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Effect of Cycloartanes on Reversal of Multidrug Resistance and Apoptosis Induction on Mouse Lymphoma Cells

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Abstract. *The ability of fifteen cycloartanes, isolated from Euphorbia species, to reverse multidrug resistance (MDR) and apoptosis induction in L5178Y mouse lymphoma cells, including its multidrug-resistant subline, was studied by flow cytometry. Reversion of MDR was investigated using a standard functional assay with rhodamine 123 as a fluorescent substrate analogue. For the evaluation of apoptosis, the cells were stained with FITC-labeled annexin V and propidium iodide. The majority of the compounds were able to reverse MDR of the tested human MDR1 gene-transfected mouse lymphoma cells. Some of the compounds were able to induce moderate apoptosis in the PAR cell line, but this effect was less effective on multidrug-resistant cells. The results indicate that cycloartanes can be substrates of ABC transporters, which might compete with certain anticancer chemotherapeutics.*

Multidrug resistance (MDR) is considered to be a major obstacle in the chemotherapeutic treatment of cancer. One of the underlying mechanisms of MDR is cellular overproduction of efflux pumps belonging to the ABC-transporter superfamily, such as P-glycoprotein (P-gp), which acts as an efflux pump for the outward transport of various cytotoxic compounds, reducing their intracellular accumulation. Most MDR inhibitors are known to interact with P-gp and thereby inhibit efflux of antitumor agents (1, 2). Therefore, the simultaneous administration of chemotherapeutic agents with resistance modifiers can be effective in anticancer therapy (3). An alternative mechanism for cancer therapy is the selective induction of programmed cell death, apoptosis, by various compounds (4). Several chemotherapeutic compounds have been found to induce apoptosis, suggesting this is the main mechanism for their anti-cancer activity and that deregulation of the apoptotic pathway can confer drug resistance in cells (4).

Euphorbia species (*Euphorbia*ceae) are well known for their biological properties. They have been used in traditional medicine for the treatment of cancers, tumors and warts for hundred of years and references to their use have appeared in the literature of many countries (5). In recent studies, several diterpenes isolated from *Euphorbia* species have been identified as effective lead compounds for the reversal of multidrug resistance (6-8). One of the characteristics of these species is the presence of a latex which is very rich in isoprenic compounds, whose major constituents are tetra and pentacyclic triterpenes. Among the tetracyclic triterpenes, cycloartanes (9,19-cyclopropyl-triterpenes), which are generally found in large amounts, have been reported as cytotoxic agents against several tumor cells (9-16). Compounds **4**, **6** and **13** have revealed cytotoxicity against Ehrlich ascite tumour cells (15) and compounds **11** and **6** against P-388 cell line (16) (Figure 1).

The purpose of the present work was to evaluate cycloartane triterpenoids isolated from *Euphorbia segetalis* (17, 18) and *Euphorbia portlandica*, as MDR modulators and apoptosis-inducers in mouse lymphoma cells.

