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Oleanolic acid derivative methyl 3,11-dioxoolean-12-en-28-olate targets multidrug resistance related to ABCB1

Anna Paszel[1], Błażej Rubiś[1], Barbara Bednarczyk-Cwynar[2], Lucjusz Zaprutko[2], Mariusz Kaczmarek[3], Johann Hofmann[4], Maria Rybczyńska[1]

[1]Department of Clinical Chemistry and Molecular Diagnostics, Poznan University of Medical Sciences, Przybyszewskiego 49, PL 60-355 Poznań, Poland

[2]Department of Organic Chemistry, Poznan University of Medical Sciences, Grunwaldzka 6, PL 60-780 Poznań, Poland

[3]Department of Clinical Immunology, Poznan University of Medical Sciences, Rokietnicka 5D, PL 60-806 Poznań, Poland

[4]Biocenter, Division of Medical Biochemistry, Innsbruck Medical University, Fritz-Pregl-St. 3, A-6020 Innsbruck, Austria

Correspondence: Maria Rybczyńska, e-mail: mrybczyn@ump.edu.pl

Abstract:
Multidrug resistance (MDR) in leukemia patients is a great incentive to the development of new drugs. In a search for potential multidrug resistance modulators we tested a group of oleanolic acid (OA) analogues modified at C-3, C-11, C-12 and C-28 using an experimental model consisting of three human acute lymphoblastic leukemia cell lines (CCRF-CEM and the multidrug resistant sublines CCRF-VCR1000 and CCRF-ADR5000).
The most effective compound, methyl 3,11-dioxoolean-12-en-28-olate (DIOXOL) was more potent in cell viability inhibition than its precursor – OA, and showed similar or even higher activity in the drug resistant than in the wild-type cells. Resistance factor (RF) values obtained for CCRF-VCR1000 and CCRF-ADR-5000 cells using MTT assay were 0.7 and 0.8 (24 h of treatment) and after 72 h of treatment 0.9 and 1.1, respectively. Moreover, 5 μM DIOXOL significantly reduced the expression of the *ABCB1* gene in MDR cells by around 30%, and also decreased the level of P-gp protein. Compared to untreated control cells, DIOXOL treatment resulted in a significant P-gp decrease (30% in CCRF-ADR5000 and 50% in CCRF-VCR1000), that was detected by western blot and confirmed by flow cytometry analysis. Moreover, DIOXOL (at 10 μM) significantly inhibited P-gp transport function by more than twofold comparing to control, untreated cells that was demonstrated using rhodamine 123-based functional test. The compound exhibited synergistic activity with ABCB1 substrate – adriamycin in CCRF-VCR1000 cells, indicating partial but significant MDR reversing ability.

Key words:
multidrug resistance, P-gp, oleanolic acid derivatives, acute lymphoblastic leukemia cells, chemotherapy

Abbreviations: ABC – ATP-binding cassette, ALL – acute lymphoblastic leukemia, DIOXOL – methyl 3,11-dioxoolean-12-en-28-olate, HIMOXOL – methyl 3-hydroxyimino-11-oxoolean-12-en-28-olate, MDR – multidrug resistance, MFI – mean fluorescence intensity, OA – oleanolic acid, P-gp (MDR1/ABCB1) – P-glycoprotein

Introduction

Chemotherapy failure in hematological malignancies is frequently related to the phenomenon of multidrug resistance (MDR). Resistance of cancer cells to chemotherapeutic agents can develop through multiple mechanisms [29] but mainly, the overexpression of genes encoding membrane transport proteins belonging to the ATP-binding cassette (ABC) superfamily is responsible for the MDR phenotype [3]. P-glycoprotein (P-gp/MDR1/ABCB1) is the first best characterized ABC transporter responsible for the drug insensitivity of tumor cells.

It is now well established that overexpression of the *ABCB1* gene causes increased excretion of anticancer drugs from cells, decreases drug accumulation and in consequence reduces intracellular drug concentration [24]. P-gp can be localized not only on the cell surface but also in intracellular membranes, including the endoplasmic reticulum and Golgi apparatus. Therefore, it causes cytoplasmic sequestration of the drug, preventing it from reaching the molecular target [19]. Several commonly used drugs interact with the substrate-binding site of P-gp *via* hydrophobic and H-bonding interactions [37] and are removed to the extracellular environment.

P-gp, encoded by the *ABCB1* gene, causes cross-resistance of cancer cells to structurally unrelated drugs, that differ widely with respect to their mechanisms of action. This drug transporter pumps a variety of hydrophobic, neutral and positively charged chemical compounds out of cells [7, 34]. Cells expressing high levels of this transmembrane protein do not respond to treatment with many currently used anticancer drugs such as anthracyclines, vinca alkaloids, taxanes [24], epipodophyllotoxins or mitoxantrone. Some of these agents are still used in acute leukemia therapy [35]. In certain human cancers, including acute lymphoblastic leukemia (ALL), elevated levels of P-gp are associated with decreased survival and poor prognosis due to decreased effective intracellular drug concentration [12, 40]. Results of prospective studies concerning the expression of the genes related to MDR indicate that prognosis for ALL patients with *ABCB1* overexpression is significantly worse than for those with low expression of this gene [17].

In recent decades, much effort has been devoted to overcome drug resistance. One of the main goals of research in the field of anticancer drug development is to find efficient modulators of multidrug resistance related proteins [2]. For this purpose several compounds have been tested but the majority of them could not be clinically applied, mostly due to serious side effects.

Recently, a group of triterpenoid compounds was found to be of some interest. The commonly known biological activity of naturally occurring oleanolic acid derivatives [8, 21, 39, 45, 46] belonging to this class of chemicals motivated us to seek such modifications of its basal structure, which could result in synthesis of more potent agents. Thus we decided to investigate several semisynthetic derivatives of oleanolic acid, in which the parental structure had been chemically modified at the C-3, C-11, C-12 and C-28 positions. The most potent of these was methyl 3,11-dioxoolean-12-en-28-olate (DIOXOL) (Fig. 1).

Materials and Methods

Compounds

Oleanolic acid was used as a starting platform for the synthesis of eight compounds: 3-oxooleanolic acid (1), 3-oxooleanolic acid oxime (2), methyl 3,11-dioxo-olean-12-en-28-olate (3; DIOXOL), methyl 3-hydroxyimino-11-oxoolean-12-en-28-olate (4), methyl 3,12-dioxoolean-28-olate (5), methyl 3-hydroxyimino-12-oxoolean-28-olate (6), 12α-bromo-3-oxoolean-28 → 13-olide (7), 12α-bromo-3-hydroxyiminoolean-28 → 13-olide (8) (Fig. 1). Oleanolic acid was isolated from an industrial by-product obtained in the process of mistletoe herb essence production. Spectral data of the resulting chemicals were consistent with data from the literature [1]. The semisynthetic oleanolic acid (OA) derivatives presented in this study were synthesized as described previously [8, 20, 38, 45]. The obtained compounds were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA) and the final concentration of the solvent was 0.28%, which was shown not to reveal any cytotoxic activity as demonstrated in MTT assay.

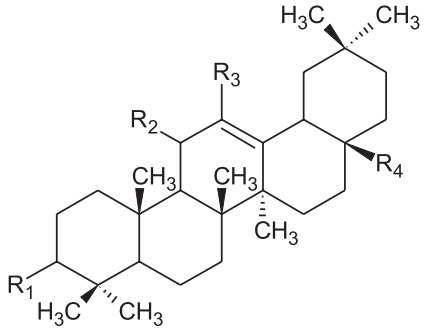
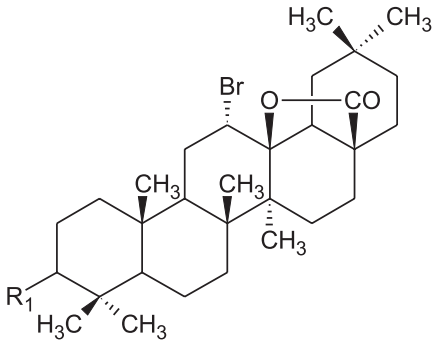


Fig. 1. Chemical structure of oleanolic acid (OA) and its semisynthetic analogues. The basic structure of parental compound was chemically modified at C-3, C-11, C-12 and C-28 by substitution of functional groups indicated in the tables

Compound	R_1	R_2	R_3	R_4
OA	–OH	–H	–H	–COOH
(1)	=O	–H	–H	–COOH
(2)	=NOH	–H	–H	–COOH
(3); DIOXOL	=O	=O	–H	$-COOCH_3$
(4); HIMOXOL	=NOH	=O	–H	$-COOCH_3$
(5)	=O	–H	=O	$-COOCH_3$
(6)	=NOH	–H	=O	$-COOCH_3$



Compound	R_1
(7)	=O
(8)	=NOH

Cell lines and culture conditions

Three human leukemia cell lines were used as an experimental model. These lines were the human acute lymphoblastic leukemia cell line CCRF-CEM (ATCC®: CCL-119™) and its multidrug resistant variants CCRF-VCR1000 and CCRF-ADR5000 overexpressing *ABCB1* [16]. MDR cells were derived from their wild-type counterparts by continuous long-term selection in increasing concentrations of vincristin sulfate and adriamycin. To maintain the resistance, CCRF-VCR1000 cells were continuously grown in the presence of 1 μg/ml vincristine sulfate, while CCRF-ADR5000 cells were grown in the presence of 5 μg/ml adriamycin (both from Sigma-Aldrich, St. Louis, MO, USA) except during the time of experiments. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (both from PAA, Pasching, Austria) and 40 μg/ml gentamicin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 5% CO_2. The cells were routinely tested for mycoplasma using PCR method.

