Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 546–554
Bioorganic & Medicinal Chemistry

Apoptosis induction and modulation of P-glycoprotein mediated multidrug resistance by new macrocyclic lathyrane-type diterpenoids

Noélia Duarte,[a] Andras Varga,[b] Georg Cherepnev,[c] Rita Radics,[d] Joseph Molnár[d] and Maria-José U. Ferreira[a,*]

[a]*CECF, Faculty of Pharmacy, University of Lisbon, Av. das Forças Armadas, 1600-083 Lisbon, Portugal*
[b]*Department of Molecular Parasitology, Humboldt University, D-10115 Berlin, Germany*
[c]*Medical Immunology (Charité), Humboldt University, D-10115 Berlin, Germany*
[d]*Department of Medical Microbiology and Immunobiology, University of Szeged, H-6720 Szeged, Hungary*

Received 29 June 2006; revised 8 September 2006; accepted 15 September 2006
Available online 10 October 2006

Abstract—The macrocyclic lathyrane diterpenes, latilagascenes D–F (**1**–**3**) and jolkinol B (**4**), were isolated from the methanol extract of *Euphorbia lagascae*, and evaluated for multidrug resistance reversing activity on mouse lymphoma cells. All compounds displayed very strong activity compared with that of the positive control, verapamil. The structure–activity relationship is discussed. The evaluation of compounds **1** and **4**, and of latigascenes A-C (**5**–**7**), isolated from the same species, as apoptosis-inducers was also carried out. Compound **1** was the most active. Furthermore, in the model of combination chemotherapy, the interaction between the doxorubicine and latilagascene B (**6**) was studied *in vitro*, on human MDR1 gene transfected mouse lymphoma cells, showing that the type of interaction was synergistic. Latilagascenes D–F (**1**–**3**) are new compounds whose structures were established on the basis of spectroscopic methods, including 2D NMR experiments (COSY, HMQC, HMBC and NOESY).

1. Introduction

Although a number of anticancer drugs have been commercialized, the need for more effective ones continues to exist, because the most common tumours are resistant to available drugs. In fact, multidrug resistance (MDR) remains a significant problem for the effectiveness of cancer treatment. Despite multiple mechanisms being involved in MDR, the most important is the typical MDR, associated with the expression of P-glycoprotein (P-gp) which transports anticancer drugs out of the cells before they reach the cytosol.[1] Therefore, inhibition of P-gp represents a promising approach for overcoming MDR. A broad structural variety of compounds can reverse P-glycoprotein mediated MDR (MDR reversers, modulators or chemosensitizers); however, a great need for new modulators with higher specificity and efficacy still remains. In order to develop effective modulators, a number of studies have been carried out to elucidate the structure–activity relationships of MDR reversing agents, being consensual that hydrophobicity and the presence of hydrogen bond-acceptor groups are important general features for the interaction of the modulator with P-gp.[2] However, the knowledge of the mechanism of the modulator–P-glycoprotein interaction, on a molecular basis, is still missing. Some P-gp modulators, such as verapamil and cyclosporin, are also P-gp substrates and inhibit drug efflux in a competitive manner, whereas recent studies suggested an allosteric mode of action for several compounds.[3]

Several studies have shown that most chemotherapeutic agents exert their anticancer activity by inducing apoptosis or programmed cell death which is an essential physiologic process required to eliminate damaged or abnormal cells. It is caused by activation of intracellular cysteine proteases, known as caspases, which are responsible for the morphological and biochemical events that characterize the apoptotic cell.[4] Consequently, resistance to apoptosis may be a major factor for the

Keywords: Macrocyclic lathyrane diterpenes; *Euphorbia lagascae*; Apoptosis; Multidrug resistance; P-glycoprotein; Antiproliferative.
* Corresponding author. Tel.: +351 21 7946475; fax: +351 21 7946470; e-mail: mjuferreira@ff.ul.pt

0968-0896/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2006.09.028

ineffectiveness of cancer treatment. In this manner, compounds which regulate and overcome apoptosis deficiency of cancer cells are of great therapeutic importance and the development of apoptosis-modulating agents has become an important approach for the discovery of new antitumour drugs.[5] There are various possible mechanisms of apoptosis such as, receptor mediated through caspase 8, mitochondrial-mediated via caspase 9, Ca activated acting by caspase 12 and caspase independent mechanisms.[6] Apoptotic response to therapy depends on mitochondrial mass, therefore, evolving malignancies with reduction of mitochondria make cancer cells less sensitive to apoptosis.[6]

Euphorbia lagascae Spreng (Euphorbiaceae) has been used in traditional medicine to treat cancer, tumours and warts.[7] *Euphorbia* plants synthesize a wide range of structurally unique macrocyclic diterpene polyesters of the jatrophane- and lathyrane-type which have revealed to be promising MDR reversing agents in cancer cells.[8–14] Polycyclic rearranged jatrophane diterpenes have also been found to be able to increase drug retention in cancer cells by interacting with P-gp.[15,16] A recent work has also considered jatrophane diterpenes new microtubule-interacting agents.[17]

In a previous study, we have described the isolation and structure characterization of lathyrane-type diterpenes from *Euphorbia lagascae* which displayed anti-MDR activity.[18] Continuing our evaluation of macrocyclic diterpenes as effective MDR modulators, and the elucidation of their structure–activity relationships,[12,14–16] herein we reported the isolation from *Euphorbia lagascae*, and structure elucidation of four lathyrane-type diterpenes (**1**–**4**, Fig. 1), three of which are new, as well as the evaluation of their ability as MDR modulators. Moreover, the antiproliferative effects of the anticancer drug doxorubicin in combination with latilagascene B (**6**) were studied.

The evaluation of the capacity of the compounds **1**, **2** and of **5**–**7**, isolated previously, as apoptosis inducers is also reported.

2. Results and discussion

2.1. Structure elucidation of compounds

The air-dried powdered plant of *Euphorbia lagascae* was exhaustively extracted with methanol. Repeated column chromatographic fractionation and further purification by HPLC of the Et_2O soluble part of the methanol extract yielded the new macrocyclic lathyrane diterpenes **1**–**3** (Fig. 1) and the known lathyrane, jolkinol B (**4**).

