Multidrug Resistance Modulation and Apoptosis Induction of Cancer Cells by Terpenic Compounds Isolated from *Euphorbia* Species

NOÉLIA DUARTE[1], CÁTIA RAMALHETE[1], ANDRAS VARGA[2], JOSEPH MOLNÁR[3] and MARIA-JOSÉ U. FERREIRA[1]

[1]*iMed.UL, Faculty of Pharmacy, University of Lisbon, 1649-003 Lisbon, Portugal;*
[2]*Department of Molecular Parasitology, Humboldt University, D-10115 Berlin, Germany;*
[3]*Department of Medical Microbiology, University of Szeged, H-6720 Szeged, Hungary*

Abstract. *Background: One of the most promising strategies to overcome multidrug resistance (MDR) is to use compounds that can modulate P-glycoprotein and restore the cytotoxicity of anticancer drugs. Furthermore, the search for compounds that regulate and overcome apoptosis deficiency of cancer cells is also of great therapeutic importance. Materials and Methods: Seven known pentacyclic triterpenes and one steroid were isolated from Euphorbia lagascae methanolic extracts and identified by physical and spectroscopic methods. These compounds, together with eleven terpenoids previously isolated from Euphorbia lagascae and E. tuckeyana were tested for their MDR-reversing and/or apoptosis induction activities by flow cytometry on L5178 human MDR1 gene-transfected mouse lymphoma cells. Results: Four taraxastane-type triterpenes: 21α-hydroxytaraxasterol, 21α-hydroxytaraxasterol acetate, 3β,30-dihydroxy-20(21)-taraxastene and 3β-hydroxy-20-taraxasten-30-al, and two steroids: stigmastane-3,6-dione and ergosterol peroxide exhibited a significant MDR-Pgp modulation activity. Some aspects of structure–activity relationships are discussed. Regarding apoptosis induction, the most significant results were obtained for the polycyclic diterpenes ent-16α,17-dihydroxykauran-3-one and ent-16α,17-dihydroxyatisan-3-one.*

The emergence of multidrug resistance (MDR) in the chemotherapy of neoplastic diseases has made many of the currently available antitumour drugs ineffective (1). The most frequent and most studied mechanism of MDR is that resulting from the overexpression of P-glycoprotein (P-gp), which acts as an efflux pump and extrudes the drugs outside the cell (2, 3). A promising strategy to overcome MDR is to administer, simultaneously with the anticancer drugs, agents that can interact with the efflux proteins, namely P-gp modulators. However, although a great number of modulators from natural and synthetic origins have been reported, there are currently no reversal agents clinically available (4, 5). Therefore, it is extremely important to continue the search in order to discover suitable modulators. Furthermore, since most chemotherapeutic agents exert their anticancer activity by inducing apoptosis or programmed cell death, the search for compounds that regulate and overcome apoptosis deficiency of cancer cells is also of great therapeutic importance (6, 7).

In previous studies, we have reported the potent MDR modulation of several macrocyclic diterpenes isolated from *Euphorbia* species (8-10). Continuing our search for bioactive compounds, we have further isolated several terpenes, which include eight pentacyclic triterpenes, several steroids with stigmastane and ergostane skeletons, and various polycyclic diterpenes with *ent*-abietane, atisane and kaurane skeletons. Herein, we describe the isolation of some of these compounds from *Euphorbia* species, as well as their evaluation as multidrug resistance modulators and apoptosis inducers on L5178 human *MDR1* gene-transfected mouse lymphoma cells.

Materials and Methods

Compounds tested. Eighteen compounds, whose structures are presented in Figure 1, were tested for their anti-MDR and/or apoptosis induction activities: the pentacyclic triterpenes **1-8**, the steroids **9-14** and the polycyclic diterpenes **15-18**. All the compounds were dissolved in dimethylsulphoxide (DMSO).

Correspondence to: Professor Maria-José U. Ferreira, iMed.UL, Faculty of Pharmacy, University of Lisbon, Av. Prof. Gama Pinto1649-003 Lisbon, Portugal. Fax: +35 1217946470, e-mail: mjuferreira@ff.ul.pt

Key Words: Euphorbiaceae, *Euphorbia*, triterpenes, steroids, multidrug resistance, P-glycoprotein, apoptosis.

