Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 68 (2007) 1587–1594
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Cytotoxicity and immunomodulating characteristics of labdane diterpenes from *Marrubium cylleneum* and *Marrubium velutinum*

Anastasia Karioti [a,*], Margarita Skopeliti [b], Ourania Tsitsilonis [b], Jörg Heilmann [c], Helen Skaltsa [a,*]

[a] *Department of Pharmacognosy and Chemistry of Natural Products, School of Pharmacy, University of Athens, Panepistimiopolis, Zografou, 157 71, Athens, Greece*
[b] *Department of Animal and Human Physiology, School of Biology, University of Athens, Panepistimiopolis, Zografou, 157 84 Athens, Greece*
[c] *Institute of Pharmacy, Department of Pharmaceutical Biology, University of Regensburg, Universitätsstrasse 31, D-93040, Regensburg, Germany*

Received 7 September 2006; received in revised form 16 March 2007

Dedicated to Professor Otto Sticher on the occasion of his 70th birthday.

Abstract

From the aerial parts of *Marrubium cylleneum*, one labdane *nor*-diterpene has been isolated together with two labdane diterpenes, hitherto not known as natural products. The structures of the isolated compounds were established by means of NMR [^{1}H–^{1}H-COSY, ^{1}H–^{13}C-HSQC, HMQC-TOCSY, HMBC, NOESY] and MS spectral analyses. Several diterpenoids from *M. cylleneum* and *M. velutinum* were tested for their cytotoxic effect against various cancer cell lines and their immunomodulating potential in human peripheral blood mononuclear cells in standard *in vitro* assays. Our results show a differential cytotoxicity of some compounds as well as their ability to improve selected lymphocyte functions.

Keywords: *Marrubium cylleneum*; *Marrubium velutinum*; Lamiaceae; Labdane diterpenes; *Nor*-diterpenes; Cytotoxicity; Lymphocyte activation

1. Introduction

The genus *Marrubium* (L.) comprises approximately 30 species, indigenous in Europe, the Mediterranean and Asia (Mabberley, 1997). In a previous communication (Karioti et al., 2005a) we studied the structural features of various labdane diterpenes, isolated from the dichloromethane extracts of the aerial parts of *M. cylleneum* and *M. velutinum*. In continuation of our phytochemical investigations onto *Marrubium* species of the Greek flora (Karioti et al., 2003, 2005a,b), we report on the isolation, structure elucidation and identification of one *nor*-diterpene from *M. cylleneum*. Furthermore, since some diterpenoids are reported to exhibit antitumor cytotoxic activity (Henry et al., 2006; Rasikari et al., 2005; Grace et al., 2006), the latter compounds, as well as previously characterized diterpenes from *M. cylleneum* and *M. velutinum* (Karioti et al., 2003, 2005a,b), were evaluated for their antiproliferative effect against a panel of human cancer cell lines comprising adherent and leukemic cells. Most importantly, the immunomodulating potential of *Marrubium* diterpenoids was tested in standard proliferation and cytolytic assays using human peripheral blood lymphocytes as responder and effector cells, respectively. The existence of some specific structural features necessary for the observed activity, as well as the possibility to

* Corresponding authors. Tel./fax: +30 210 7274593.
E-mail address: skaltsa@pharm.uoa.gr (H. Skaltsa).

0031-9422/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2007.03.027

generate eventually molecules of improved biological activity is discussed.

2. Results and discussion

Further phytochemical investigation of the aerial parts of *M. cylleneum* revealed three additional diterpenoids (**1**, **2** and **13**). The HR ESIMS spectrum of compound **1** (cyllenine C) revealed a pseudomolecular peak at *m/z* 293.1746 $[M+H]^+$ indicating a molecular weight corresponding to a molecular formula of $C_{17}H_{24}O_4$. The IR spectrum of **1** contained absorption bands characteristic of aliphatic groups (2920 cm^{-1}, 1460 cm^{-1}) and γ-lactone (1769 cm^{-1}).

The ^{13}C NMR spectrum exhibited resonances of 17 carbons: two tertiary methyl groups (at δ 22.1, 22.4), one secondary methyl group (at δ 15.4), six methylenes (at δ 17.7, 24.5, 27.8, 28.3, 29.3 and 31.2), three methines (at δ 32.3, 45.3 and 75.6), as well as five quaternary carbons (at δ 38.6, 44.0, 91.2, 177.0, and 183.3), two belonging to an ester carbonyl group (at δ 177.0 and 183.3, respectively).