Viability test

Viability of the human acute lymphoblastic leukemia cells treated with the tested triterpenoid compounds were assessed by the MTT assay [31]. In viable cells, MTT is reduced to blue formazan dye. The color intensity of the product is proportional to the number of viable, metabolizing cells. Briefly, 2×10^4 cells were seeded per well in 96-well plates. After 2 h the cells were exposed for 24 and/or 72 h to OA in a concentra-

tion range of 1–100 μM, compounds (1), (2), (3, DIOXOL), (4, HIMOXOL), (6) and (8) in a concentration range of 0.5–50 μM, compounds (5) and (7) in a concentration range of 0.5–25 μM and adriamycin in a concentration range of 1 nM – 100 μM. The solvent, DMSO in concentration of 0.28% was also applied as a control. Subsequently, 20 μl of MTT solution (5 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. The plate was incubated at 37°C for 4 h followed by 100 μl of solubilization buffer (10% SDS in 0.01 M HCl) addition. After overnight incubation, absorbance at 570 nm and 690 nm was measured using a Multiscan RC microplate reader (Labsystems Inc., Helsinki, Finland). The IC_{50} values were calculated using CalcuSyn software (Biosoft, Cambridge, UK). The experiment was performed in duplicates.

Isobologram analysis

To analyze the combined effects of DIOXOL and adriamycin, the CalcuSyn program (Biosoft, Cambridge, UK) was used. Data from the viability test (MTT) were expressed as the fraction of affected cells (FA) in drug-treated *versus* untreated cells. CalcuSyn is based on the Chou and Talalay method [9] according to the following equation:

Combination index (CI) = (D)1/(Dx)1 + (D)2/(Dx)2 + (D)1(D)2/(Dx)1(Dx)2

where (D)1 and (D)2 are the doses of drug 1 and drug 2 that have "x" effect when used in combination and (Dx)1 and (Dx)2 are the doses of drug 1 and drug 2 that have the same "x" effect when used alone. When CI = 1 this equation represents the conservation isobologram indicating an additive effect. CI values below 1.0 indicate synergism while results above 1 indicate antagonism.

Accumulation of rhodamine 123

Five times 10^5 per ml of logarithmically growing CCRF-CEM, CCRF-VCR1000 or CCRF-ADR5000 cells were washed with phosphate-buffered saline (PBS) and re-suspended in 1 ml of Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) without serum, and supplemented with 20 mM MOPS (Sigma-Aldrich, St. Louis, MO, USA). The cells were preincubated with DIOXOL (1 μM and 10 μM) and verapamil (10 μM) at 37°C for 1 h before the addition of 60 μl of 0.5 mg/ml rhodamine 123 (Sigma-Aldrich, St. Louis, MO, USA). Flow cytometry (FACSCalibur, BD, Franklin Lakes, NJ, USA) analysis using excitation at 488 nm was performed 0, 30, 60 and 90 min after the addition of the fluorochrome. Emission was detected with a 530 nm filter and fluorescence intensity was expressed as the mean of 10,000 cells gated by forward and side scattered light.

Quantitative real-time RT-PCR assay

Total RNA was extracted with TriPure (Roche Diagnostics, Indianapolis, IN, USA) following 24 h treatment with DIOXOL (0.5 μM and 5 μM) and verapamil (10 μM). cDNA was synthesized using Transcriptor First Strand cDNA synthesis kits (Roche Diagnostics, Indianapolis, IN, USA) using 2 μg of total RNA and oligo dT primers. The real-time polymerase chain reaction (PCR) was carried out using a LightCycler 2.0 instrument (Roche Diagnostics, Indianapolis, IN, USA). The oligonucleotide primers were ABCB1-fw: 5'- TGCAATAGCAGGAGTTGTTG-3', ABCB1-rev: 5'- ACCGGAAACATCCAG CATAG-3', GAPDH-fw: 5'-TTCGTCATGGGTGTGAACC-3'and GAPDH-rev: 5'-GATGATGTTCTGGAGAGCCC-3'. Amplification products were detected *via* intercalation of the fluorescent dye SYBR green (from LightCycler FastStart DNA Master SYBR Green 1 kit, Roche Diagnostics, Indianapolis, IN, USA). Cycling conditions for *ABCB1* and *GAPDH* were as follows: initially 95°C for 10 min, followed by 35 cycles at 94°C for 25 s, 60°C for 25 s, and 72°C for 15 s. All cycling reactions were performed in the presence of 2.5 mM $MgCl_2$. Gene specific products were confirmed by melting curve analysis. *ABCB1 g*ene expression was normalized by the expression of the housekeeping gene *GAPDH*.

Western blot analysis

Five times 10^5 cells per ml were treated for 24 h with 5, 10 or 20 μM of DIOXOL. To obtain cellular extracts, cells were lysed on ice in the lysis buffer (consisting of 50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% NP40, pH 8.0). Protein concentration was measured with Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA). Hundred micrograms of total protein was separated by SDS-polyacrylamide gel electrophoresis at 200 V for 50 min and transferred to PVDF membranes (Thermo Fisher Scientific Inc., Rockford, IL, USA). The primary antibodies were:

Tab. 1. IC_{50} values of oleanolic acid (OA), its semisynthetic derivatives and adriamycin (ADR), a P-gp substrate. IC_{50} was calculated using CalcuSyn software. Cells were treated for 24 or 72 h (results of the short-term treatment are shown only for the most active compound, DIOXOL). The resistant factors (RF) in square brackets, indicating the resistance compared with the parental cell line, were calculated by the IC_{50}-resistant/IC_{50}-sensitive ratio. The means ± SD of five independent experiments performed in duplicates are shown

Compounds		IC_{50} (μM); [RF]				
		CCRF-CEM	CCRF-VCR1000		CCRF-ADR5000	
OA	72 h	5.12 ± 0.41	8.46 ± 0.25	[1.7]	12.88 ± 1.78	[2.5]
(1)	72 h	3.71 ± 0.44	7.85 ± 0.60	[2.1]	10.25 ± 0.30	[2.8]
(2)	72 h	1.28 ± 0.15	2.61 ± 0.41	[2.0]	3.58 ± 0.21	[2.8]
(3); DIOXOL	24 h	20.6 ± 0.5	15.1 ± 2.0	[0.7]	16.3 ± 2.5	[0.8]
	72 h	1.7 ± 0.04	1.56 ± 0.3	[0.9]	1.95 ± 0.45	[1.1]
(4); HIMOXOL	72 h	1.93 ± 0.36	3.54 ± 0.34	[1.8]	3.34 ± 0.48	[1.7]
(5)	72 h	> 25	> 25	[–]	> 25	[–]
(6)	72 h	6.37 ± 0.61	7.98 ± 0.55	[1.3]	10.11 ± 1.65	[1.6]
(7)	72 h	> 25	> 25	[–]	> 25	[–]
(8)	72 h	1.67 ± 0.62	3.61 ± 0.8	[2.2]	4.54 ± 0.22	[2.7]
ADR	72 h	0.021 ± 0.002	1.40 ± 0.05	[66.7]	0.79 ± 0.1	[37.6]

mouse anti-Mdr1 (D-11) and rabbit anti-GAPDH (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc.) antibodies were used for chemiluminescence detection. All antibodies were used at 1:1000 dilution. The films were developed and quantified by scanning with a Bio Imaging System Epi-Chemi^3 Darkroom (UVP, Upland, CA, USA).

Flow cytometry analysis of the P-gp level

To examine the influence of the most active compound – DIOXOL on P-gp levels in the cell membranes of multidrug resistant CCRF-VCR1000 and CCRF-ADR5000 cells flow cytometry analysis was performed. Five times 10^5 cells per ml were treated with the triterpenoid in three different concentrations (0.5, 5 and 10 μM), verapamil (10 μM), adriamycin (5 μg/ml) or DIOXOL (5 μM) and adriamycin (5 μg/ml) in a combination for 24 h. Then, cells were washed twice with PBS and fixed with 4% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA). After further washes, the cells were incubated in 5% BSA (Sigma-Aldrich, St. Louis, MO, USA) for 30 min and subsequently in an anti-Mdr1 (UIC2) primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) solution in 3% BSA (1:200) for 30 min. Next, cells were washed with PBS and labeled with a FITC-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 3% BSA (1:500) for 30 min. The cells were washed once again with PBS and analyzed by flow cytometer (Facs-Canto, BD, Franklin Lakes, NJ, USA).