Compound **1**, named latilagascene D, was obtained as a colorless oil with $[\alpha]_D^{25}$ −158.0° ($CHCl_3$, *c* 0.2). Its IR spectrum displayed absorption bands for hydroxyl (3475 cm^{-1}), ester carbonyl (1715 cm^{-1}) and α,β-unsaturated ketone (1655 cm^{-1}) groups, as well as characteristic absorptions of the aromatic ring (1450 and

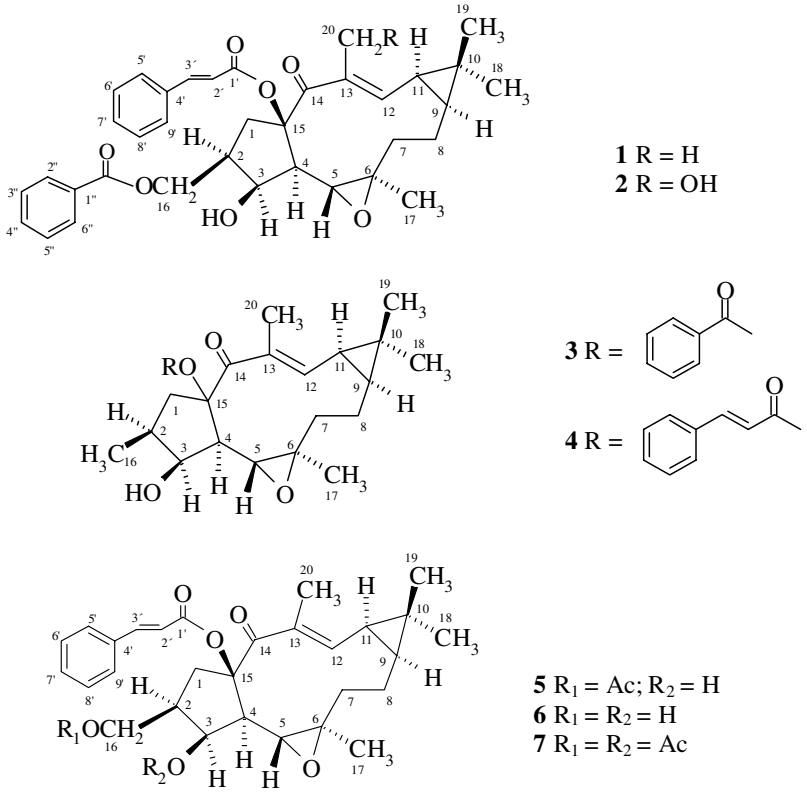

Figure 1. Structures of latilagascenes D–F (**1**–**3**), jolkinol B (**4**) and latilagascenes A–C (**5**–**7**).

714 cm^{-1}). Its HRSIMS exhibited a pseudomolecular ion at *m/z* 585.2865 $[M+1]^+$ (calcd for $C_{36}H_{41}O_7$: 585.2852) that was in agreement with the molecular formula $C_{36}H_{40}O_7$, from which seventeen degrees of unsaturation was deduced. In the FABMS, the ions at *m/z* 454 $[M+1-C_9H_7O]^+$, 437 $[M+1-C_9H_8O_2]^+$, 154 $[C_9H_7O+Na]^+$, 131 $[C_9H_7O]^+$ and 105 $[C_7H_5O]^+$ pointed to the existence of cinnamoyl and benzoyl moieties in the molecule. These features were also supported by the NMR spectra of **1**, which showed signals for one benzoyl group (δ 7.36–7.95, 5H; δ_C 167.5, 133.3, 2× 129.8, 129.7, 2× 128.4) and one cinnamoyl group (δ 7.33–7.42, 5H, 6.41 and 7.64 doublets, J = 15.6 Hz; δ_C 165.7, 146.8, 133.9, 2 × 130.8, 129.0, 2 × 128.2, 117.3). The ^{1}H NMR spectrum of **1** also showed signals for three tertiary methyl groups, resonating as singlets at δ 0.79, 1.04 and 1.09, one vinylic methyl (δ 1.81) and one diastereotopic methylene group bounded to an oxygen (δ 4.17, dd, J = 4.8 and 10.8 Hz; 4.68, t, J = 10.8 Hz). In addition, two oxymethine protons (δ 4.17, br s; δ 3.61, d, J = 9.6 Hz) and one olefinic proton at δ 6.96 (br d, J = 10.8 Hz) could also be observed. Moreover, the presence of two shielded methines on a cyclopropane ring was supported by two signals at δ 1.06 (m) and 1.42 (dd, J = 8.0 and 11.2 Hz). A broad singlet at δ 3.24, without correlation in the HMQC spectrum, provided evidence for the hydroxyl group.

Besides the signals due to the ester groups, the ^{13}C NMR spectrum displayed 20 carbon resonances discriminated by a DEPT experiment as four methyl groups, four methylenes (one oxygen bearing carbon at δ_C 63.2), seven methines (two oxygenated at δ_C 58.1 and 74.7, and one olefinic at δ_C 144.8) and five quaternary carbons (a carbonyl group at δ_C 194.9, one olefinic carbon at 133.9 and two oxygenated carbons at 63.9 and 91.2). The presence of an α,β-unsaturated ketone was confirmed by the particularly low chemical shift value of the carbonyl signal at δ_C 194.9 together with a downfield vinylic proton resonance at δ 6.96 evidencing mesomeric effects due to a conjugated system. Furthermore, the chemical shifts of the oxygenated carbons at δ_C 58.1 and 63.9, taking into account the signal of the oxygenated methine at δ 3.61, evidenced the presence of an oxacyclopropane ring.

The above data indicated the molecular formula, $C_{20}H_{30}O_5$, for the parent polyol structure of **1**, with six degrees of unsaturation corresponding to a diterpenoid of the lathyrane-type, carrying a rare 5,6-epoxy ring and bearing an unusual acylation at C-16. These structural features were corroborated by two-dimensional NMR experiments (COSY, HMQC, and HMBC) which allowed the unambiguous assignment of all the proton and carbon signals (Table 1). Analysis of the HMQC and ^{1}H–^{1}H COSY spectra provided evidence for two proton spin-systems –CH_2–CH(CH_2OR)–CH(OR)–CH(R)–CH(OR)–(A); –CH_2–CH_2–CH(R)–CH(R)–CH=C(C)–(B). Furthermore, the heteronuclear $^2J_{C-H}$ and $^3J_{C-H}$ connectivities displayed in the HMBC spectrum of **1** allowed the establishment of its structural frame and the location of functional groups. In particular, the position of the benzoate ester at C-16 was clearly deduced by observation of a long-range correlation between the carbonyl signal of the benzoyl group (δ_C 167.5) and the diastereotopic proton signals at δ 4.17 and 4.68.