Detailed examination of COSY, HSQC and HMBC spectra showed that compound **1** is a *nor*-diterpenoid with labdane features bearing an oxygen on C-9 and two (6, 16 and 9, 13) lactone functions. COSY along with TOCSY and HSQC-TOCSY experiments revealed the presence of three main spin-systems: H-1a,b/H-2a,b/H-3a,b (fragment A), H-5/H-6/H-7/H-8/H-14 (fragment B) and H-11a,b/H-12a,b (fragment C). To confirm these findings extra series of spectra were recorded at 600 MHz. Diagnostic HMBC correlations between H-1a, H-1b, H-6, H-8, H-11a, H-17/C-10, H-2a, H-3a, H-3b, H-15/C-4 and H-1a, H-7a, H-14, H-17/C-9 were helpful to establish the gross structure of **1**. From the HMBC spectrum it was evident (H-11a, H-11b, H-12/C-13, H-11a/C-9) that these two methylenes should be placed between two tertiary oxygenated carbons forming a lactone ring.

NOESY experiment as well as chemical shifts and coupling constants of the non overlapped protons established the relative stereochemistry of compound **1** (Figs. 1 and 2). The coupling constant of proton H-6 (*dd*, $J = 5.0$, 5.4 Hz) showed its equatorial position, while NOE crosspeaks between H-1a/H-5, H-3b/H-5, H-5/H-6, H-5/H-7b, H-6/H-15, H-5/H-15, indicated that these are on the same side (α), while strong interactions between H-1b/H-17, H-3a/H-17, H-8/H-17 and H-11a/H-17 showed that these are on the opposite side (β). Moreover, the orientation of C-11 with respect to ring B was determined to be equatorial, as revealed by NOE crosspeaks between H-11a/H-17, H-11b/H-14 and H-12b/H-14. Cyllenine C (**1**) is a new compound.

Compounds **2** and **13** were isolated for the first time as natural products. A literature survey indicated that both compounds were recently synthesized (Rigano et al., 2006). Our spectroscopic data are identical with those previously described. As compound **13** was rapidly decomposed, it was not feasible to submit it to bioassays.

Using the MTT dye reduction assay, the aforementioned purified diterpenes (compounds **1**–**12**) were screened for their cytotoxicity against five leukemic and three solid-tumor human cell lines. The 50% inhibitory concentrations (IC_{50}) of the compounds on each cell line are presented in Table 2. Vincristine was used as a standard cytotoxic agent and showed IC_{50} values of less than 1 μM for all cell lines tested. As noticed, differential patterns of inhibition were observed, with some compounds exhibiting remarkable to strong activity against some tumor cell lines, whereas others showed moderate or no cytotoxicity. More specifically, compounds **4/5** and **7** were the most potent diterpenes, as they showed remarkable cytotoxicity in the majority of cancer cell lines tested, with IC_{50} values ranging from 114 to 2.85 μM. Interestingly, **4/5** exhibited their activity only in leukemic cell lines, whereas compound **7** was toxic against all cancer cell lines with the exception of the monocytic leukemia cells U937. Compounds **3**, **8/9** and **10/11** exhibited weak to moderate activity against the three solid-tumor-forming cell lines (MCF-7, HeLa and FM3), but were nearly ever inactive in inhibiting leukemic cell growth. Like **4/5**, also compound **1** presented a similar effect, by being able to significantly reduce leukemic cell survival (IC_{50} ranging from 10.3 to 185.3 μM for **1** and 2.85–57.1 μM for **4/5**), but being not cytotoxic against breast, cervix carcinoma and melanoma. Finally, compound **2** was inactive against all cell lines tested, whereas **12** was significantly active only against T cell leukemia (MOLT-4). As an additional observation, we noticed that MCF-7, HeLa and FM3 cells were more resistant to the assayed compounds, showing clearly lower IC_{50} values (in comparison to the leukemic cells) and no compound showed strong activity in these cell lines. These results are partly in agreement with other reports showing increased resistance, particularly of MCF-7 cells, to the cytotoxic effects of clerodane (Rasikari et al., 2005), brominated (Iliopoulou et al., 2003) and kaurene (Henry et al., 2006) diterpenes. For HeLa cells relatively moderate diterpene-induced cytotoxicity is reported (Iliopoulou et al., 2003).

It is of interest that the tested diterpenes, especially compounds **1**, **4/5** and **12** showed in contrast to the adherent cells differential effects against the leukemic cell lines. Compound **12** is nearly inactive against all tested cancer cells, but exhibited relatively strong activity against MOLT-4 cells (15.4 μM). Compound **1** showed moderate to weak activity against the leukemic cells MOLT-4, DAUDI, K562 and U937 cells, but a strong activity against RAJI B cell leukemia (10.3 μM). Only compound **4/5** was able to significantly reduce the growth of the monocytic U937 cells, by exhibiting an IC_{50} value of 14 μM, whereas all other compounds were not active or showed very weak activity (**6** and **12**, 188 and 175 μM, respectively). In addition, the high cytotoxicity (2.85 μM) of **4/5** against RAJI B cell leukemia is noteworthy, especially due to the fact that the effect against MOLT-4, DAUDI and K562 cells is moderate. No compound showed high activity against the K562

