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Phenothiazine maleates stimulate MRP1 transport activity in human erythrocytes

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Abstract

The expression of multidrug resistance-associated protein (MRP1) results in ATP-dependent reduction of drugs' concentration in cancer cells, i.e., multidrug resistance (MDR). Since the majority of projects are concentrated on the search of the new MDR modulators, there are very few reports on drug-induced stimulation of MDR transporters activity. In the present work, by means of functional fluorescence assay we have shown that MRP1-mediated efflux of 2′,7′-bis-(3-carboxypropyl)-5-(and-6)-carboxyfluorescein (BCPCF) out of human erythrocytes is stimulated by phenothiazine maleates that have been already identified as P-glycoprotein inhibitors. Phenothiazine maleates-induced stimulation of ATP-dependent uptake of 2′,7′-bis-(3-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) into inside-out membrane vesicles prepared from erythrocyte membranes has been also demonstrated. Moreover, it was shown that phenothiazine maleates exerted stimulating effect on ATPase activity measured in erythrocyte membranes. To our best knowledge, this report is the first one demonstrating that compounds able to inhibit transport activity of P-glycoprotein can stimulate MRP1 transporter. We conclude that phenothiazine maleates probably exert their stimulatory effect on MRP1 by direct interaction with the protein at the site different from the substrate binding site.

Keywords: Multidrug resistance (MDR); Multidrug resistance-associated protein (MRP); Transport stimulation; ATPase activity; Phenothiazine derivative; Erythrocyte

1. Introduction

Overexpression of multidrug resistance-associated protein (MRP1; ABCC1) confers multidrug resistance (MDR) on tumour cells, the phenomenon characterized by ATP-dependent reduction of anticancer drug concentration inside the cells that leads to frequent chemotherapy failures. MRP1 is a 190-kDa protein that belongs to ATP-binding cassette (ABC) superfamily [1]. Composed of two hydrophobic transmembrane domains, each followed by a cytoplasmic nucleotide binding domain (NBD), MRP1 shares core structure with many other ABC superfamily members (e.g., P-glycoprotein). The characteristic feature of MRP1 is the presence of additional NH_2-proximal transmembrane domain. Glutathione, glucuronate and sulfate conjugates of many endogenous and xenobiotic substances were shown to be MRP1 substrates [2]. MRP1 is expressed not only in tumours but it is also physiologically present in many cell types, e.g., hepatocytes, erythrocytes, mast cells, eosinophils and cardiac cells [1]. In human erythrocytes, apart from MRP1, two other ABC-transporters were identified, namely MRP4 and MRP5 [3], whereas no P-glycoprotein expression was found in this cell type [4].

Since the pioneer work of Homolya et al. [5], who have demonstrated that fluorescent dyes can be the substrates of P-glycoprotein, functional tests are widely used to study MDR transporters activity. Besides acetoxymethyl ester of calcein

Abbreviations: MRP1 (ABCC1), multidrug resistance-associated protein 1; MDR, multidrug resistance; BCECF, 2′,7′-bis-(3-carboxyethyl)-5-(and-6)-carboxyfluorescein; P-gp, P-glycoprotein; BCPCF, 2′,7′-bis-(3-carboxypropyl)-5-(and-6)-carboxyfluorescein; PhMs, phenothiazine maleates; PBS, phosphate-buffered saline; GSH, glutathione

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(calcein-AM) [6] and free calcein [7], free acid form of 2′,7′-bis-(3-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) was found to be MRP1 substrate [4,8]. The method used in the present work and in previous studies [4,9] takes an advantage of the fact that the non-fluorescent acetoxymethyl ester of BCECF (or its structural analogue BCPCF) rapidly penetrates into erythrocytes where cellular esterases cleave the ester bond thus releasing highly fluorescent, free acid form of BCECF (or BCPCF) which in turn is actively pumped out of the cell. Rychlik et al. [4] have shown that BCECF efflux out of erythrocytes was inhibited by indomethacin, probenecid and benzbromarone, typical set of organic anion transport inhibitors. The authors have also demonstrated that QCRL3, specific anti-MRP1 monoclonal antibody, inhibited BCECF uptake into inside-out membrane vesicles prepared from red blood cells. Virtually the same inhibition profile was observed by Klokouzas et al. [10] in their study of dinitrophenyl S-glutathione transport in human erythrocytes. Both groups concluded that MRP1 was responsible for transport activity observed by them in red blood cells. However, the participation of MRP5 in BCECF efflux from human erythrocytes cannot be excluded as this protein was also reported to transport BCECF [11].