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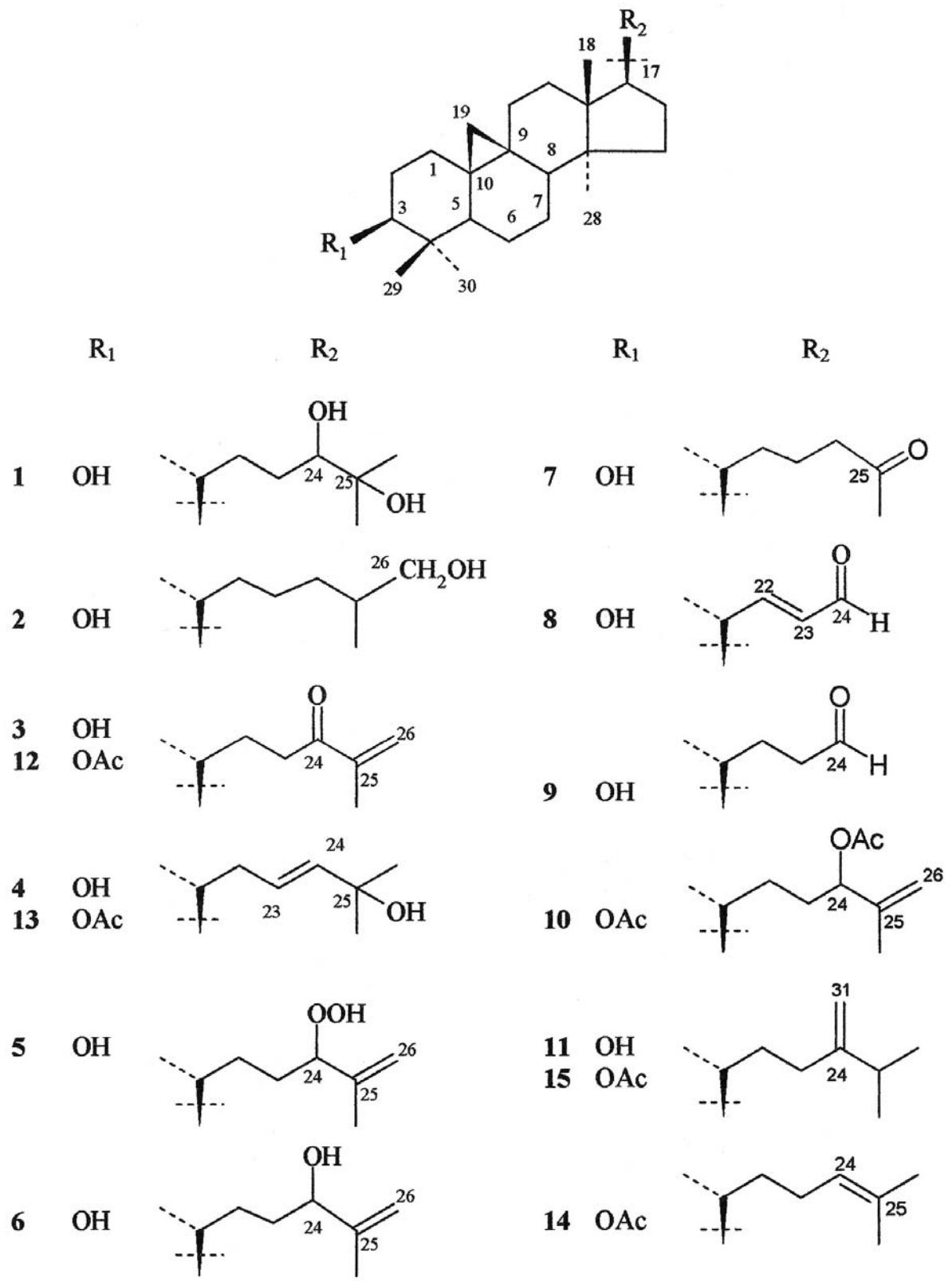

Figure 1. *Chemical structures of cycloartane triterpenes.*

Table I. *Effect of compounds **1-5** on reversal of multidrug resistance (MDR) on human MDR1 gene-transfected mouse lymphoma cells.*

Samples	Concentration (μg/mL)	FSC[a]	SSC[a]	FL-1[a]	Fluorescence activity ratio
PAR+R123 [b]	-	445.47	177.93	994.47	
PAR-R123	-	436.21	176.45	2.13	
MDR+R123 [c]	-	491.55	238.71	10.19	
Verapamil	10	519.73	257.24	38.39	3.76
1	4	484.60	222.02	24.34	2.39
	40	477.87	237.59	472.46	46.37
2	4	464.96	179.58	24.03	2.36
	40	420.31	185.81	399.97	39.25
3	4	430.02	191.13	6.91	0.68
	40	431.70	203.13	7.61	0.75
4	4	460.38	204.40	14.18	1.39
	40	457.75	221.17	556.46	54.61
5	4	465.87	222.77	8.67	0.85
	40	444.14	230.11	296.58	29.11
DMSO	20 μl	441.08	230.65	6.83	0.67

[a] FSC: Forward scatter count; SSC: Side scatter count; FL-1: Fluorescence intensity.
[b] Par: a parental cell without MDR gene.
[c] MDR: a parental cell line transfected with human MDR1 gene.

Table II. *Effect of compounds **6-15** on reversal of multidrug resistance (MDR) on human MDR1 gene-transfected mouse lymphoma cells.*

