Bioorganic & Medicinal Chemistry 16 (2008) 9323–9330

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synergistic interaction between p-glycoprotein modulators and epirubicine on resistant cancer cells

Noélia Duarte [a], Anett Járdánházy [b], Joseph Molnár [b], Andreas Hilgeroth [c], Maria-José U. Ferreira [a,*]

[a] iMed.UL, Faculty of Pharmacy, University of Lisbon, Av. das Forças Armadas, 1600-083 Lisbon, Portugal
[b] Department of Medical Microbiology and Immunobiology, University of Szeged, H-6720 Szeged, Hungary
[c] Institute of Pharmacy, Martin Luther University, Wolfgang-Langenbeck Straße, 06120 Halle, Germany

ARTICLE INFO

Article history:
Received 14 May 2008
Revised 21 August 2008
Accepted 28 August 2008
Available online 31 August 2008

Keywords:
Multidrug resistance
P-glycoprotein
Lathyrane and jatrophane diterpenes
Molecular orbital energies

ABSTRACT

The macrocyclic lathyrane diterpene latilagascene B, previously isolated from *Euphorbia lagascae*, was acylated to afford three new diterpene esters, latilagascenes G (**1**), H (**2**), and I (**3**), whose structures were assigned by spectroscopic methods. These acyl derivatives, and the macrocyclic diterpenes of the jatrophane-type, tuckeyanols A (**4**) and B (**5**), and euphotuckeyanol (**6**), isolated from *Euphorbia tuckeyana*, were tested for P-gp modulating properties on human *MDR1* gene-transfected and parental L5178 mouse lymphoma cell lines. All the compounds displayed very strong activity. The molecular orbital energies (HOMO and LUMO) of diterpenes **1**–**6** and **7**–**13**, previously isolated, have also been calculated in order to estimate their probable charge transfer interactions with P-gp. Structure–activity relationships (SAR) are discussed. Furthermore, compounds (**1**–**6**) were assayed, in vitro, for their antiproliferative effects in combination with epirubicine and all of them synergistically enhance the effect of the antitumor drug.

1. Introduction

During recent decades, chemotherapy has become one of the most important tools against cancer. However, the emergence of drug resistance has resulted in many of the available anticancer drugs becoming ineffective.[1] Drug resistance, which can be natural or acquired, occurs in the latter case when a previously effective drug dose is no longer effective. Multidrug resistance (MDR), an acquired form of drug resistance, observed in tumor cells and microorganisms, consists in simultaneous resistance to chemically different drugs with different targets.[2] MDR is a complex multifactorial phenomenon that can result from a number of biochemical mechanisms, including a decreased drug uptake or an increased drug efflux; the overproduction of target enzymes or altered target enzymes; the altered metabolism of drugs; the increased repair of drug-induced DNA damage; or a failure to undergo apoptosis.[2,3] Although multiple mechanisms can be involved, the most significant mechanism of MDR, referred to as typical or classical MDR, is that resulting from the overexpression of ABC transporter proteins which act as energy-dependent pumps for chemotherapeutic agents, thereby decreasing their intracellular concentration. ABC proteins are expressed not only in cancer cells, but also in many normal tissues, such as the kidney, lung, liver, intestine, and blood–brain barrier, where one of their physiological roles is related to the cell detoxification processes.[4] The most important and best-studied ABC transporter is P-glycoprotein (P-gp), characterized by a broad substrate spectrum, which comprises mainly neutral or cationic amphiphilic molecules.[3]

Many strategies for the overcoming of P-gp-mediated MDR have been proposed. The strategy based on the development of compounds (MDR modulators, inhibitors or chemosensitizers) able to restore the cytotoxicity of the available anticancer drugs against MDR tumor cells, when used in combination with the latter, is among the most promising.[5] Consequently, since the discovery of verapamil as a P-gp inhibitor,[6] many studies have been carried out to find new and effective MDR modulators. However, although a large number of compounds, with a wide spectrum of chemical structures and biological activities, have been found to be able to reverse MDR, there are currently no reversal agents available clinically. Thus, the discovery of potent and specific P-gp modulators remains a great challenge. In order to achieve this goal, a number of structure–activity relationship (SAR) studies have been carried out. Nevertheless, only general molecular properties have been recognized as important factors, such as the amphiphilic character of the modulators, the presence of aromatic rings, and a positive charge at neutral pH. Hydrophobic and hydrogen bond acceptor interactions have also been considered important.[7] HOMO (highest occupied molecular orbital) energies have been calculated and discussed for numerous MDR modulators. High energy values of about −9 eV suggest electron donor activities of the modulators in charge transfer (CT) complexes with amino acids of a P-gp bind-

* Corresponding author. Tel.: +351 21 7946475; fax: +351 21 7946470.
E-mail address: mjuferreira@ff.ul.pt (M.U. Ferreira).

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.08.071

ing site.[8] The development of a general and conclusive SAR has been hampered by various limiting factors, mainly the lack of a high-resolution 3D structure of P-gp. Its co-expression with other ABC transporters in MDR tumor cells and its presence in normal tissues has posed a great challenge in the development of agents able to overcome the phenomenon of MDR selectively.

In a previous study, we reported the isolation and structure characterization of lathyrane-type diterpenes from *Euphorbia lagascae* which displayed strong antiMDR activity.[9,10] Continuing our search for effective MDR modulators,[9–13] and with the aim of finding a structure–activity relationship for macrocyclic diterpenes, we report here the preparation and structure elucidation of three new ester derivatives of the lathyrane diterpene latilagascene B, previously isolated from *E. lagascae*,[9] and their evaluation as MDR-reversal agents, together with three jatrophane diterpenes, isolated from *Euphorbia tuckeyana*.[14] Moreover, their combinations with the cytostatic epirubicine were tested in order to obtain evidence as to additive or synergistic interactions.