Phenothiazine maleates (PhMs) are newly synthesized compounds that have been shown previously to inhibit P-glycoprotein in multidrug resistant cancer cells [12,13], to possess antibacterial and antifungal activity [14] and to modulate biophysical properties of model membranes [13].

In the present study, we demonstrate the stimulation of 2′,7′-bis-(3-carboxypropyl)-5-(and-6)-carboxyfluorescein (BCPCF) efflux out of human erythrocytes by phenothiazine maleates. Also the enhanced ATP-dependent uptake of 2′,7′-bis-(3-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) into inside-out vesicles prepared from red blood cell membrane is shown in the presence of PhMs. The stimulation of Mg^{2+}-dependent ATPase activity measured in erythrocyte membrane by phenothiazine derivatives has also been observed. To our best knowledge, this report is the first to show that P-glycoprotein inhibitors can stimulate transport activity of MRP1. The systematic studies of this phenomenon are performed. We conclude that phenothiazine derivatives probably interact with MRP1 at the site different from the substrate binding site. Our data support the presumption that MRP1 possess at least two binding sites at which substrates or modulators can interact with protein.

2. Materials and methods

2.1. Materials

2′,7′-bis-(3-carboxypropyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCPCF-AM) and 2′,7′-bis-(3-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) were purchased from Molecular Probes (Eugene, OR). Sodium orthovanadate, benzbromarone, ouabain, phenylmethylsulphonylfluoride (PMSF), AMP, ATP, phosphocreatine, and creatine phosphokinase were from Sigma (St. Louis, MO). Sodium dodecyl sulfate (SDS) was from Amresco (Solon, OH) and Triton X-100 was purchased from Merck (Whitehouse Station, NJ). All other reagents used were of analytical grade.

Phenothiazine maleates (PhMs) were synthesized as described previously [14]. The chemical structure of PhMs is shown in Fig. 1. Six PhMs studied differ in the type of substituent at position 2 (H-, Cl- or CF_3-) and in the length of the alkyl bridge connecting the phenothiazine nucleus with side chain amino group (3 or 4 carbon atoms). Stock solutions of BCPCF-AM, BCECF, PhMs and benzbromarone were prepared in DMSO. Orthovanadate, Triton X-100 and SDS were dissolved in water.

2.2. Erythrocyte preparation

Human blood was obtained from healthy volunteers by vein puncture. The blood was washed (2000×*g*, 5 min, 4 °C) with isoosmotic PBS solution. The precipitate was resuspended in PBS and passed through the α-cellulose column to remove leukocytes and platelets [15]. Subsequently, the erythrocytes were washed three more times and resuspended in the transport buffer containing 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.

2.3. BCPCF transport studies

Measurement of BCPCF efflux out of the cells was performed using the functional test developed by Rychlik et al. [4]. Shortly, the erythrocytes suspended in transport buffer (at 5% hematocrit) were incubated with an appropriate concentration of PhMs and/or inhibitor (15 min, room temperature, darkness). The samples were then mixed with an equal volume of ice-cold 2 μM BCPCF-AM solution in the same buffer and cells were allowed to load for 10 min on ice. The cells were incubated at 37 °C for 0, 20, 40 and 60 min in darkness. The incubation was stopped by putting the samples on ice and subsequent centrifugation (14,000×*g*, 3 min, 4 °C). MRP1 transport activity was monitored by measuring BCPCF fluorescence in supernatant (excitation and emission wavelengths were 475 nm and 525 nm, respectively). Percentage of MRP1 stimulation/inhibition was determined by comparing the slopes of fluorescence intensity versus time dependencies for control sample and the samples containing PhMs and/or inhibitor. It was checked that PhMs themselves did not influence BCPCF fluorescence. DMSO concentration in the samples never exceeded 0.5%. Care was also taken to maintain hemolysis level in the samples below 1%. Experiments were performed in triplicate, for each repetition blood from different donor was used.