The spectroscopic data of **1** clearly resemble those of latilagascene B (**6**), which was previously isolated from *Euphorbia lagascae*.[17] When comparing the NMR spectra, the diastereotopic methylene group at C-16, which appears as a pair of double duplets at δ 3.85 (J = 4.0 and 11.0 Hz) and 3.80, (J = 7.4 and 11.0 Hz) and has a ^{13}C signal at δ_C 61.2 in **6**, is displayed at lowerfield in compound **1** (δ 4.68, t, J = 10.8 Hz; and 4.17, dd, J = 4.8 and 10.8 Hz; δ_C 63.2); the upfield shift of 1.0 ppm at C-2 (β-carbon), observed for compound **1**, is also in agreement with the β-effects expected for the replacement of a hydroxyl group by an acyl group. Furthermore, a significant shielding effect at C-3 was also verified for compound **1** (δ_C 74.7) relative to latilagascene B (δ_C 76.4), which may be due mainly to the decrease of the intermolecular H-bonding potential of **1** resulting from the acylation of the hydroxyl group at C-16.

The relative configuration of **1** was deduced from coupling constant values and a NOESY spectrum assuming α orientation for H-4, characteristic of all macrocyclic diterpenes derivatives isolated to date.[19,20] The stereochemistry of all tetrahedral stereocenters was found to be identical to that of latilagascene B.

Compound **2**, named latilagascene E, was obtained as colorless oil, $[\alpha]_D^{25}$ −103.0° ($CHCl_3$, *c* 0.12) and was established to have the molecular formula of $C_{36}H_{40}O_8$, by its HRESIMS, which exhibited a pseudomolecular ion at m/z 601.2796 $[M+1]^+$. Like latilagascene D, its IR spectrum exhibited the characteristic absorption bands for ester carbonyl, α,β-unsaturated ketone and hydroxyl functions, as well as for the benzene ring. In turn, the MS spectrum also showed fragments at *m/z* 131 $[C_9H_7O]^+$ and 105 $[C_7H_5O]^+$ giving evidence for the presence in the molecule of the benzoyl and cinnamoyl groups.

Comparison of the NMR data of compound **2** with those of latilagascene D (**1**) showed that both were very similar except for the singlet signal of the vinylic methyl protons in the spectra of **1** (δ 1.81 and δ_C 12.4), which was absent in the NMR spectra of **2**, and replaced by an AB system represented by a couple of doublets at δ 4.44 and 4.25 (J = 12.3 Hz), and a carbon resonance at δ_C 58.1 (Table 2). The above data suggested that, in comparison with latilagascene D, compound **2** bears another free hydroxyl group, which was located at C-20, on the basis of observed differences in carbon and proton chemical shifts of compounds **2** and **1**. Besides the expected downfield shifts resulting by the introduction of a hydroxyl at C-20 ($\Delta\delta_C$ = +47.5; α-carbon) and at C-13 ($\Delta\delta_C$ = +2.9; β-carbon), inspection of the ^{13}C data of **2** revealed that the γ carbons C-12 ($\Delta\delta_C$ = +3.3) and C-14 ($\Delta\delta_C$ = +1.3) were also shifted downfield when compared with those of latilagascene D (**1**). This unexpected paramagnetic effect at the γ-carbons may be explained by the intermolecular hydrogen

Table 1. NMR data of latilagascene D (**1**), (*J* in Hz)

Position	^{1}H	^{13}C	DEPT	HMBC (C → H)	NOESY
1α	3.50 dd (7.2; 13.2)	39.9	CH_2	—	H-1β, H-2
1β	1.81 t (12.8)	—	—	—	H-1α, H-16a
2	2.26 m	44.5	CH	1α, 1β, 3, 16a, 16b	H-1α, H-3, H-4, H-16a, 3-OH
3[a]	4.17 br s	74.7	CH	1α, 16a, 16b	H-2, H-4
4	1.51 dd (3.6; 9.6)	51.7	CH	1α, 5	H-2, H-3,H-5,H-12, Me-17
5	3.61 d (9.6)	58.1	CH	7α, 4, 17	H-7β/8β, H-12, 3-OH
6	—	63.9	C	4, 17	—
7α[b]	1.98 m	38.7	CH_2	17	H-7β/8β
7β[c]	1.56 m	—	—	—	H-7α/8α
8α[b]	1.98 m	23.3	CH_2	9	H-7β/8β
8β[c]	1.56 m	—	—	—	H-7α/8α
9	1.06 m	33.9	CH	18, 19	H-7α/8α, H-11, Me-20
10	—	26.5	C	9, 18, 19	—
11	1.42 dd (8.0; 11.2)	29.8	CH	18, 19	H-7α/8α, H-9, Me-20
12	6.96 br d (10.8)	144.8	CH	20	H-5, H-7β/8β, H-19
13	—	133.9	C	11, 20	—
14	—	194.9	C	1α, 4, 12, 20	—
15	—	91.2	C	1α, 1β	—
16a	4.68 t (10.8)	63.2	CH_2	1β	H-1β, H-2, H-7β, H-8β, H-16b, 3-OH
16b[a]	4.17 dd (4.8; 10.8)			—	H-2, H-16a
17	1.09 s	20.0	CH_3	—	H-4, H-7α/8α
18	1.04 s	29.1	CH_3	9, 11, 19	H-9, H-11
19	0.79 s	16.3	CH_3	18	H-12, H-2′
20	1.81 s	12.4	CH_3	12	H-9, H-11
3-OH	3.24 br s	—	—	—	H-1β, H-5, H-7β/8β, H-16a
15-OCin					
1′	—	165.7	C	2′, 3′	—
2′	6.41 d (15.6)	117.3	CH	3′	Me-19, H-3′, H-5′, H-6′, H-7′,H-8′, H-9′
3′	7.64 d (15.6)	146.8	CH	5′, 9′	H-2′, H-5
4′	—	133.9	C	2′, 5′, 6′, 8′, 9′	—
5′, 9′	7.42 m	128.2	CH	3′, 5′, 6′, 8′, 7′, 9′	H-2′
6′, 8′	7.33 m	130.8	CH	5′, 9′	H-2′
7′	7.33 m	129.0	CH	5′, 9′	H-2′
16-OBz		167.5	C	2′′, 6″, 16a, 16b	—
1″	—	129.7	C	2″, 3″, 5″, 6″	—
2″, 6″	7.95 dd (1.2; 7.2)	129.8	CH	4″	H-3″,H- 5″
3″, 5″	7.36 m	128.4	CH	2″,4″, 6″	H-2″,H- 6″
4″	7.49 m	133.2	CH	2″, 6″	H-3″,H- 5″

[a,b,c]Overlapped signals.

bonding between the hydroxyl group and the ketone at C-14 conjugated with the double bond at C-12.

