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# Inhibition of the MDR1 transporter by new phenothiazine derivatives

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**Abstract**

The MDR1 transporter mediated efflux of different xenobiotics out of the cells serves as the most important mechanisms of the multidrug resistance in cancer cells, thus inhibition of the MDR1 transporter may increase the efficiency of anticancer drugs in the therapy. Here we describe some new phenothiazine derivatives, which possess strong in vitro MDR1 inhibitory activity. The effectiveness of the compounds on the MDR1 mediated calcein-AM efflux, ATPase activity, and colchicine resistance was proven by microplate assays and flow cytometry using recombinant and control cell lines. Some of these derivatives were more active than verapamil and one of them was at least as active as cyclosporin A. According to our results the new structural elements built in these phenothiazine type compounds increased their MDR1 inhibitory activity, which may serve as a basis of the development of an effective MDR1 inhibitor drug.


*Keywords:* MDR1 transporter; Phenothiazine; Calcein; MDCK cells; Flow cytometry; Colchicine resistance

Living organisms are continuously exposed to the potentially harmful effects of various xenobiotics, which are present in their environment. One of the defense mechanisms developed against these compounds is mediated by the various transporters in the cell membranes, which accept xenobiotics as their substrates and pump them out of the cells. Several transporters belonging to the ABC (ATP binding cassette) protein family take part in the detoxification of the cells [1] using ATP hydrolysis as energy source [2]. MDR1 is the first described and until now the most well-characterized human ABC transporter [3] playing an important role in the formation of different barriers of the human body. The transporter is highly expressed on the canalicular surface of hepatocytes, on the apical surface of epithelial cells of the intestine and renal tubules, and on the luminal surface of capillary endothelial cells in the brain [4,5] making the penetration of some drugs across these barriers difficult. The most serious therapeutic problem caused by MDR1 transporter is the multidrug resistance of cancer cells, because several cytotoxic drugs are also substrates of MDR1 [6] and therefore the activity of the transporter in cancer cells makes the chemotherapy ineffective. Restoring sensitivity of multidrug-resistant cancer cells to chemotherapeutic agents by the inhibition of the transporter is an attractive approach to fight against cancer. Since the discovery of the transporter several MDR1 inhibitors have been described and are in different phases of clinical investigations, but as none of them have been approved in cancer chemotherapy yet [7–9] the interest in new and more effective compounds has not declined and several studies are in progress in this field. The multidrug resistance reversal ability [10,11] and the MDR1 modulator property [12] of phenothiazine type compounds were investigated earlier and some of them proved to be effective modulators. However, the previously published findings were obtained with multidrug-resistant cell lines, in which the pattern of the expressed ABC transporters was not identified [10,13] and it was not investigated which of these transporters were inhibited by phenothiazines. After the isolation of the MDR1 transporter and sequencing its gene [14], more selective assays were developed for MDR1, but the efficacy of the investigated phenothiazine compounds was found to be insufficient for further development [12,15]. In this paper, we describe the MDR1 inhibitory

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activity of certain phenothiazine derivatives with new structural elements, which were more effective in inhibiting the transporter than the compounds investigated earlier.

## Materials and methods

*Chemicals.* Calcein-AM, cyclosporine A, and verapamil were purchased as Vybrant™ Multidrug Resistance Assay Kit from Molecular Probes (Eugene, OR, USA). Bluescript II SK+ and pCMV-Script plasmids were purchased from Stratagene (La Jolla, CA, USA). MLLV reverse transcriptase was purchased from Promega (Madison, WI, USA). Restriction endonucleases, T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA). Lipofectin was purchased from Invitrogen (Carlsbad, CA, USA). PCR Readymix with *Taq* polymerase, TRI-REAGENT™, colchicine, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and geneticin were purchased from Sigma (St. Louis, MO, USA). All the other chemicals used in the experiments were of analytical grade.

*Cell lines.* Caco-2 (human colorectal adenocarcinoma), MDCK (Madin–Darby Canine Kidney), MDCK:pCMV (MDCK cells transfected with pCMVscript plasmid), and MDCK:mdr1 (MDCK cells transfected with pCMVscript plasmid carrying the human mdr1 gene) cells were cultured in minimal essential medium (MEM) supplemented with penicillin–streptomycin, L-glutamine, non-essential amino acid solution, and 10% FBS at 37 °C in 5% $CO_2$ atmosphere.