2.4. Membrane preparation

Erythrocytes were prepared as described above. PBS was replaced by isoosmotic Tris buffer (20 mM Tris–HCl, 155 mM NaCl, pH 7.0) when membranes were used for ATPase activity measurements. Erythrocyte ghosts and inside-out vesicles were prepared according to modified procedure of Steck and Kant [16]. Erythrocytes harvested after passage through α-cellulose column were lysed in 50 volumes of ice-cold buffer containing 20 mM Tris–HCl, 1 mM $EDTANa_2$, 0.575 mM phenylmethylsulphonylfluoride (pH 7.4 at 4 °C). The lysate was centrifuged (23,000×*g*, 12 min, 4 °C) and supernatant removed. This step was repeated twice. The pellet after last wash was

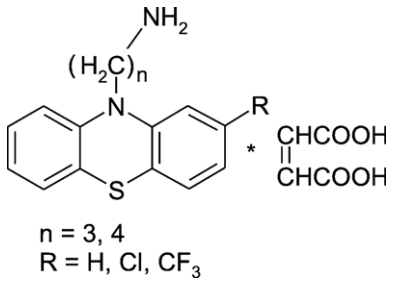

Fig. 1. Chemical structure of phenothiazine maleates. The ring substituent is: H- in 3HPhM and 4HPhM; Cl- in 3ClPhM and 4ClPhM; CF_3- in 3FPhM and 4FPhM. The length of the alkyl bridge connecting the phenothiazine nucleus with side chain amino group is 3 carbon atoms in 3HPhM, 3ClPhM, 3FPhM; and 4 carbon atoms in 4HPhM, 4ClPhM, 4FPhM.

suspended in 50 volumes of ice-cold vesiculation buffer (VB) containing 0.5 mM Tris–HCl, 1 mM $EDTANa_2$, 250 mM sucrose (pH 8.1 at 4 °C) and incubated overnight on ice. After the incubation the suspension was centrifuged (29,000×*g*, 30 min, 4 °C) and the vesicle pellet was resuspended in VB by vigorous syringing with a 21-gauge needle. The protein concentration in the membrane preparations was determined according to Lowry et al. [17]. The percentage of inside-out vesicles in preparation was assessed by the comparison of accessible and total activity of acetylcholinesterase using method of Ellman et al. [18]. On average 88±2% of vesicles were inside-out. Erythrocyte ghosts and inside-out vesicles were snap-frozen and stored at −80 °C until use.

2.5. Uptake studies in inside-out vesicles

The ATP-dependent accumulation of BCECF inside membrane vesicles was measured in buffer containing 10 mM Tris–HCl, 250 mM sucrose, 10 mM $MgCl_2$, 0.66 mM ouabain, 10 mM phosphocreatine, 1 U/ml creatine phosphokinase (pH 7.4 at 37 °C) with addition of 2.5 mM ATP or 2.5 mM AMP in control samples. Inside-out vesicles (protein concentration 0.5 mg/ml) were incubated with an appropriate concentration of PhMs (15 min, room temperature, darkness). The samples were then mixed with an equal volume of ice-cold BCECF solution (final concentration of BCECF was 12.5 μM) and ATP or AMP was added. The cells were incubated at 37 °C for 60 min in darkness. The reaction was stopped by the addition of 1.2 ml of ice-cold VB and subsequent centrifugation (18,000×*g*, 5 min, 4 °C). The supernatant was removed and the vesicle pellet was washed twice with 1 ml of VB. After last wash, the pellet was dissolved in 1 ml of 1% (v/v) Triton X-100 in 10 mM Tris (pH 10.0). After 20 min (room temperature, darkness) the fluorescence of BCECF was measured (excitation and emission wavelengths were 475 nm and 525 nm, respectively). BCECF vesicle uptake was determined as a difference of the fluorescence value measured in the presence of ATP and AMP. It was checked that PhMs themselves did not influence BCECF fluorescence. DMSO concentration in the samples never exceeded 1%. Experiments were performed in triplicate.

2.6. ATPase activity determination

Erythrocyte ghosts (protein concentration 0.36 mg/ml) were incubated with an appropriate concentration of PhMs (15 min, room temperature, darkness) in buffer containing 100 mM Tris–HCl, 10 mM $MgCl_2$, 0.1 mM ouabain (pH 7.4 at 37 °C). Then ATP was added (final concentration 1 mM) and the samples were incubated at 37 °C (30 min, darkness) or on ice (control samples). The reaction was stopped by the addition of an equal volume of 0.6 M trichloroacetic acid and subsequent centrifugation (18,000×*g*, 5 min, 4 °C). The amount of inorganic phosphate in the supernatant was determined as the complex of phosphomolybdate and malachite green by method of Van Veldhoven and Mannaerts [19]. ATPase activity was determined as a difference of absorbance value (at 610 nm) measured in the samples incubated at 37 °C and on ice. DMSO concentration in the samples never exceeded 1%. Experiments were performed in triplicate.