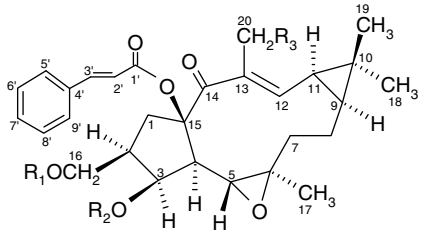
2. Results and discussion

2.1. Structure elucidation of compounds

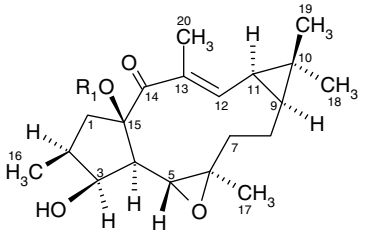
The lathyrane diterpene latilagascene B (**8**), previously isolated from the methanolic extract of *E. lagascae*,[9] was derivatized with several acylating reagents (see Section 3), to afford three new lathyrane esters: latilagascene G (**1**), H (**2**), and I (**3**), (Fig. 1). The structure elucidation of these new compounds was essentially based on a comparison of their IR, MS, and NMR spectra with those of latilagascene B.

Benzoylation of latilagascene B (**8**), followed by acetylation, afforded compound **1**, which was named latilagascene G. This compound was obtained as a colorless oil with $[\alpha]_D^{20}$ −40.4° (*c* 0.20, $CHCl_3$). Its IR spectrum displayed absorption bands of ester carbonyl groups (1740 and 1720 cm^{-1}) and α,β-unsaturated ketone (1655 cm^{-1}) groups, as well as characteristic absorptions of the aromatic ring (1450 and 714 cm^{-1}). Comparison of the ^1H and ^{13}C NMR spectra (Table 1) of **1** with those of latilagascene B (**8**)[9] revealed additional signals of benzoyl [δ_H 8.05 d (6.6 Hz); 7.56 m; 7.44 m, and δ_C 166.4 (CO), 133.0, 129.9, 129.8, and 128.3] and acetyl [δ_H 2.19 s, and δ_C 170.9 (CO) and 20.9] ester residues in the spectra of **1**. As expected, the most noteworthy differences for the diterpenic scaffold in the ^1H NMR spectra of the two compounds are the downfield chemical shift of the diastereotopic methylene group at C-16, which appears as a pair of double doublets at δ 4.39 (J = 11.2 and 6.4 Hz) and 4.27 (J = 11.0 and 8.8 Hz), and the chemical shift of H-3, which is also displayed downfield as a triplet at δ 5.78 (J = 3.2 Hz). These differences are in agreement with the effects expected for acylation of the hydroxyl groups. The HMBC experiment indicated the location of the benzoate ester at C-16, due to the observation of a long-range correlation between the carbonyl signal of the benzoyl group (δ_C 166.4) and the diastereotopic proton signals at δ 4.39 and 4.27. In the same way, the presence of the acetyl group at C-3 was confirmed by the existence of a cross-peak between the carbonyl group (δ_C 170.9) and H-3 (δ 5.78).

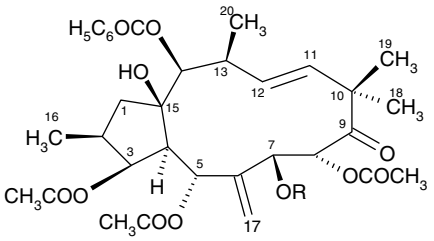
Compounds **2** and **3** were obtained through acylation of latilagascene B (**8**) with propionic and butyric anhydride, respectively. Compound **2**, named latilagascene H was a colorless oil, with $[\alpha]_D^{20}$ −120.2° (*c* 0.20, $CHCl_3$). Compound **3**, which was named latilagascene I, was also obtained as a colorless oil, with $[\alpha]_D^{20}$ −120.7° (*c*



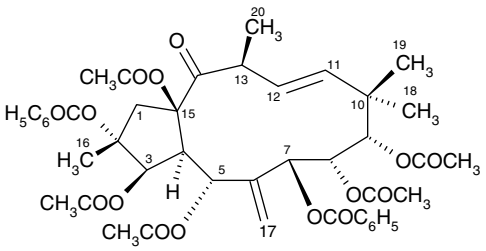
1 (Lat.G): R_1 = COC_6H_5; R_2 = $COCH_3$; R_3 = H
2 (Lat.H): R_1 = R_2 = $COCH_2CH_3$; R_3 = H
3 (Lat.I): R_1 = R_2 = $COCH_2CH_2CH_3$; R_3 = H
7 (Lat. A): R_1 = $COCH_3$; R_2 = R_3 = H
8 (Lat. B): R_1 = R_2 = R_3 = H
9 (Lat. C): R_1 = $COCH_3$; R_2 = $COCH_3$; R_3 = H
10 (Lat. D): R_1 = COC_6H_5; R_2 = R_3 = H
11 (Lat. E): R_1 = COC_6H_5; R_2 = H; R_3 = OH



12 (Lat.F): R_1 = COC_6H_5
13 (Jolk.B): R_1 = $COCHCHC_6H_5$



4 (Tuckeyanol A): R = $COCH(CH_3)CH_2CH_3$
5 (Tuckeyanol B): R = $COCH(CH_3)_2$



6 (Euphotuckeyanol)

Figure 1. Chemical structures of compounds **1**–**13**.

Table 1
NMR data of latilagascenes G (**1**), H (**2**), and I (**3**), ($CDCl_3$, *J* in Hz)