The diterpenes latilagascene D (**1**) and latilagascene E (**2**), together with latilagascenes A–C (**5**–**7**), previously isolated from *Euphorbia lagascae*,[18] were the first macrocyclic lathyrane diterpenes showing oxidation at C-16.

The known lathyrane diterpene jolkinol B (**4**), previously isolated from *Euphorbia jolkini*, was identified by comparison of its ^{1}H NMR spectrum with that reported in the literature,[21] and by combined inspection of the ^{13}C NMR, DEPT and 2D-NMR spectra (COSY, HMQC, HMBC, and NOESY) which allowed the unambiguous assignment of the ^{13}C resonances (see Section 3) which are reported here for the first time.

Compound **3**, named latilagascene F, was a colorless oil with $[\alpha]_D^{25}$ −97° ($CHCl_3$, *c* 0.10). Its molecular formula ($C_{27}H_{34}O_5$) was assigned on the basis of HRSIMS, whose spectrum showed a pseudomolecular ion at *m*/*z* 439.2482 $[M+1]^+$, indicative of 11 unsaturation degrees. IR absorptions at 3470, 1713, 1655, and 716 cm^{-1} implied the presence of hydroxyl, ester carbonyl, an enone system and a benzenic ring, respectively. The ^{1}H NMR spectrum of **3** showed, besides the signals for a benzoate ester (see Section 3), an olefinic proton at δ 6.97 (d, J = 10.4 Hz), three tertiary methyl groups (δ 1.21, 1.05 and 0.28), a methyl group bounded to a sp^2 carbon (δ 1.90) and two oxygenated methine protons at δ 4.19 (t, J = 3.4 Hz) and 3.75 (d, J = 9.2 Hz). Furthermore, it also provided evidence for the presence of the characteristic cyclopropane ring of lathyrane diterpenes by the high field methine signals at δ 1.09 (m) and 1.42 (m). The spectroscopic data of compound **3** were very similar to those found for jolkinol B (**4**), evidencing the presence of the same parent alcohol, but with a benzoic acid moiety instead of cinnamic acid at C-15. This feature was also corroborated by the FABMS spectrum which exhibited a prominent peak at *m*/*z* 105 $[C_6H_5CO]^+$, whereas evidence for the cinnamate ester was missing. An important difference between the ^{1}H NMR spectrum of jolkinol B and compound **3** is the resonance of the β orientated Me-19, at the cyclopropane ring, which appears at

Table 2. NMR data of latilagascene E (**2**), (*J* in Hz)

Position	^{1}H	^{13}C	DEPT
1α	3.52 dd (7.2; 13.6)	39.5	CH_2
1β	1.82 t (13.2)	—	—
2	2.26 m	44.6	CH
3[a]	4.24 br s	74.7	CH
4	1.57 dd (4.4; 9.6)	51.7	CH
5	3.61 d (9.6)	57.8	CH
6	—	63.1	C
7α[b]	2.00 m	38.6	CH_2
7β[c]	1.59 m	—	—
8α[b]	2.00 m	23.3	CH_2
8β[c]	1.59 m	—	—
9	1.08 m	34.9	CH
10	—	27.4	C
11	1.47 dd (8.4; 11.6)	29.7	CH
12	7.08 br d (11.2)	148.1	CH
13	—	136.8	C
14	—	196.2	C
15	—	91.0	C
16a	4.67 t (10.8)	63.7	CH_2
16b[a]	4.24 m	—	—
17	1.00 s	20.2	CH_3
18	1.04 s	29.0	CH_3
19	0.80 s	16.2	CH_3
20a	4.25 d (12.3)	58.1	CH_2
20b	4.44 d (12.3)	—	—
15-OCin			
1′	—	165.7	C
2′	6.46 d (16.0)	117.0	CH
3′	7.71 d (16.0)	147.2	CH
4′	—	133.8	C
5′, 9′	7.42 m	128.2	CH
6′, 8′	7.34 m	130.9	CH
7′	7.34 m	129.0	CH
16-OBz		167.5	C
1″		129.6	C
2″, 6″	8.02 d (7.2)	129.8	CH
3″, 5″	7.38 m	128.4	CH
4″	7.54 m	133.3	CH

[a,b,c]Overlapped signals.

$\delta = 0.28$ in the spectrum of **3** and at $\delta = 0.78$ in that of jolkinol B (**4**). This remarkable diamagnetic shift, also found in jolkinol A′ and other diterpene with lathyrane skeleton,[22,23] may be due to the shielding effect of the benzene ring which is located in the same β-side of the molecule.[22]

2.2. Biological activity

The MDR-reversing activities of lathyrane diterpenes **1**–**4**, characterized above, were investigated using the rhodamine 123 exclusion test. The effects of the compounds **1** and **4** and of **5**–**7**, isolated previously from the same species,[18] were also evaluated on apoptosis induction on resistant mouse lymphoma cells. Results for MDR are summarized in Table 3. The well-known MDR modifier verapamil was applied for control purposes. Two concentrations (4 and 40 μg/mL) were used in the experiments. All compounds were shown to enhance drug retention in the cells by inhibiting the efflux-pump activity, mediated by P-gp. Compounds **1**, **2**, and **4** were found to be very strong inhibitors (fluorescence activity ratio $R = 110.4$–216.8 at 4 μg/mL concentration) exhibiting latigascene E (**2**) the highest effect, and a manifold activity when compared to that of the positive control verapamil ($R = 2.8$ at 10 μg/mL concentration). The results showed concentration dependence for compounds **1**, **3**, and **4**. However, for latilagascene E (**2**) the effect was somewhat lower at higher concentration meaning that both of the applied concentrations were in the saturation zone. The maximum effect was found at the applied lowest dose.