3. Results

The efflux of BCPCF out of erythrocytes remained linear for at least 60 min under the conditions of functional test used in the present study (data not shown) that is in agreement with previous observations made by Rychlik et al. [4] in the same experimental system. The outward BCPCF transport in our test was inhibited by such nonspecific inhibitors of organic anion transport as indomethacin, probenecid, fluoride ions and benzbromarone, by ATPase inhibitor orthovanadate and by MK-571 regarded as a specific MRP1 inhibitor (data not shown). The IC_{50} values for all the inhibitors were similar to the values obtained by other authors in BCPCF-erythrocyte system [4,20,21]. Metabolic depletion also caused almost complete inhibition of BCPCF efflux (data not shown). Following the reasoning of Rychlik et al. [4] (see Introduction), we accepted the BCPCF transport test in erythrocyte system to be indicative of MRP1 transport activity.

All six phenothiazine maleates studied stimulated MRP1 transport activity (Table 1). The maximal stimulation varied between 15% and 70% being the highest in case of 4HPhM and 3ClPhM. Typically, phenothiazine maleates caused concentration-dependent increase of BCPCF efflux out of erythrocytes (see example in Fig. 2). However, above certain concentration of PhMs, their stimulatory activity stopped increasing and even a small decrease was observed. Such an effect was observed for all compounds studied. As PhMs caused erythrocyte hemolysis start at different concentrations, the concentration range studied was different for each compound. To compare MRP1 stimulating effect of phenothiazine maleates the concentration of 20 μM was chosen as it was the highest concentration that could be studied for all PhMs. At this concentration the strongest stimulation was observed for 4HPhM and 4ClPhM. Other compounds were weaker MRP1 stimulants and activity of 3FPhM at this concentration was negligible. At 20 μM phenothiazine maleates possessing 4 carbon atom alkyl bridge connecting the phenothiazine nucleus with side chain amino group were more effective MRP1 stimulating agents than compounds with 3 carbon atom alkyl bridge.

The increased BCPCF efflux out of erythrocytes in the presence of phenothiazine maleates is likely to be the result of increased MRP1 transport activity. As phenothiazines in high concentrations are supposed to exert detergent-like activity on cell membranes we decided to check if the presence of free BCPCF outside the erythrocytes could result from the reason other than MRP1 stimulation, e.g., increased membrane permeability. To investigate this problem we have examined the influence of MRP1 inhibitors on BCPCF efflux in the presence of 4HPhM, the most active compound studied. In the presence of 25 μM of orthovanadate 4HPhM was not able to stimulate outward BCPCF transport (Fig. 2). Similar effect was observed when 50 μM of benzbromarone was used (data not shown). The influence of 50 μM of 4HPhM on MRP1 inhibition by orthovanadate (Fig. 3A) and benzbromarone

Table 1
Stimulation of BCPCF efflux out of erythrocytes by phenothiazine maleates (mean±S.D. of three independent experiments) measured in buffer containing 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) at 5% hematocrit

Compound	Stimulation at 20 μM [% of control]	Maximal stimulation	
		[% of control]	Concentration [μM]
3HPhM	130.1±20.3	152.6±16.0	100
4HPhM	154.9±4.5	171.1±15.1	50
3ClPhM	126.4±18.9	171.6±18.4	50
4ClPhM	153.1±11.9	155.0±2.7	15
3FPhM	107.9±10.5	114.3±3.2	15
4FPhM	143.0±2.2	143.0±2.2	20

Fluorescent probe concentration: 2 μM. Percentage of stimulation was determined by comparing the slopes of fluorescence intensity versus time dependencies for control sample and the samples containing PhMs.