Statistical analysis

All results are the means of at least three separate experiments made in duplicates. Statistical analysis was performed by one-way ANOVA followed by Tukey's *post-hoc* test using Graph Pad Prism 5 software. Results were considered as statistically significant when $p < 0.05$.

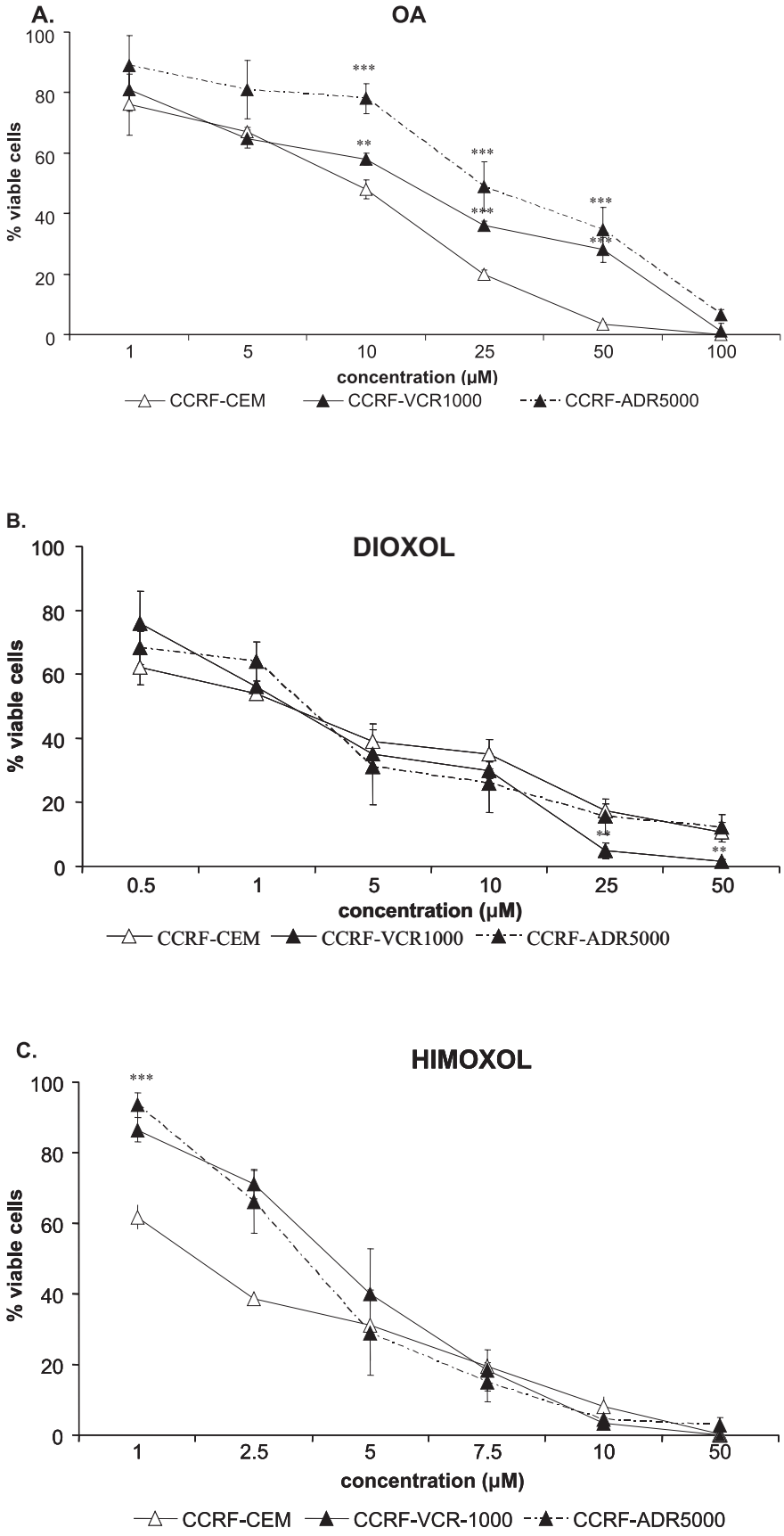


Fig. 2. Dose-effect curves for leukemic cells treated with (**A**) OA, (**B**) DIOXOL and (**C**) HIMOXOL. CCRF-CEM and its two MDR variants: CCRF-ADR5000 and CCRF-VCR1000 were treated with different concentrations of compounds (concentration range of 1–100 μM for OA, 0.5–50 μM for DIOXOL and 1–50 μM for HIMOXOL) for 72 h. Each point represents the mean ± SD of four independent experiments performed in duplicates (** $p < 0.01$; *** $p < 0.001$)

Results

OA derivative modified at C-3, C-11 and C-28 - DIOXOL is most potent against MDR leukemia cells

First we determined whether OA derivatives affect the viability of leukemic blasts. Cytotoxic activity of DMSO in concentration of 0.28% was also checked. Viability of the leukemic cells after 24 h and 72 h treatment with this solvent was 99 ± 1% and 98 ± 2%, respectively. Thus the compound was considered as nontoxic in the used concentration.

Cells used in the project differed with the expression level of the *ABCB1* gene. A high cellular content of this gene's product is responsible for chemoresistance in many malignancies, including acute leukemia. We used an experimental model, consisting of the sensitive (wild type) human acute lymphoblastic leukemia cell line – CCRF-CEM and its two resistant variants: CCRF-VCR1000 and CCRF-ADR5000. This cellular model allows for the assessment of the chemicals' potential for cell viability reduction, with respect to the MDR phenotype. To confirm the status of MDR in CCRF-VCR1000 and CCRF-ADR5000 lines we treated cells with the P-gp substrate, adriamycin and performed the MTT test. The data from this test were used for calculations of the IC_{50} values and the resistance factor (RF) which was defined as the IC_{50} of the resistant cells divided by the IC_{50} of the sensitive (wild type) cells. The IC_{50} values and RF obtained for adriamycin demonstrated that CCRF-VCR1000 and CCRF-ADR5000 cells are highly resistant to this drug (RF values: 66.7 and 37.6, respectively) (Tab. 1). The most active OA analogue, chemically modified at C-3, C-11 and C-28 – DIOXOL showed similar or higher cytotoxic activity against multidrug resistant cells in comparison to the wild-type after 24 h and 72 h of treatment, with RF values of 0.7 and 0.8 (24 h treatment) or 0.9 and 1.1 (72 h treatment) for CCRF-VCR1000 and CCRF-ADR5000, respectively (Tab. 1).

To emphasize the beneficial effect of the chemical modifications resulting in DIOXOL a comparison of OA, DIOXOL and HIMOXOL dose-effect curves was shown (Fig. 2 A–C). DIOXOL strongly inhibited both, sensitive and multidrug resistant leukemic cells viability after 72 h of treatment. It decreased the percentage of living cells in a dose- and time-dependent manner and it was the most effective of all other tested oleanolic acid derivatives comparing to the parental compound. This effect was observed particularly in MDR cell lines. It was also demonstrated that DIOXOL differing from HIMOXOL only in C-3 moiety is more active in cell viability inhibition, especially in lower concentrations, that might have important clinical implications. Interestingly, the difference in cytotoxicity effect (IC_{50} values) observed in the wild type CCRF-CEM and the two resistant sublines CCRF-VCR1000 and CCRF-ADR5000 after 72 h of treatment with HIMOXOL is significant (1.93 ± 0.36 μM, 3.54 ± 0.34 μM and 3.34 ± 0.48 μM, respectively; $p < 0.05$) (Tab. 1). OA and all of the tested derivatives (except DIOXOL) were significantly less active against the multidrug resistant cells than to the wild type cells. Short-term treatment (24 h) of the cells with all of the tested compounds was much less effective (data not shown, except the data for DIOXOL).

The MTT test was used to determine cells viability reduction by the tested compounds. However, this assay does not provide any information about the molecular mechanism of drug resistance or sensitivity. Thus, to explain the results obtained in this part, investigation of the mechanism was performed. Based on the obtained results, the most efficient compound – DIOXOL was applied in further experiments.

