

New Chalcone Derivative Inhibits ABCB1 in Multidrug Resistant T-cell Lymphoma and Colon Adenocarcinoma Cells

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Abstract. *Background/Aim:* Development of new potential drugs to overcome multidrug resistance to chemotherapy is a big challenge for cancer treatment. Attention is also given to the natural compounds and their derivatives. The study aimed at evaluating the impact of a new chalcone derivative (IC) on multidrug resistant cell lines, focusing on P-glycoprotein (P-gp, ABCB1) inhibition, as well as IC-doxorubicin interaction *in vitro*. *Materials and Methods:* Cytotoxic and antiproliferative effects of the IC compound were assessed by thiazolyl blue tetrazolium bromide (MTT) method in mouse T-cell lymphoma and human colon adenocarcinoma cells expressing ABCB1. Alterations in ABCB1 activity were evaluated by rhodamine 123 accumulation assay using flow cytometry. Drug-drug interaction was studied using combination assay. *Results:* Our results confirmed antiproliferative, cytotoxic, as well as ABCB1 inhibitory potential of IC in both tested ABCB1-expressing cancer cell lines. Furthermore, IC displayed synergistic interaction with doxorubicin. *Conclusion:* Our results suggest the IC chalcone derivative as a promising compound against

resistant lymphoma and colon cancer, which could be used in monotherapy or in combination with other chemotherapeutics.

Several studies in many cancers have shown an association between overexpression of P-glycoprotein (P-gp, MDR1, lately named as ABCB1) and resistance to a number of structurally and functionally unrelated chemotherapeutics (multidrug resistance) (1, 2). This membrane protein can expel drugs from the cancer cells, preventing their pharmacological action (3). Currently, many cytostatics are considered as ABCB1 substrates (*e.g.* anthracyclines, antimetabolites) (4).

Until now, several ABCB1 inhibitors (*e.g.* verapamil, tariquidar) have been discovered to increase the sensitivity of multidrug-resistant cancer cells. However, due to their poor potency, high risk of side effects, and adverse interactions with concomitant chemotherapy, no ABCB1 inhibitor has been suitable for use in the clinic. Therefore, many researchers have focused their attention on searching for new molecules to overcome multidrug resistance (5-7). Within the last years, interest intensified in the group of natural phytochemicals and their derivatives as the fourth generation of multidrug resistance reversal agents (4, 8). Chalcones (1,3-diarylprop-2-en-1-ones) are precursors in the biosynthesis of flavonoids with a wide spectrum of biological activities (*e.g.* anti-inflammatory, antimicrobial, anticancer) (9, 10) and they represent one of the promising substances. Although their multidrug resistance reversal effect and potential to modulate ABCB1 activity have been evaluated in several cancer cell lines (11-18), their impact on colon cancer has not been studied yet.

In our recent study, we showed a strong antiproliferative activity of the new synthetic acridine chalcone derivative IC on human colon adenocarcinoma cells and T-cell lymphoma

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(19). Therefore, the present work was focused on the investigation of antiproliferative, cytotoxic, and ABCB1 inhibitory effects of this compound in human colon adenocarcinoma cells and T-cell lymphoma cell lines overexpressing ABCB1. Additionally, the type of *in vitro* drug-drug interaction between compound 1C and doxorubicin was assessed.

Materials and Methods

Tested compound. (2E)-3-(acridin-9-yl)-1-(2,6-dimethoxyphenyl)prop-2-en-1-one (1C) was synthesized at the Faculty of Natural Sciences of the P. J. Safarik University in Kosice.