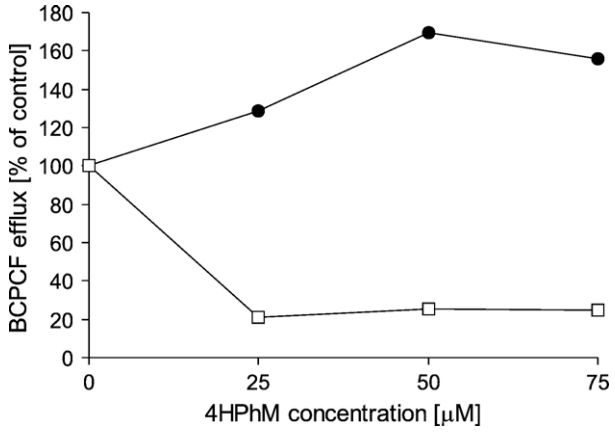

Fig. 2. The influence of phenothiazine derivative 4HPhM (black symbols) and 4HPhM plus 25 μM of orthovanadate (open symbols) on BCPCF efflux out of human erythrocytes. Buffer: 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 138 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose (pH 7.4). Hematocrit: 5%. Fluorescent probe concentration: 2 μM. Percentage of stimulation/inhibition was determined by comparing the slopes of fluorescence intensity versus time dependencies for control sample and the samples containing 4HPhM or 4HPhM/inhibitor.

(Fig. 3B) was also studied. Such an amount of 4HPhM increased BCPCF efflux by 70% in the absence of inhibitors. In the presence of orthovanadate the stimulatory effect of 4HPhM was not observed but the inhibitory activity of orthovanadate in the presence of 4HPhM was lower as compared to pure inhibitor. Similar results were obtained for benzbromarone, however in this case the stimulation of BCPCF efflux could be observed at the lowest concentration of inhibitor used (1 μM). This stimulation (c.a. 50%) was, however, smaller than the effect of 4HPhM alone. Increasing benzbromarone concentration abolished MRP1 stimulation by the studied compound. At benzbromarone concentration of 50 μM the BCPCF efflux inhibition was the same in the sample containing pure inhibitor and in the one with the addition of 4HPhM.

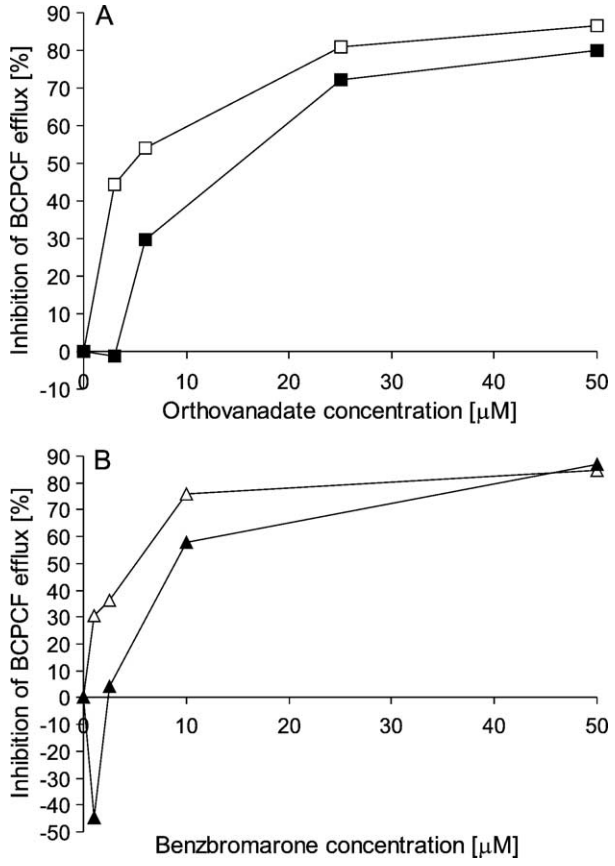

Fig. 3. Inhibition of BCPCF efflux out of human erythrocytes by orthovanadate (A) and benzbromarone (B). Pure inhibitors were used (open symbols) and inhibitors plus 50 μM of 4HPhM (black symbols). Experimental conditions as in Fig. 2.

Table 2
Stimulation of BCECF uptake into inside-out vesicles prepared from erythrocyte membrane by phenothiazine maleates (mean±S.D. of three independent experiments) measured in buffer containing 10 mM Tris–HCl, 250 mM sucrose, 10 mM $MgCl_2$, 0.66 mM ouabain, 10 mM phosphocreatine, 1 U/ml creatine phosphokinase (pH 7.4 at 37 °C)

Compound	Stimulation at 20 μM [% of control]
3HPhM	115.5±11.0
4HPhM	157.3±7.8
3ClPhM	126.1±5.2
4ClPhM	139.1±14.0
3FPhM	120.7±10.9
4FPhM	133.8±2.4

Incubation: 60 min at 37 °C. Fluorescent probe concentration: 12.5 μM. Protein concentration: 0.5 mg/ml. BCECF uptake into vesicles was determined as a difference of the fluorescence intensity value (excitation at 475 nm, and emission at 525 nm) measured in the presence of ATP (2.5 mM) and AMP (2.5 mM).