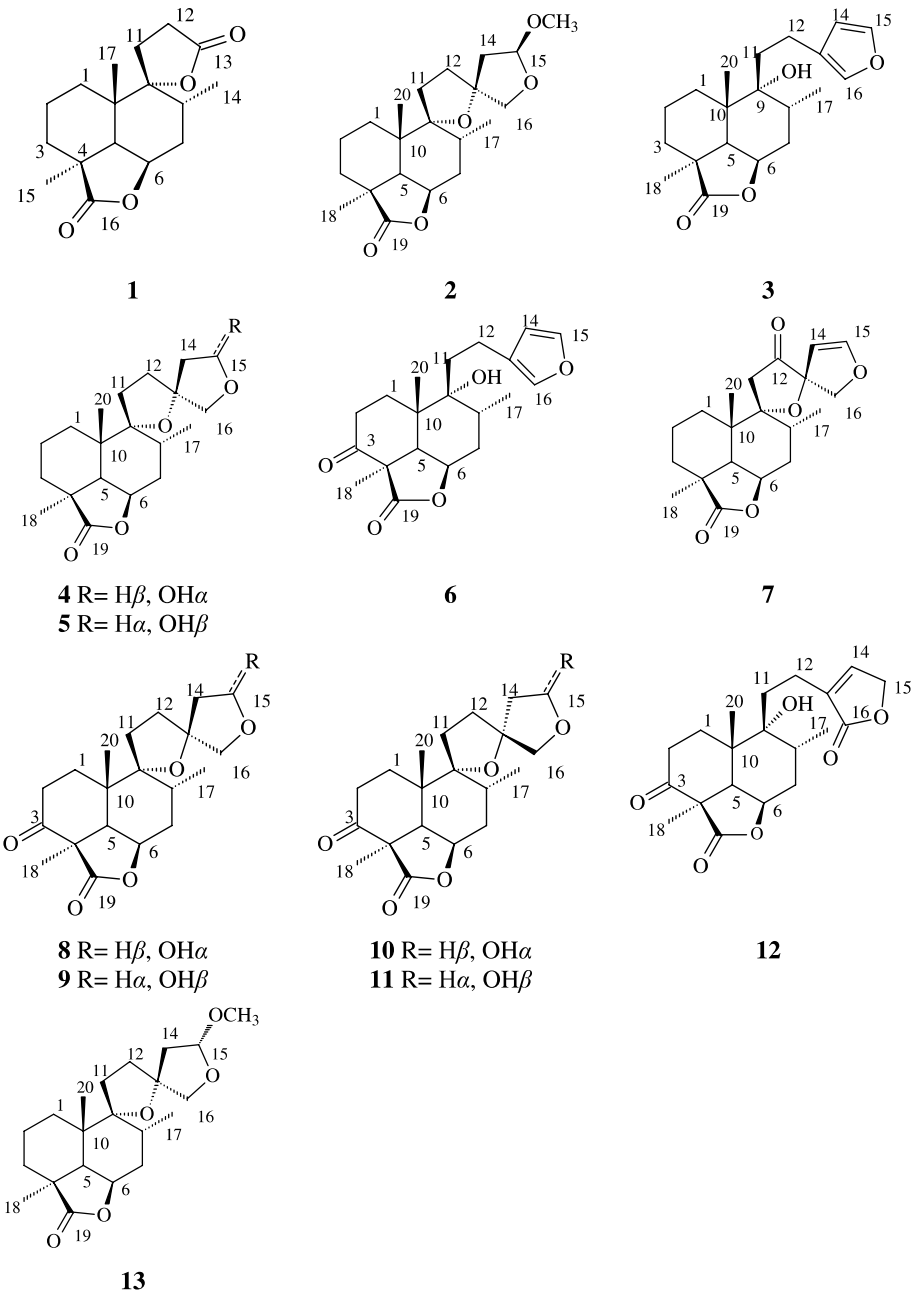

Fig. 1. Structures of compounds **1**–**13**.

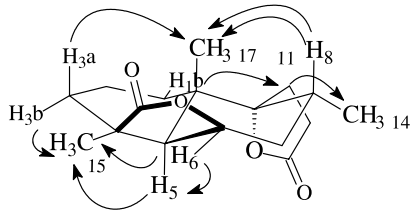

Fig. 2. Key NOESY correlations of compound **1**.

granulocytic leukemia cells. K562 has already been reported to resist against the cytotoxic activity of other plant-derived compounds, such as naturally isolated or synthetically modified flavonoids (Dimas et al., 1999, 2000). In the former study, U937 were also reported to show moderate growth inhibition, whereas most of T and B cell leukemias were very sensitive.