In further experiments, the *in vitro* effects of latilagascene B (**6**), which was isolated in major quantity, in combination with doxorubicin on human MDR1 gene transfected mouse lymphoma cells, were examined. As can be observed (Table 4 and Fig. 2), a synergistic interaction between doxorubicin and compound **6** was found (fractional inhibitory index, FIX = 0.292). In the antiproliferative assay, the ID_{50} for compound **6** was 4.58 μg/mL and for doxorubicin was 0.35 μg/mL. The ID_{50} value for doxorubicin was decreased to 0.095 μg/mL in the presence of latilagascene B (1.25 μg/mL).

The structures of the diterpenes **1**–**7** differ in the substitution pattern of ring A, having compound **2** also a different substitution at C-20. Therefore, they may be considered as a homogeneous set of compounds allowing considerations of structure–activity relationships. As can be observed in Table 5, by its calculated log *P* values, all compounds are lipophilic and have a molecular weight comprised between 600 and 438. It is interesting to note that, latilagascene E (**2**), the most active, has the highest value of topological polar surface area (TPSA) and the highest molecular weight value. However, the correlation between the MDR reversing effects and calculated molecular orbital parameters must be multifactorial (including H donators and possibly dipoles) because none of the calculated parameters were directly correlated alone. As an example, we can refer to the role of the hydroxyl group at C-3 (Table 5). The functionally important molecular conformation can be distorted after acetylation of the hydroxyl group.

Thus, taking in account the results for compounds **1**–**4** and previous ones found for latilagascenes A-C (**5**–**7**),[18] it can be concluded that the presence of a free hydroxyl group at C-3 is very important for the interaction of lathyrane diterpenes with P-gp, since the acetylation of the hydroxyl function at that position decreased significantly the fluorescence activity ratio value as was demonstrated in the case of latilagascene C (**7**). This conclusion was also found for jatrophane derivatives.[24] Additionally, the presence at C-16 of a second aromatic ring in compounds **1** and **2** seems to be a relevant structural feature for the activity. The hydroxyl group at C-20 also appears to play an important role being responsible for the higher activity of compound **2** when compared to that of **1** ($R = 168.5$ at 4 μg/mL).

The results obtained in this study, together with our previous ones, led to support the importance of the involvement of ring A of lathyranes and jatrophanes, including the rearranged derivatives, in P-gp modulation.

Table 3. Effect of compounds **1**–**4** on reversal of multidrug resistance (MDR) in human MDR1 gene transfected mouse lymphoma cells

Samples	Concentration (μg/mL)	FSC[a]	SSC[a]	FL-1[a]	Fluorescence activity ratio
PAR + R123[b]	—	528.7	190.7	925.1	—
PAR − R123	—	525.0	189.9	917.4	—
MDR + R123[c]	—	524.3	223.6	4.4	—
Verapamil	10	516.0	222.4	11.2	2.8
1	4	512.8	211.6	685.9	168.5
	40	508.8	209.6	714.0	175.4
2	4	500.4	207.4	882.2	216.8
	40	497.7	206.9	810.8	199.3
3[d]	4	647.4	213.7	212.6	15.3
	40	674.6	230.7	1029.4	74.0
4	4	518.9	213.1	449.2	110.4
	40	527.1	211.2	649.3	159.5
DMSO	10 μL	470.4	199.6	3.9	0.95

[a] FSC, forward scatter count; SSC, side scatter count; FL-1, mean fluorescence intensity of the cells. Fluorescence activity ratio values were calculated by using the equation given in Section 3.

[b] PAR, a parent cell without MDR gene.

[c] MDR, a parent cell line transfected with human MDR gene.

[d] The results of compound **3** were obtained from a different assay (PAR + R123: FL−1 = 711.4; MDR+R123: FL−1 = 11.0; Verapamil: FL−1 = 47.5, FAR = 3.4).

Table 4. The evaluation of the interaction between latilagascene B(**6**) and doxorubicine on human mdr1-gene transfected mouse lymphoma cells

Sample	ID_{50}(μg/mL)	FIX[a]	Interaction
Doxorubicine	0.35		
Latilagascene B (**6**)	4.58		
Latilagascene B + doxorubicine	0.095	0.292	Synergism

[a] FIX, fractional inhibitory index.

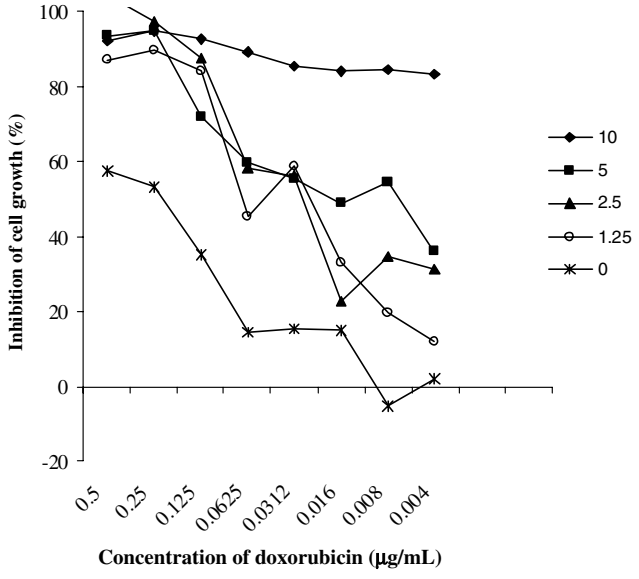

Figure 2. Effect of latilagascene B (**6**) in combination with doxorubicin on human MDR1 gene transfected mouse lymphoma cell line (treatments with 10, 5, 2.5, 1.25, and 0 μg/mL, latilagascene B concentration).

The apoptosis-inducing activity of the compounds **1** and **4** and of **5**–**7** is summarized in Table 6. As can be observed, at low concentration the most active compound was the potent P-gp modulator latilagascene D (**1**). However, it is interesting to note that latilagascene B (**6**), which has shown the lowest activity at low concentration, was the most active at the highest concentration. The comparison of the effect of low- and high-concentrations possibly reflects somehow the *in vitro* model of 'therapeutic window' and nothing else, showing the lowest active concentration and the lowest toxic dose. According to these results and considering together our previous data, we can conclude that lathyrane diterpenes can be considered not only as effective anti-MDR agents but also as apoptosis inducers, reinforcing the importance of macrocyclic diterpenes as antitumour agents.