DIOXOL inhibits transport activity of P-gp

To determine the ability of DIOXOL to inhibit P-gp transport function, the intracellular accumulation of the fluorescent P-gp substrate rhodamine 123 was assessed (Fig. 3A, B). Cells treated with 10 μM of the known P-gp inhibitor verapamil were used as positive control. It was noted that short-term pretreatment (1 h) of the resistant CCRF-ADR5000 cells with DIOXOL inhibited the P-gp function. The efficacy of this compound was time- and dose- dependent (Fig. 3B). This OA derivative in a concentration of 10 μM caused a 124% increase (relative to control, untreated cells) in mean fluorescence intensity (MFI) in CCRF-ADR5000 cells after 30 min of incubation with rhodamine 123. After 60 min, rhodamine 123 accumulation increased up to 182% and after 90 min the fluorescence intensity of treated cells increased more than twofold compared to the control (untreated cells). Similar effects were observed in CCRF-VCR1000 cells (data not shown). DIOXOL at concentrations ten times lower (1 μM) did not exert any significant influence on the P-gp functional activity even at the maximal time-point of experiment (90 min).

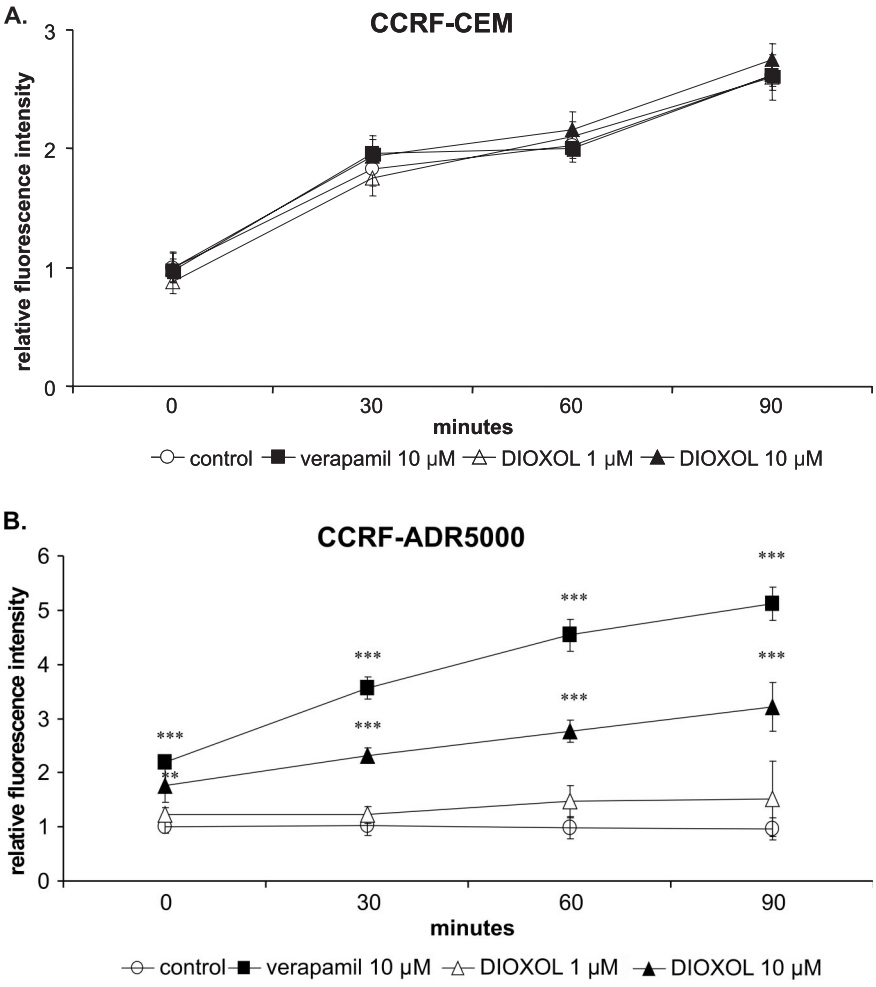


Fig. 3. Quantitative analysis of P-gp inhibition induced by DIOXOL. Intracellular accumulation of the P-gp substrate rhodamine 123 was measured in (**A**) sensitive acute lymphoblastic leukemia cells CCRF–CEM and (**B**) multidrug resistant CCRF-ADR5000 pretreated with 1 and 10 μM DIOXOL or 10 μM verapamil (positive control) for 1 h. Fluorescence intensity was detected using a flow cytometer immediately after adding the fluorochrome (0 min) and at the indicated time intervals. Results are the means ± SD of three independent experiments performed in duplicates (** $p < 0.01$; *** $p < 0.001$)

Short-term treatment (1 h) with the same concentrations of this triterpenoid compound and verapamil did not alter accumulation of rhodamine 123 in sensitive CCRF-CEM cells (Fig. 3A).

DIOXOL decreases the expression of the *ABCB1* gene

To investigate whether DIOXOL is able to reduce the expression of *ABCB1* mRNA, CCRF-ADR5000 and CCRF-VCR1000 cells were exposed to 0.5 and 5 μM of DIOXOL for 24 h. The results confirmed that DIOXOL not only decreased the transport function of P-gp, but also downregulated *ABCB1* gene expression in MDR cells. This illustrates that the compound, at a concentration of 5 μM, is able to act at the transcriptional level, leading to a significant reduction of *ABCB1* transcript by around 30% ($p < 0.05$) in treated cells (Fig. 4).

DIOXOL reduces the level of P-gp

To determine whether the decrease of *ABCB1* gene expression induced by DIOXOL is accompanied by corresponding changes in P-gp levels, western blots and flow cytometry were carried out. Both techniques confirmed significant P-gp overexpression in multidrug resistant cell lines. No expression of the multidrug transporter was detected in parental cells CCRF-CEM. The results prove that treatment of MDR cells with DIOXOL resulted in a dose-dependent reduction of the P-gp level. The lowest concentration (5 μM) used for western blot analysis led to a 30 and 50% reduction in the total amount of P-gp in CCRF-ADR5000 and CCRF-VCR1000 cells, respectively, reflecting data obtained with qPCR. Increasing the concentration to 10 or 20 μM augmented the observed effect. We did not detect any signal from the protein for CCRF-CEM cells (Fig. 5).

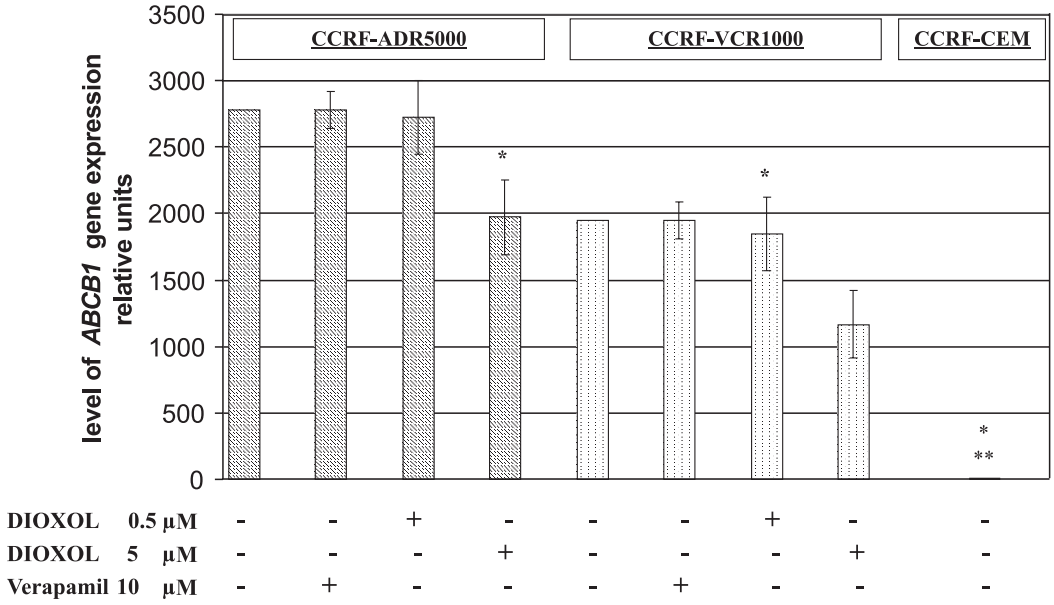


Fig. 4. Quantitative real-time PCR analysis of *ABCB1* gene expression in CCRF-CEM, CCRF-ADR5000 and CCRF-VCR1000 cells. Transcript levels were determined following 24 h incubation with or without compounds (0.5 and 5 µM DIOXOL, 10 µM verapamil). *ABCB1* mRNA in CCRF-CEM cells was detectable but the signal was significantly weaker than that coming from MDR cells. The data were normalized to *GAPDH* expression level. Data are the means ± SD of three independent experiments performed in duplicates (* $p < 0.05$; ** $p < 0.001$)