We further assayed the effect of the isolated diterpenes on human lymphocytes, in order to reveal which of these compounds, besides their antitumor activity, could also act as potential modulators of specific immune functions. Thus, we isolated PBMC from healthy volunteers and cultured them for 18 h (~1 day) to 90 h (~4 days) in the presence of various concentrations of compounds **1**–**12**. As for controls, the same cells were incubated in plain medium, with vincristine (1 μg/ml) and interleukin (IL)-2 (500 IU/ml). Enhancement of NK and LAK cell lysis was assayed

Table 1
^{1}H NMR and ^{13}C NMR data for compound **1** (600 MHz, $CDCl_3$, *J* in Hz)

Position	δ_H	δ_C
1a (α)	1.51–1.41 *m*	27.8
1b (β)	1.31–1.27[a]	
2a (β)	1.76 *m*	17.7
2b (α)	1.50 *ddd* (13.8, 13.3, 4.8)	
3a (β)	2.09 *ddd* (14.9, 13.0, 4.9)	28.3
3b (α)	1.47 *m*	
4	–	44.0
5	2.16 *d* (4.6)	45.3
6	4.72 *dd* (5.0, 5.4)	75.6
7a (β)	2.26 *dd* (16.4, 6.6)	31.2
7b (α)	1.69 *dddd* (16.4, 12.1, 6.4, 5.6)	
8	2.15[a]	32.3
9	–	91.2
10	–	38.6
11a	2.20 [a]	24.5
11b	1.93 *td* (11.2, 4.6)	
12a	2.63–2.55[a]	29.3
12b	2.57–2.49[a]	
13	–	177.0
14	0.90 *d* (6.7)	15.4
15	1.28 *s*	22.4
16	–	183.3
17	1.06 *s*	22.1

[a] Signal pattern unclear due to overlapping.

in standard ^{51}Cr-release cytotoxicity assays, whereas induction of T cell proliferation was determined by thymidine incorporation (Tsavaris et al., 2004).

Following the effects on PBMC cytolytic activity (Fig. 3), compounds **3**, **4/5** and **7** were able to significantly augment NK cell-mediated killing of target cells (specific lysis of K562 >40%); Compound **7**, marrubinone B, was the most potent (50.3%) NK cell-stimulator, followed by marrubiin (**3**) (47.2%) and compounds **4/5** (43.1%). All other diterpenes only moderately upregulated NK cell cytotoxicity and % of specific lysis ranged from 30% to 40%.

LAK cell activity was significantly upregulated only by compounds **3**, **8/9** and **10/11** (specific lysis of Daudi targets >35%; Fig. 3), whereas compounds **4/5** and **7** did not enhance LAK cell-mediated target lysis. Most interestingly, compounds **3**, **8/9** and **10/11** were able to equally induce both, NK and LAK cell activity (47.2% and 50.5% for compound **3**, 37.5% and 41.3% for compounds **8/9** and 37.5% and 38.6% for compounds **10/11**, for NK and LAK specific lysis, respectively). Thus, using the latter compounds, we next performed dose and time–responses studies, in order to determine the optimal concentration and incubation time for inducing maximal cytolytic responses. Indeed, the immunoenhancing effect of compounds **3**, **8/9** and **10/11** on NK cell cytotoxicity was most pronounced upon a short-term (ca. 2 days) incubation of PBMC with relatively low concentrations (100 ng^{-1} μg/ml, equivalent to 0.3–3 μM) compared to those used in MTT assays (Fig. 4a). Prolonged incubation (>2 days) did not further increase specific target lysis, whereas higher or lower concentrations of all compounds did not significantly affect NK cell cytotoxicity (Fig. 4a). On the contrary, optimal LAK cell cytotoxicity was observed between 2 and 3 days of exposure and, in some cases, persisted until day 4 (Fig. 4b, compound **3** and **10/11**). No significant further increase in LAK-induced cell lysis could be noticed when PBMC were subjected to prolonged exposure (>4 days; data not shown). Although the mode of action of diterpenoids on NK and LAK cell activity is still unexplored, our results show that at least some of the assayed compounds can induce rapid (18–42 h) activation of NK and/or LAK cell effectors, when administered at low doses (100 ng^{-1} μg/ml).

Proliferation assays revealed that PBMC exposed to compounds **1**, **4/5** and **7** (100 ng/mL) for 72 h, exhibited moderately increased thymidine incorporation (SI values >1), and in the case of compound **10/11**, the SI determined was comparable to that induced by high doses of interleukin-2 (500 IU/ml), which is the most potent soluble mediator of lymphocyte proliferation (Ellery and Nicholls, 2002). More specifically, compounds **1** and **10/11** were most active in inducing high levels of lymphocyte proliferation, with SI values of 2.7 and 7.25, respectively (Fig. 5). It is noteworthy that compounds **10/11** are the only ones among the isolated spiro-diterpenes with opposite stereochemistry at chiral center C-13. These results give evidence that the stereochemistry of C-13 could be important, since compounds **8/9** when assayed in parallel were inactive.

