated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum supplemented with 2 mM L-glutamine, 1 mM Na-pyruvate and 100 mM HEPES. The L5178 mouse T-cell lymphoma cells were transfected with the pHa *MDR1/A* retrovirus, as previously described (2). The MDR1-expressing cell line was selected by culturing the infected cells with 60 ng/mL colchicine to maintain the expression of the MDR phenotype. L5178 (parental) mouse T-cell lymphoma cells and the human *MDR1*-transfected subline were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics. Each cell line was kept at 37˚C in a humidified atmosphere (5% CO_2, 95% air).

Assay for reversal of MDR in mouse lymphoma cells. After propagation, the cells in suspension were adjusted to a density of $2x10^6$/mL, resuspended in serum-free McCoy's 5A medium and distributed as 0.5-ml aliquots into Eppendorf centrifuge tubes. The test compounds were added at various concentrations of different volumes (0.5-20 μl) of the 1.0-10.0 mg/mL stock solutions and the samples were incubated for 10 min at room temperature. Next, 10 μL (5.2 μM final concentration) of the indicator rhodamine 123 (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was added to the samples and the cells were incubated for a further 20 min at 37˚C, washed twice and resuspended in 0.5 mL of phosphate-buffered saline (PBS) for analysis. The fluorescence of the cell population was measured with a Becton Dickinson FACS flow cytometer.

Verapamil (EGIS, Hungarian Pharmaceutical Company, Budapest, Hungary) was used in order to get a positive control in the rhodamine 123 exclusion experiments. The mean fluorescence intensity was calculated for the treated MDR and parental cell lines and compared to the untreated control cells. The activity ratio R for verapamil or phenothiazines (treated) was calculated by the following equation (6), on the basis of the measured fluorescence values:

$$R = \frac{\text{MDR-treated/MDR control}}{\text{parental-treated/parental control}}$$

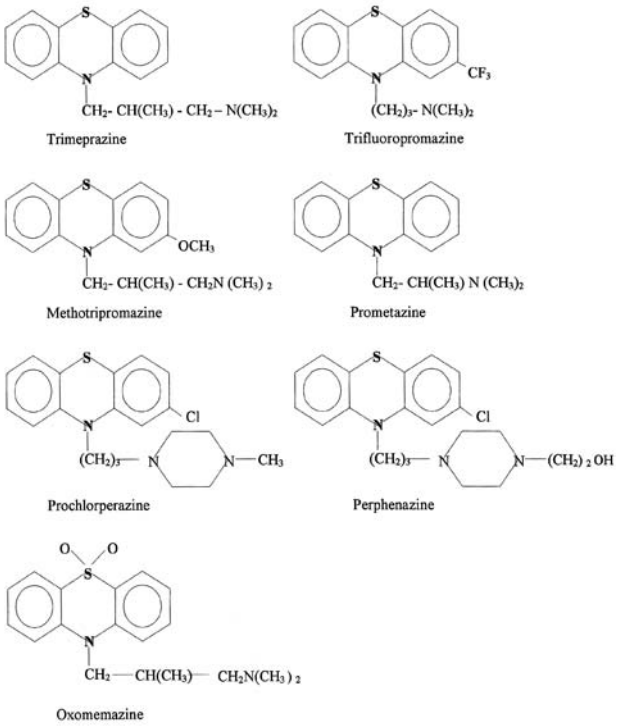

Figure 1. *The chemical structures of phenothiazines.*

Assay for reversal of MDR in COLO 320 cell line. After confluence, the cells were adjusted to a density of $2x10^6$/mL and resuspended in a serum-free medium. The following procedure was carried out in the same way as that described for mouse lymphoma cells. Finally, the activity ratio was calculated by making comparison of the experimental value with the fluorescence value of the negative control, without rhodamine 123.

Results

The standard assay to evaluate the potency of chemical compounds as MDR modulators is based on the differential accumulation of rhodamine 123 by parental and multidrug-resistant mouse lymphoma L5178 cell lines. In the MDR/COLO 320 cell line, the drug accumulation was compared to the negative control without dye. The potency of cells to accumulate rhodamine 123 was studied by flow cytometry. Seven phenothiazine derivatives were tested. Two concentrations of drugs (4 and 40 μg/mL) were used in the experiments. In both tested cell lines, at 4 μg/mL, all the phenothiazines used were moderately active, however, only two of them (perphenazine and prochlorperazine) were considerably more powerful MDR inhibitors than verapamil. At the higher concentration of 40 μg/mL, some phenothiazines (prochlorperazine, trifluoropromazine, trimeprazine, prometazine) were found to be toxic to the

Table I. *The effects of phenothiazines on rhodamine 123 accumulation in MDR mouse lymphoma cells.*

Compound	Concentration (μg/mL)	Fluorescence activity (ratio)
Verapamil (positive control)	10	8.34
Oxomemazine	4	1.14
	40	4.58
Perphenazine	4	10.66
	40	1.14[x]
Prometazine hydrochloride	4	1.23
	40	4.03[x]
Methotripromazine maleate	4	2.18
	40	5.03[x]
Prochlorperazine dimaleate	4	11.74
	40	1.24[x]
Trifluoropromazine hydrochloride	4	4.11
	40	1.59[x]
Trimeprazine	4	2.04
	40	1.20[x]
DMSO (control)	0.01% (v/v)	0.53

Numbers with the superscript x indicate the cytotoxic effect induced by the particular phenothiazine derivative.