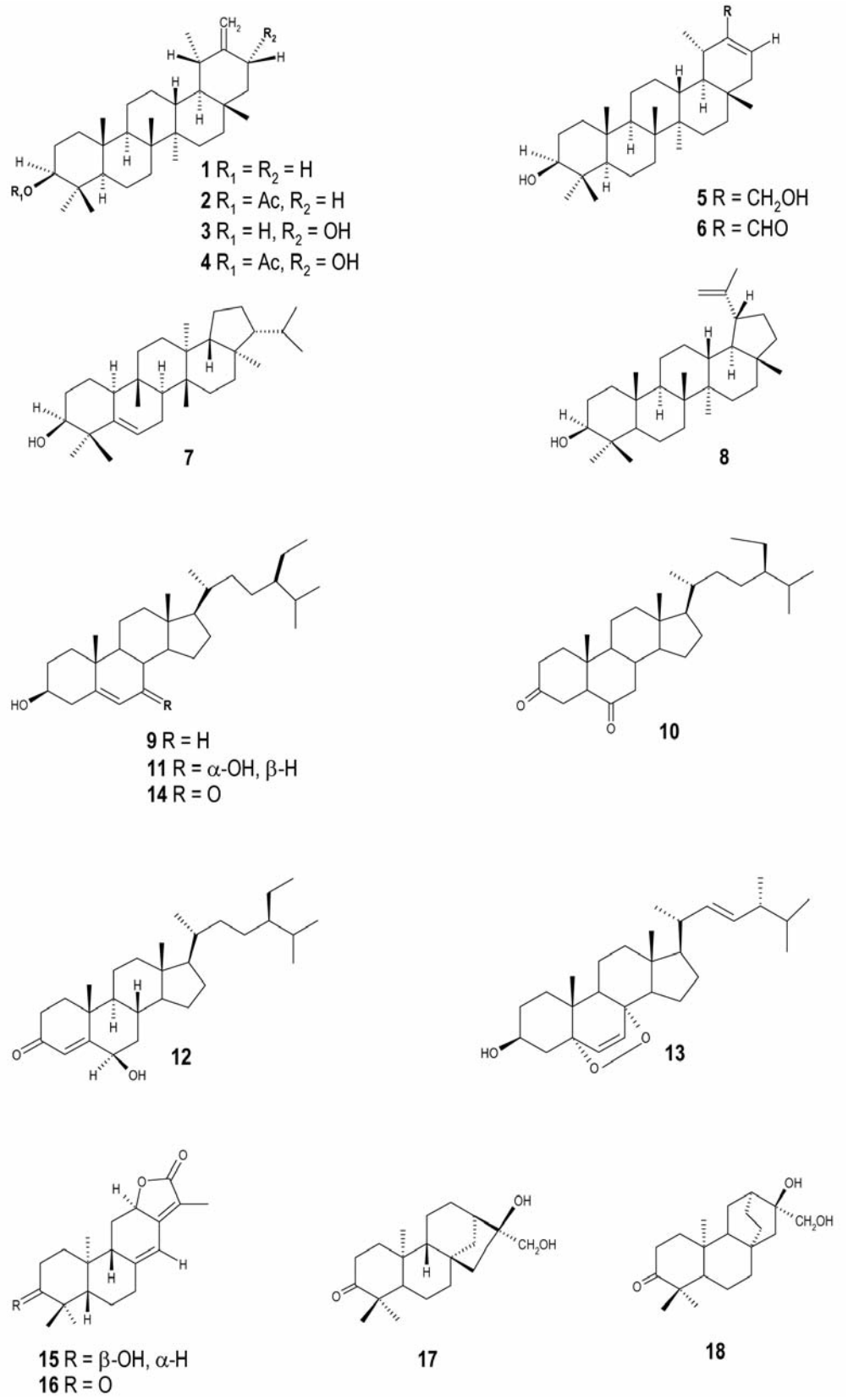

Figure 1. *Structures of compounds **1-18**.*

Compounds **6, 10-14** and **17-18** were isolated from the methanolic extract of the aerial parts of *E. lagascae* (8, 11, 12). Compounds **15** and **16** were isolated from the methanolic extract of the aerial parts of *E. tuckeyana* (13). Compounds **1-5** and **7-9** were isolated from *E. lagascae* as described below. The purity of the isolated compounds was more than 95%.