*ATPase assay.* Purified membrane suspension from Sf9 (*Spodoptera frugiperda*) cells, expressing high levels of the human MDR1 transporter protein, was purchased from Solvo Biotechnology (Budapest, Hungary). The assay was performed according to the instruction of the manufacturer. The purified membrane was diluted to fivefold (1 mg/ml protein concentration) with assay mix (50 mM Mops–Tris, pH 7.0; 50 mM KCl; 5 mM Na-azide; 2 mM DTT; 0.1 mM EGTA–Tris, pH 7.0; 1 mM Ouabain in distilled water). Forty microliters of diluted membrane suspension was loaded into the wells of a 96-well microplate. One microliter of the tested compound dissolved in DMSO or/and 1 μl of verapamil dissolved in DMSO was added to the membrane suspension. The same volume of DMSO was added to the control wells. The mixtures were pre-incubated at 37 °C for 5 min and the reaction was started with addition of 10 μl of 25 mM ATP-Mg solution. The reaction was performed at 37 °C for 20 min then it was stopped by the addition of 40 μl of 5% SDS solution. The amount of liberated inorganic phosphate was determined by colorimetric reaction adding 200 μl detection reagent (8.75 mM ammonium molybdate; 3.75 mM zinc acetate; 7.5% ascorbic acid in distilled water, pH 5.0). After incubation at 37 °C for 30 min, the absorbance was read at 600 nm in a microplate reader and the concentration of the liberated phosphate was calculated from calibration curve [16].

*Cloning of the MDR1 gene.* RNA was isolated from MDR1 protein expressing Caco-2 cells using TRI-REAGENT™ and cDNA was synthesized from mRNA using MLLV reverse transcriptase. The mdr1 gene was multiplied from cDNA by polymerase chain reaction (PCR) using *Taq* polymerase in five fragments. The primer pairs were designed according to the sequence of the gene (GenBank Accession No. M14758) in such a manner that the size of the amplified fragments was 700–1100 bp and except for the first and the last primer the sequence of the primers contained recognition sites of restriction endonucleases, which were unique sites in the gene. The sequences of the applied primer pairs with the restriction endonuclease sites were the following:

mdr1/1fw: 5′CAGTTTCTCGAGGAATCAGC3′;
mdr1/1rev (*Apa*LI): 5′AACATCGTGCACATCAAACC3′
mdr1/2fw (*Apa*LI): 5′TTTGATGTGCACGATGTTGG3′,
mdr1/2rev (*Eco*RI): 5′TTTCTGAATTCCAAATTTCC3′
mdr1/3fw (*Eco*RI): 5′TTTGGAATTCAGAAATGTTC3′,
mdr1/3rev (*Hin*dIII): 5′ACTAAGCTTTCTGTCTTGGG3′
mdr1/4fw (*Hin*dIII): 5′CAGAAAGCTTAGTACCAAAG3′,
mdr1/4rev (*Kpn*I): 5′TATGGTACCTGCAAACTCTG3′
mdr1/5fw (*Kpn*I): 5′AGTTTGCAGGTACCATACAG3′,
mdr1/5rev: 5′TACAGTCAGAGTTCACTGGC3′

The parameters of the polymerase chain reaction were the following: 25 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, elongation at 72 °C for 2 min, and final elongation at 72 °C for 6 min.

The multiplied DNA fragments were purified by precipitation with ethanol and the products were inserted with T4 DNA ligase into a T-tagged [17] pBluescript II SK+ (Stratagene, La Jolla, CA, USA) cloning vector, with which *Escherichia coli* XL-1 strain was transformed. After isolation of the plasmids, the five fragments of the gene were cut out with restriction endonucleases, the recognition sites of which were present in the primers designed for the amplification. Because the first and the last primer did not contain restriction endonuclease recognition site, we selected restriction endonuclease sites (*Hin*dIII) from the multiple cloning site of the bluescript plasmid to cut the first and the last fragment of the gene. After purification of the fragments by agarose gel electrophoresis, the mdr1 gene was joined in mammalian expression vector pCMV-Script plasmid with T4 DNA ligase obtaining the plasmid designated pCMV-script:mdr1. The identity of the cloned gene was verified by sequencing (Central Labs of the Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary). Molecular biology techniques were performed according to the manufacturer's instructions or by standard methods [18].