Samples	Concentration (μg/mL)	FSC[a]	SSC[a]	FL-1[a]	Fluorescence activity ratio
PAR+R123 b	-	344.17	123.20		
PAR-R123	-	350.61	135.15		
MDR+R123 c	-	422.55	168.50	10.17	
Verapamil	10	482.73	207.49	25.41	2.50
6	4	484.32	198.35	18.96	1.86
	40	329.25	210.71	Toxic	
7	4	398.28	152.67	13.58	1.34
	40	339.80	198.29	Toxic	
8	4	410.39	160.12	28.82	2.83
	40	317.06	146.00	203.25	19.99
9	4	431.22	171.10	14.37	1.41
	40	377.45	224.95	Toxic	
10	4	460.64	182.21	15.72	1.55
	40	379.85	149.74	10.60	1.04
11	4	371.14	154.76	16.75	1.65
	40	365.98	146.73	9.32	0.92
12	4	333.38	187.07	23.54	2.31
	40	336.28	167.01	Toxic	
13	4	380.55	148.12	10.58	1.04
	40	368.92	151.09	15.11	1.49
14	4	374.86	145.39	15.24	1.50
	40	343.60	178.25	176.76	17.38
15	4	357.75	147.02	10.59	1.04
	40	365.02	156.45	16.06	1.58
DMSO	20 μl	361.73	152.42	11.27	1.11

[a] FSC: Forward scatter count; SSC: Side scatter count; FL-1: Fluorescence intensity.
[b] Par: a parental cell without MDR gene.
[c] MDR: a parental cell transfected with human MDR1 gene.

Materials and Methods

Compounds. Fifteen different cycloartane triterpenes, whose structures are presented in Figure 1, were tested for reversal of MDR and apoptosis induction. The tested set differ by the substitution and length of their side-chain and/or substitution at C-3: 9,19-cyclolanostane-3β,24,25-triol **(1)**, 9,19-cyclolanostane-3β,26-diol **(2)**, 3β-hydroxy-9,19-cyclolanost-25-en-24-one **(3)**, 9,19-cyclolanost-23-ene-3β,25-diol **(4)**, 24-hydroperoxy-9,19-cyclolanost-25-en-3β-ol **(5)**, 9,19-cyclolanost-25-ene-3β,24-diol **(6)**, 3β-hydroxy-4,4,14-trimethyl-26-*nor*-9,19-cyclolanostan-25-one **(7)**, (22*E*)-3β-hydroxy-4,4,14-trimethyl-9,19-cyclochol-22-en-24-al **(8)**, 3β-hydroxy-4,4,14-trimethyl-9,19-cyclocholan-24-al **(9)**, 9,19-cyclolanost-25-ene-3β,24-diacetate **(10)**, 24-methylene-9,19-cyclolanostan-3β-ol **(11)**, 3β-acetoxy-9,19-cyclolanost-25-en-24-one **(12)**, 3β-acetoxy-9,19-cyclolanost-23-en-25-ol **(13)**, 9,19-cyclolanost-24-en-3β-acetate **(14)**, 24-methylene-9,19-cyclolanostan-3β-acetate **(15)**.

Compounds **10**, **12**, **13-15** were isolated from the acetone extract of *Euphorbia segetalis* L. and identified as previously described (17, 18). Compounds **1-9** and **11** were isolated from the acetone extract of *Euphorbia portlandica* L. and identified by spectroscopic methods. Their isolation and identification will be reported elsewhere. The purity of the compounds was more than 95 % by GC or HPLC analysis. 12H-benzo(α)-phenothiazine, used as an apoptosis inducer, was synthesized by Motohashi *et al.* (19). All compounds were dissolved in DMSO.

Chemicals. Annexin-V labeled with FITC (Alexis Company, Grunberg, Germany); propidium iodine, rhodamine 123, verapamil and colchine (Sigma). All other chemicals used were of analytical grade.

Cells. The L5178 Y mouse T-lymphoma parental cell line was transfected with the pHa MDR1/A retrovirus as previously described (20). The L5178 MDR cell line and the L5178 Y parental cell line (obtained from Prof. M. Gottesmann, NCI and FDA, USA) were grown in McCoy's 5A medium with 10% heat-inactivated horse serum, L-glutamine and antibiotics. MDR1-expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine, to maintain expression of the MDR phenotype. Cell viability was determined by trypan blue.

