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Differential Effect of Phenothiazines on MRP1 and P-Glycoprotein Activity

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Abstract. *Background: Overexpression of ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1) or breast cancer resistance protein (BCRP) accounts for majority of cases of multidrug resistance (MDR) of cancer cells. Materials and Methods: In the present work, the interactions of seven commercially available phenothiazine derivatives, known P-glycoprotein inhibitors, with this transporter and MRP1 were compared. By flow cytometry, it was shown that all the drugs increased the accumulation of rhodamine 123 in the P-gp-overexpressing lymphoma cell line L5178 MDR. On the other hand, phenothiazine derivatives stimulated MRP1-mediated efflux of fluorescent probe (BCPCF) out of human erythrocytes. Results: In this way, these phenothiazine derivatives were identified as a group of atypical MDR modulators that differently interact with P-gp (as inhibitors) and MRP1 (as stimulators). Conclusion: This observation clearly shows that the activity of all new modulators should be tested for their effects towards different ABC transporters as a standard procedure.*

The occurrence of primary or therapy-induced drug resistance in cancer cells is one of the most common reasons for tumor chemotherapy failure. Multiple multidrug-resistance (MDR) mechanisms were described. Among them, however, MDR associated with the expression of transporters belonging to ATP-binding cassette (ABC) superfamily is considered to be of primary importance. These transmembrane proteins function as ATP-fueled pumps transporting anticancer drugs out of the cells. To date, 48 human ABC transporters are known (1), 12 of which have been recognized as putative drug transporters (2). However, overexpression of only three proteins: P-glycoprotein (MDR1, ABCB1), multidrug resistance-associated protein 1 (MRP1, ABCC1) and breast cancer resistance protein (BCRP, MXR, ABCG2), appears to account for almost all clinical cases of multidrug resistance (3). P-glycoprotein (P-gp) and MRP1 share only 15% amino acid sequence homology (4) and display preference for transport of dissimilar substrates. P-gp pumps out mostly amphiphilic drugs that are positively charged in physiological pH (reviewed in (5)), while MRP1 prefers glutathione, glucuronate and sulfate conjugates of many endogenous and xenobiotic compounds (6). The search for compounds able to reduce multidrug resistance (modulators) both *in vitro* and *in vivo* is an unceasing task.

During chemotherapy, drugs are introduced into extremely complex cellular environments in order to modify one of the metabolic or signalling pathways. The complexity of the 'inner world' of cells causes, however, drugs aimed to interact with a given cellular target also to interact with other proteins not anticipated by the drug designers. For example, newly introduced anticancer drugs Iressa™ and Gleevec™ were found not only to inhibit tyrosine kinases as expected, but also to interact with ABC transporter ABCG2 (7). Usually, during *in vitro* studies of MDR modulators, well-defined cancer cell lines are used, in which only one of the ABC transporters is overexpressed. The usage of such models for MDR modulators testing results in selection of specific inhibitors of a given ABC transporter. It should be noted, however, that malignant cells that express more than one type of ABC transporter are very likely to occur *in vivo*.

Phenothiazine derivatives are widely used as antipsychotic drugs due to their interaction with many types of ion channels (8). They are also calmodulin antagonists (9), inhibitors of protein kinase C (10) and adenylate cyclase (11). Phenothiazines are able to modulate physicochemical properties of cell membranes and model lipid bilayers (reviewed in (12)). They were among the first group of recognized modifiers of P-gp transport activity (13, 14). At least two independent biological processes are likely to be

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responsible for the reversal of MDR by phenothiazines – reduced activity of the efflux pump and down-regulation of the MDR gene (15). Previously, we showed that the ability of phenothiazine methoxycarbonylamides and methanesulfonylamides to interact with model membranes was correlated with their ability to inhibit P-gp activity (16). Such a correlation was not, however, noticed when phenothiazine maleates were studied (17). Apart from our recent study on phenothiazine maleates (18), the interaction of phenothiazine derivatives with MRP1 does not appear to have been studied.

In the present study, we have examined the influence of seven commercially available phenothiazine derivatives on two main ABC transporters associated with multidrug resistance: P-gp and MRP1.