Summing up, of the series of *Marrubium* diterpenoids assayed, compound **3** can selectively act as NK and LAK cell stimulator, by significantly increasing their lytic activity against tumor targets. Its overall cytotoxicity in a MTT based assay is low or insignificant. Compound **4/5** was shown to modulate both, NK cell lysis and PBMC proliferation. Its significant to strong cytotoxic activity in the MTT test is limited to leukemic cell lines. Compound **7**, showed significant cytotoxicity in a broad range of tumor cells, including leukemia, breast and cervix carcinoma, and, at much lower concentrations, it was also capable to regulate the functional programs of NK cells, T cells and probably other mononuclear cell populations. Noteworthy is also the strong stimulation of PBMC by compounds **10/11** which is a multiple higher compared to the other diterpenes. Additionally, the cytotoxicity in leukemic cell lines is negligible in the MTT assay. Due to the limited number of compounds with varying structural features, a correlation between the structure and a specific activity is in the moment not possible. Isolation and synthesis of further diterpenoids with the same skeleton combined with the pharmacological testing, will give more insights into the structural requirements for the interesting activity reported for compounds **3**, **4/5**, **7** and **10/11**. An important issue that should be considered in future investigations for the evaluation of the biologic activity of diterpenes, is that the optimal concentrations required for NK/LAK cell activation and T cell proliferation are much lower compared to the those required for eliminating cancerous cell growth.

Table 2
In vitro cytotoxicity of *Marrubium* diterpenoids **1–12** on human tumor cell lines

$IC_{50} \pm SD$ (in μM)[a]									
Cell lines (origin)	Compounds								
	1	**2**	**3**	**4/5**	**6**	**7**	**8/9**	**10/11**	**12**
MOLT-4 (T cell leukemia)	185.3 ± 5.1	>200	65.3 ± 4.9	38.8 ± 5.6	>200	19.5 ± 1.4	>200	>200	15.8 ± 2.3
DAUDI (B cell leukemia)	67.5 ± 12.2	>200	>200	46.2 ± 7.8	56.4 ± 3.8	13.5 ± 3.2	>200	>200	>200
RAJI (B cell leukemia)	10.3 ± 3.8	>200	>200	2.85 ± 0.5	>200	14.3 ± 4.1	>200	>200	>200
K562 (granulocytic leukemia)	88 ± 7.9	>200	>200	57.1 ± 10.4	>200	114 ± 15.3	>200	>200	>200
U937 (monocytic leukemia)	>200	>200	>200	14 ± 2.1	188 ± 23.7	>200	>200	>200	175 ± 5.2
MCF-7 (breast cancer)	>200	>200	116 ± 15.2	>200	147 ± 19.5	86.3 ± 11.9	65.5 ± 6.8	155.3 ± 21.5	>200
HeLa (cervix cancer)	>200	>200	100 ± 1.3	>200	128 ± 12.9	92.5 ± 8.7	76.1 ± 12.8	95.9 ± 18.5	>200
FM3 (melanoma)	>200	>200	166.1 ± 21.1	>200	159 ± 26.1	75.8 ± 5.7	50.2 ± 3.1	85 ± 13.2	>200

IC_{50} values (expressed in μM) were determined after 72 h of exposure to each compound and represent means ± standard deviation (SD) of three independent experiments performed.
[a] Vincristine was used as positive control and showed $IC_{50} < 1$ μM for all cell lines assayed.

Therefore, our *in vitro* data imply that any future therapeutic administration of high diterpenoid doses in cancer-bearing individuals could result in tumor regression, but could concomitantly cause severe side-effects, such as lymphocyte toxicity. Finally, the use of diterpenes to enhance deficient lymphocyte responses caused by various immune disorders (e.g. malignancies and/or immunodeficiencies) should be further investigated.

3. Experimental

3.1. General experimental procedures

^1H, ^{13}C and 2D NMR spectra were recorded in $CDCl_3$ on Bruker Avance 600, Bruker DRX 400 and Bruker AC 200 (50.3 MHz for ^{13}C NMR) instruments at 295 K. Chemical shifts are given in ppm (δ) and were referenced to the solvent signals at 7.24 ppm and 77.0 ppm for ^1H and ^{13}C NMR, respectively. IR spectra were obtained on a Perkin–Elmer PARAGON 500 FT-IR spectrophotometer. HR ESI mass spectra were measured on a TSQ 7000 spectrometer with a spray voltage of 4 kV. The $[\alpha]_D$ values were obtained in $CDCl_3$ at 20 °C on a Perkin–Elmer 341 polarimeter.