Flow cytometry was used to evaluate the impact of lower concentrations of DIOXOL (0.5–5 µM) on cell membrane P-gp levels in multidrug resistant cells (Fig. 6A–H). Cytometric detection, using a specific monoclonal antibody showed that 5 µM DIOXOL significantly ($p < 0.001$) diminished the amount of P-gp in both CCRF-VCR1000 and CCRF-ADR5000 cells (Fig. 6B and Fig. 6F). In concentrations below 5 µM, the compound exerted only minimal influence on the drug transporter content in CCRF-VCR1000 cells (Fig. 6D–E). The decreased P-gp membrane content was seen on the histograms as a left-shift in

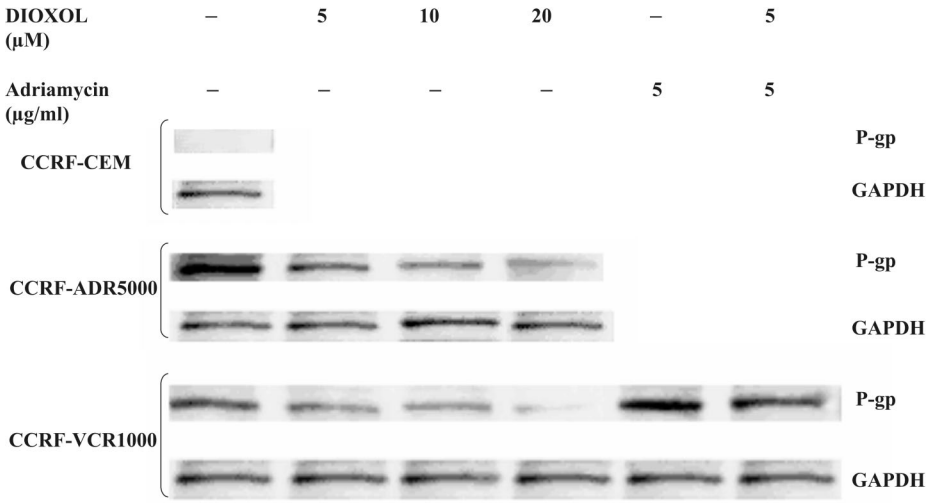
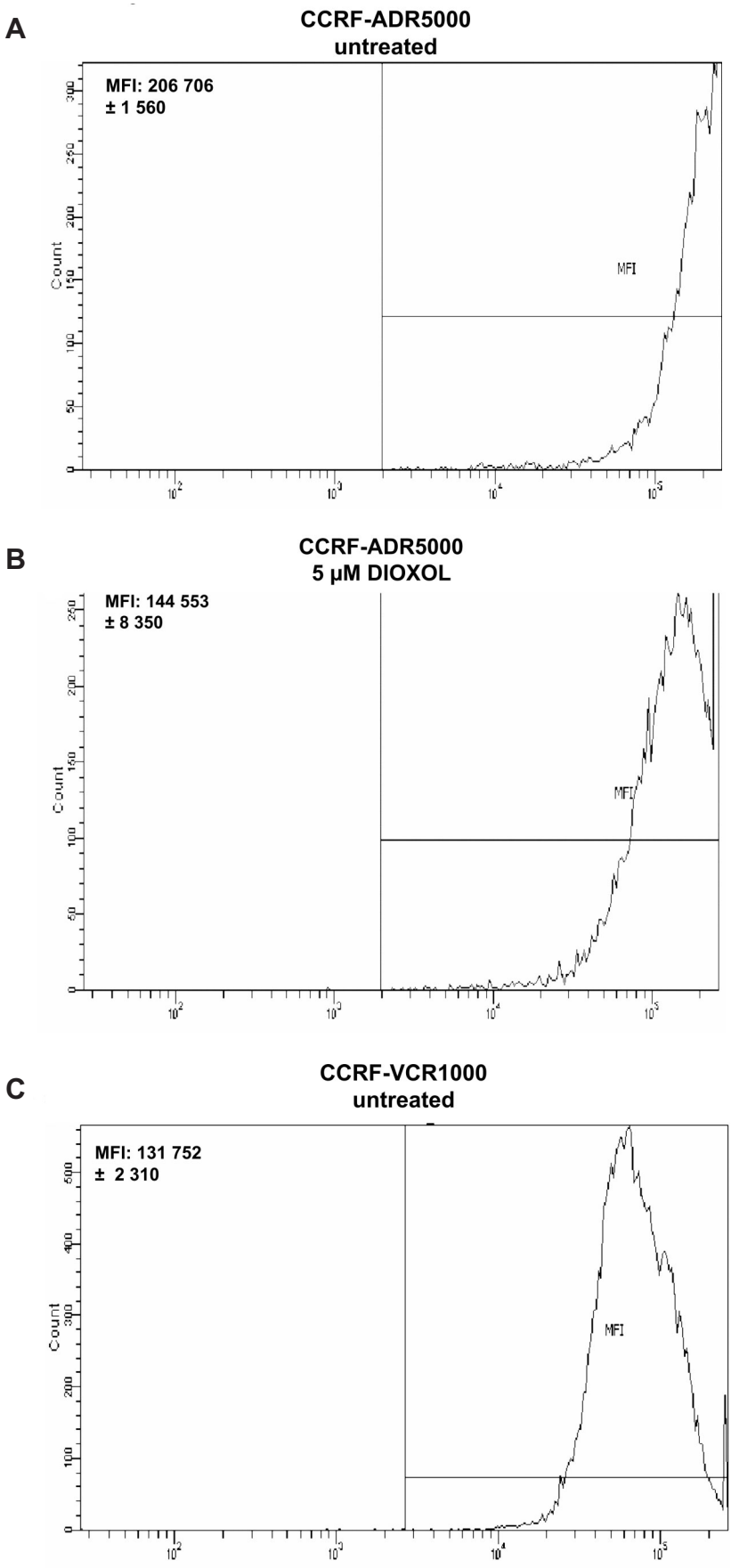


Fig. 5. Western blot analysis of P-gp levels. CCRF-CEM, CCRF-ADR5000 and CCRF-VCR1000 cells were treated with DIOXOL (5, 10, 20 µM) for 24 h. CCRF-VCR1000 cells showing a lower basal level of P-gp than CCRF-ADR5000 cells but more resistant to adriamycin were treated with 5 µg/ml adriamycin and with a combination of 5 µg/ml adriamycin and 5 µM DIOXOL. *GAPDH* was used as loading control. The data show one representative out of three experiments


A
CCRF-ADR5000
untreated
MFI: 206 706
± 1 560
MFI
Count
B
CCRF-ADR5000
5 µM DIOXOL
MFI: 144 553
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MFI
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C
CCRF-VCR1000
untreated
MFI: 131 752
± 2 310
MFI
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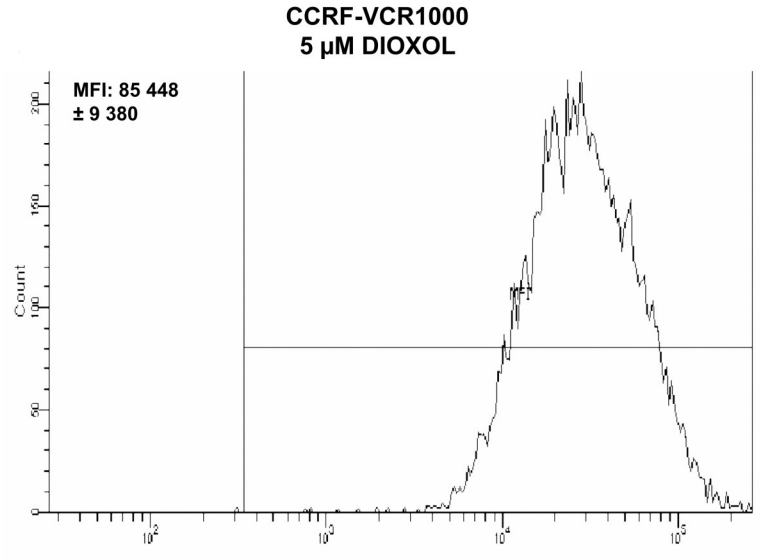
CCRF-VCR1000
5 µM DIOXOL
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MFI