Table II. *The effects of phenothiazines on rhodamine 123 accumulation in COLO 320 cells.*

Compound	Concentration (μg/mL)	Fluorescence activity (ratio)
Verapamil (positive control)	10	4.52
Oxomemazine	4	1.74
	40	3.04
Perphenazine	4	2.77
	40	2.17[x]
Prometazine hydrochloride	4	1.58
	40	2.41
Methotripromazine maleate	4	2.29
	40	2.10
Prochlorperazine dimaleate	4	3.45
	40	2.12[x]
Trifluoropromazine hydrochloride	4	2.56
	40	0.83[x]
Trimeprazine	4	1.91
	40	2.09
DMSO (control)	0.01% (v/v)	1.08

Numbers with the superscript x indicate the cytotoxic effect induced by the particular phenothiazine derivative.

Table III. *The effects of perphenazine and prochloperazine on rhodamine 123 accumulation in MDR mouse lymphoma cells – dose-response experiment.*

Compound	Concentration (μg/mL)	Fluorescence activity (ratio)
Verapamil (positive control)	10	12.30
Perphenazine	0.5	1.73
	1	5.52
	2	9.00
	4	12.60
Prochlorperazine dimaleate	0.5	1.44
	1	7.28
	2	7.97
	4	9.45
DMSO (control)	0.01% (v/v)	1.10

Table IV. *The effects of perphenazine and prochloperazine on rhodamine 123 accumulation by COLO 320 cells – dose-response experiment.*

Compound	Concentration (μg/mL)	Fluorescence activity (ratio)
Verapamil (positive control)	10	6.89
Perphenazine	0.5	1.02
	1	3.21
	2	4.48
	4	4.50
Prochlorperazine dimaleate	0.5	0.99
	1	3.04
	2	3.38
	4	4.47
DMSO (control)	0.01% (v/v)	1.07

extent that it was not able to examine the cells under the test conditions. However, the cytotoxicity affected mouse lymphoma cells (Table I) more profoundly than the COLO 320 cell line (Table II). Two of the most active compounds were shown to modify the MDR in a dose-dependent manner (Table III, Table IV). At four concentrations used (0.5, 1, 2 and 4 μg/mL), the effect of perphenazine and prochlorperazine on MDR reversal was accentuated in mouse lymphoma cells. Moreover, the perphenazine-stimulating effect on rhodamine accumulation was stronger than that of verapamil (Table III). On the other hand, in the COLO 320 cell line the observed effect of phenothiazines did not differ from that of the verapamil positive control, while both phenothiazines had stimulated rhodamine efflux in cytotoxic concentrations from these cells due to their membrane disintegration (Table IV).

Discussion

The phenothiazines tested in this study were moderately active resistance modulators, especially at the lower concentration (4 μg/mL). The higher concentration, 40

μg/mL, appeared cytotoxic, however, this effect was more profoundly accentuated in the MDR1-expressing mouse T-cell lymphoma cell line than in the COLO 320 cell line. The toxic effect of phenothiazine derivatives on tumor cells has been described previously by Wesolowska *et al*. (15), who reported that the phenothiazine maleates were more toxic than other derivatives tested. These observations are in agreement with our results, where methotripromazine maleate and prochlorperazine dimaleate caused decreased cell survival of the two cell lines studied. According to the observations of Flores *et al*. (4), phenothiazine hydrochlorides are also characterized by high cytotoxicity. Similarly, in our experiment, trifluoropromazine hydrochloride was toxic. The above-mentioned authors claimed that the phenothiazines, exemplified by phenothiazine hydrochlorides, are able to modify cell membrane properties by disorganization of the phospholipids and translocation of antitumor drug complexes through the membrane. On the other hand, the same ability could also benefit in the modulation of MDR. Actually, if the drug concentration were reduced, obviously it would behave as an effective MDR inhibitor and not cause cell death. In our study, within a series of tested compounds, perphenazine and prochlorperazine dimaleate appeared as rhodamine 123 efflux modifiers. Moreover, in the multidrug-resistant mouse T-cell lymphoma cell line, the observed effect was more spectacular, probably because these cells overexpress the *MDR1* gene. Moreover, it was clearly shown that the effect of non-toxic phenothiazines is dose-dependent (Table III, Table IV). According to the Flores *et al*. (4), the MDR reversal in L5178Y and MDR/COLO 320 cell lines by phenothiazines might be mediated by two alternative mechanisms: by changing the conformation of lipids in the membranes, and, when combined with other drugs, by acting as carriers of insoluble antitumor agents to target proteins of the cell interior. Furthermore, it was previously reported that chlorine-substituted phenothiazine derivatives, exemplified by prochlorperazine in our experiment, possessed higher biological activity than other type of derivatives (15). It is clear that the non-toxic compounds tested are good candidates to reverse MDR in tumors and potentiate the action of antineoplastic drugs. New MDR modifiers, like phenothiazines, offer a practical approach to improve the efficacy of chemotherapy. However, one has to be cautious that the dose is tolerable and restricted to a non-toxic level, both for new phenothiazine chemosensitizers or phenothiazine antitumor drug complexes.

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