Cell lines. The ABCB1-expressing T-cell lymphoma cell line (MDR) was obtained from parental L5178Y mouse T lymphoma cells (PAR) (ECACC Cat. No. 87111908, FDA, Silver Spring, MD, USA) after transfection with pHMDR1/A retrovirus and culturing of both cell lines, as previously described (7). The Colo 205, doxorubicin-sensitive (CCL-222) and Colo 320/MDR1-LRP multidrug resistant, overexpressing ABCB1-LRP (CCL-220.1) human colon adenocarcinoma cell lines were purchased from LGC Promochem (Teddington, UK). The cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 1 mM Na-pyruvate, 100 mM HEPES (Sigma-Aldrich), nystatin and a penicillin-streptomycin mixture (Sigma-Aldrich) at concentrations of 100 U/l and 10 mg/l, respectively. The NIH/3T3 mouse embryonic fibroblast cell line (ATCC CRL-1658) (LGC Promochem, Teddington, UK) was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich), containing 4.5 g/l glucose, supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine (Sigma-Aldrich), 1 mM Na-pyruvate (Sigma-Aldrich), nystatin (Sigma-Aldrich) and a penicillin-streptomycin mixture (Sigma-Aldrich) in concentrations of 100 U/l and 10 mg/l, respectively. The MRC-5 human embryonic lung fibroblast cell line (ATCC CCL-171) (LGC Promochem, Teddington, UK) was cultured in Eagle's Minimal Essential Medium (EMEM, Sigma-Aldrich), containing 4.5 g/l glucose, supplemented with a non-essential amino acid (NEAA) mixture (Sigma-Aldrich), a selection of vitamins and 10% heat-inactivated FBS, 2 mM L-glutamine (Sigma-Aldrich), 1 mM Na-pyruvate (Sigma-Aldrich), nystatin (Sigma-Aldrich) and a penicillin-streptomycin mixture (Sigma-Aldrich) at concentrations of 100 U/l and 10 mg/l, respectively.

Cytotoxic and antiproliferative assay. The effects of decreasing concentrations of the synthetic chalcone derivative 1C on cell growth were determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay in 96-well cell culture microplates. The maximum concentration of tested compound was 20 μ M. Cells (PAR, MDR, Colo 205 and Colo 320) were seeded at a density of 1×10^4 cells/well or 6×10^3 cells/well for cytotoxic and antiproliferative assay, respectively. After a 24-h (cytotoxic assay) or 72-h (antiproliferative assay) incubation at 37°C, 20 μ l of MTT (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) solution (5 mg/ml) were added to each well. After an additional incubation at 37°C for 4 h, 100 μ l of 10% sodium dodecyl sulphate (SDS, Sigma-Aldrich Chemie GmbH) were added to each well to dissolve the produced

formazan, and after 12 h optical density (OD) was measured. Additionally to 1C, the effects of decreasing concentrations of doxorubicin (TEVA Pharmaceuticals, Debrecen, Hungary; maximum concentration 10 μ M), verapamil (Sigma; maximum concentration 100 μ M), which is known as ABCB1 substrate and inhibitor (20, 21), and DMSO (Sigma-Aldrich Chemie GmbH; maximum concentration 1% v/v) on cell growth were evaluated. Cytotoxic assay was also performed for the two non-cancerous cell lines NIH/3T3 and MRC-5 (2×10^4 cells/well). Overall, the cell growth was then determined by measuring the OD at 550 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). Results obtained from MTT assay were used to determine the percentage of inhibition of cell growth. Absorbance of control wells (untreated cells) was taken as 100%, and the inhibition of cell growth was expressed as a percent of control.

Flow cytometric analysis. The fluorescence-based rhodamine 123 accumulation assay was performed as previously described (7) in order to investigate the activity of 1C as ABCB1 modulator on both parental cancer cell lines (PAR, Colo 205) and the corresponding ABCB1-overexpressing sublines (MDR, Colo 320). Cells were adjusted to 2×10^6 cells/ml and tested compound was added (10 μ l). Different concentrations (0.5, 1, 2.5 and 10 μ M) of 1C as well as doxorubicin (5 μ M) were tested. Verapamil (Sigma; 20 μ M) used in both cancer cell lines (MDR, Colo 320) served as reference inhibitor (positive control). DMSO at 1% v/v was used as solvent control. Rhodamine 123 fluorescent dye (Sigma) was added to the samples at a final concentration of 5.2 μ M. The fluorescence of the cell population was measured with a Partec CyFlow® flow cytometer (Partec, Münster, Germany). The mean fluorescence and SD values were calculated from three independent experiments.

Drug combination assay. Serial dilutions of doxorubicin ranging from 17.24 μ M to 0.14 μ M were made in a horizontal direction (100 μ l), while serial dilutions of the drug resistance modifier were made vertically (50 μ l), in a microtiter plate. The MDR and Colo 320 cells were re-suspended in culture medium (6×10^3 cells/well) and seeded at a density 50 μ l/well. The plates were incubated for 72 h at 37°C in a 5% CO₂ incubator, and cell growth was determined by the MTT assay.