Atom	**1**		**2**		**3**	
	^{1}H	^{13}C	1H	^{13}C	^{1}H	^{13}C
1α; **1β**	3.75 dd (13.6; 7.6); 1.57 m	41.1	3.65 dd (13.2; 7.2); 1.55 m	41.1	3.64 dd (13.6; 7.6); 1.55 m	41.1
2	2.59 m	42.5	2.46 m	42.5	2.45 m	42.5
3	5.78 t (3.2)	77.2	5.65 t (3.6)	76.4	5.64 t (3.6)	76.4
4	1.81 dd (9.2; 4.4)	51.0	1.75 m	50.9	1.74 m	51.0
5	3.38 d (9.2)	57.1	3.33 d (9.2)	57.1	3.32 d (9.2)	57.1
6		63.6		63.5		63.5
7α; **7β**	1.98 m; 1.74 m	38.5	2.05 m; 1.75 m	38.5	2.05 m; 1.74 m	38.6
8α; **8β**	1.98 m; 1.74 m	23.4	2.05 m; 1.55 m	23.4	2.05 m; 1.55 m	23.4
9		34.0	1.14 m	34.0	1.14 m	34.0
10		26.6		26.6		26.6
11	1.54 dd (11.2; 8.0)	29.8	1.55 m	29.8	1.51 dd (10.8; 7.6)	29.8
12	7.04 d (10.8)	145.0	7.02 d (10.8)	144.9	7.02 d (11.2)	144.9
13		134.0		134.0		134.0
14		194.4		194.4		194.4
15		91.0		91.0		91.0
16a	4.39 dd (11.2; 6.4)	62.9	4.14 dd (11.2; 8.8)	62.3	4.11 dd (11.2; 8.8)	62.2
16b	4.27 dd (11.0; 8.8)		4.00 dd (11.0; 6.2)		4.01 dd (10.8; 6.4)	
17	1.21 s	20.0	1.19 s	20.0	1.18 s	20.0
18	1.15 s	29.1	1.14 s	29.1	1.13 s	29.1
19	0.90 s	16.3	0.89 s	16.3	0.88 s	16.3
20	1.92 s	12.4	1.90 s	12.4	1.90 s	12.4
15-OCin						
1′		165.4		165.4		165.4
2′	6.47 d (16.0)	117.0	6.44 d (16.0)	117.0	6.43 d (16.0)	117.0
3′	7.64 d (16.0)	147.1	7.74 d (16.0)	147.0	7.73 d (16.0)	147.0
4′		133.8		133.8		133.8
5′, **9′**	7.56 m	128.2	7.50 m	128.2	7.50 m	128.1
6′, **8′**	7.44 m	131.0	7.44 m	131.0	7.44 m	131.0
7′	7.44 m	129.1	7.44 m	129.1	7.44 m	129.1
16-OBz						
1″		166.4				
2″		129.9				
3″, **7″**	8.05 d (6.8)	129.8				
4″, **6″**	7.44 m	128.3				
5″	7.56 m	133.0				
3-OAc		170.9				
	2.19 s	20.9				
3-OPr						
1″				174.3		
2″			2.46 m	27.7		
3″			1.22 t (7.6)	9.4		
16-OPr						
1‴				172.7		
2‴			2.32 td (7.6)	27.4		
3‴			1.13 t (7.6)	9.0		
3-OBu						
1″						173.5
2″					2.40 td (7.6)	18.6
3″					1.63 q (7.4)	36.3
4″					1.00 t (7.4)	13.8
16-OBu						
1‴						171.8
2‴					2.26 br t (7.2)	18.3
3‴					1.74 m	36.1
4‴					0.93 t (7.4)	13.7

0.14, $CHCl_3$) Their IR and NMR spectra (see Table 1) closely resemble those recorded for latilagascene G (**1**), from which they differed in having two propanoyl (latilagascene H, **2**) or two butanoyl (latilagascene I, **3**) residues instead of the benzoyl and acetyl groups.

2.2. Biological activity

The lathyranes latilagascenes G, H, and I (**1**–**3**), and jatrophane diterpenes tuckeyanols A (**4**) and B (**5**) and euphotuckeyanol (**6**) were investigated for their MDR-reversal activity on *MDR1* gene-transfected L15178 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. The anti-MDR activity results are summarized in Table 2. All compounds were found to highly increase the drug retention in the cells by inhibiting the efflux pump activity mediated by P-gp. All of them seemed to be strong inhibitors of the MDR P-gp, with a manifold activity than that of the positive control verapamil. For latilagascenes G, H, and I (**1**–**3**), this inhibition was slightly dose-dependent because both of the two concentrations were at the saturation zone of biological effect. The antiproliferative effects of compounds **1**–**6** on a doxorubicin-sensitive mouse lymphoma cell line (PAR) and on its MDR subline are summarized in Table 3. All of the tested com-

Table 2
Effect of latilagascenes G–I (**1**–**3**), tuckeyanols A and B (**4**–**5**), and euphotuckeyanol (**6**) on reversal of multidrug resistance (MDR) on human *MDR1* gene-transfected mouse lymphoma cells

Compound	Concentration (μg/mL)	FSC[a]	SSC[a]	FL-1[a]	FAR
PAR + R123[b]	—	623.6	158. 9	711.4	
MDR + R123[c]	—	698.5	185.0	11.0	
Verapamil	10	612.5	193.6	69.3	13.7
1	4.0	634.3	459.9	478.3	68.9
	40.0	584.8	458.0	496.6	71. 6
2	4.0	604.7	463.3	427.4	61.6
	40.0	563.7	465.4	541.3	78.0
3	4.0	620.0	452.8	433.4	62.4
	40.0	563.9	465. 8	376.5	54.2
4	4.0	668.8	240.4	554.0	39.8
	40.0	664.0	226.8	1375.6	98.9
5	4.0	669.5	245.7	347. 6	25.0
	40.0	670.5	230.8	1353.2	97.3
6	4.0	670.9	230.2	1127.4	81.0
	40.0	665.7	242.4	1476. 7	106.2
DMSO	20 μL	655.8	268.7	15.1	1.1

[a] FSC, forward scatter count of cells in the samples; SSC, side scatter count of cells in the samples; FL-1, mean fluorescence intensity of the cells; FAR, fluorescence activity ratio: values were calculated by using the equation given in Section 3.
[b] PAR control: a parental cell without MDR gene.
[c] MDR: a parental cell line transfected with human MDR1 gene.

Table 3
Antiproliferative effects of compounds **1**–**6** on parental (PAR) and multidrug resistance (MDR) mouse lymphoma cell lines (L5178)

Compound	PAR ID_{50} (μg/mL)	MDR ID_{50} (μg/mL)
1	32.2 ± 0.02	28.0 ± 0.04
2	25.3 ± 0.01	17.5 ± 0.01
3	23.9 ± 0.02	17.3 ± 0.02
4	25.8 ± 0.04	17.6 ± 0.01
5	19.4 ± 0.02	12.0 ± 0.01
6	45.0 ± 0.04	22.8 ± 0.01

pounds exhibited moderate antiproliferative effects on the two cell lines.