Extraction and isolation. *E. lagascae* was collected in Cova da Beira, Coimbra, Portugal, and identified by Dr. Teresa Vasconcelos of Instituto Superior de Agronomia, University of Lisbon (voucher specimen no. 323). The air-dried aerial parts were extracted with methanol and fractionated as previously described (8, 9) to obtain eight crude fractions (Fr A-H). Fraction A was recrystallized from Me_2CO to give 535 mg of compound **2** and 131 mg of compound **7**. Fraction B was recrystallized from Me_2CO yielding 5 mg of compound **1**. Fraction B was successively rechromatographed to give 17 mg of compound **8** and 5 mg of compound **4**. Column chromatography of fraction C afforded 354 mg of compound **9**. Compounds **3** (24 mg) and **5** (24 mg) were obtained from chromatography of fraction D.

Cell cultures. L5178 mouse T-cell lymphoma cells were transfected with pHa MDR1/A retrovirus, as previously described (14). MDR1-expressing cell lines were selected by culturing the infected cells with 60 ng/ml colchicine to maintain the expression of the MDR phenotype. L5178 (parent) mouse T-cell lymphoma cells and the human *MDR1*-transfected subline (obtained from Prof. M. Gottesmann, NCI and FDA, USA) were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum L-glutamine and antibiotics.

Assay for rhodamine-123 accumulation test (8, 9). Briefly, the harvested cells were resuspended in serum-free McCoy's 5A medium and distributed into Eppendorf tubes. Volumes of 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 rhodamine 123 to the samples, the cells were incubated, washed and resuspended in phosphate-buffered saline (PBS) for flow cytometry. The fluorescence uptake of the cells was measured by flow cytometry using a Beckton Dickinson FACScan instrument equipped with an argon laser and fluorescence excitation and emission wavelengths of 488 nm and 520 nm, respectively. Verapamil was used as a positive control. 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 (FAR) was calculated on the basis of the measured fluorescence values (FL-1) measured *via* the following equation:

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

Assay for apoptosis induction. The assay was carried out according to the protocol of Alexis Bichemicals (15). The cells were incubated in the presence of the compounds for 40 min at 37˚C, and 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 in 5% CO_2. The treated cells were transferred into small centrifuge tubes, centrifuged, washed in 0.5 ml PBS and resuspended in 195 μl binding buffer. Annexin V-FITC (4.5 μl) was added to the samples, which were incubated at room temperature for 10 min in the dark. Finally, the cells were washed in PBS, resuspended in 190 μl binding buffer and 10 μl of a 20 μl propidium iodide stock solution were added to the samples (final concentration 1 mg/ml). The fluorescence activity (FL-1, FL-2) of the cells was measured and analyzed on a Becton Dickinson FACScan instrument. 12H-Benzo(α)phenothiazine (M-627) was used as positive control.

Results

The triterpene 3β-hydroxy-20-taraxasten-30-al (**6**), the steroids stigmastane-3,6-dione (**10**), 7α-hydroxysitosterol (**11**), 6β-hydroxysitostenone (**12**), ergosterol peroxide (**13**) and stigmast-5-en-3β-ol-7-one (**14**), along with the *ent*-abietane lactones helioscopinolides B (**15**) and E (**16**), and the polycyclic diterpenes *ent*-16α,17-dihydroxykauran-3-one (**17**) and *ent*-16α,17-dihydroxyatisan-3-one (**18**) were previously isolated from *E. lagascae* and *E. tuckeyana* as reported (8, 11, 12). Further phytochemical studies of the ether-soluble fraction of the methanolic extract of *E. lagascae* have led to the isolation of eight pentacyclic triterpenes: taraxasterol (**1**), taraxasterol acetate (**2**), 21α-hydroxytaraxasterol (**3**), 21α-hydroxytaraxasterol acetate (**4**), 3β,30-dihydroxy-20(21)-taraxastene (**5**), simiarenol (**7**) and lupeol (**8**), and the steroid β-sitosterol (**9**). Compounds were identified by comparison of their physical and spectroscopic data with those reported in the literature (16-18).