3. Experimental

3.1. General experimental procedures

Optical rotations were obtained using a Perkin Elmer 241 polarimeter. IR spectra were determined 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), using $CDCl_3$ as solvent. Low resolution mass spectrometry was taken on a Kratos MS25RF spectrometer (70 eV); HRSIMS and HRESIMS were recorded on a Micromass Autospec spectrometer and on an ESI-TOF spectrometer (Biotof II Model, Brucker), respectively. Column chromatography was carried out on SiO_2 (Merck 9385). TLC were performed on precoated SiO_2 F_{254} plates (Merck 5554 and 5744) and visualized under UV light and by spraying with sulphuric acid–acetic acid–water (1:20:4) or sulphuric acid–water (1:1) followed by heating. HPLC was carried out on a Merck-Hitachi instrument, with UV detection (254 nm), using a Merck LiChrospher 100 RP-18 (10 μm, 250 × 10 mm) column.

3.2. Plant material

Euphorbia lagascae Spreng. was cultivated in Cova da Beira, Coimbra, Portugal, and identified by Dr. Teresa

Table 5. Anti-MDR activity and physico-chemical properties[a] of compounds **1**–**7** (number of hydrogen bond donors and acceptors, molecular weight, molecular volume, octanol/water partition coefficient and topological polar surface area)

Compound	FAR (4 μg/ml)	FAR[b] (40 μg/ml)	N° H/acceptors	N° H/donators	MW	MV	log *P*	TPSA
1	168.5	175.4	7	1	584	543	7.2	102.4
2	216.8	199.2	8	2	600	551	5.9	122.7
3	15.3	74.0	5	1	438	415	5.3	76.1
4	110.4	159.5	5	1	464	443	5.8	76.1
5	13.0	46.0	7	1	522	488	5.5	102.4
6	28.1	102.1	6	2	480	451	4.8	96.4
7	12.2	13.8	8	0	564	524	6.2	108.5

[a] Physico-chemical parameters were determined by using the JME molecular editor. (version April 2006, http://www.molinspiration.com/).
[b] FAR, fluorescence activity ratio.

Table 6. Effect of compounds **1** and **4**–**7** on apoptosis induction in human MDR1 gene transfected mouse lymphoma cells

Sample	Concentration (μg/mL)	Early apoptosis (%)	Total apoptosis (%)	Necrosis (%)
Cell control without staining	—	0.02	0.04	0.10
Cell control annexin-V + PI[a]+	—	2.98	18.11	2.26
Cell control annexin-V−PI − 1.0% DMSO	—	0.08	18.31	0.01
M-627[b] control +	5	1.72	65.01	16.30
	50	0.02	98.00	1.81
1	7	12.7	34.7	1.54
	75	2.96	67.97	6.83
4	7	6.91	27.00	1.16
	75	4.06	38.83	0.41
5	7	7.79	22.70	0.85
	75	33.88	55.24	2.16
6	7	5.87	18.96	0.79
	75	1.38	96.31	1.82
7	7	7.80	21.90	0.69
	75	1.04	41.31	3.93

[a] PI, propidium iodide.
[b] M-627: 12H-benzo(α)phenothiazine.

Vasconcelos (plant taxonomist) of Instituto Superior de Agronomia, University of Lisbon. A voucher specimen (no. 323) has been deposited at the herbarium of Instituto Superior de Agronomia.

3.3. Extraction and isolation

The air-dried powdered plant (5.9 kg) was extracted with methanol (6× 12 L) at room temperature. Evaporation of the solvent (under vacuum, 40 °C) from the crude extract yielded a residue of 284 g which was suspended in boiling MeOH and frozen, to give a precipitate (55 g) consisting mainly of waxes, that was eliminated by filtration. The filtrate was evaporated, and the residue (233 g) resuspended on a MeOH/H_2O solution (1:2, 3 L), and extracted with Et_2O (6× 2 L). The ether extract was dried (Na_2SO_4) and evaporated under vacuum, yielding a residue (68.3 g) that was chromatographed over SiO_2 (2 kg), using mixtures of *n*-hexane–EtOAc (1:0 to 0:1) and EtOAc–MeOH (19:1 to 1:4) as eluents. According to differences in composition as indicated by TLC, eight crude fractions were obtained (Fr A–H). The more apolar crude fractions containing mainly waxes and triterpenes were not further investigated.

The crude Fr D (10.4 g) eluted with *n*-hexane–EtOAc (7:3 to 2:3) was subjected to CC on SiO_2 with mixtures of *n*-hexane–EtOAc. After TLC monitoring, chromatographic fractions were combined into five secondary fractions (Fr D_A–D_E).

The residue of Fr D_D (1.5 g, *n*-hexane–EtOAc, 13:7 to 3:2) was successively rechromatographed by CC using gradients of *n*-hexane–EtOAc and *n*-hexane–CH_2Cl_2, by preparative TLC ($CHCl_3$–MeOH, 19:1) and HPLC yielding 14 mg of compound **1** (MeOH–H_2O, 4:1, 4 mL/min, t_R = 17 min), 2 mg of compound **3** (MeOH/H_2O, 7:3, 4 mL/min, t_R = 19 min) and 7 mg of compound **4** (MeOH–H_2O, 13:7, 4 mL/min, t_R = 55 min).

The residue (7.2 g) of the crude Fr E eluted with *n*-hexane–EtOAc (13:7 to 1:9) was chromatographed on SiO_2 (285 g) with mixtures of *n*-hexane–EtOAc of increasing polarity, giving four secondary fractions (Fr E_A–E_D). The residue (2.0 g) of fraction E_C eluted with *n*-hexane–EtOAc (13:7 to 9:1) was chromatographed twice on silica gel using mixtures of *n*-hexane–EtOAc, *n*-hexane–CH_2Cl_2 and CH_2Cl_2–MeOH of increasing polarity. After TLC monitoring, fractions eluted with *n*-hexane–EtOAc, 1:1 (1.71 g) were submitted to CC as above. The residue of fractions eluted with CH_2Cl_2–MeOH 19:1 (374 mg) shows the presence of a spot with a strong absorption at 254 nm, which was separated by CC and preparative TLC ($CHCl_3$–MeOH 19:1, 3×), and purified by HPLC to give 6 mg of compound **2** (MeOH–H_2O 7:3, 4 ml/min, t_R = 25 min)