Vacuum liquid chromatography (VLC): silica gel 60H (Merck, Art. 7736); Column chromatography (CC): silica gel 60 (SDS, 40–63 μm), gradient elution with the solvent mixtures indicated in each case; Sephadex LH-20 (Pharmacia). TLC: Merck siliga gel 60 F_{254} (Art. 5554). Detection: UV-light, spray reagent [vanillin-H_2SO_4 on silica gel].

3.2. Plant material

Aerial parts of *M. velutinum* Sibth. & Sm. were collected from Kellaria-Parnassos mountain (Sterea Hellas) in July 1998 and of *M. cylleneum* were collected from Mainalon mountain (Peloponnese) in May 1999. Voucher specimens have been kept in the Herbarium of Patras University (UPA) under the numbers Skaltsa & Lazari 114 and Skaltsa and Lazari 111b-99.

3.3. Extraction and isolation

The air-dried powdered aerial parts of *M. cylleneum* (1.3 kg) were successively extracted at room temperature with petroleum ether, dichloromethane, EtOAc and MeOH (2 l of each solvent, twice, 48 h). The dried dichloromethane extract (26.8 g) was subjected to vacuum liquid chromatography (VLC) over silica gel (8×6.0 cm) with CH_2Cl_2–EtOAc mixtures (95:5–10:90) of increasing polarity to yield 13 fractions (A-M) of 500 ml. Fraction B (2.0 g, eluted with CH_2Cl_2 100%) was further purified by CC over silica gel using cyclohexane–EtOAc (95:5–0:100) and afforded 13 fractions (B_1–B_{13}). Fraction B_{10} (89.3 mg, eluted with cyclohexane–EtOAc 65:35) was further subjected to repeated Sephadex LH 20 (MeOH) and

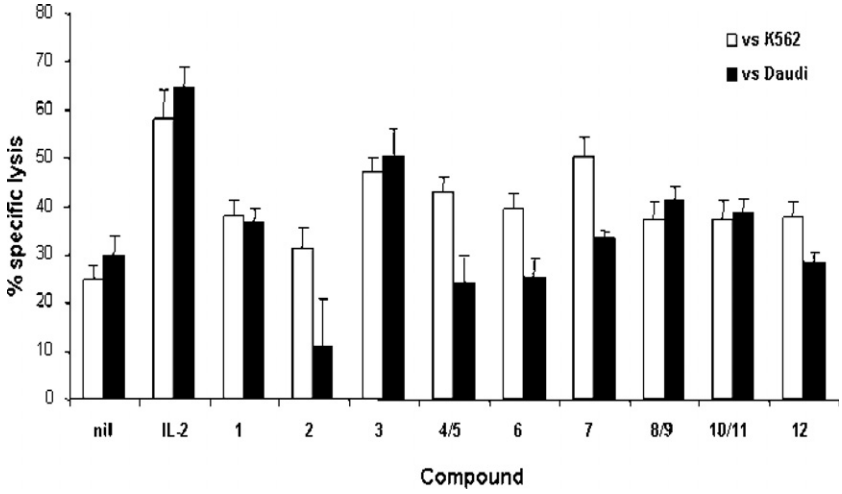

Fig. 3. Effect of purified diterpenes **1**–**12** on PBMC cytolytic activity. PBMC were incubated for 1 day (for NK cell lysis), or preincubated for 3 days with 100 IU/ml IL-2 and then incubated for 1 day (for LAK cell lysis), in the presence of 100 ng/ml of compounds **1**–**12** and for an additional 18 h with ^{51}Cr-labeled K562 or Daudi targets, respectively. Values of % specific lysis represent means ± SD from three individual experiments performed. nil: PBMC incubated in plain complete medium; VIN: PBMC incubated with 1 μg/ml vincristine; IL-2: PBMC incubated with 500 IU/ml of interleukin-2.