The pentacyclic triterpenes **1-8** and the steroids **9-14**, were investigated for their MDR-reversal activity on *MDR1* gene-transfected L5178 mouse lymphoma cells, by flow cytometry, using the rhodamine-123 exclusion test. Verapamil, a well-known MDR modifier, was used as a positive control. Two concentrations (4.0 and 40.0 μg/ml) were applied in the experiments in order to find out significant effective compounds and obtain some information about the dose-dependent effects for further studies. The results are summarized in Table I. At the tested concentrations, the taraxastane-type triterpenes 21α-hydroxytaraxasterol (**3**; FAR=5.8 and 100.0), 21α-hydroxytaraxasterol acetate (**4**; FAR=2.2 and 49.1), 3β,30-dihydroxy-20(21)-taraxastene (**5**; FAR=4.6 and 12.1) and 3β-hydroxy-20-taraxasten-30-al (**6**; FAR=3.5 and 14.4) were able to reverse MDR, displaying strong activity at the highest concentration (Table I). However, taraxasterol (**1**) and its acetylated derivative (**2**) were found to be inactive in the MDR assay. On the other hand, the pentacyclic triterpenes simiarenol (**7**) and lupeol (**8**), both characterized by an E ring with five carbons, were also ineffective in modulating P-gp, even when tested at 40.0 μg/ml.

Concerning the steroid set (Table I), stigmastane-3,6-dione (**10**) and ergosterol peroxide (**13**), an ergostane-type steroid characterized by an unusual peroxide function, also enhanced drug retention in the cells in a dose-dependent mode, as shown by the increase of the FAR at the higher concentration (for **10**, FAR=3.7 and 37.0; for **13**, FAR=6.6 and 39.9 at 4.0 μg/ml and

Table I. *Effect of triterpenes **1-8** and steroids **9-14** on the reversal of multidrug resistance (MDR) in human MDR1 gene-transfected mouse lymphoma cells and their physicochemical properties (octanol/water partition coefficient and number of hydrogen bond acceptors and donors)*[a].

	logP	Number of H-bond		Conc. (μg/ml)	FSC	SSC	FL-1	FAR
		Acceptor	Donor					
PAR+R123					508.1	137.0	866.4	–
MDR+R123					521.4	166.6	5.1	–
Verapamil				10	520.4	168.7	69.3	13.7
Triterpenes								
1	8.1	1	1	4	525.4	172.7	6.6	1.3
				40	538.4	191.7	8.0	1.6
2	8.6	1	0	4	515.9	171.1	6.4	1.3
				40	524.7	166.5	5.9	1.2
3	7.2	2	2	4	591.3	197.5	43.0	5.8
				40	592.8	215.8	668.8	100.0
4	7.9	3	1	4	482.7	149.4	12.9	2.2
				40	490.9	157.7	282.1	49.1
5	7.1	2	2	4	551.8	182.9	54.9	4.6
				40	545.6	155.5	144.3	12.1
6	7.5	2	1	4	524.7	177.3	17.6	3.5
				40	559.0	171.5	73.0	14.4
7	8.6	1	1	4	518.7	170.0	5.7	1.1
				40	520.2	173.6	8.0	1.6
8	8.3	1	1	4	519.0	167.3	6.5	1.3
				40	513.8	169.6	7.6	1.5
Steroids								
9	8.6	1	1	4	473.0	203.2	4.0	1.0
				40	474.2	200.4	3.8	0.9
10	7.8	2	0	4	541.7	182.3	44.1	3.7
				40	533.4	192.9	440.0	37.0
11	7.9	2	2	4	477.6	208.9	5.2	1.3
				40	479.6	214.8	7.9	1.9
12	7.8	2	1	4	570.4	181.0	10.3	1.4
				40	564.6	189.6	11.2	1.5
13	6.7	3	1	4	492.1	208.7	26.8	6.6
				40	490.5	213.5	162.4	39.9
14	7.8	2	1	4	484.4	212.8	3.8	0.9
				40	479.2	213.9	4.0	1.0
DMSO				10 μl	513.6	167.9	5.2	1.0