3.3.1. Latilagascene D [(2R*, 3S*, 4R*, 5R*, 6R*, 9S*,11S*,15R*)-16-benzoyloxi-15β-cinnamoyloxy-5α,6β-epoxy-3β-hydroxy-14-oxolathyr-12E-ene] (1). colorless oil; $[\alpha]_D^{25}$ −158.0° ($CHCl_3$, *c* 0.2); HRSIMS: *m/z* 585.2865 $[M+1]^+$, (calcd for $C_{36}H_{41}O_7$: 585.2852); IR (film), ν_{max}: 3475, 2944, 2864, 1715, 1655, 1632, 1450, 1276, 1167, 714 cm^{-1}; FABMS *m/z* (rel. int.): 607 $[M+Na]^+$ (5), 585 $[M+1]^+$ (14), 584 $[M]^+$ (3), 454 $[M+1-C_9H_7O]^+$ (8), 437 $[M+1-C_9H_8O_2]^+$ (3), 315 (14), 307 (33), 154 $[C_9H_7O+Na]^+$ (100) 131 $[C_9H_7O]^+$ (54), 105 $[C_7H_5O]^+$ (31), ^1H and ^{13}C NMR see Table 1.

3.3.2. Latilagascene E [(2R*, 3S*, 4R*, 5R*, 6R*, 9S*,11S*,15R*)-16-benzoyloxi-15β-cinnamoyloxy-5α,6β-epoxy-3β,20-dihydroxy-14-oxolathyr-12E-ene] (2). Colorless oil; $[\alpha]_D^{25}$ −103.0° ($CHCl_3$, *c* 0.12); HRESIMS: *m/z* 601.2796 $[M+1]^+$, (calcd for $C_{36}H_{41}O_8$: 601.2801); IR (film), ν_{max}: 3475, 2944, 2864, 1715, 1655, 1632, 1450, 1276, 1167, 714 cm^{-1}; FABMS *m/z* (rel. int.): 624 $[M+Na+1]^+$ (3), 518 $[M+Na-C_7H_5O]^+$ (1), 476 $[M+Na-C_9H_8O_2]^+$ (1), 131 $[C_9H_7O]^+$ (81), 105 $[C_7H_5O]^+$ (94); ^1H and ^{13}C NMR see Table 2.

3.3.3. Latilagascene F [(2R*, 3S*, 4R*, 5R*, 6R*, 9S*, 11S*, 15R*)-15β-benzoyloxy-5α,6β-epoxy-3β-hydroxy-14-oxolathyr-12E-ene] (3). colorless oil; $[\alpha]_D^{25}$ −97° ($CHCl_3$, *c* 0.10); HRSIMS: *m/z* 439.2482 $[M+1]^+$, (calcd for $C_{27}H_{33}O_5$: 439.2484); IR (film), ν_{max}: 3470, 2944, 2864, 1713, 1655, 1632, 1276, 1167, 716 cm^{-1}; FABMS *m/z* (rel. int.): 461 $[M+Na]^+$ (1), 439 $[M+1]^+$ (1), 438 $[M]^+$ (0.3), 421 $[M+1-H_2O]^+$ (0.3), 105 $[C_7H_5O]^+$ (68), 77 $[C_6H_5]^+$; ^1H NMR (400 MHz, $CDCl_3$): 8.05 (d, *J* = 7.6 Hz, H-2′/H-6′), 7.59 (t, *J* = 7.6 Hz, H-4′), 7.45 (t, *J* = 7.6 Hz, H3′/H-5′), 6.97 (d, *J* = 10.4 Hz, H-12), 4.19 (t, *J* = 3.4 Hz, H-3), 3.75 (d, *J* = 9.2 Hz, H-5), 3.60 (dd, *J* = 7.2/13.2 Hz, H-1α), 2.06 (m, H-2), 1.96 (m, H-7α/H-8α), 1.90 (s, H-20), 1.80 (t, *J* = 13.2 Hz), 1.65 (m, H-7β/H-8β), 1.42 (m, H-4/H-11), 1.21 (s, H-17), 1.12 (d, *J* = 6.4 Hz, H-16), 1.09 (m, H-9), 1.05 (s, H-18), 0.28 (s, H-19).

3.3.4. Jolkinol B [(2R*, 3S*, 4R*, 5R*, 6R*, 9S*, 11S*,15R*)-15β-cinnamoyloxy-5α,6β-epoxy-3β-hydroxy-14-oxolathyr-12E-ene] (4). Colorless oil; $[\alpha]_D^{25}$ −121.0° ($CHCl_3$, *c* 0.14), (lit.[20] $[\alpha]_D^{20}$ −120.0° (*c* 0.40 $CHCl_3$); IR (film), ν_{max}: 3514, 2944, 2864, 1718, 1655, 1632, 1450, 1276, 1167, 714 cm^{-1}; FABMS *m/z* (rel. int.): 488 $[M+Na+1]^+$ (1), 154 $[C_9H_7O+Na]^+$ (100), 131 $[C_9H_7O]^+$ (66); ^{13}C NMR (100.61 MHz, $CDCl_3$): 195.1 (C-14), 165.7 (C-1′), 146.5 (C-3′), 144.3 (C-12), 134.0 (C-13, C-4′), 130.7 (C-7′), 129.0 (C-6′, C-8′), 128.2 (C-5′, C-9′), 117.4 (C-2′), 92.0 (C-15), 78.6 (C-3), 63.9 (C-6), 58.2 (C-5), 52.2 (C-4), 44.9 (C-1), 38.8 (C-7), 38.5 (C-2), 33.8 (C-9), 29.7 (C-11), 29.1 (C-18), 26.3 (C-10), 23.3 (C-8), 20.0 (C-17), 16.2 (C-19), 13.2 (C-16), 12.4 (C-20).

3.4. Assay for MDR reversal effect

3.4.1. Cells. The L5178 Y mouse T-lymphoma parental cell line was transfected with the pHa MDR1/A retrovirus as previously described.[25] 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.