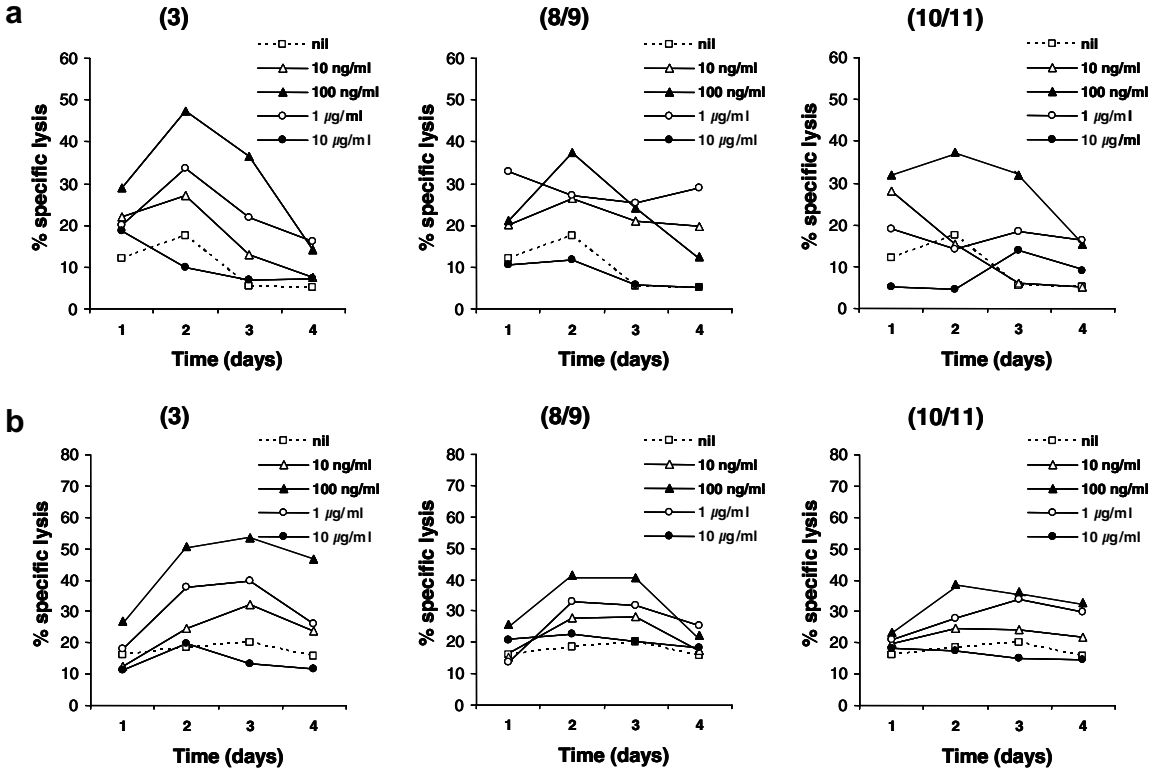

Fig. 4. Dose and time–response curves of PBMC incubated with selected diterpenes inducing both, NK and LAK cell lysis (compounds **3**, **8/9** and **10/11**). PBMC were treated with 10 ng–10 μg/ml of compounds, for 1–4 days and tested in standard ^{51}Cr-release assays against the NK-sensitive K562 (a) and the LAK-sensitive Daudi (b) targets. Values represent means from two individual experiments performed. SD is omitted for clarity reasons and in all cases did not exceed 15% of the mean. nil: PBMC incubated in the absence of compounds.

yielded compound **13** (5.1 mg) and **2** (4.3 mg), as well as a mixture of compounds **2** and **13**. Fraction B_{12} (151.7 mg, eluted with cyclohexane–EtOAc 30:70) was further purified on Sephadex LH 20 (MeOH) and yielded cyllenine C (**1**) (15.1 mg). Fraction C (5.2 g; eluted with CH_2Cl_2–EtOAc 95:5–90:10) was further applied to VLC over silica gel (8 × 5.5 cm) with cyclohexane-EtOAc mixtures of increasing polarity (95:5–0:100) to yield 10 fractions (C_1–C_{10}) of 500 ml. Fraction C_5 (688.9 mg, eluted with cyclohexane-EtOAc 80:20) was subjected to CC over silica gel using cyclohexane–EtOAc (95:5–0:100) and yielded cyllenine D (**2**) (6.6 mg). Isolation procedure of compounds **3**–**12** is

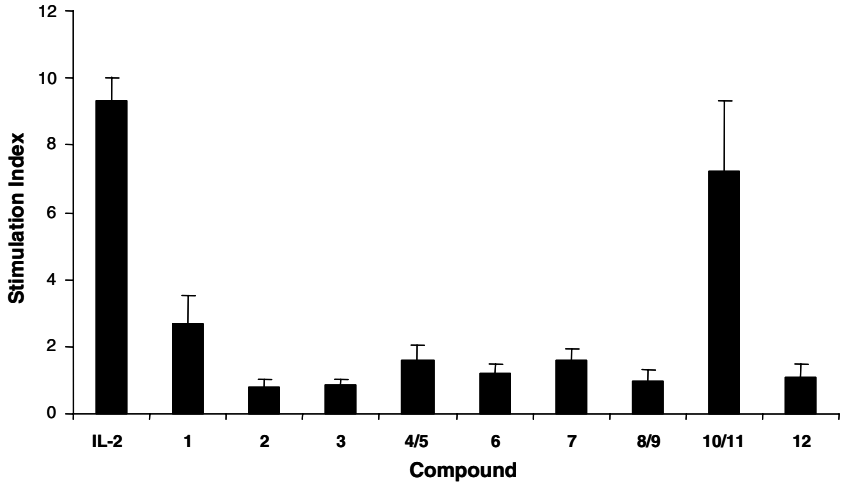

Fig. 5. Effect of compounds **1**–**12** on PBMC proliferation. PBMC were incubated for 3 days in the presence of each compound (100 ng/ml) and for an additional 18 h with ^{3}H-thymidine. Stimulation index (SI) values are calculated as described in Section 3. SI values represent means ± SD from two individual experiments performed. VIN: PBMC incubated in the presence of 1 μg/ml vincristine; IL-2: PBMC incubated in the presence of 500 IU/ml of interleukin-2.

described in a previous communication (Karioti et al., 2003).