The results of compounds **3, 5, 10** and **12** were obtained from different assays: for **3** and **12**: PAR+R123: FL-1=924.5; MDR+R123: FL-1=8.0; verapamil: FL-1=137.3, FAR=18.7; for **5** and **10**: PAR+R123: FL-1=971.9; MDR+R123: FL-1=12.8; verapamil: FL-1=128.8, FAR=10.8). [a]Physicochemical parameters were determined by using the JME molecular editor, version June 2009, http://www.molinspiration.com/. FSC: Forward scatter count; SSC: side scatter count; FL-1: mean fluorescence intensity of the cells. FAR: fluorescence activity ratio; PAR: a parent cell without *MDR1* gene; MDR: a parent cell line transfected with human *MDR1* gene; logP: log of the octanol-water partition coefficient.

40.0 μg/ml, respectively). Both compounds were much more active at the highest concentration tested. β-Sitosterol (**9**), 7α-hydroxysitosterol (**11**), 6β-hydroxysitostenone (**12**) and stigmast-5-en-3β-ol-7-one (**14**) exhibited no significant activity.

The apoptosis-inducing activity of triterpenes **1-8**, steroids **11-13** and polycyclic diterpenes **15-18**, was studied on *MDR1*-transfected L5718 mouse lymphoma cell line, by flow cytometry, using the FITC-annexin-V/propidium iodide assay described in the experimental section. The compounds were tested at 65 μg/ml (triterpenes and steroids) and 50 μg/ml (diterpenes). 12H-Benzo-(α)-phenothiazine, an apoptosis inducer, was used as positive control. The results are summarized in Table II. As can be seen, none of the evaluated compounds showed strong apoptosis induction activity. Regarding the effects of the triterpenes and steroids studied, at the tested concentration, the most significant results were obtained for the triterpenes 21α-hydroxytaraxasterol (**3**) and 3β,30-dihydroxy-(20)21-taraxastene (**5**), which induced 25.3% and 19.7% apoptosis, respectively. Concerning the diterpene set, the most effective compounds were *ent*-16α,17-dihydroxykauran-3-one (**17**) and *ent*-16α,17-dihydroxyatisan-3-one (**18**) reaching values of 31.4% and 26.5%, respectively.

Table II. *Effect of triterpenes **1-5** and **7-8**, steroids **11-13** and polycyclic diterpenes **15-18** on apoptosis induction in human MDR1 gene-transfected mouse lymphoma cells.*

	Conc. μg/ml	Early apoptosis %	Total apoptosis %	Necrosis %
Cell control without staining		0.02	0.04	0.10
Cell control annexin-V$^+$PI$^+$		3.0	18.1	2.3
Cell control annexin-V$^-$PI$^-$		0.1	18.3	0.01
M-627[a]	50	0.02	98.0	1.8
Triterpenes				
1	65	3.8	10.7	0.50
2	65	1.3	17.0	2.8
3	65	1.1	25.3	3.7
4	65	6.6	17.4	0.8
5	65	1.3	19.7	4.0
7	65	0.9	13.9	1.7
8	65	1.0	14.9	1.7
Steroids				
11	65	1.5	17.5	1.2
12	65	1.1	13.7	1.6
13	65	1.4	18.2	2.2
Polycyclic diterpenes				
15	50	4.8	14.5	0.6
16	50	3.8	17.7	0.8
17	50	1.7	31.4	6.2
18	50	1.3	26.5	5.7

PI: Propidium iodide; [a]positive control: M-627 (12H-benzo(α)phenothiazine).