Additionally, the influence of nonionic detergent Triton X-100 and anionic detergent SDS on BCPCF efflux out of erythrocytes was investigated. SDS was found to inhibit MRP1 transport activity (IC_{50} < 18 μM). On the other hand, Triton X-100 was found to stimulate outward BCPCF efflux by 50% at the concentration of 0.0009% (v/v) and by 65% at 0.0018% (v/v).

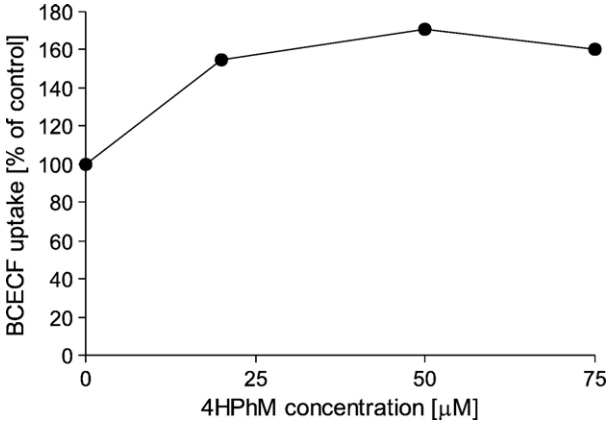

Fig. 4. The influence of phenothiazine derivative 4HPhM on BCECF uptake into inside-out vesicles prepared from erythrocyte membrane. Buffer: 10 mM Tris–HCl, 250 mM sucrose, 10 mM $MgCl_2$, 0.66 mM ouabain, 10 mM phosphocreatine, 1 U/ml creatine phosphokinase (pH 7.4 at 37 °C). Incubation: 60 min at 37 °C. Fluorescent probe concentration: 12.5 μM. Protein concentration: 0.5 mg/ml. BCECF uptake into vesicles was determined as a difference of the fluorescence intensity value (excitation at 475 nm, and emission at 525 nm) measured in the presence of ATP (2.5 mM) and AMP (2.5 mM).

Table 3
Stimulation of ATPase activity in erythrocyte membrane by phenothiazine maleates (mean±S.D. of three independent experiments) measured in buffer containing 100 mM Tris–HCl, 10 mM $MgCl_2$, 0.1 mM ouabain, 1 mM ATP (pH 7.4 at 37 °C)

Compound	Stimulation at 20 μM [% of control]	Maximal stimulation	
		[% of control]	Concentration [μM]
3HPhM	112.6±10.9	116.0±2.3	50
4HPhM	145.7±8.7	145.7±8.7	20
3ClPhM	124.0±15.6	124.0±15.6	20
4ClPhM	105.2±3.2	110.2±5.6	5
3FPhM	173.3±18.0	187.6±30.0	50
4FPhM	125.8±14.3	125.8±14.3	20

Protein concentration: 0.36 mg/ml. ATPase activity was determined as a difference of inorganic phosphate amount released by samples incubated at 37 °C and on ice (incubation: 30 min).

MRP1 transport activity was also studied using inside-out vesicles prepared from erythrocyte membrane. ATP-dependent uptake of BCECF into the vesicles was linear for at least 60 min under the conditions of the experiment (data not shown). All phenothiazine maleates studied were able to stimulate BCECF transport into the vesicles (Table 2). The percentage of stimulation of fluorescent probe transport by a given PhM was similar in whole erythrocytes and in inside-out vesicles. Similarly as in erythrocytes, the compounds 4HPhM and 4ClPhM (tested at 20 μM concentration) induced the strongest MRP1 stimulation. Also in inside-out vesicles phenothiazine maleates possessing 4 carbon atom alkyl bridge connecting the ring system with side chain amino group were found to be more effective MRP1 stimulating agents than compounds with 3 carbon atom alkyl bridge. The dependence of BCECF uptake into the vesicles and BCPCF efflux out of erythrocytes on PhMs concentration were also similar (compare Figs. 2 and 4).