Latilagascenes G–I (**1**–**3**) presented in this study, together with the lathyrane diterpenes previously isolated from *E. lagascae* (**7**–**13**, Fig. 1)[9,10] represent a set of compounds that may allow considerations of structure–activity relationship. As can be observed in Table 4, all lathyranes are lipophilic (log*P* values between 4.8 and 8.0) and all of them are H-bond acceptors (between 5 and 8). Some of them are also H-bond donors (between 1 and 2). Taking in account the results obtained with latilagascenes A–F (**7**–**12**) and jolkinol B (**13**), the importance of a free hydroxyl group (H-bond donor/acceptor) at C-3 seems to be highlighted by the marked decrease of activity observed after acetylation, as was reported earlier.[10] This feature was also concluded for jatrophane derivatives.[15,16] Nevertheless, as is clear from Table 2, at low concentration, the acylation of the free hydroxyl groups of latilagascene B (**8,** FAR = 28.1) at C-16 and C-3 drastically enhanced the antiMDR activity in latilagascenes G–I (**1**–**3**, FAR = 68.9, 61.6, and 62.4, respectively, at 4.0 μg/mL), (Table S1, Suppl. data)**.** The acyl groups in latilagascenes G–I (**1**–**3**) have a higher number of carbons that led to different physico-chemical properties from those of compound **8** (Table 4), for example, the octanol/water partition coefficient (log*P*), which was 4.8 for latilagascene B (**8**) and 7.9, 6.9, and 8.0 for the acylated derivatives latilagascenes G–I (**1**–**3**), respectively, corroborating the importance of lipophilicity in the modulation of P-gp.

The most active of the three lathyrane derivatives (**1**–**3**), latilagascene G (**1**) (FAR = 68.9 at 4.0 μg/mL), has an aromatic ring at C-16. The importance of the aromatic ring at C-16 was demonstrated by a comparison between latilagascenes D (**10**), A (**7**), and C (**9**): replacement of the aromatic ring at C-16 by an acetyl group resulted in a decrease of activity (FAR = 168.5, 13.0, and 12.2, respectively, at 4.0 μg/mL).[9,10] However, latilagascene G (**1**) has a much lower FAR value than that found for latilagascene D (**10**, FAR = 168.5 at 4.0 μg/mL)[10] differing the two compounds only in the presence of a free hydroxyl group at C-3, in the latter. Thus, the highest activity for these ester derivatives of latilagascene B (**8**) was obtained with a benzoyl moiety on C-16 and a free hydroxyl group on C-3. This feature is also supported by the results on latilagascene E (**11**), which differs from latilagascene D (**10**) in having an extra hydroxyl group at C-20. In fact, latilagascene E (**11**) showed the lowest log*P* value (5.9) of all the lathyrane esters discussed, exhibiting however the highest activity (FAR = 216.8 at 4 μg/mL).[10] Latilagascene I (**3**) (FAR = 62.4 at 4.0 μg/mL), has butanoyl esters at both C-3 and C-16 positions resulting in a combination of acyl residues that origins the highest lipophilicity (log*P* = 8.0), but not the highest activity.

Table 4
HOMO and LUMO energies[a] [eV] and physico-chemical properties[b] of lathyrane diterpenes (**1**–**3** and **7**–**13**) and jatrophane diterpenes (**4**–**6**)

Compound	HOMO	LUMO	N° H		MW	log*P*
			Acceptors	Donators		
Lathyranes						
Latilagascene A (**7**)	−9.5745	−0.8448	7	1	522	5.5
Latilagascene B (**8**)	−9.6145	−0.8930	6	2	480	4.8
Latilagascene C (**9**)	−9.5941	−0.9029	8	0	564	6.2
Latilagascene D (**10**)	−9.5708	−0.8419	7	1	584	7.2
Latilagascene E (**11**)	−9.7007	−1.0002	8	2	600	5.9
Latilagascene F (**12**)	−9.6035	−0.6718	5	1	438	5.3
Latilagascene G (**1**)	−9.5885	−0.9029	8	0	626	7.9
Latilagascene H (**2**)	−9.6006	−0.8892	8	0	592	6.9
Latilagascene I (**3**)	−9.5955	−0.8819	8	0	620	8.0
Jolkinol B (**13**)	−9.6066	−0.9327	5	1	464	5.8
Jatrophanes						
Tuckeyanol A (**4**)	−9.5755	−0.0291	12	1	698	4.5
Tuckeyanol B (**5**)	−9.7114	−0.2649	12	1	684	4.0
Euphotuckeyanol (**6**)	−9.5581	−0.4455	15	0	818	6.7

[a] The structures were drawn using MOE v2006.08. Subsequently, a stochastic conformation search was performed for each molecule using MOE v2006.08. For each molecule, the conformation possessing the lowest energy was optimized with MOPAC using the semiempirical AM-1 method (gradient convergence: <0.001 kcal/mol). Then, HOMO and LUMO energies were computed using MOE v2006.08.
[b] Physico-chemical parameters were determined by using the JME molecular editor (version May 2007, http://www.molinspiration.com/).

Table 5
In vitro effects of compounds **1–6** in combination with epirubicine on human *MDR1* gene-transfected mouse lymphoma cell line

Compounds	FIX value[a]	Interaction
1	0.14	Synergism
2	0.07	Synergism
3	0.14	Synergism
4	0.18	Synergism
5	0.25	Synergism
6	0.08	Synergism

[a] FIX, fractional inhibitory index.