Discussion

Continuing our search for bioactive compounds from *Euphorbia* species, in this study, we isolated and identified eight pentacyclic triterpenes (**1-5** and **7-8**) and one stigmastane steroid (**9**). These compounds and the previously isolated ones (**6**, **10-18**) were investigated for their MDR-reversing and apoptosis induction activities by flow cytometry on L5178 human *MDR1* gene-transfected mouse lymphoma cells. The taraxastane-type triterpenes 21α-hydroxytaraxasterol (**3**), 21α-hydroxytaraxasterol acetate (**4**), 3β,30-dihydroxy-20(21)-taraxastene (**5**) and 3β-hydroxy-20-taraxasten-30-al (**6**), and the steroids stigmastane-3,6-dione (**10**) and ergosterol peroxide (**13**) were shown to be significant MDR-Pgp modulators in a dose-dependent manner. The major differences between taraxastane triterpenes are in rings A and E. With respect to the A ring, the presence of an acetyl group at C-3 strongly reduced the ability to modulate P-gp, as can be concluded by comparison of the FARs found for 21α-hydroxytaraxasterol (**3**; FAR=5.8 and 100), and 21α-hydroxytaraxasterol acetate (**4**; FAR=2.2 and 49.1), at 4.0 μg/ml and 40.0 μg/ml, respectively. Moreover, taking into account the results obtained at the two concentrations tested for the pairs of compounds **1** and **3**, and **2** and **4**, it could also be deduced that the introduction of a hydroxyl group in the E ring (C-21) strongly increases the MDR-reversing ability of these triterpenes. These conclusions are corroborated by the results obtained for 3β,30-dihydroxy-20(21)-taraxastene (**5**) and 3β-hydroxy-20-taraxasten-30-al (**6**), which, besides the location of the double bond (C-20/C-30 for **1** and C-20/C-21 for **5** and **6**), differ from taraxasterol by an aldehyde or hydroxymethylene group at C-30. In fact, by increasing the H-bonding potential, these extra functional groups may be responsible for considerably improving the ability to modulate P-gp in compounds **5** (FAR=4.6 and 12.1 at 4.0 μg/ml and 40.0 μg/ml, respectively) and **6** (FAR=3.5 and 14.4 at 4.0 μg/ml and 40.0 μg/ml, respectively).

The steroids **9-12** and **14** revealed a large difference of FAR values. Nevertheless, they have very similar structures which differ only in the substitution pattern of rings A and B, and in the location of the double bond, which is absent from compound **10**. Therefore, this part of the steroid skeleton would seem to play an important role in their activity. Concerning the H-bonding potential, compounds **10-12** and **14** have in common the same number of H-bond acceptor groups. Apart from compound **10**, the number of H-bond donor groups is also common in **9-14**. Taking into account that stigmastane-3,6-dione (**10**), one of the compounds that showed significant activity, has two H-bond acceptor groups and no H-donor group, these results seem to corroborate the statements that the modulators should be H-bond acceptors rather than H-bond donors, since P-gp is a H-bond donor (19). As can be observed in Table I, all tested compounds are lipophylic (logP values between 6.7 and 8.6), supporting the importance of lipophilicity in the modulation of P-gp. However, it should be noted that among the fourteen compounds tested, and with the exception of compound **10**, those with the highest logP values (logP≥8), were inactive or exhibited weak activity. The most active compound (21α-hydroxytaraxasterol, **3**) has a logP value of 7.2. On the other hand, ergosterol peroxide (**13**), which has the lowest logP value (6.7), was also one of the most active compounds. Therefore, no apparent correlation between logP and MDR-reversing ability was found.

In conclusion, these results suggest that some triterpenes and steroids may be valuable as lead compounds for the development of P-gp modulators in multidrug-resistant cancer cells.

Acknowledgements

The Science and Technology Foundation, Portugal (FCT) and the Szeged Foundation for Cancer Research supported this work.

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Received June 30, 2009
Revised September 30, 2009
Accepted October 5, 2009