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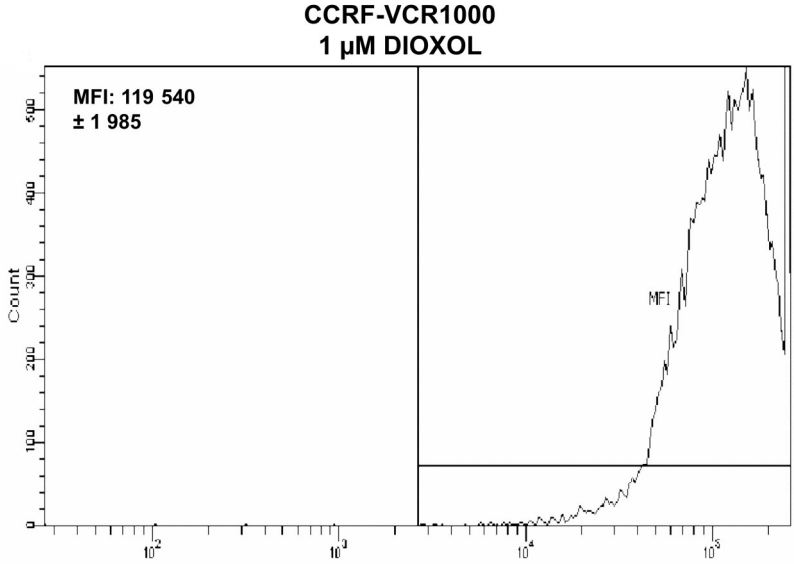
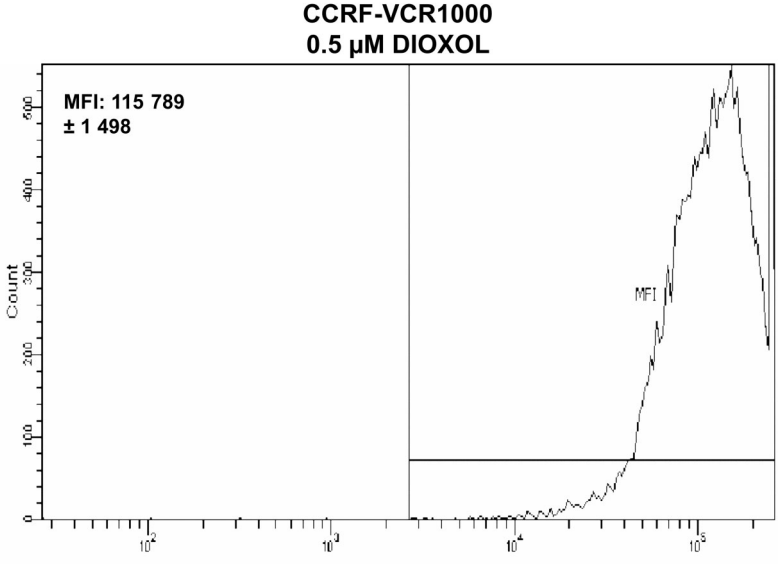

CCRF-VCR1000
1 µM DIOXOL
MFI: 119 540
± 1 985
Count
MFI

F


CCRF-VCR1000
0.5 µM DIOXOL
MFI: 115 789
± 1 498
Count
MFI

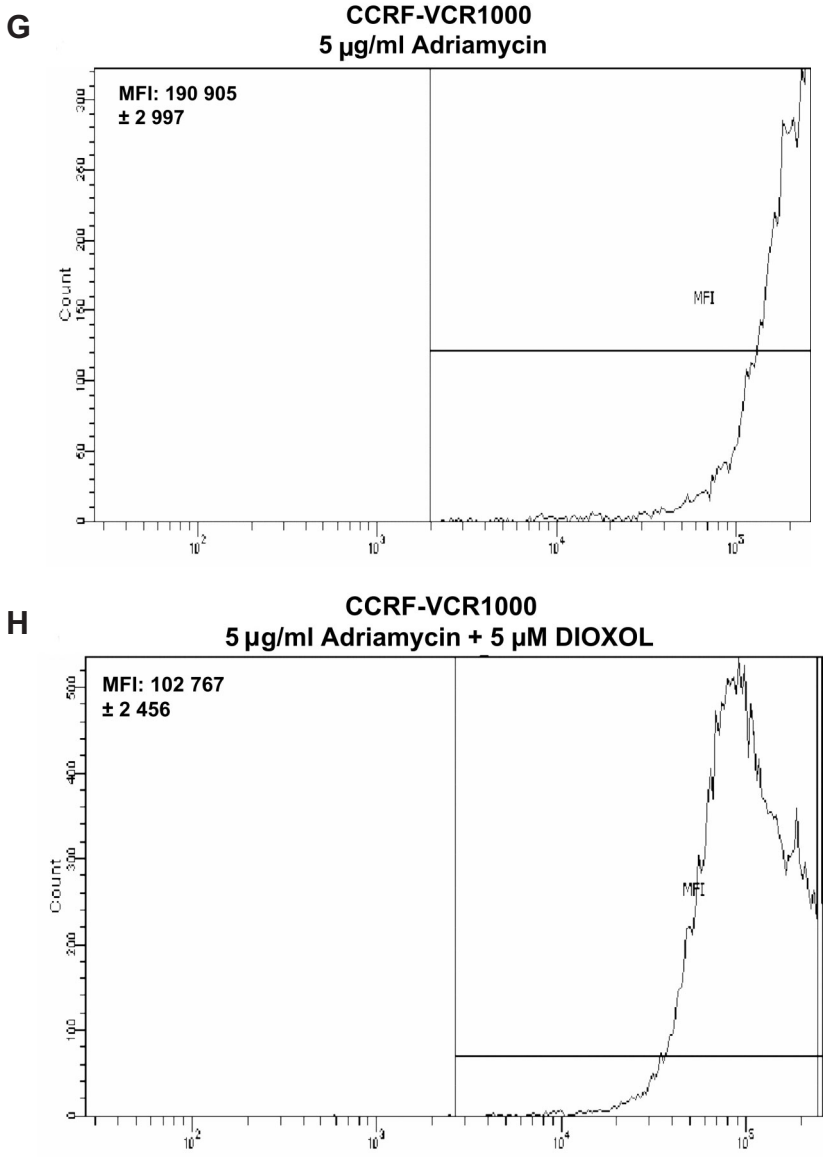
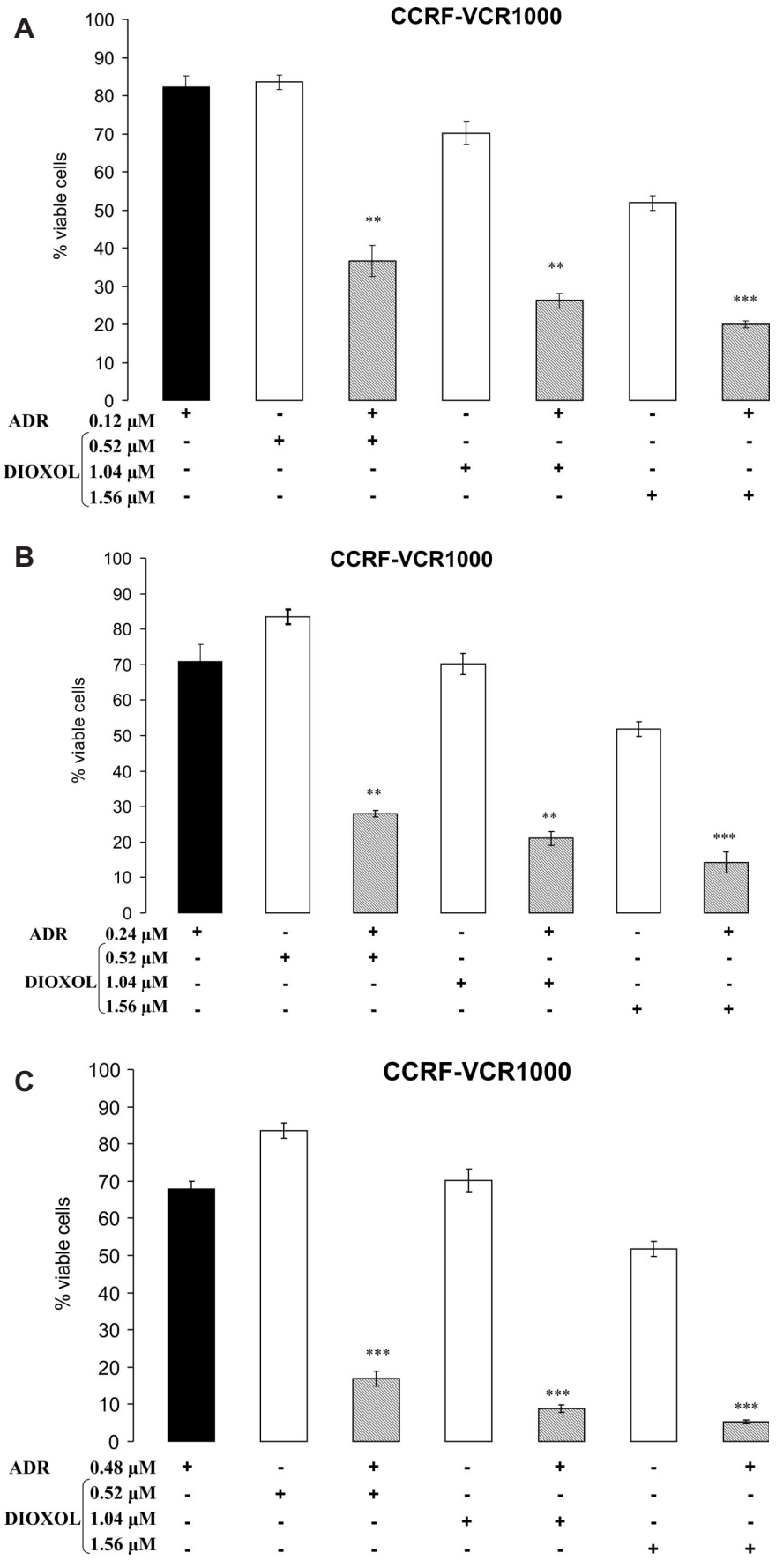