Assay for rhodamine 123 accumulation test. The harvested cells were resuspended in serum-free McCoy's 5A medium and distributed into Eppendorf tubes at a density of 2 x 10^6 cell/mL. Then, 2 to 20 μl of the stock solution (1 mg/mL in DMSO) of the tested compounds were added and the samples were incubated for 10 min at room temperature. Following the addition of 10 μl of rhodamine 123 to the samples (5.5 μM final concentration), the cells were further incubated for 20 min at 37˚C, washed twice and resuspended in 0.5 mL phosphate-buffered saline (PBS) for analysis. The fluorescence uptake of the cells was measured by flow cytometry using a Beckton Dickinson FACScan instrument equipped with an argon laser. The fluorescence excitation and

Table III. *Effect of compounds **6-9**, **12** and **14** on reversal of multidrug resistance (MDR) on human MDR1 gene-transfected mouse lymphoma cells at 10 and 20 μg/mL concentration.*

Samples	Concentration (μg/mL)	FSC[a]	SSC[a]	FL-1[a]	Fluorescence activity ratio
PAR+R123	-	496.11	151.66	882.85	
PAR-R123	-	506.17	159.71		
MDR+R123	-	614.39	210.31	13.06	
Verapamil	10	618.47	214.82	139.85	10.71
6	10	622.75	219.03	213.87	16.38
	20	598.39	231.42	488.02	37.37
7	10	641.02	217.60	32.96	2.52
	20	623.22	233.80	146.46	11.21
8	10	648.48	220.38	531.59	40.70
	20	590.07	196.50	675.48	51.72
9	10	636.97	202.08	24.28	1.86
	20	636.22	216.32	44.32	3.39
12	10	621.93	218.59	9.77	0.75
	20	615.18	211.39	1.08	0.08
14	10	667.20	204.13	162.44	12.43
	20	669.04	205.36	169.21	12.96
DMSO	10 μl	632.36	201.55	22.26	1.7

[a] FSC: Forward scatter count; SSC: Side scatter count; FL-1: Fluorescence intensity.
[b] Par: a parental cell without MDR gene.
[c] MDR: a parental cell transfected with human MDR1 gene.

Table IV. *Effect of compounds **1-15** on apoptosis induction in PAR cell line.*

Samples	Concentration (μg/mL)	Early apoptosis (%)	Apoptosis (%)	Cell death (%)
Cell control without staining		0.1	0.21	0.002
Cell control + Propidium Iodide		0.001	0.33	5.43
Cell control +AnnexinV		6.33	0.2	0.03
Cell -control – Double Staining		3.75	2.21	0.79
DMSO-control Annexin V + Propidium Iodide		0.17	0.21	0.02
12H-benzo(α)-phenothiazine	50.0	12.34	10.14	2.06
1	10.0	6.15	3.80	0.58
2	10.0	6.36	3.68	0.35
3	10.0	4.44	2.75	0.65
4	10.0	5.72	3.77	0.47
5	10.0	8.40	3.91	0.52
6	10.0	6.11	4.49	0.61
7	10.0	3.43	2.34	3.30
8	10.0	12.13	5.91	0.76
9	10.0	8.39	2.43	0.58
10	10.0	5.20	3.84	0.52
11	10.0	4.71	4.39	1.64
12	10.0	7.58	4.02	0.51
13	10.0	4.41	2.29	2.38
14	10.0	4.88	2.99	0.92
15	10.0	5.77	4.02	1.01
Second control An-PI-		0.26	0.27	0.01

emission wavelengths were 488 nm and 520 nm, respectively. Verapamil was used as a positive control and the influence of DMSO on the cells was monitored. The mean fluorescence intensity was calculated as a percentage of the control for the parental (PAR) and MDR cell lines as compared to untreated cells. An activity ratio (R) was calculated on the basis of the measured fluorescence values (FL-1) measured using the following equation (21, 22):

$R = (FL\text{-}1_{MDR\ treated}/FL\text{-}1_{MDR\ control})/(FL\text{-}1_{parental\ treated}/FL\text{-}1_{parental\ control})$

Assay for apoptosis induction. The assay was carried out according to the protocol of Alexis Bichemicals (23) with little modification. The cells were incubated in the presence of the compounds for 40 min at 37˚C, then the samples were washed in PBS. The harvested cells were resuspended in culture medium and distributed to 26-well tissue culture plate in 1 mL aliquots, followed by the incubation of the plate for 24 h at 37˚C, 5% CO_2. The treated cells were then transferred into small centrifuge tubes, centrifuged, washed in 0.5 mL PBS and resuspended in 195 μL binding buffer. 4.5 μL Annexin V-FITC were added to the samples, which were incubated at room temperature for 10 min in dark. Finally, the cells were washed in PBS, resuspended in 190 μL binding buffer and 10 μL of a 20 μg/mL propidium iodide stock solution were added to the samples (final conc. 1μg/mL). The fluorescence activity (FL-1, FL-2) of the cells was measured and analysed on a Becton Dickinson FACScan instrument.