*Transfection and selection of the MDCK cells.* MDCK cells ($5 \times 10^5$ cells/well) were transferred into the wells of a 6-well plate. After 4 h incubation, the cells were transfected with the pCMVscript:mdr1 and with pCMV-script plasmids, purified by cesium-chloride ultracentrifugation, using lipofectin according to the instruction of the manufacturer. After 24 h incubation, the medium was changed to selection medium containing 800 μg/ml geneticin and the selection was carried out for 10 days. Geneticin-resistant clones, which grew after transfection with pCMV-script plasmid, were isolated and were used as control cells (MDCK:pCMV) in the further investigation. Individual colonies grown in the plate after transfection with pCMVscript:mdr1 plasmid were isolated and were investigated for the expression of the MDR1 protein by determining their calcein-AM uptake. Clones, which had lower calcein uptake than the control (MDCK:pCMV) cells but the uptake could be increased to the control level after inhibition of the MDR1 transporter by verapamil, were selected. One of the selected clones, the calcein uptake of which was the lowest, was chosen and the expression level of the recombinant MDR1 protein was enhanced by further selection in 30 ng/ml colchicine containing medium.

*Inhibition of the MDR1 mediated calcein-AM efflux.* From semiconfluent cultures made on 75 $cm^2$ plates the cells (MDCK:pCMV and MDCK:mdr1) were collected using trypsin-EDTA and $5 \times 10^5$ cells/well were transferred into the wells of 6-well plates where they were further incubated at 37 °C for 2 days. Test compounds dissolved in DMSO were added to the wells (final DMSO concentration did not exceed 1%) and the plates were incubated at 37 °C for 30 min then calcein-AM was added in 0.25 μM concentration into the wells. After 30 min incubation at 37 °C, the medium was discarded, the cells were washed twice with PBS and then lysed with 600 μl of 10 mM Hepes (pH 7.4) containing 0.2% SDS. The fluorescence of calcein was determined from the supernatant (ex: 492 nm, em: 520 nm) and the measured fluorescence values were corrected with the cell number in the well.

For the calculation of $IC_{50}$ value for the inhibition of MDR1 mediated calcein-AM efflux, the calcein uptake of the two cell lines (MDCK:pCMV and MDCK:mdr1) was investigated simultaneously in the presence of different concentrations (0–10 μM) of the test compounds. Curves were fitted (with Prism 4 for Windows software) to the measured points obtained with the control and the recombinant MDR1 expressing cells. Kinetic curves of the difference between the calcein uptakes of the two cell lines in the absence and in the presence of different concentrations of the experimental compounds were generated by subtracting MDCK:mdr1 calcein uptake curves from those of MDCK:pCMV and were plotted (as % of maximal difference) against concentrations of the examined compounds. This curve reflects the activity of the expressed human recombinant MDR1 protein and converges to zero with the increase of the concentration of an active compound while it is parallel with the *X* axis in case of an inactive compound. We determined the concentration of the test compounds, which caused 50% decrease in the activity of the recombinant