Materials and Methods

Materials. Rhodamine 123, verapamil, chlorpromazine, perphenazine, benzbromarone and orthovanadate were obtained from Sigma (Poznan, Poland). Trifluoperazine and thioridazine were the products of ICN Biochemicals (Solon, OH, USA). Diethazine, levomepromazine and thiethylperazine were from Egis Pharmaceutical Works (Budapest, Hungary). 2',7'-*Bis*-(3-carboxypropyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCPCF-AM) was purchased from Molecular Probes (Eugene, OR, USA). All other reagents used were of analytical grade. Stock solutions of rhodamine 123 (260 μM) and verapamil (10 μg/ml) were prepared in water. Chlorpromazine, trifluoperazine and thioridazine were dissolved in phosphate-buffered saline (PBS). All other compounds were dissolved in dimethylsulfoxide (DMSO).

Cell culture. Sensitive mouse T lymphoma cell line L5178Y and its resistant subline L5178 MDR, obtained previously by transfection with pHa MDR1/A retrovirus (19), were used to study P-gp transport activity. Cells were maintained in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics at 37˚C and 5% CO_2. Colchicine (at 60 ng/ml) was constantly present in the medium of the resistant subline to maintain P-gp expression.

Accumulation of rhodamine 123 by cancer cells. The cells (2×10^6/ml in serum-free medium) were incubated with phenothiazine derivatives (at 4 μg/ml; molar concentrations were 7.7-11.9 μM depending on the compound) for 10 minutes at room temperature. Rhodamine 123 was then added (final concentration 5.2 μM) and the cells were incubated for 20 minutes at 37˚C. After this incubation, the cells were washed twice and resuspended in PBS for flow cytometric analysis. A Becton Dickinson FACScan instrument equipped with an argon laser was used for measurement of the fluorescence of the cell populations, using fluorescence excitation and emission wavelengths of 488 nm and 520 nm, respectively. Verapamil was used as a positive control. The influence of DMSO (concentration in samples 0.8%) on the cells was also monitored. Fluorescence intensity ratio (FIR) was the ratio of fluorescence values for modulator-treated and untreated samples. Resistant cells treated with medium only (no modulator) had an FIR value equal to 1 (MDR control). Experiments were performed in triplicate.

Human erythrocyte preparation. Blood of healthy volunteers, obtained by venous puncture with the use of EDTA as an anticoagulant, was diluted with iso-osmotic PBS and centrifuged (2,000 × g, 5 min, 4˚C). The sediment of blood cells was resuspended in PBS and passed through an α-cellulose column to remove leukocytes and platelets. After three more washes, the erythrocytes were resuspended in transport buffer (6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 138 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose; pH 7.4). Cells were stored at 4˚C and used within 36 h.

Efflux of BCPCF from erythrocytes. Human erythrocytes can serve as a model for MRP1-transport activity studies with the use of a functional test developed by Rychlik *et al*. (20). Phenothiazine derivatives at an appropriate concentration and/or inhibitors were added to the erythrocytes suspended in transport buffer (5% hematocrit). After incubation (15 minutes, room temperature, darkness), the samples were mixed with an equal volume of ice-cold 2 μM BCPCF-AM solution in the same buffer and incubated for 10 minutes on ice. During that time, hydrophobic non-fluorescent acetoxymethyl ester of BCPCF passively penetrated into erythrocytes where its ester bond was cleaved by cellular esterases. Highly fluorescent, free acid form of BCPCF was thus released which can only leave the erythrocyte interior by being pumped out by MRP1. After loading with BCPCF-AM the cells were incubated at 37˚C for 0, 20, 40 and 60 minutes in darkness. Putting the samples on ice followed by rapid centrifugation (14,000 × g, 3 minutes, 4˚C) terminated the incubation. MRP1 transport activity was monitored by measuring BCPCF fluorescence intensity in the supernatant (excited at 475 nm and read at 525 nm). The percentage of MRP1 stimulation/inhibition was determined by comparing the slopes of fluorescence intensity *versus* time plots for the control samples and the samples containing the studied compounds. The influence of phenothiazine derivatives on BCPCF fluorescence was negligible. The DMSO concentration in samples was kept under 0.5%. Care was also taken to maintain hemolysis in the samples below 1%. All experiments with phenothiazine derivatives were performed in triplicate. Experiments with the use of orthovanadate and benzbromarone, and their mixtures with thiethylperazine were performed only once. The uncertainty of the values measured by the functional test employed in the present work was typically less than 20%.

Results

Apart from their wide use as antipsychotic drugs, phenothiazine derivatives were among the first known modulators of P-gp-mediated multidrug resistance in cancer cells (13, 14). We showed that the sensitive mouse T lymphoma cell line accumulated more rhodamine 123 than its drug-resistant subline (Figure 1). The addition of verapamil or thiethylperazine shifted the fluorescence of the population of resistant cells towards higher values (Figure 1), thus increasing rhodamine 123 retention inside L5178 MDR cells. Our results confirmed that all seven phenothiazine derivatives studied inhibited P-gp transport activity in this mouse T lymphoma cell line (Figure 2). Four compounds: thiethylperazine, thioridazine, trifluoperazine and perphenazine were shown to inhibit P-gp transport activity to a greater extent than verapamil, used as a positive control.