3.4.2. Assay for rhodamine 123 accumulation test. The harvested cells were resuspended in serum-free McCoy's 5A medium and distributed into Eppendorf tubes at the density of 2×10^6 cell/mL. Then, 2–20 μL of the stock solution (1 mg/mL in DMSO) of the tested compounds was 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 emission wavelengths were 488 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 via the following equation.[26,27]

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

3.5. Assay for antiproliferative effect

The effects of increasing concentrations of the compounds alone 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 (thiazolyl 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, St. Louis, MO, USA) 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 a Dynatech MRX vertical beam ELISA reader. Inhibition of cell growth (as a percentage) was determined according to the formula:

$$100 - \left[\frac{\mathrm{ODsample} - \mathrm{ODmedium\ control}}{\mathrm{ODcellcontrol} - \mathrm{ODmediumcontrol}}\right] \times 100$$

3.6. Checkerboard microplate method as a model for combination therapy

The effects of the anticancer drug doxorubicin and compound **6** in combination were studied on mouse lympho-

ma cancer cells. The dilutions of doxorubicin (A) were made in a horizontal direction, meanwhile the dilutions of resistance modifier (B) vertically in the microtitre plate in 100 μL volume. The cell suspension in the tissue culture medium was distributed into each well in 100 μL 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 colour was measured on a Dynatech MRX vertical beam 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 = fractional inhibitory index, $FIX = FIC_A + FIC_B$, FIX = 0.51–1 additive effect, FIX < 0.5 synergism, FIX = 1–2 indifferent effect, FIX > 2 antagonism.

3.7. Assay for apoptosis induction

The assay was carried out according to the protocol of Alexis Biochemicals[28] with little modification. 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, 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. 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 μL propidium iodide stock solution were added to the samples (final concn 1 mg/mL). The fluorescence activity (FL-1, FL-2) of the cells was measured and analysed on a Becton–Dickinson FACScan instrument.

Acknowledgments

Science and Technology Foundation, Portugal (FCT) supported this work. The authors thank Dr. Teresa Vasconcelos (ISA, University of Lisbon, Portugal) for identification of the plant, Prof. M. Gottesmann and Prof. A. Aszalos (Food and Drug Administration, USA) for cell lines.

References and notes

1. Szakacs, G.; Paterson, J. K.; Ludwig, J. A.; Booth-Genthe, C.; Gottesman, M. M. *Nat. Rev. Drug Discov.* **2006**, *5*, 219.
2. Wiese, M.; Pajeva, I. *Curr. Med. Chem.* **2001**, *8*, 685.
3. Maki, N.; Silver, C.; Ghosh, P.; Chattopadhyay, A.; Dey, S. *Biochemistry* **2006**, *45*, 2739.
4. Reed, J. C. *Trends Mol. Med.* **2001**, *7*, 314.
5. Tolomeo, M.; Simoni, D. *Curr. Med. Chem. Anticancer Agents* **2002**, *2*, 387.
6. Anderson, K. M.; Harris, J. E. *Med. Hypotheses* **2001**, *57*, 87.
7. Hartwell, J. L. *Lloydia* **1969**, *62*, 153.
8. Hohmann, J.; Molnár, J.; Redei, D.; Evanics, F.; Forgo, P.; Kalman, A.; Argay, G.; Szabo, P. *J. Med. Chem.* **2002**, *45*, 2425.
9. Appendino, G.; Della Porta, C.; Conseil, G.; Sterner, O.; Mercalli, E.; Dumontet, C.; Di Pietro, A. *J. Nat. Prod.* **2003**, *66*, 140.
10. Valente, C.; Pedro, M.; Duarte, A.; Nascimento, M. S.; Abreu, P. M.; Ferreira, M. J. *J. Nat. Prod.* **2004**, *67*, 902.
11. Valente, C.; Pedro, M.; Ascenso, J. R.; Abreu, P. M.; Nascimento, M. S.; Ferreira, M. *J. Planta Med.* **2004**, *70*, 244.
12. Valente, C.; Ferreira, M. J.; Abreu, P. M.; Gyémánt, N.; Ugocsai, K.; Hohmann, J.; Molnár, J. *Planta Med.* **2004**, *70*, 81.
13. Valente, C.; Ferreira, M. J.; Abreu, P. M.; Pedro, M.; Cerqueira, F.; Nascimento, M. S. *Planta Med.* **2003**, *69*, 361.
14. Ferreira, M. J.; Gyémánt, N.; Madureira, A. M.; Tanaka, M.; Koos, K.; Didziapetris, R.; Molnár, J. *Anticancer Res.* **2005**, *25*, 4173.
15. Madureira, A. M.; Ferreira, M. J.; Gyémánt, N.; Ugocsai, K.; Ascenso, J. R.; Abreu, P. M.; Hohmann, J.; Molnár, J. *Planta Med.* **2004**, *70*, 45.
16. Madureira, A. M.; Gyémánt, N.; Ascenso, J. R.; Abreu, P. M.; Molnár, J.; Ferreira, M. J. *J. Nat. Prod.* **2006**, *69*, 950.
17. Miglietta, A.; Gabriel, L.; Appendino, G.; Bocca, C. *Cancer Chemother. Pharmacol.* **2003**, *51*, 67.
18. Duarte, N.; Gyémánt, N.; Abreu, P. M.; Molnár, J.; Ferreira, M. J. U. *Planta Med.* **2006**, *72*, 162.
19. Appendino, G.; Jakupovic, S.; Tron, G. C.; Jakupovic, J.; Milon, V.; Ballero, M. *J. Nat. Prod.* **1998**, *61*, 749, and references cited therein.
20. Corea, G.; Fattorusso, E.; Lanzotti, V.; Di Meglio, P.; Maffia, P.; Grassia, G.; Ialenti, A.; Ianaro, A. *J. Med. Chem.* **2005**, *48*, 7055, and references cited therein.
21. Uemura, D.; Nobuhara, K.; Nakayma, Y.; Shizuri, Y.; Hirata, Y. *Tetrahedron Lett.* **1976**, 4593.
22. Adolf, W.; Hecker, E.; Becker, H. *Planta Med.* **1984**, *50*, 259.
23. Vasas, A.; Hohmann, J.; Forgo, P.; Szabó, P. *Tetrahedron* **2004**, *60*, 5025.
24. Corea, G.; Fattorusso, E.; Lanzotti, V.; Motti, R.; Simon, P. N.; Dumontet, C.; Di Pietro, A. *J. Med. Chem.* **2004**, *47*, 988.
25. Coenwell, M. M.; Pastan, I.; Gottesmann, M. M. *J. Biol. Chem.* **1987**, *262*, 2166.
26. Weaver, J. L.; Szabo, G.; Pine, P. S.; Gottesman, M. M.; Goldenberg, S.; Aszalos, A. *Int. J. Cancer* **1993**, *54*, 456.
27. Kessel, D. *Cancer Commun.* **1989**, *1*, 145.
28. Koopmann, G. *Blood* **1994**, *84*, 1115.