Statistical analysis. Results obtained from three independent MTT measurements were used to determine a half maximal inhibitory concentration (IC₅₀) and the mean \pm SD for each cell line. The results from rhodamine 123 assay were obtained from three independent flow cytometry experiments, in which at least 20,000 individual cells/measurement of the overall population were evaluated for rhodamine 123 retention. The percentage of mean fluorescence intensity was calculated for the treated MDR and Colo 320 cells as compared with the untreated cells. Rhodamine 123 accumulation was expressed as a fluorescence activity ratio (FAR) that was calculated based on the equation mentioned previously (7). Drug interactions were evaluated using CompuSyn software (www.combosyn.com, ComboSyn, Inc., Paramus, NJ, USA). Combination index (CI) values at 50% of the growth inhibition dose (ED₅₀) with corresponding SD values were determined using the software to plot four to five data points to each ratio. CI values were calculated by means of the median-effect equation, where CI < 1, CI = 1, and CI > 1 represent synergism, additive effect (or no interaction), and antagonism, respectively (22). Student's t-test

Table I. Cytotoxic effect of tested compounds on different cell lines [IC_{50} (μM) \pm SD].

Compound	Cell lines			
	PAR	MDR	Colo 205	Colo 320
1C	4.64 \pm 0.24	5.02 \pm 0.38	9.20 \pm 1.37	10.97 \pm 1.86
Verapamil	15.81 \pm 1.18	23.41 \pm 0.31***	43.21 \pm 2.28	>100***
Doxorubicin	0.49 \pm 0.04	11.15 \pm 0.56***	1.56 \pm 0.03	6.45 \pm 0.19***

PAR, Parental L5178Y mouse T-cell lymphoma cells; MDR, ABCB1-transfected L5178Y cells. The results are presented from three independent experiments. *** p <0.001 (MDR vs. PAR, Colo 320 vs. Colo 205).

Table II. Antiproliferative effect of tested compounds on different cell lines [IC_{50} (μM) \pm SD].

Compound	Cell lines			
	PAR	MDR	Colo 205	Colo 320
1C	3.47 \pm 0.40	4.78 \pm 0.71	3.84 \pm 0.25	4.49 \pm 0.42
Verapamil	24.13 \pm 1.41	25.82 \pm 0.28	>100	>100
Doxorubicin	0.03 \pm 0.01	0.39 \pm 0.04***	0.24 \pm 0.03	0.14 \pm 0.03*

PAR, Parental L5178Y mouse T-cell lymphoma cells; MDR, ABCB1-transfected L5178Y cells. The results are presented from three independent experiments. * p <0.05, *** p <0.001 (MDR vs. PAR, Colo 320 vs. Colo 205).

(GraphPad Software, La Jolla, CA, USA) was employed to determine statistical significance. Differences were considered significant when p -values were lower than 0.05.

Results

Effect of synthetic chalcone derivative 1C on cell growth. The effect of the synthetic chalcone derivative 1C on cell growth was determined on mouse T-cell lymphoma cells and human colon adenocarcinoma cells expressing ABCB1 (MDR and Colo 320) or not (PAR and Colo 205) by MTT analysis. Overall, compared to the parental cell lines, 1C exerted weaker cytotoxic effects on multidrug resistant cell lines; nevertheless, 1C compound was still effective on these sub-lines (PAR vs. MDR: IC_{50} 4.64 μM vs. 5.02 μM , $p \geq 0.05$; Colo 205 vs. Colo 320: IC_{50} 9.20 μM vs. 10.97 μM , $p \geq 0.05$). Similarly, antiproliferative activity was comparable (PAR vs. MDR: IC_{50} 3.47 μM vs. 4.78 μM , $p \geq 0.05$; Colo 205 vs. Colo 320: IC_{50} 3.84 μM vs. 4.49 μM , $p \geq 0.05$). On the other hand, in the cytotoxic assay, verapamil (ABCB1 substrate, inhibitor or inducer) and doxorubicin (ABCB1 substrate or inducer) (4) exhibited significantly decreased inhibition of cell growth in the resistant cell lines (p <0.001). All IC_{50} values obtained from MTT assays (cytotoxic and antiproliferative) on cancer cell lines together

Table III. Cytotoxic effect of tested compounds on non-cancerous and cancer cell lines [IC_{50} (μM) \pm SD].