As can be observed in Table 2, the jatrophane polyesters tuckeyanols A (**4**) and B (**5**) and euphotuckeyanol (**6**), also exhibited high rhodamine-123 accumulation, displaying a concentration dependence and a very strong activity when compared to that of the positive control verapamil. Euphotuckeyanol (**6**) proved to be the most active (FAR = 81.0 at 4.0 μg/mL). This compound, with seven ester residues has the highest values of log*P* (6.7), molecular weight (818), and the highest number of hydrogen bond acceptor groups (15 H-bond acceptors), all of them considered by several authors, as important requirements to P-gp modulation.[17,18] Two aromatic moieties (C-2 and C-7), regarded as well, as an important

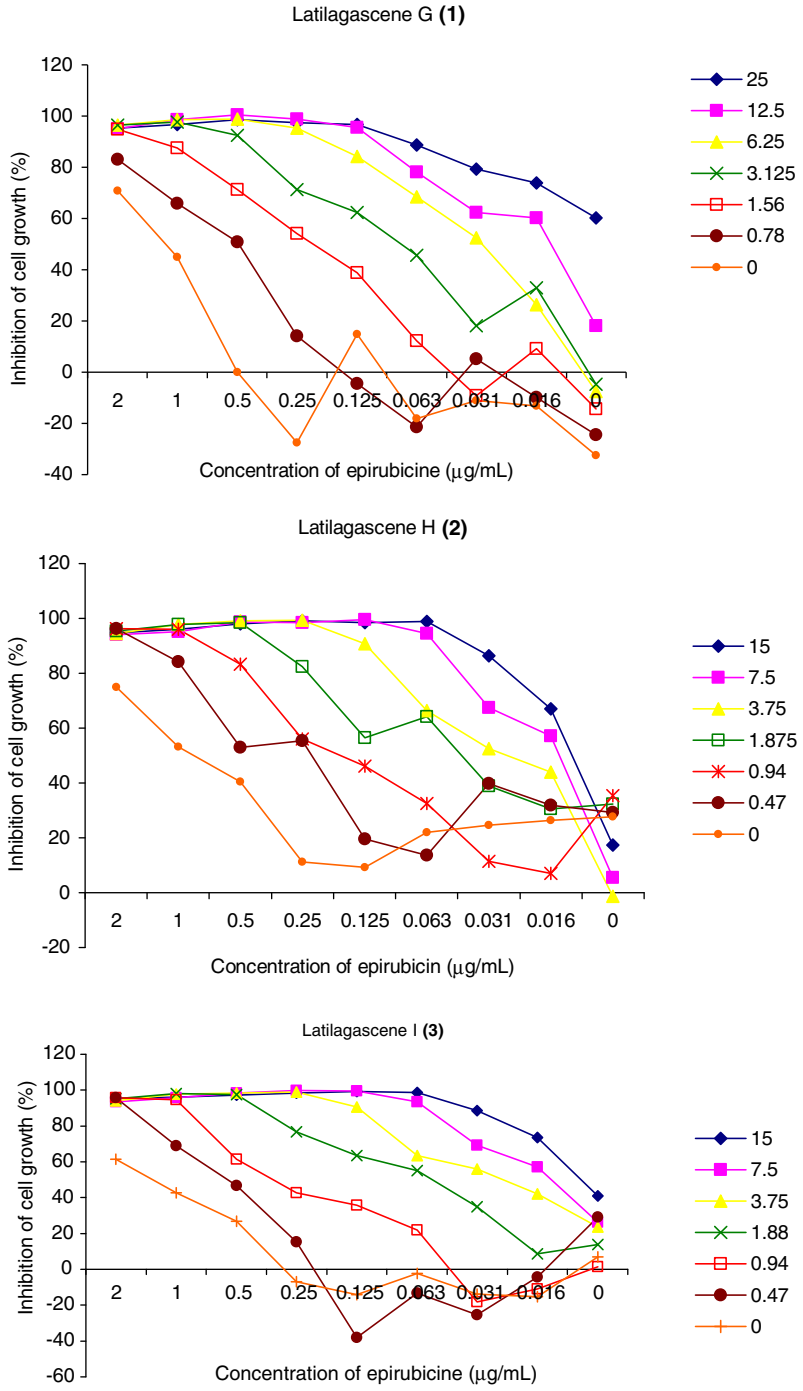


Figure 2. Effect of latilagascenes G [(**1**), 25–0 μg/mL], H (**2**), and I [(**3**), 15–0 μg/mL] in combination with epirubicine on L5178 MDR cell line.

structural requirement, are also present, whereas tuckeyanol A (**4**) and B (**5**) have one single benzene ring. Therefore, concerning all these factors, it is difficult to explain which of them has the most relevant role for the high MDR-reversal activity. Furthermore, it should be noted that euphotuckeyanol (**6**) has a strong preference to remain in the *endo*-type conformation, whereas tuckeyanols A (**4**) and B (**5**) adopt the *exo*-type conformation.[14] These different conformations are dependent on the spatial orientation of the exo-methylene group at C-6 with respect to the plane of the macrocycle,[19–21] and may also be an important factor in MDR modulation.

The HOMO and LUMO energy values have been calculated for lathyrane diterpenes (**1**–**3** and **7**–**13**) and jatrophane diterpenes (**4**–**6**), (Table 4). All the diterpenes showed HOMO (highest occupied molecular orbital) energy values in the range of reported MDR modulators and thus cannot help to understand the differences in activities.[8] The set of jatrophane diterpenes (**4**–**6**) exhibited the lowest calculated LUMO (lowest unoccupied orbital) energy values of all the lathyrane and jatrophane diterpenes tested (**1**–**13**). High energy values of the LUMO are of favor for charge transfer (CT) interactions with electron donors in CT complexes.[22,23] Aromatic amino acids like phenylalanine or tyrosine may serve as electron donors in CT complexes. They can be found in the first extracellular loop of P-gp which has been discussed as a potential P-gp binding site for MDR modulators of the phenothiazine type.[23] Euphotuckeyanol (**6**) shows the highest LUMO energy value within the jatrophane series **4**–**6** and proves to have the strongest activity especially at the lowest concentration. This highest energy value favors such CT interactions and thus may contribute to the observed activity of the compound in the sense of higher binding affinities to the P-gp binding region which may consist of aromatic amino acids like the extracellular P-gp loop.