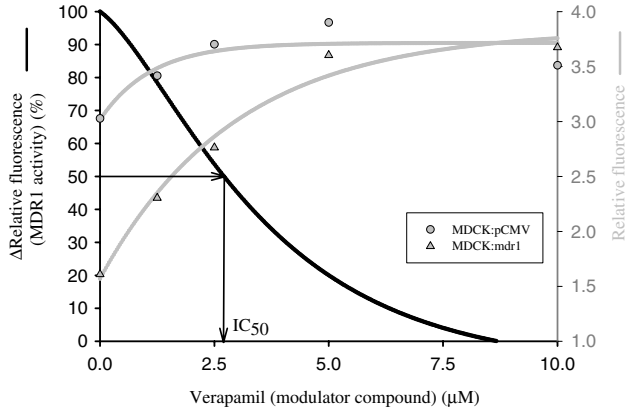



Fig. 1. Calculation of $IC_{50}$ values for the inhibition of MDR1 mediated calcein-AM efflux. Control cells (MDCK:pCMV) (○) and cells transfected with the human mdr1 gene (MDCK:mdr1) (△) were incubated with 0.25 μM calcein-AM and with the tested compounds in 0–10 μM concentration for 25 min. The fluorescence of calcein in the cell lysates was determined and normalized with the number of the cells. Curves were fitted to the measured values and the curve for the recombinant human MDR1 expressing cells was subtracted from the curve for the control cells (gray curves) obtaining the third curve (black), which converged to zero as a function of the concentration of verapamil. The $IC_{50}$ value was calculated as the concentration of the test compound, which caused 50% decrease in the curve.

MDR1 protein ($IC_{50}$) and the tested compounds were characterized with this concentration value (Fig. 1).

*Cell number determination.* During the determination of the effect of the tested compounds on the MDR1 mediated calcein-AM efflux, cell number was used for the correction of the calcein fluorescence intensity values. The number of the cells was calculated from the DNA content of the lysed supernatant, which was determined with bisbenzimide. Twenty microliters of the supernatant was added to 180 μl TNE buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 2 M NaCl) containing 20 μg/ml bisbenzimide. The fluorescence of bisbenzimide was measured (ex: 355 nm, em: 460 nm) and the cell number was calculated from a calibration curve.

*Inhibition of colchicin resistance.* Cells (MDCK:pCMV and MDCK:mdr1) were collected from 75 $cm^2$ plates and $10^5$ cells/well were transferred to the wells of 24-well plates. Colchicine in 30 ng/ml concentration and the investigated compounds in 0–10 μM concentration were added to the medium. The medium was replaced every day for 3 days and then the proliferation of the cells was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) method [19].

*Flow cytometry.* MDCK:mdr1 and MDCK:pCMV cells were transferred to 6-well plates at $10^5$ cells/well density. After 2 days cultivation, test compounds dissolved in DMSO were added to the wells. The plates were incubated for 30 min and then 0.25 μM calcein-AM was added to the wells. After 30 min incubation, the medium was discarded, cells were washed with PBS buffer, removed from the plate by trypsin-EDTA, and suspended in PBS buffer. Live MDCK cells were analyzed with a FACSCalibur equipped with a 488-nm argon-ion laser (Becton–Dickinson, San Jose, CA, USA). For each sample 10,000 events were measured using linear amplification for forward- and side angled scattered lights, and logarithmic amplification for green fluorescence. Fluorescence signal of calcein was collected through a 530 ± 30 nm band-pass filter. CellQuest Pro software from BD was used for acquisition and analysis of data. Graphic representation was performed using WinMDI (version 2.5) and SigmaPlot (version 8.0) software.

## Results and discussion

Compounds with different chemical structures from the molecular library of the Institute were investigated for their MDR1 modulatory activity. In the screening program the capacity of the compounds either to activate or to inhibit the ATPase activity of the transporter were studied. In the activation studies the compounds alone were added to the membrane preparation and the increase in ATPase activity was measured. With this test the chemicals, which are substrates of the transporter, were selected and these molecules were excluded from further studies. In the inhibition studies the ATPase activity of the transporter was increased by addition of 40 μM verapamil and the ability of the tested compounds to inhibit this ATPase activity was investigated. Among the tested molecules phenothiazine derivatives were found to have the strongest MDR1 inhibitory activity therefore molecules containing this structure group were selected for detailed analysis.