Mg^{2+}-dependent ATPase activity was also measured in human erythrocyte membrane. In our experimental system, this activity was inhibited by orthovanadate and fluoride ions and stimulated by indomethacin, probenecid, benzbromarone and MK-571 (data not shown). All phenothiazine maleates studied stimulated ATPase activity when tested at the concentration of 20 μM (Table 3). The maximal stimulation varied between 10% and 80% being the highest in case of 3FPhM and 4HPhM. No correlation between the ability of a given phenothiazine derivative to stimulate MRP1 transport activity and ATPase activity in erythrocytes was observed.

4. Discussion

In the present study, we have demonstrated that phenothiazine maleates stimulate MRP1 transport activity in human erythrocytes. PhMs increased both efflux of fluorescent probe BCPCF out of intact erythrocytes and ATP-dependent uptake of BCECF into inside-out vesicles prepared from erythrocyte membrane. The similarity of the results obtained in the two experimental procedures indicated that in both systems the same transport activity was measured, i.e., MRP1-mediated transport of 2′,7′-bis-(3-carboxypropyl)- or 2′,7′-bis-(3-carboxyethyl)-5-(and-6)-carboxyfluorescein.

PhMs have been previously recognised as P-glycoprotein inhibitors in cancer cells [12,13]. Neither P-gp inhibition studied previously [13] nor MRP1 stimulation efficiency of PhMs seemed to be correlated with compounds' lipophilicity as determined in [13].

The increased BCPCF efflux out of erythrocytes observed in our experiments could not be caused by the changes in MRP1 transporter gene expression. Such an effect was excluded because of the short time of the functional test used (60 min) and by the use of mature erythrocytes as a model system as these cells lack nuclei. The other possible reason of elevated free BCPCF concentration outside the erythrocytes could be the putative detergent-like activity of PhMs. It could be suspected that these compounds in concentrations below hemolysis start could produce some local defects in cell membranes thereby increasing membrane permeability for small molecules like BCPCF. We have, however, demonstrated that the use of known MRP1 inhibitors such as benzbromarone and orthovanadate was able to abolish the stimulating effect of phenothiazine maleates. We therefore concluded that the observed BCPCF efflux was caused by the stimulation of an active transport system rather than by increasing membrane permeability for BCPCF.

The influence of phenothiazine maleates on Mg^{2+}-dependent ATPase activity in human erythrocyte membrane was also studied. All the compounds studied caused ATPase activity stimulation. The lack of correlation between the degree of MRP1 transport activity stimulation and ATPase activity stimulation may the result of the complexity of model system that is constituted by erythrocyte membrane. Rychlik et al. [4] have demonstrated previously that MRP1 was the transporter responsible for BCPCF and BCECF translocation across the red blood cell membrane. However, in case of drug-stimulated ATPase activity in erythrocyte membrane probably the activity of more ATP-dependent transporters, such as MRP4 and MRP5, was recorded and not only that of MRP1. Also, the contribution of other, non-ABC transporters such as RLIP76 [22] and aminophospholipid flippase [23], to ATPase activity measured in erythrocyte membrane could not be excluded.

Stimulation of ATPase activity of both MRP1 and P-glycoprotein by their substrates is well characterized [24,25]. Phenothiazines have been previously demonstrated to stimulate ATPase activity of P-gp [26]. The stimulating effect exerted by phenothiazine maleates on ATPase activity in erythrocyte membranes may suggest that PhMs bind to MRP proteins and may be substrates of these transporters.

Contrary to ATPase activity studies, reports on stimulation of ABC-proteins transport activity are scarce in literature because the majority of projects concentrate on the search of the new inhibitors of MDR transporters. Bobrowska-Hagerstand et al. [20] observed that cationic detergents stimulated BCPCF efflux from human erythrocytes, whereas anionic detergents were inhibitors of MRP1 transport activity. We were able to repeat these observations in our system using Triton X-100 and SDS as exemplary detergents from both classes. The

authors concluded that the relationship between the charge of amphiphiles and their impact on BCPCF efflux might reflect direct charge interaction between polyanionic BCPCF substrate and detergents. Such an interaction could hinder or facilitate BCPCF transport by MRP1 depending on modulator's character. Nguyen et al. [27] have shown that flavonoids apigenin, fisetin, galangin, luteolin, myricetin and rhoifolin were able to decrease daunomycin accumulation in MRP1-overexpressing cancer cell line. However, only two compounds from this group (fisetin and myricetin) were able to decrease accumulation of vinblastine, too. It seemed therefore likely that the stimulatory effect of a given compound on MRP1 transport activity could be dependent on the substrate used.