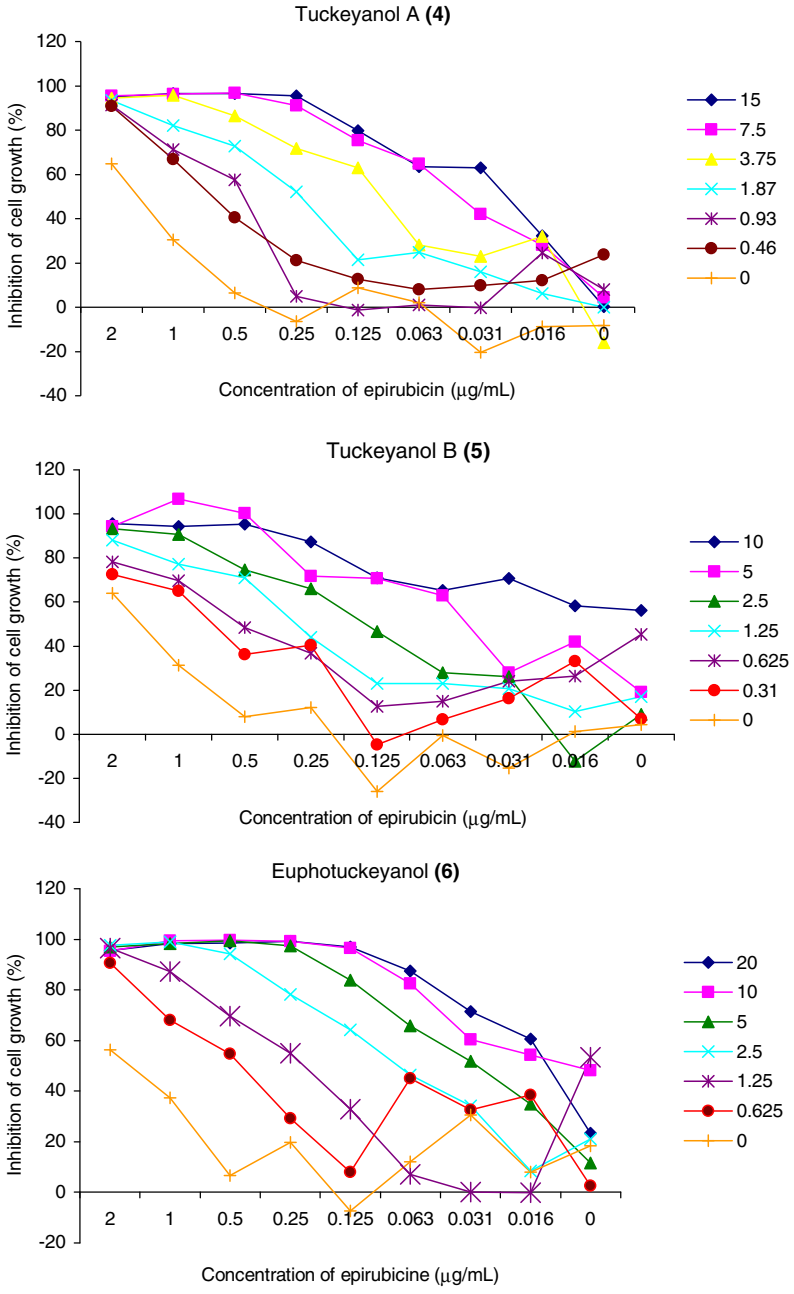


Figure 3. Effect of tuckeyanol A [(**4**), 15–0 µg/mL)], tuckeyanol B [(**5**), 10–0 µg/mL)] and euphotuckeyanol [(**6**), 20–0 µg/mL] in combination with epirubicine on L5178 MDR cell line.

In further experiments, the in vitro antiproliferative effects of the lathyranes latilagascenes G–I (**1**–**3**) and jatrophanes tuckeyanols A (**4**) and B (**5**) and euphotuckeyanol (**6**), were studied in combination with epirubicine, on human *MDR1* gene-transfected mouse lymphoma cells, using the checkerboard microplate method. Several concentrations of the compounds and the antitumor drug were tested. As can be observed at Table 5 and in Figures 2 and 3, all the tested compounds exhibited a synergistic interaction with epirubicine on the studied cell line (fractional inhibitory index, FIX = 0.07–0.25). The most effective compounds were latilagascene H (**2**) and euphotuckeyanol (**6**), which expressed a very low fractional inhibitory index (0.07 and 0.08, respectively), in the checkerboard experiments. Latilagascene B (**8**) has also shown synergistic interaction with doxorubicine.[10] Therefore, these diterpenes are very promising compounds for further studies. Synergistic interaction was also found for one jatrophane diterpene in other cancer cell line.[24]

In summary, according to our and other[1,15,16,25,26] studies, macrocyclic lathyrane, and jatrophane diterpenes may be valuable as lead compounds for the development of P-gp modulators in different multidrug-resistant cancer cells.

3. Experimental

3.1. General experimental procedures

Optical rotations were determined with a Perkin-Elmer 241 polarimeter. IR spectra were recorded on a FTIR Nicolet Impact 400, and NMR spectra recorded on a Bruker ARX-400 NMR spectrometer (^{1}H 400 MHz; ^{13}C 100.61 MHz), in $CDCl_3$ as solvent. The signal of the deuterated solvent was taken as reference. MS were taken on a Kratos MS25RF spectrometer (70 eV). TLC was performed on precoated SiO_2 F_{254} plates (Merck 5554 and 5744), with visualization under UV light and by spraying with sulfuric acid/acetic acid/water (1:20:4) or sulfuric acid/water (1:1), followed by heating.

3.2. Plant material

Euphorbia tuckeyana (Euphorbiaceae) was collected at a Garcia d' Orta garden in Lisbon, Portugal, and *E. lagascae* in Cova da Beira, Coimbra, Portugal. Both species were identified by Dr. Teresa Vasconcelos of Instituto Superior de Agronomia (ISA), University of Lisbon. Voucher specimens (Nos. 139/2005 and 323, respectively) have been deposited at the herbarium of ISA.

3.3. Compounds studied

Latilagascenes A (**7**), B (**8**), and D–F (**10**–**12**), and jolkinol B (**13**) were isolated from the aerial parts of *E. lagascae*.[9,10] Latilagascene C (**9**) was prepared through acetylation reaction of latilagascene B.[9] Tuckeyanol A (**4**), tuckeyanol B (**5**), and euphotuckeyanol (**6**) were isolated from the aerial parts of *E. tuckeyana*.[14]

3.3.1. Acylation of latilagascene B

[(2*R**,3*S**,4*R**,5*R**,6*R**,9*S**,11*S**,15*R**)-15β-Cinnamoyloxy-5α,6β-epoxy-3β,16-dihydroxy-14-oxolathyr-12*E*-ene] was derivatized with several acylating reagents as described below, to afford three new lathyrane derivatives (**1**–**3**).