To investigate the activity of the compounds in a bioassay, recombinant human MDR1 transporter expressing cells were produced. The mdr1 gene was cloned from Caco-2 cells, ligated into pCMV-script plasmid and MDCK cells were transfected with the construct. After selection with geneticin, the clones, in which the level of the expression of the human recombinant MDR1 protein was high and stable, were isolated. The expression level was checked by calcein-AM uptake of the cells. The acetoxymethyl ester derivative of the fluorescent dye calcein is cell-permeable non-fluorescent molecule and a substrate of the MDR1 transporter. The calcein-AM, which is not pumped out of the cells by the MDR1 transporter, was cleaved by intracellular esterases forming fluorescent, non-cell-permeable calcein that is not a substrate for MDR1 [20,21]. The fluorescence intensity of calcein in the cells is inversely proportional to the activity of the MDR1 transporter. MDCK cells transfected with pCMV-script plasmid (MDCK:pCMV) were used as control cell line in the experiments. As it was expected the calcein level of the MDR1-transfected cell lines (for example, MDCK:mdr1/D5 in Fig. 2) was lower than that of the control cells (MDCK:pCMV) and it could be restored to the control level by the inhibition of the expressed transporter with 40 μM verapamil. One clone, which had the lowest calcein uptake among the transfected, geneticin-selected cell lines, was selected and the expression of the recombinant transporter was further enhanced by culturing the clone on medium containing 30 ng/ml colchicine. This selection procedure resulted in the cell line (MDCK:mdr1) (Fig. 2), which was used in the examination of the transporter modulating effects of the selected group of phenothiazine derivatives in cell based assays.

The inhibitory activity of the compounds on the MDR1 mediated calcein-AM efflux was determined, the $IC_{50}$ values are shown in Table 1. The selective examination of the effect of the compounds on the human MDR1 transporter was possible by the simultaneous usage of the human MDR1 expressing cells (MDCK:mdr1) and the control cells (MDCK:pCMV). With this method the activity of the endogen transporters of the MDCK cells did not influence the obtained results. Cyclosporine A and

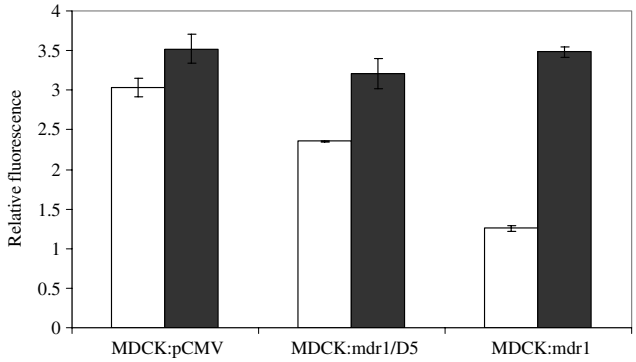
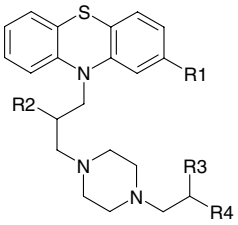


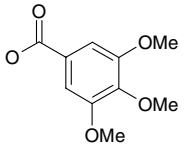
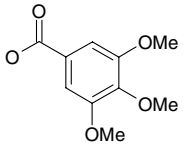

Fig. 2. Investigation of the calcein uptake of the cell lines. Control cells (MDCK:pCMV), the transfected, geneticin-selected clone (MDCK:mdr1/D5), and the transfected, geneticin- and colchicine-selected clone (MDCK:mdr1) were incubated with 0.25 μM calcein-AM without verapamil (□) and with 40 μM verapamil (■) for 25 min. The fluorescence of calcein was determined in the cell lysate and was corrected with the number of the cells obtaining the relative fluorescence values.

verapamil as known MDR1 modulators were used as positive controls. The $IC_{50}$ values of these drugs obtained in our system showed good correlation with those described in the literature [22]. In this assay compound T158 had the strongest inhibitory activity, in which methoxy group was in R1, carbonyl group in R2, methyl group in R3, and trimethoxy benzyl group in R4 position. The replacement of the carbonyl group with hydrogen atom or methyl group in the R2 position decreased the inhibitory activity of these compounds. The incorporation of methoxy group into the R1 position resulted in similar inhibitory activity as chlorine atom, while the presence of hydrogen atom in that position decreased the activity. There was no significant difference between the effects of methyl group and hydrogen atom in the R3 position. The presence of trimethoxy benzyl moiety in the R4 position was essential for the inhibitory activity, compound T157 without this group did not show any MDR1 inhibitory effect up to 10 μM concentration. Without esterification of the hydroxyl group with trimethoxy benzoic acid the molecule became too hydrophilic and its MDR1 inhibitory effect was abolished. The investigated compounds contained new structural elements namely methoxy group in the R1, carbonyl group in the R2, and trimethoxy benzyl group in the R4 positions, which were not tested earlier in multidrug reversal assays but these moieties were found to be beneficial for inhibiting the MDR1 activity in our assays.