Compound	Cell lines		
	NIH/3T3	PAR	MDR
1C	>20	4.64 \pm 0.24***	5.02 \pm 0.38***
Verapamil	>100	15.81 \pm 1.18***	23.41 \pm 0.31***
Doxorubicin	5.71 \pm 0.50	0.49 \pm 0.04***	11.15 \pm 0.56***
	MRC-5	Colo 205	Colo 320
	1C	>20	9.20 \pm 1.37***
Verapamil	>100	43.21 \pm 2.28***	>100***
Doxorubicin	>10	1.56 \pm 0.03***	6.45 \pm 0.19***

PAR, Parental L5178Y mouse T-cell lymphoma cells; MDR, ABCB1-transfected L5178Y cells. The results are presented from three independent experiments. ** p <0.01, *** p <0.001 (PAR/MDR vs. NIH/3T3, Colo 205/Colo 320 vs. MRC-5).

with IC_{50} of verapamil and doxorubicin are summarized in Table I and Table II, respectively. Moreover, it was demonstrated that 1C had significantly decreased inhibitory effects on non-cancerous cell lines (mouse NIH/3T3 and human MRC-5 cells) than on the tested cancer cell lines (p <0.001 or p <0.01; Table III). DMSO did not show any inhibitory activity on cell growth at a concentration of 1% v/v in all experiments.

To sum up, in addition to potent cytotoxic and antiproliferative effects on cell lines expressing ABCB1 or not, the tested compound 1C showed lower toxicity against non-cancer cells.

Effect of synthetic chalcone derivative 1C on ABCB1 transporter. Due to the potent cell growth inhibitory effect of 1C on cell lines expressing ABCB1, we next analysed whether compound 1C affects ABCB1 efflux activity in cancer cells. Thus, rhodamine 123 accumulation assay was performed using rhodamine 123 as an ABCB1-specific substrate. Briefly, parental cells (PAR, Colo 205) and their sub-lines expressing ABCB1 (MDR, Colo 320) were treated with 1C, doxorubicin, as well as verapamil as positive control and DMSO as negative control. The accumulation of rhodamine 123 was evaluated by flow cytometry. The resulting values of mean fluorescence intensity of the cells (FL-1) \pm SD values and fluorescence activity ratio (FAR) are summarized in Table IV (for mouse T-lymphoma cells) and Table V (for human colon adenocarcinoma cells). The potential of compounds to modulate ABCB1 was estimated from FAR values, which were calculated using the formula presented previously (7). Our compound 1C at concentrations ≥ 1 μM was able to significantly increase rhodamine 123 accumulation in MDR cell line, compared to untreated MDR

Table IV. Rhodamine 123 accumulation in mouse T-lymphoma cells.

Samples	Concentration (μM)	FL-1±SD	FAR
Untreated PAR	-	92.60±1.40	-
Untreated MDR	-	0.52±0.02	1
Treated MDR			
Verapamil	20	0.99±0.05	1.90***
Doxorubicin	5	0.82±0.03	1.58***
1C	0.5	9.58±6.72	18.42
1C	1	23.10±5.12	44.42** ++
1C	2.5	66.60±1.57	128.08*** +++
1C	10	92.20±4.72	177.31*** +++
DMSO (% v/v)	1.0	0.44±0.13	0.85

FL-1, Mean fluorescence intensity of the cells; SEM, standard error of the mean; FAR, fluorescence activity ratio, PAR, parental L5178Y mouse T-cell lymphoma cells; MDR, ABCB1-transfected L5178Y cells. All data represent the means±SD of three independent experiments. ** $p < 0.01$, *** $p < 0.001$ (treated MDR vs. untreated MDR); ++ $p < 0.01$, +++ $p < 0.001$ (1C vs. verapamil).

Table V. Rhodamine 123 accumulation in human colon adenocarcinoma cells.