3.3.1.1. Benzoylation and acetylation. One hundred microliters of benzoyl chloride and 100 μL of triethylamine were added to a dichloromethane solution of latilagascene B (10 mg). After stirring at room temperature for 48 h, the mixture was purified by preparative TLC ($CHCl_3$/MeOH, 9:1) to give 8 mg of a benzoylated compound, which was suspended in Ac_2O (1.0 mL) and pyridine (1.0 mL). After stirring at room temperature for 36 h, the excess of reagents was eliminated with N_2, and the product obtained was purified by preparative TLC ($CHCl_3$/MeOH, 19:1) to afford 4 mg of compound **1**.

*Latilagascene G, 3β-acetoxy-16-benzoyloxy-15β-cinnamoyloxy-5α,6β-epoxy-14-oxolathyr-12E-ene (**1**):* a colorless oil; $[\alpha]_D^{20}$ −40.4° (*c* 0.20, $CHCl_3$); IR, ν_{max} cm^{-1} (film): 1740, 1720, 1655, 714; FABMS, *m*/*z* (rel. int): 649 $[M+Na]^+$; ^{1}H NMR and ^{13}C NMR (see Table 1).

3.3.1.2. Acylation with propionic anhydride. Latilagascene B (15 mg) was suspended in propionic anhydride (1.0 mL) and pyridine (1.0 mL). After stirring at room temperature for 48 h, the excess of pyridine was eliminated with N_2 and the product obtained was purified twice by preparative TLC, using $CHCl_3$/MeOH (19:1) and CH_2Cl_2/acetone (49:1, 2×) as eluants, to afford 17 mg of compound **2**.

*Latilagascene H, 3β,16-dipropanoyloxy-15β-cinnamoyloxy-5α,6β-epoxy-14-oxolathyr-12E-ene (**2**):* a colorless oil; $[\alpha]_D^{20}$ −120.2° (*c* 0.10, $CHCl_3$); IR, ν_{max} cm^{-1} (film): 1735, 1718, 1655, 714; FABMS, *m*/*z* (rel. int): 592 $[M]^+$; ^{1}H NMR and ^{13}C NMR (see Table 1).

3.3.1.3. Acylation with butyric anhydride. Latilagascene B (30 mg) was suspended in butyric anhydride (1.0 mL) and pyridine (1.0 mL). After stirring at room temperature for 48 h, the excess of pyridine was eliminated with N_2 and the product obtained was purified twice by preparative TLC, using CH_2Cl_2/acetone (19:1) and CH_2Cl_2/acetone (49:1, 2×) as eluants, to afford 40 mg of compound **3**.

*Latilagascene I, 3β,16-dibutanoyloxy-15β-cinnamoyloxy-5α,6β-epoxy-14oxolathyr-12E-ene (**3**):* a colorless oil; $[\alpha]_D^{20}$ −120.7° (*c* 0.14, $CHCl_3$); IR, ν_{max} cm^{-1} (film): 1746, 1715, 1655, 714; FABMS, *m*/*z* (rel. int): 620 $[M]^+$; ^{1}H NMR and ^{13}C NMR (see Table 1).

3.4. Biological assays

3.4.1. Cell cultures

L5178 mouse T-cell lymphoma cells were transfected with pHa MDR1/A retrovirus, as previously described.[27] MDR-1-expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain the expression of the MDR phenotype in all cells of the population. L5178 (parent) mouse T-cell lymphoma cells and the human mdr-1-transfected subline were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics. This cell line was cultured at 37 °C, and maintained in a 5% CO_2 atmosphere.

3.4.2. Assay for antiproliferative effect

The effects of increasing concentrations of the drugs alone and their combinations with resistance modifiers on cell growth were tested in 96-well flat-bottomed microtitre plates. The compounds were diluted in a volume of 50 μL medium. Then, 1×10^4 cells in 0.1 mL of medium were added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37 °C for 72 h; at the end of the incubation period, 20 μL of MTT (thiazoil blue, Sigma, St. Louis, MO, USA) solution (from a 5 mg/mL stock) was added to each well. After incubation at 37 °C for 4 h, 100 μL of sodium dodecyl sulfate (SDS) (Sigma) solution (10%) was measured into each well and the plates were further incubated at 37 °C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with the Dynatec MRX vertical beam ELISA reader. Inhibition of the cell growth was determined according to the formula:

$$100 - \left[\frac{\text{OD sample} - \text{OD medium control}}{\text{OD cell control} - \text{OD medium control}}\right] x100$$

The ID_{50} values are expressed as means ± SD from three experiments.

3.4.3. Assay for reversal of MDR in tumor cells

The cells were adjusted to a density of 2×10^6/mL, resuspended in serum-free McCoy's 5A medium and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. The test compounds were added at various concentrations in different volumes (2.0–20.0 μL) of the 1.0–10.0 mg/mL stock solutions, and the samples were incubated for 10 min at room temperature. Next, 10 μL (5.2 mM final concentration) of the indicator rhodamine-123 was added to the samples and the cells were incubated for a further 20 min at 37 °C, washed twice and resuspended in 0.5 mL phosphate-buffered saline (PBS) for analysis. The fluorescence uptake of the cell population was measured with a Beckton–Dickinson FACScan flow cytometer. Verapamil was used as a positive control in the rhodamine-123 exclusion experiments. The percentage mean fluorescence intensity was calculated for the treated MDR and parental cell lines as compared to untreated cells. An activity ratio FAR was calculated via the following equation, on the basis of the measured fluorescence values:

$$\mathrm{FAR} = \frac{\text{MDR treated/MDR control}}{\text{parental treated/parental control}}$$

3.4.4. Checkerboard microplate method

The microplate method was applied to study the effects of drug interactions between resistance modifiers and epirubicine on cancer cells. The effects of the anticancer drug epirubicine and the resistance modifiers in combination were studied on *MDR1* gene-transfected mouse lymphoma cells. The dilution of epirubicine (A) was made in a horizontal direction, and the dilutions of resistance modifiers (B) vertically in the microtiter plate, in a volume of 100 μL. The cell suspension in the tissue culture medium was distributed into each well in 100 mL containing 5×10^4 cells. The plates were incubated for 72 h at 37 °C in a CO_2-incubator. The cell growth rate was determined after MTT staining and the intensity of the blue color was measured on a micro ELISA reader. Drug interactions were evaluated according to the following system:

$FIC_A = ID_{50A\ \text{in combination}}/ID_{50A\ \text{alone}}$	
$FIC_B = ID_{50B\ \text{in combination}}/ID_{50B\ \text{alone}}$	
ID = inhibitory dose	
FIC = fractional inhibitory concentration	
$FIX = FIC_A + FIC_B$	
FIX = 0.5–1	additive effect
FIX < 0.5	synergism
1 < FIX < 2	indifferent effect
FIX > 2	antagonism
FIX = fractional inhibitory index	

Acknowledgments

The Science and Technology Foundation, Portugal (FCT) and the Szeged Foundation for Cancer Research supported this work. The authors thank Mrs. Vigyikán Várady Aniko for technical assistance with the tissue cultures, Dr. Teresa Vasconcelos (ISA, University of Lisbon, Portugal) for plant identification and Rene Meier for assisting the molecular orbital calculations.

Supplementary data

Comparison of the antiMDR activities and octanol/water partition coefficients (log*P*) of lathyrane diterpenes. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.08.071.

References and notes

1. Molnár, J.; Gyémánt, N.; Tanaka, M.; Hohmann, J.; Bergmann-Leitner, E.; Molnár, P.; Deli, J.; Didiziapetris, R.; Ferreira, M. J. U. *Curr. Pharm. Des.* **2006**, *12*, 287.
2. Teodori, E.; Dei, S.; Martelli, C.; Scapecchi, C.; Gualtieri, F. *Curr. Drug Targets* **2006**, *7*, 893.
3. Szakács, G.; Paterson, J.; Ludwig, J.; Genthe, C.; Gottesman, M. *Nat. Rev. Drug Discov.* **2006**, *5*, 219.
4. Srinivas, E.; Murthy, J.; Rao, A.; Sastry, G. *Curr. Drug Metab.* **2006**, *7*, 205.
5. Borowski, E.; Bontemps-Gracz, M.; Picokowska, A. *Acta Biochim. Pol.* **2005**, *52*, 609.
6. Tsuruo, T.; Iida, H.; Tsukagoshi, S.; Sakurai, Y. *Cancer Res.* **1981**, *41*, 1967.
7. Pajeva, I. K.; Wiese, M. *J. Med. Chem.* **2002**, *45*, 5671.
8. Wang, R. B.; Kuo, C. L.; Lien, L. L. *J. Clin. Pharm. Ther.* **2003**, *28*, 203.
9. Duarte, N.; Gyémánt, N.; Abreu, P. M.; Molnár, J.; Ferreira, M. J. U. *Planta Med.* **2006**, *72*, 162.
10. Duarte, N.; Varga, A.; Radics, R.; Molnár, J.; Ferreira, M. J. U. *Bioorg. Med. Chem.* **2007**, *15*, 546.
11. Ferreira, M. J. U.; Duarte, N.; Gyémánt, N.; Radics, R.; Chepernev, G.; Varga, A.; Molnár, J. *Anticancer Res.* **2006**, *26*, 3541.
12. Ferreira, M. J. U.; Gyemant, N.; Madureira, A. M.; Tanaka, M.; Koos, K.; Didziapetris, R.; Molnár, J. *Anticancer Res.* **2005**, *25*, 4173.
13. Madureira, A. M.; Ferreira, M. J.; Gyemant, N.; Ugocsai, K.; Ascenso, J. R.; Abreu, P. M.; Hohmann, J.; Molnár, J. *Planta Med.* **2004**, *70*, 45.
14. Duarte, N.; Lage, H.; Ferreira, M. J. U. *Planta Med.* **2008**, *74*, 61.
15. Corea, G.; Fattorusso, E.; Lanzotti, V.; Taglialatela-Scafati, O.; Appendino, G.; Ballero, M.; Simon, P. N.; Dumontet, C.; Di Pietro, A. *J. Med. Chem.* **2003**, *46*, 3395.
16. Corea, G.; Fattorusso, E.; Lanzotti, V.; Motti, R.; Simon, P. N.; Dumontet, C.; Di Pietro, A. *J. Med. Chem.* **2004**, *47*, 988.
17. Wiese, M.; Pajeva, I. *Curr. Med. Chem.* **2001**, *8*, 685.
18. Robert, J.; Jarry, C. *J. Med. Chem.* **2003**, *46*, 4805.
19. Marco, J.; Sanz-Cervera, J.; Yuste, A.; Jakupovic, J.; Jeske, F. *Phytochemistry* **1998**, *47*, 1621.
20. Jakupovic, J.; Morgenstern, T.; Bittner, M.; Silva, M. *Phytochemistry* **1998**, *47*, 1601.
21. Jakupovic, J.; Morgenstern, T.; Marco, J.; Berendohn, W. *Phytochemistry* **1998**, *47*, 1611.
22. Kuroda, H.; Kobayashi, M.; Kinoshita, M.; Takemoto, S. *J. Chem. Phys.* **1962**, *36*, 457.
23. Hilgeroth, A.; Molnár, A.; Molnár, J.; Voigt, B. *Eur. J. Med. Chem.* **2006**, *41*, 548.
24. Engi, H.; Vasas, A.; Rédei, D.; Molnár, J.; Hohmann, J. *Anticancer Res.* **2007**, *27*, 3451.
25. Hohmann, J.; Molnar, J.; Redei, D.; Evanics, F.; Forgo, P.; Kalman, A.; Argay, G.; Szabo, P. *J. Med. Chem.* **2002**, *45*, 2425.
26. Barile, E.; Borriello, M.; Di Pietro, A.; Doreau, A.; Fattorusso, C.; Fattorusso, E.; Lanzotti, V. *Org. Biomol. Chem.* **2008**, *6*, 1756.
27. Cornwell, M. M.; Pastan, I.; Gottesmann, M. M. *J. Biol. Chem.* **1987**, *262*, 2166.