The inhibitory effect of the compound designated T158 that was the most active in the previous test was also investigated using flow cytometry. MDCK:pCMV and MDCK:mdr1 cells were incubated with calcein-AM without any inhibitor or in the presence of 40 μM verapamil or 10 μM compound T158. The distribution of the cells on the basis of their calcein level measured by flow cytometer is shown in Fig. 3. The calcein uptake of the MDR1 expressing cells was lower than that of the control cells (Fig. 3A), which proved that the recombinant human MDR1 transporter was expressed in functionally active form in the transfected cells. In the presence of verapamil the pumping of calcein-AM

Table 1
Chemical structure of the investigated compounds and the measured $IC_{50}$ values for the tested compounds in the MDR1 mediated calcein-AM efflux assay




| No. | R1 | R2 | R3 | R4 | $IC_{50}$ (μM) ± SE (n) |
|---|---|---|---|---|---|
| T5 | Cl | H | H |  | 1.35 ± 0.27 (10) |
| T114 | H | O | H | | 2.13 ± 0.25 (8) |
| T116 | OMe | O | H | | 1.45 ± 0.22 (9) |
| T145 | Cl | O | Me | | 0.87 ± 0.19 (8) |
| T157 | OMe | O | Me | OH | No inhibition effect up to 10 μM |
| T158 | OMe | O | Me |  | 0.71 ± 0.19 (8) |
| T172 | Cl | Me | H | | 1.65 ± 0.2 (9) |
| T174 | Cl | Me | Me | | 1.59 ± 0.27 (9) |
| Cyclosporine A | | | | | 0.4 ± 0.1 ($n = 5$) |
| Verapamil | | | | | 3.6 ± 0.5 ($n = 5$) |

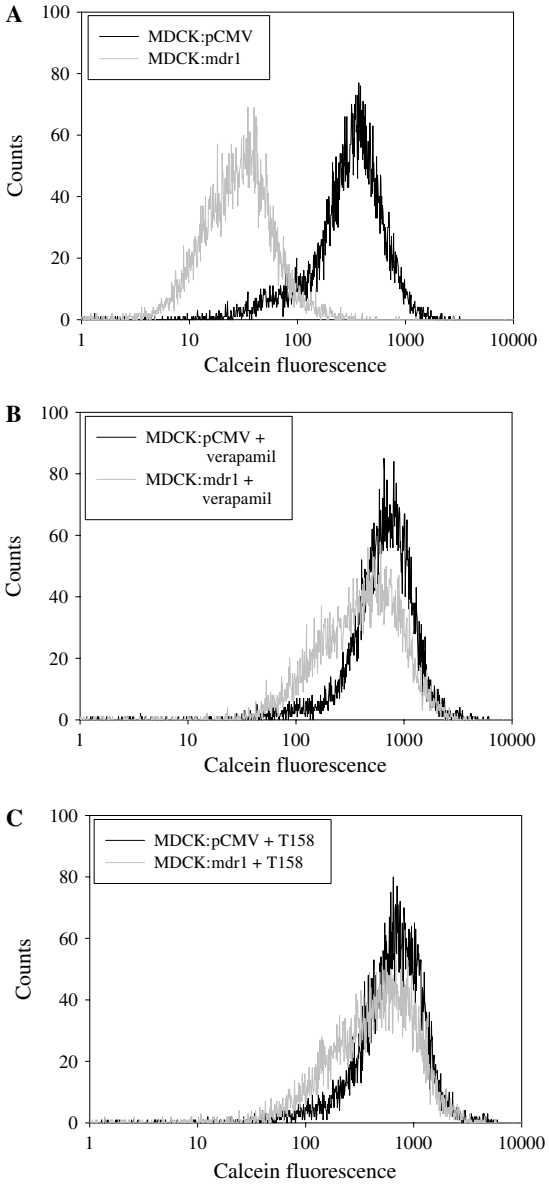



Fig. 3. Investigation of the MDR1 mediated calcein-AM efflux by flow cytometry. Control cells (MDCK:pCMV) (black line) and cells transfected with the human mdr1 gene (MDCK:mdr1) (gray line) were incubated with 0.25 μM calcein-AM without any MDR1 inhibitor (A), with 40 μM verapamil (B), and with 10 μM compound T158 (C) for 25 min. Cells were washed and removed from the plates and then were analyzed with a FACSCalibur equipped with a 488-nm argon-ion laser. Fluorescence signal of calcein was collected through a 530 ± 30 nm band-pass filter.