Fig. 6. Flow cytometric analysis of P-gp in (**A**, **B**) CCRF-ADR5000 and (**C**–**H**) CCRF-VCR1000. Cells were treated with DIOXOL (0.5–5 µM), adriamycin (5 µg/ml), a combination of both compounds (5 µM and 5 µg/ml, respectively) and 10 µM verapamil (data not shown). Values of MFI are shown as small inserts within the histograms. Results are the means ± SD of three independent experiments performed in duplicates

the fluorescence peak associated with the FITC-conjugated antibody. Compared to untreated control cells, samples incubated with 5 µM DIOXOL showed 30% (CCRF-ADR5000) and 47% (CCRF-VCR1000) lower P-gp levels. The percentage was calculated using the mean fluorescence intensity (MFI) values measured by cytometric analysis by comparison of MFI obtained from treated cells to that of untreated cells.

Both methods proved that 5 µg/ml adriamycin induced significant upregulation of P-gp in CCRF-VCR1000 cells. On the histogram obtained for this sample, the fluorescence peak was situated further to the right than for the untreated cells (45% increase compared to untreated cells in the cytometry assay, Fig. 6G). Co-treatment with 5 µM DIOXOL completely abolished adriamycin-induced P-gp activity and even decreased the P-gp level below that detected


A
CCRF-VCR1000
% viable cells
100
90
80
70
60
50
40
30
20
10
0
**
**
***
ADR 0.12 μM + - + - + - +
DIOXOL 0.52 μM - + + - - - -
1.04 μM - - - + + - -
1.56 μM - - - - - + +
B
CCRF-VCR1000
% viable cells
**
**
***
ADR 0.24 μM + - + - + - +
DIOXOL 0.52 μM - + + - - - -
1.04 μM - - - + + - -
1.56 μM - - - - - + +
C
CCRF-VCR1000
% viable cells
***
***
***
ADR 0.48 μM + - + - + - +
DIOXOL 0.52 μM - + + - - - -
1.04 μM - - - + + - -
1.56 μM - - - - - + +

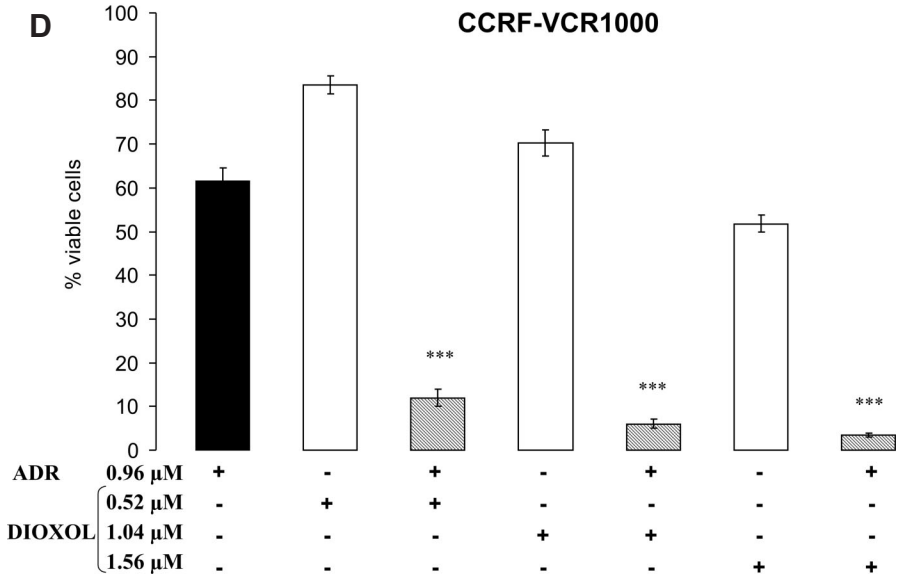


Fig. 7. Sensitising of multidrug resistant CCRF-VCR1000 cells to adriamycin by DIOXOL. Leukemic cells were treated with four concentrations of adriamycin (ADR): (**A**) 0.12 µM, (**B**) 0.24 µM, (**C**) 0.48 µM and (**D**) 0.96 µM and three concentrations of DIOXOL: (**A**–**D**) 0.52 µM, 1.04 µM and 1.56 µM, alone or in a combination, for 72 h. Changes of cell viability were assessed with the use of MTT test. Results are the means ± SD of three independent experiments performed in duplicates (** $p < 0.01$, *** $p < 0.001$)

for untreated cells (22% decrease compared to untreated cells in cytometry test, Fig. 6H) and was related to a leftward shift on the histogram. Neither CCRF-ADR5000 cells nor CCRF-VCR1000 cells, treated with 10 µM of the model P-gp inhibitor verapamil, showed any changes in P-gp level. We did not note any significant difference in measured MFI values between control (untreated) cells and verapamil treated samples (data not shown).

DIOXOL acts synergistically with adriamycin

To determine whether DIOXOL is able to increase the antiproliferative activity of a P-gp transported anticancer drug, a combination with adriamycin was tested in CCRF-VCR1000 cells. As demonstrated, CCRF-VCR1000 cells were more resistant to this anthracycline than CCRF-ADR5000 (Tab. 1). Therefore, these cells were selected for further experiments. CCRF-VCR1000 cells were almost 70 times more resistant to adriamycin than wild-type CCRF-CEM cells (Tab. 1). Drug combination studies were based on dose-response curves. The synergistic decrease of cell viability by DIOXOL and adriamycin was documented by MTT assays, computing the combination index (CI) and isobologram analysis. Cells were treated with each agent alone and in combination for 72 h and the percentage of living cells was evaluated. It was revealed that DIOXOL sensitized the multidrug resistant cells to adriamycin (Fig. 7A–D). The combination of the two compounds significantly decreased cell viability when compared to adriamycin or DIOXOL used separately. Even the two lowest concentrations of both compounds (adriamycin 0.12 µM, DIOXOL 0.52 µM, Fig. 7A) decreased the viability of CCRF-VCR1000 cells by 63%, while adriamycin alone reduced it by 28%, and DIOXOL by 27% (relative to control, untreated cells). Higher triterpenoid concentrations were much more potent (Fig. 7B–D). We observed almost completely reduced cells viability treated with 1.56 µM DIOXOL and 0.92 µM adriamycin (4% of living cells comparing to untreated control cells, Fig. 7D). To explain the type of interaction between the drugs, the Chou and Talalay equation was used. Two experimental points (combinations of DIOXOL and adriamycin in concentrations indicated in the figure caption) are plotted alongside the curve and are located below the dotted line at CI = 1 (Fig. 8). In our experiment CI values were 0.14, 0.1 and 0.08 at drug concentrations that induced 50, 75 or 90% cell death, respectively.

Treatment of sensitive parental CCRF-CEM cells with drug combinations did not enhance the antiproliferative activity of adriamycin (data not shown).

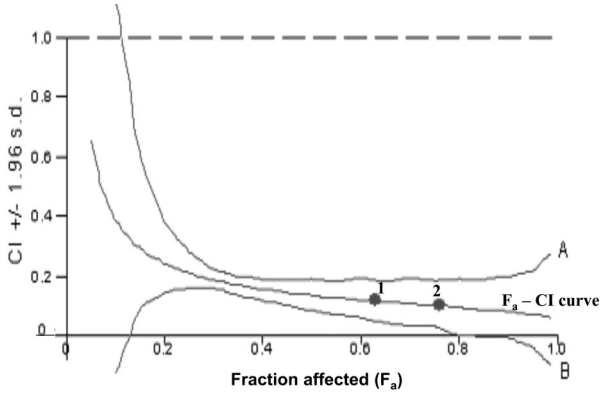


Fig. 8. Isobologram graph of DIOXOL and adriamycin synergy. The two points marked on the curve correspond to combinations of DIOXOL and adriamycin in concentrations: 0.52 and 0.12 μM (1); 1.04 and 0.24 μM (2), respectively. The graph was drawn using CalcuSyn software. Additional curves (indicated as **A** and **B** on the graph) limit the range of standard deviation

Discussion

Acquired or intrinsic MDR is an important impediment in the chemotherapy of malignant diseases [22], including leukemia [26]. ALL is the most common oncological disease diagnosed in children and, despite the great progress of medicine during recent decades, there is still great number of therapeutic failures in leukemia treatment [10] and the majority of patients who relapse die of leukemia. This indicates that the treatment regimen used in ALL patients is not always effective [43]. Frequently unsatisfactory outcomes are due to the MDR1/P-gp/ABCB1 phenotype of transformed leukemia cells [17]. Therapy of multidrug resistant leukemia is a serious clinical problem because several anti-neoplastic drugs are substrates of ABC proteins (etoposide, mitoxantrone, daunorubicin) and thus, they cannot exert their tumoricidal effects [28]. Therefore, the development of P-gp modulators which would overcome this type of drug resistance is a great challenge.