Results and Discussion

Recently, a large number of compounds, either naturally occurring products or synthetic, have been reported as potential MDR modifiers *in vitro*. However, clinical trials of these MDR inhibitors have revealed unacceptable side-effects or toxicity at the doses required for effectiveness. Therefore, a promising strategy is to search for potent modulators without side-effects and low toxicity (24). It is widely accepted that, in cancer cells, many anticancer agents act as apoptosis-inducers and that the major reason for the unresponsiveness of cancer cells is the insufficiency of these drugs to trigger apoptosis (25).

The effects of cycloartanes **1-15** were tested on the reversion of multidrug resistance and apoptosis induction in the L5178Y mouse T-cell lymphoma drug-sensitive (parent) and MDR cells. Results for MDR are displayed in Table I, Table II and Table III. The well-known MDR modifier verapamil was applied as a control. Two concentrations (4 and 40 μg/mL; Tables I and II) were used in the experiments. Toxic compounds were also studied at 10 and 20 μg/mL concentration (Table III). Compounds **1**, **2**, **4-8** and **14** were shown to enhance drug retention in the cells by

Table V. *Effect of compounds **1-15** on apoptosis induction in MDR cell line.*

Samples	Concentration (μg/mL)	Early apoptosis (%)	Apoptosis (%)	Cell death (%)
Cell control without staining		0.04	0.13	0.08
Cell control +Propidium Iodide		0.0	1.12	3.38
Cell control+AnnexinV		1.56	0.65	0.11
Cell -control –Double Staining		0.74	1.56	2.24
DMSO-control AnnexinV + Propidium Iodide		0.0	1.95	4.50
12H-benzo(α)-phenothiazine	50.0	3.20	96.35	0.04
1	10.0	0.78	3.09	2.22
2	10.0	0.61	2.02	1.92
3	10.0	0.72	3.55	2.51
4	10.0	1.18	4.06	3.05
5	10.0	0.38	2.71	2.58
6	10.0	1.55	4.89	3.31
7	10.0	0.99	2.58	2.15
8	10.0	0.51	1.84	2.59
9	10.0	0.90	2.48	2.85
10	10.0	0.64	1.88	2.20
11	10.0	0.52	2.45	3.47
12	10.0	0.49	2.18	2.93
13	10.0	0.43	1.56	2.02
14	10.0	0.67	2.23	1.67
15	10.0	0.55	1.55	2.68
Second control An-PI-		0.25	0.30	0.18

inhibiting the efflux pump activity, mediated by P-glycoprotein. Compounds **3**, **10**, **11**, **13** and **15** were found to be ineffective in the MDR reversal assay and compounds **9** and **12** revealed to be toxic as well as **6** and **7** at 20 μg/mL, as indicated by the decreased cell size as expressed in the lower forward scatter count (FSC). The results showed concentration dependence for all the effective compounds. The compounds cycloartane-3β,24,25-triol (**1**) and cycloartane-23-ene-3β,25-diol (**4**) exhibited the highest effect in reversing MDR [fluorescence activity ratios R = 46.37 (**1**) and 54.61 (**4**) in 40 μg/mL concentration].

In order to investigate a structure-activity relationship, theoretical octanol/water partition coefficients for the tested compounds were calculated (26). No apparent correlation between $Log_{10} P$ and MDR-reversing ability was found. A free hydroxyl group at C-3 seems to be a significant feature for the effectiveness of the compounds. The acetyl derivatives at C-3 are inactive (**10, 13, 15**) or toxic (**12**). In the side chain, the existence of a tertiary hydroxyl group at C-25 also seems to lead to higher MDR reversal activity in **4** and **1**. These features are in accordance with the concept that the presence of hydrogen bond donor/acceptor groups and their spatial arrangement in the modulators correlate with anti-MDR potency (27).

The effects on apoptosis are summarized in Table IV and Table V. As can be observed, the death rate in the presence of the compounds is extremely low. The parent mouse lymphoma cells are more sensitive in the induction of early apoptosis by cycloartane triterpenes than the human MDR1 transfected subline. According to this data, compounds **5**, **8**, **9** and **12** can be considered as moderate apoptosis inducers. Since the initiated early apoptosis was not completed, the annexin positivity of the cells could be due to a membrane destabilisation (maybe induced as transient state between the liquid-crystalline phase), resulting in phosphatidyl serine translocation from the inner side of the membrane to the outer side.

These results indicate that cycloartane triterpenes can be considered as substrates for the ABC transporters operating in the lymphoma cells, and these compounds might compete with some anticancer drugs in the MDR-reversal and antiproliferative action.

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