out of the cells was inhibited and the fluorescence intensity of the transfected cells increased to the control level (Fig. 3B). After addition of T158, the calcein uptake of the transfected cells increased also to the control level indicating that the transporter was inhibited by this compound (Fig. 3C).

The efficacy of T158 in inhibiting MDR1 transporter was also verified by the inhibition of colchicine transport. Colchicine is toxic for the cells and also a substrate of MDR1 transporter, so the higher the level of the MDR1 expression is, the higher concentration of colchicine can be tolerated by the cells. Because of the activity of the recombinant MDR1 protein, the recombinant human MDR1 expressing cell line was able to proliferate in a medium containing up to 50 ng/ml colchicine, while the control cells can tolerate only 10–20 ng/ml colchicine. We investigated the colchicine resistance diminishing ability of the compounds T158 and T157 (Fig. 4). Compound T158 fully restored the colchicine sensitivity of the transfected cell line in 5 μM concentration, while compound T157 did not affect the colchicine resistance of the cell line up to 10 μM concentration, which confirmed the results obtained in calcein efflux studies, in which T158 proved to be a strong inhibitor of the transporter, while compound T157 without the trimethoxy benzyl moiety had not any MDR1 inhibitory effect.

Similar phenothiazines were investigated earlier, but the potency of the compounds was increased considerably with the incorporation of new structural elements. The most effective compound T158 was found to be an effective chemosensitizer, its activity was better than that of the known MDR modulator verapamil and was comparable to that of cyclosporine A in our assays.

According to the results described in the paper, further derivatives of the investigated phenothiazine compounds may be developed to an effective MDR1 inhibitor, which might be applied for cancer chemotherapy.

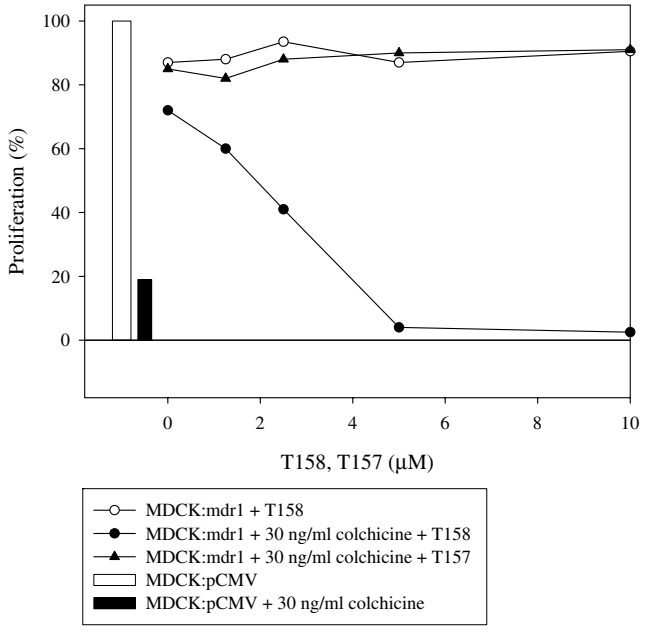



Fig. 4. Reversal of colchicine resistance. Control cells (MDCK:pCMV) were incubated in medium without colchicine (empty column) and with 30 ng/ml colchicine (filled column) for 3 days and the proliferation was determined with MTT. Cells transfected with human mdr1 gene (MDCK:mdr1) was incubated in medium without colchicine (empty marks) and with 30 ng/ml colchicine (filled marks) supplemented with compounds T157 (▲) or T158 (○, ●) in 0–10 μM concentration for 3 days and then the proliferation was determined with MTT.

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