Samples	Concentration (μM)	FL-1±SD	FAR
Untreated Colo 205	-	96.20±5.05	-
Untreated Colo 320	-	1.95±0.47	1
Treated Colo 320			
Verapamil	20	13.50±3.40	6.92**
Doxorubicin	5	1.78±0.08	0.91
1C	0.5	9.93±3.51	5.90*
1C	1	18.60±3.12	9.54***
1C	2.5	29.50±2.87	15.13*** ++
1C	10	61.50±5.20	31.54*** +++
DMSO (% v/v)	1.0	1.45±0.52	0.74

FL-1, Mean fluorescence intensity of the cells; SEM, standard error of the mean; FAR, fluorescence activity ratio. All data represent the means±SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (treated Colo 320 vs. untreated Colo 320); ++ $p < 0.01$, +++ $p < 0.001$ (1C vs. verapamil).

cells (1 μM: $p < 0.01$, 2.5 μM: $p < 0.001$, and 10 μM: $p < 0.001$) (Table IV). A similar effect of 1C-induced increase in rhodamine 123 accumulation was observed in Colo 320 cells treated with 1C, compared to the same untreated cell line (0.5 μM: $p < 0.05$, 1, 2.5, and 10 μM: $p < 0.001$) (Table V). Moreover, accumulation of rhodamine 123 was significantly higher in 1C-treated resistant cancer cell lines in comparison with the same cell lines treated with verapamil (MDR: $p < 0.001$ at concentration of 1C≥2.5 μM, $p < 0.01$ at concentration of 1C=1 μM; Colo320: $p < 0.001$ at concentration of 1C=10 μM, $p < 0.01$ at concentration of 1C=2.5 μM) (Table IV and Table V).

Table VI. The type of interaction between the synthetic chalcone derivative 1C and doxorubicin.

Cell lines	Best ratio ¹	CI at ED ₅₀ ²	SD	Interaction
MDR	4.64:1	0.62	0.09	Synergism
Colo 320	4.64:1	0.50	0.07	Synergism

¹Data are shown as the best combination ratio between 1C and doxorubicin. ²Combination index (CI) values at the 50% growth inhibition dose (ED₅₀) were determined by the CompuSyn software to plot four to five data points at each ratio. CI values were calculated by means of the median-effect equation, where CI<1, represent synergism. MDR, ABCB1-transfected L5178Y mouse T-cell lymphoma cells; SD, standard deviation.

Overall, the tested chalcone 1C enhanced accumulation of known ABCB1 substrate (rhodamine 123) more strongly than verapamil.

Combination assay between synthetic chalcone derivative 1C and doxorubicin. In order to find out the type of *in vitro* drug-drug interaction between the compound 1C and the cytostatic drug doxorubicin (known ABCB1 substrate) (23), a combination assay was carried out on the resistant MDR and Colo 320 cell lines. Several concentrations of the compound 1C and doxorubicin were assessed. The type of interaction between tested substances was calculated and expressed using the combination index (CI) value±standard deviation. Tested chalcone derivative displayed synergistic interaction with doxorubicin, being able to enhance inhibition of cell growth of both drug-resistant cancer cell lines (MDR: CI at ED₅₀=0.62, Colo 320: CI at ED₅₀=0.50). The best combination ratios between 1C and doxorubicin were also determined (Table VI).

In conclusion, the tested chalcone increased cell growth inhibition of doxorubicin in both tested cell lines.

Discussion

To our knowledge, this is the first study that assessed ABCB1 reversal effect of chalcone derivative in human colon adenocarcinoma cells, in addition to the effect on mouse T-lymphoma cells that has been tested (14). The choice of tested cancer cell lines (PAR, MDR, Colo 205 and Colo 320) and selection of chalcone derivative was in accordance to our recent study, which demonstrated a strong antiproliferative activity of the chalcone derivative 1C in human colorectal HCT116 cells and human T-cell lymphoma (19).