Our current study was performed in order to determine whether semisynthetic derivatives of OA are able to overcome resistance and, if so, what is the mechanism of their action. To our knowledge the group of compounds investigated in our laboratory have not yet been tested in MDR malignant cells. However, there are some circumstances giving hope for clinical application in anticancer practice. Data presented in the literature indicate that some natural triterpenoids probably are not transported *via* P-gp because they show similar cytotoxic activity in both sensitive and resistant cells [11]. OA and its synthetic derivative 2-cyano-3,12-dioxoolean-1,9(11)- dien-28--olic acid (CDDO) have been widely investigated in recent years [23, 25] but, in spite of this, very little is known about their effect on MDR cells. Therefore, we analyzed whether compounds derived from OA possess the ability to kill MDR cells and, for the first time, we proved their structure-dependent multidrug resistance modulating ability.

It was reported by Fernandes et al. [13] that OA caused a cytotoxic effect in vincristine- resistant Lucena 1 cells expressing P-gp. We also observed the reduction of MDR cells viability after treatment with OA, but the potency of the compound was significantly higher against sensitive CCRF-CEM cells, what is in accordance with the finding of the research group investigating the activity of OA isolated from *C. edulis* in parental and multidrug resistant mouse T-cell lymphoma cells [30]. The results obtained in our laboratory prove that chemical modifications at positions C-3, C-11 and C-28 leading to DIOXOL creation significantly improve the biological activity of parental compound in the context of MDR modulation. Substitution of O= atoms at C-3 and C-11 positions as well as esterification of COOH- group at C-28 position gave the compound with a higher potency against resistant than sensitive leukemic cells. Moreover, it should be pointed out that two completely inefficient compounds (considering cell viability inhibition activity) i.e., (5) and (7) possessed the chemical modification at C-12. The substitution of O= atom at position C-3 of those compounds with =NOH group increased activity of compounds (6) and (8). These data indicate that the type of functional group and position(s) of substitution play an important role in the biological activity of OA derivatives.

With regard to the obtained data, we decided to investigate whether the most active OA derivative, DIOXOL, possesses the ability to modulate *ABCB1* gene expression and/or to influence the level or transport *via* P-gp. Using quantitative real-time PCR we confirmed *ABCB1* overexpression in CCRF-ADR5000 and CCRF-VCR1000 cells. As described previously, the level of *ABCB1* mRNA in CCRF-ADR5000 is higher than in CCRF-VCR1000 [36], which was also confirmed in our studies by Ct determination (ΔΔCt) in qPCR analysis. In general, our results concerning

the basal level of the *ABCB1* transcript correspond to a previous study [36]. The only difference is that we detected a very low *ABCB1* mRNA level in parental CCRF-CEM cells, but the authors of the mentioned report used a significantly less sensitive semiquantitative RT-PCR method. As described by Yagüe et al. [44] selection for drug resistance by long-term exposure to the drug leads to the resistant line emerge, in which the translational block is overcome – *ABCB1* mRNA is stabilized and P-gp is synthesized. The *ABCB1* transcript in native (sensitive) cells has a very short half-life (1–2 h), which can account for the low steady-state levels of P-gp, whereas in resistant cells *ABCB1* mRNA half-life increases 10–20 fold. We have proven for the first time that the semisynthetic OA-derivative, DIOXOL acts as the *ABCB1* gene expression modulator. Results of the real-time PCR assay suggest that the compound may be able to act at the transcriptional level or affect *ABCB1* mRNA stability. Until now the type of interaction with the gene is unknown.

It was previously found that OA used in the concentration range of 1 μg/ml (ca. 2.2 μM) – 10 μg/ml (ca. 22 μM) exerted inhibitory effect on ABCC1 (MRP1) transporter protein activity, but it had no effect on the activity of P-gp in embryonic Ma104 monkey kidney epithelial cells [5]. Performing a functional test, we provided another evidence for the beneficial effect of OA basal structure modification. It should be pointed out that 10 μM DIOXOL was able to inhibit transport activity of P-gp in multidrug resistant leukemia cells. However, we noticed significantly higher activity of 10 μM verapamil compared to 10 μM DIOXOL. Verapamil acts as a model P-gp inhibitor [15]. Cells treated with this ion channel blocker were used as positive controls. Verapamil is a substrate of P-gp and inhibits drug transport in a competitive manner without interrupting the catalytic turnover of the protein [27]. Thus, some authors claim that verapamil, as a multidrug reversing agent, given in combination with other ABCB1 substrates is preferentially transported to the extracellular environment [28]. The difference in the potency of DIOXOL and verapamil in the same concentration of 10 μM may be due to their distinct mechanisms of action (in this concentration). Our current results indicate that 10 μM verapamil was able to inhibit rhodamine 123 transport *via* P-gp, but contrary to the tested triterpenoid – DIOXOL, did not change *ABCB1* expression or P-gp level. However, in the literature there are described some cases, which prove that verapamil used in higher concentrations (15 μM and higher) may affect the expression of *ABCB1* gene. Muller et al. [32] demonstrated that this agent in concentration of 15 μM induced 3-fold reduction in P-gp expression after 72 h exposure of K562/ADR cells. It was also shown that a decrease in *ABCB1* mRNA levels after 24 h verapamil treatment was dose related at concentrations between 15 and 50 μM [33]. It should be pointed out that verapamil in concentration of 2 mg/ml (ca. 4.4 μM) is cardiotoxic [30], thus its clinical use for the treatment of MDR cancers is limited.

It was interesting to test whether DIOXOL was able to improve the activity of other antineoplastic drugs. To answer this question, CCRF-VCR1000 cells were treated with a combination of DIOXOL and adriamycin. This anthracycline belongs to a group of P-gp substrates [18] and is one of the principal drugs in the chemotherapy protocols for ALL [6]. The main side effect of adriamycin is cardiotoxicity [42]. Thus, the development of drugs which can be used in combination, and thereby reducing unwanted toxic effects by decreasing the dose, is of great importance.

Although *ABCB1* expression in CCRF-1000VCR cells is lower than in CCRF-ADR5000, somehow cells showing lower MDR status are selectively more resistant to adriamycin than CCRF-ADR5000 cells, whereas cells with higher level of *ABCB1* gene expression (CCRF-ADR5000) were more resistant to vinblastine and miltefosine, that was found out previously [36].

We proved that DIOXOL and adriamycin act synergistically in CCRF-VCR1000 cells. This interaction seems to result from the multidrug resistance reversing ability of DIOXOL. Possibly the concomitant application of the drugs could reduce the required effective dosage and the side effects of adriamycin, but further studies concerning DIOXOL pharmacokinetic are needed.

Our experiments showed that short-term (60–150 min) treatment of MDR cells with an intermediate concentration of DIOXOL (10 μM) inhibited the transport function of P-gp (Fig. 3B), whereas exposure of these cells for a longer time (24 h) to lower concentration (5 μM) caused a reduction in the expression of the *ABCB1* gene (Fig. 4), leading to lower P-gp levels (Figs. 5 and 6). This observation is very important from the clinical point of view, because it indicates that even a short exposure to the drug results in a beneficial effect. The experimental procedure was designed to allow demonstration of the biological activity of the investigated triterpenoid at different time intervals.

Most pre-clinical and clinical efforts to overcome MDR have been focused on the modulation of P-gp activity, but compounds which inhibited the function of the protein had limited success and led to undesired side effects [14]. For this reason, some authors have indicated that *ABCB1* expression may be a more appropriate target [41, 47]. Moreover, recently the strategy of identification of new, non-P-gp substrates, anticancer agents becomes a new standard approach in the field of new drug design and development [15]. The outcomes of clinical trials have demonstrated the involvement of ABCB1 protein in treatment failure of leukemia patients [4, 44]. Thus, our findings may be of great importance. DIOXOL ability to modulation of molecular target at different levels (the reduction of cell survival, down-modulation of the *ABCB1* gene, inhibition of P-gp transport activity and synergism with adriamycin) make it an interesting compound for the modulation of MDR. Altogether, these observations make DIOXOL a candidate compound for the treatment of leukemia and further studies are under progress addressing its potential application for combating this disease.

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