Stimulation of P-glycoprotein transport activity was also reported (reviewed in [28]). Flavonoids quercetin, kaempferol and galangin upregulated activity of P-gp that resulted in markedly decreased accumulation and increased efflux of adriamycin in multidrug resistant cancer cells [29]. The stimulatory effect of flavonoids was completely blocked by known P-gp inhibitors such as verapamil, vinblastine and quinidine. Sharom et al. [30] demonstrated that some linear hydrophobic peptides (NAc-Leu_n-Tyr-amide) and colchicine mutually stimulated the transport of each other by P-glycoprotein in membrane vesicles and reconstituted proteoliposomes. Also, rhodamine and Hoechst 33342 were reported to stimulate the P-gp-mediated transport of each other reciprocally [31]. Such an observation led the authors to propose the functional model of P-glycoprotein containing at least two positively cooperative sites (R- and H-site) for drug binding and transport. According to this model, P-gp substrates like rhodamine 123 and anthracyclines bound to R-site, whereas Hoechst 33342, flavonoid quercetin and colchicine bound to H-site. The results reported by Kondratov et al. [32] could also be explained basing on the above model. The authors observed that the series of small aromatic molecules stimulated efflux of rhodamine 123, adriamycin and daunorubicin by P-gp, and inhibited efflux of taxol and Hoechst 33342. Verapamil blocked the stimulatory effect of these molecules completely. The above literature reports point very clearly to the existence of two or more allosteric binding sites on P-glycoprotein. This is probably why the stimulatory effect of a given class of modulators is usually observed only for a given substrate (or class of substrates) and inhibition of transport of other substrates is recorded.

The presence of multiple binding sites on MRP1 has also been suggested basing on the experiments on interaction of photoreactive-iodinated analogue of rhodamine 123 with this transporter [33]. The situation in case of MRP1 is additionally complicated by glutathione (GSH) that, besides substrate and inhibitor, constitutes the third player in modulation of MRP1 transport activity. MRP1 translocates glutathione and glucuronate conjugates of many xenobiotics [2], whereas free forms of anticancer drugs are postulated to be co-transported with GSH [34]. Glutathione also influences the modulatory action of many MRP1 inhibitors and transport of substrates. Verapamil was reported to inhibit MRP1 transport activity only in GSH presence [35]. Lowering intracellular levels of glutathione resulted in decreased efflux of calcein from MRP1-expressing cancer cells but had no effect on BCECF transport [36]. These observations prompted Evers et al. [37] to propose a working model of MRP-family transporters. According to this model, two binding sites on the protein exist (G- and D-site) that act in positively cooperative manner. The sites can be occupied either by GSH or by the drug.

Such a model could explain also the stimulation of MRP1 transport activity observed in the present work. We propose phenothiazine maleates to interact directly with MRP1 at the site other than occupied by BCPCF and to stimulate BCPCF transport. However, as PhMs were observed to stimulate ATPase activity in erythrocyte membrane, it is also possible that these compounds interact with ATP-binding site of MRP1, stimulate ATP hydrolysis and in this way stimulate the transport of fluorescent substrate across the erythrocyte membrane. It remains also to be solved, whether phenothiazine derivatives themselves can be transported by MRP1. Since, at least in case of P-gp, modulating agents usually are able to stimulate transport of some substrates only while to inhibit the transport of others, it would be interesting to study the influence of PhMs on transport of other MRP1 substrates. Rychlik et al. [4] have studied in details transport of different fluorescent anions across the human erythrocyte membrane. The authors clearly demonstrated that among fluorescein, calcein, carboxyfluorescein and BCECF, only the transport of the last one could be attributed to MRP1. Therefore, from the above fluorescent substrates only BCECF seems to be rational choice to study MRP1 activity in human erythrocytes. To fully elucidate the mechanism of PhMs-induced MRP1 stimulation further studies are required employing MRP1-expressing cell lines. Also studying the influence of phenothiazine derivatives on glutathione transport could provide additional information.

In summary, our results demonstrate that phenothiazine maleates, compounds known as P-glycoprotein inhibitors, stimulate BCPCF efflux from human erythrocytes, BCECF uptake into inside-out vesicles, and Mg^{2+}-dependent ATPase activity in erythrocyte membrane. The stimulation of MRP1 transport activity is probably caused by PhMs interaction with this transporter at the site different from the substrate binding site.

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