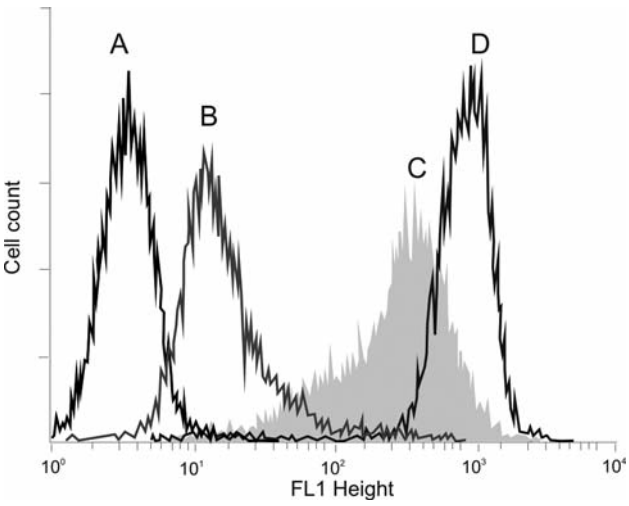

Figure 1. *Rhodamine 123 accumulation in the resistant mouse T lymphoma cell line L5178 MDR not treated by a modulator (A), treated by 10 μg/ml of verapamil (B), treated by 4 μg/ml of thiethylperazine (C), and in the parental sensitive cell line L5178Y (D).*

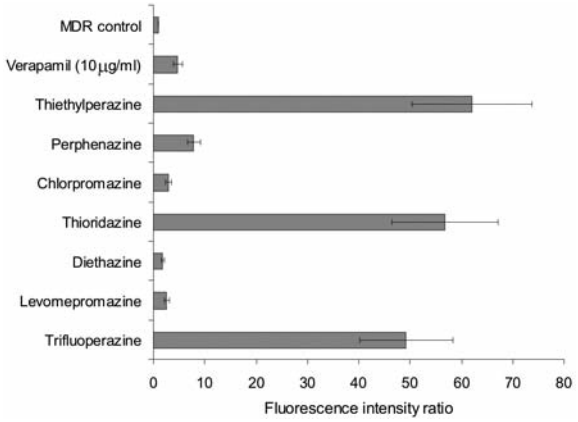

Figure 2. *The influence of phenothiazine derivatives (at 4 μg/ml) on rhodamine 123 accumulation by P-glycoprotein-expressing L5178 MDR cell line (mean±S.D. of three independent experiments). MDR control was not treated with modulator.*

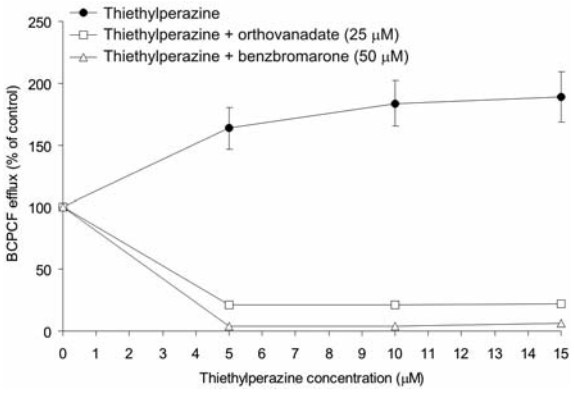

Figure 3. *The influence of thiethylperazine, thiethylperazine plus 25 μM of orthovanadate, and thiethylperazine plus 50 μM of benzbromarone on BCPCF efflux out of human erythrocytes (mean±S.D. of three independent experiments for thiethylperazine alone, see Materials and Methods for other experiments).*