In the present work, we found that compound 1C had potent antiproliferative and cytotoxic effects on parental cancer cell lines (PAR, Colo 205), as well as on their resistant sub-lines expressing ABCB1, with comparable IC₅₀

values. Therefore, our findings suggest that expression of ABCB1 was not able to abolish the cytotoxic effect of 1C. The important factor influencing the cytotoxicity of chalcones against multidrug resistant cancer cell lines is considered to be a presence of a methoxy substituent in the scaffold, whereas chalcones with two methoxy groups show better activity than chalcones with only one (24). The fact that chalcone 1C contains two methoxy groups may be critical for the mediation of its strong antiproliferative activity toward the tested cell lines. Previous studies have demonstrated inhibition of cell growth induced by methoxy chalcones in cells from various cancer types (*e.g.* synovial sarcoma, prostate cancer, colon cancer, bladder cancer, and human oral squamous cell carcinoma) (25, 26), as well as in multidrug resistant cells (mouse T-cell lymphoma-L5178, human leukemia-CEM/ADR5000 or colon cancer-LoVo/DX) (14, 24, 27).

Interestingly, toxicity of 1C against non-cancerous mouse NIH/3T3 and human MRC-5 cells was markedly reduced, which is a crucial parameter to ensure safety of anticancer treatment. Such a preferential action of chalcones against cancer cells and not non-cancer cells has also been documented in other studies (26, 28-30).

Furthermore, our analysis showed that 1C could inhibit ABCB1 mediated efflux of rhodamine 123 (ABCB1 substrate). In line with our findings, there are several additional studies reporting the ability of chalcones to bind to ABCB1 and inhibit its transport activity (12, 31). Multidrug resistance reversal activity of chalcones, including inhibition of other membrane transporters, has been previously demonstrated for example in human breast cancer (15, 32, 33) or human glioblastoma cells (13). Herein, the tested chalcone 1C was even more potent inhibitor of ABCB1 than verapamil. In literature, other chalcones have been shown to inhibit ABCB1 activity more potently than verapamil (14, 15). The precise mechanism by which chalcones inhibit ABCB1 is still not fully understood; however, according to several authors, chalcones can overlap two binding sites of the nucleotide-binding domain (NBD2) of ABCB1 (17, 34). The key factors influencing ABCB1 inhibitory activity of chalcones are believed to be lipophilicity as well as the size and shape of molecules (17). Structure-activity relationship (SAR) studies have shown that the presence of methoxy groups (-OCH₃) on ring A or basic functional groups (*i.e.* nitrogen-containing heterocycles) on ring B of chalcone scaffold is favourable for an interaction with ABCB1 (12, 13, 16, 35). The substitution of ring A by two methoxy groups and ring B by a basic acridine group in 1C molecule probably played an important role in facilitation of ABCB1 inhibition; however, further experiments are necessary to clarify the precise mechanism of its action.

Combination therapy in cancer treatment is an essential approach used to reduce drug resistance (36). Thus, in order

to know the type of drug-drug interaction at the cellular level, *in vitro* combination chemotherapy model was performed. Compound 1C was combined with the anticancer drug doxorubicin, a well-known ABCB1 substrate and inducer. Herein, we observed that the tested chalcone derivative displayed synergistic interaction with doxorubicin leading to enhancement of cell growth inhibition in both cancer cell lines expressing ABCB1. Similarly, Arianingrum *et al.* observed synergistic effects of a synthetic chalcone with doxorubicin in breast cancer cells (37), while another synthetic chalcone was shown to sensitize epithelial cancer to cisplatin also displaying synergism (38). However, pre-clinical *in vivo* studies are needed to evaluate drug-drug interactions because of a greater impact of ABCB1 on drug intestinal absorption and distribution.

To conclude, results presented here demonstrated dual potential of the chalcone 1C to act as a chemotherapeutic, as well as a chemosensitizing agent in resistant T-cell lymphoma and colon cancer, even with higher sensitivity for cancer cells. Furthermore, due to the ABCB1 inhibitory activity, the compound could be potentially used as an ABCB1 inhibitor in lab analyses.

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

Authors' Contributions

M. Cizmarikova and P. Takac contributed to the study design, experimental work, article drafting, statistical analysis and data interpretation. G. Spengler contributed to the study design, experimental work, statistical analysis and data interpretation, provided critical revision of the article and obtained funding. A. Kincses and M. Nové contributed to the experimental work, statistical analysis and data interpretation. M. Vilková synthesized the studied compound. J. Mojzís revised the paper, obtained funding and gave final approval.

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