Human erythrocytes physiologically express MRP1 (21), MRP4 and MRP5 (22), but not P-gp (20). It was demonstrated (18, 20) that the functional test based on the efflux of carboxyfluorescein derivative BCPCF or BCECF out of human erythrocytes can be employed to reliably monitor MRP1 transport activity. It was found that all phenothiazine derivatives studied, in concentrations that did not cause erythrocyte hemolysis, stimulated BCPCF efflux out of human erythrocytes. Each drug was studied at several concentrations. The magnitude of the effect was different for different compounds, ranging from 50% up to almost 90% (Table I). The stimulation of outward BCPCF transport by phenothiazines was generally concentration dependent as shown in Figure 3, using thiethylperazine as an example. However, for some compounds (*e.g.* levomepromazine and trifluoperazine), a reduction of the MRP1 stimulation at higher concentrations was observed. Because each drug caused hemolysis at a different concentration, the concentration of 15 μM was the highest that could be tested for all phenothiazines. Therefore, it was chosen to compare the strength of the stimulatory effect exerted by the different compounds (Table I). At this concentration thiethylperazine, followed by perphenazine and chlorpromazine were identified to be the most effective MRP1 stimulators.

To rule out the possibility that the observed effect of phenothiazine derivatives was the result of factors other than increased MRP1 activity (*e.g.* increased membrane permeability), MRP1 inhibitors orthovanadate and benzbromarone were used to show that thiethylperazine was not otherwise able to increase BCPCF efflux out of human

Table I. *Stimulation of BCPCF efflux out of human erythrocytes by phenothiazine derivatives (mean±S.D. of three independent experiments).*

Compound	Stimulation at 15 μM (% of control)	Maximal stimulation	
		Concentration (μM)	(% of control)
Thiethylperazine	189.0±20.6	15	189.0±20.6
Perphenazine	163.1±9.5	15	163.1±9.5
Chlorpromazine	160.5±9.1	20	165.9±7.0
Thioridazine	147.4±13.0	10	155.6±7.2
Diethazine	145.6±18.9	20	154.0±15.8
Levomepromazine	134.6±9.5	40	193.0±9.9
Trifluoperazine	127.9±12.1	5	151.1±32.6

erythrocytes (Figure 3). The effect exerted by other MRP1 inhibitors, such as MK-571 and flavonoid morin, were similar (data not shown).

Discussion

The results obtained here brought us to the conclusion that the observed increased efflux of BCPCF out of human erythrocytes was thus not an artifact but was the result of true stimulation of MRP1 transport activity by phenothiazines. The possible mechanisms of this stimulation were discussed in our previous work (18). Here, we only wish to bring attention to the fact that the increased BCPCF efflux observed in our experiments could not be caused by the changes in gene expression, since the use of mature human erythrocytes (which lack nuclei) excluded such an effect. We conclude that phenothiazine derivatives likely interact with MRP1 at a site different from the BCPCF binding site and in this way stimulate the transport of this substrate. It should be noted, however, that the stimulatory effect exerted by phenothiazines on MRP1 might be substrate-dependent, *i.e.* the results obtained with other substrates could be different. For example, Nguyen *et al.* (23) identified six flavonoids that were able to reduce the accumulation of daunomycin in MRP1-expressing PANC-1 cells but only two also reduced the accumulation of vinblastine.

In the present work, phenothiazine derivatives were shown to inhibit P-gp and to enhance transport activity of MRP1. Such behavior of modulators seems rather atypical. Examples of antagonistic action exerted by small molecules on two different ABC transporters are seldom to be found in literature. Gyemant *et al.* (24) studied the influence of sixteen flavonoids on BCECF accumulation in MRP1-expressing cell line MDA-MB-231 and on rhodamine 123 accumulation in mouse T lymphoma cell line L5178 MDR transfected with human *ABCB1* gene. Three compounds (rotenone, catechin and neohesperidin) were observed to act similarly to phenothiazines, whereas others either inhibited both ABC transporters, or stimulated both, or were not active. Sulfinpyrazone and penicillin G were found to stimulate the transport of *N*-ethylmaleimide glutathione by MRP2 and to inhibit the transport of this substrate by MRP1 (25). Indomethacin at low concentrations stimulated transport activity of both ABC proteins, inhibiting it at higher concentrations. Other organic anions, probenecid and methotrexate, were identified as inhibitors of both transporters. Substrate specificities of P-gp and MRP1 partially overlap. As many MDR modulators inhibit ABC transporters by competition with substrates, it is not surprising that many instances of modulators inhibiting both P-gp and MRP1 were reported (*e.g.* (26, 27)). Tetrahydrocurcumin was shown to inhibit the function of not only P-gp and MRP1 but also BCRP (28).

In summary, we have shown that phenothiazine derivatives constitute a group of atypical MDR modulators that act on P-gp and MRP1 in antagonistic way, inhibiting the former and stimulating the latter. This observation clearly shows that the activity of putative modulators should be routinely tested towards different ABC transporters before their usage *in vivo* be attempted.

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