3.4. Cyllenine C (*1*)

Yellowish oil (15.1 mg); $[\alpha]_D^{20}$: +11.82 (*c* 0.33, CH_2Cl_2); IR (CaF_2): $\nu_{max} = 2920$ (C–H), 1769 (C=O), 1460 (CH_2) cm^{-1}; HR ESIMS (pos.) *m/z*: 293.1746 $[M+H]^+$ (calcd. for $C_{17}H_{25}O_4$ 293.1753); ^{1}H and ^{13}C NMR spectral data see Table 1.

3.5. Cytotoxicity determination

The human tumor cell lines MCF-7 (breast), HeLa (cervix), FM3 (melanoma), and the leukemic cell lines MOLT-4 (T cell), Daudi and Raji (B cells), K562 (granulocytic) and U937 (monocytic) were maintained in RPMI-1640 (Gibco, Grand Island, NY), supplemented with 10% heat inactivated fetal calf serum (FCS; Gibco), 2 mM L-glutamine (Sigma Chemical Co., St. Louis, MO), 10 mM Hepes (Gibco) and 100 U/ml penicillin–streptomycin (Sigma) (thereafter referred to as complete medium) in a humidified 5% CO_2 incubator. Adherent cells (MCF-7, HeLa and FM3) were grown as monolayers and passaged by trypsinization every 3–5 days, in order to keep them in log phase. The leukemic cell lines were exponentially grown as suspension under the same culture conditions and passaged twice a week.

Cells were seeded into 96-well flat-bottom microplates (Costar, Cambridge, MA; 100 μl/well; 1×10^5 cells/ml) and pre-incubated for 12–18 h to acquire stabilization. Compounds **1**–**12** (Fig. 1) were initially dissolved in DMSO (Sigma; 10 mg/ml), further serially diluted at 5 concentrations (2×10^{-4}–2×10^{-8} M) and added to the wells. All cultures were set in triplicate, whereas cultures containing an equivalent amount of DMSO or 1 μg/ml vincristine (Vincristine sulfate, Sigma V8388) were used as negative and positive controls, respectively. Cells were cultured for 72 h and the cytotoxic activity of each compound was determined by the MTT dye reduction assay as modified by Papazafiri et al. (2005). Absorption was measured by an ELISA reader (WellScan Denley Instruments Inc., UK) at 545 nm, with reference filter at 690 nm. IC_{50} was calculated according to the formula: $100(A_0 - A)/A_0 = 50$, where A and A_0 are optical densities of wells exposed to the compounds and control wells, respectively.

3.6. Effect on PBMC activity

Mononuclear cells were isolated from the peripheral blood (PBMC) of healthy donors by Ficoll-Hypaque density centrifugation (Sigma) as previously described (Skopeliti et al., 2006). PBMC were immediately suspended in complete medium at a density of 2×10^6 cells/ml, seeded into 96-well U-bottom microplates (Costar; 100 μl/well) and exposed to the compounds.

To determine natural killer (NK) cell or lymphokine-activated killer (LAK) cytotoxicity, PBMC were incubated with 10 ng/ml to 10 μg/ml (approx. 0.03–30 μM) of each compound for 18–90 h. LAK cells were generated by preincubating PBMC with 100 IU/ml human recombinant IL-2 (Cetus Corp., Los Angeles, CA) for 3 days. PBMC cultured in complete medium (background cytotoxicity), in the presence of 1 μg/ml vincristine or 500 IU/ml IL-2 were used as negative and positive controls. At the selected time points, for the last 18 h of culture ^{51}Cr-labeled K562 or Daudi targets (radio-labeling performed according to Skopeliti et al., 2006), at an effector (E) to target (T) ratio of 40:1 were added to the respective wells. To determine spontaneous and total ^{51}Cr release, targets were incubated in complete medium or with 3 N HCl, respectively. Radioactivity (in counts per minute; cpm) was measured in cultures'

supernatant using a γ-counter (Minigamma, LKB-Wallac, Finland) and % cytotoxicity was determined according to the formula: (cpm experimental − cpm spontaneous)/(cpm maximum − cpm spontaneous) × 100. Three experiments using PBMC from three different normal donors were conducted.

For the determination of lymphocyte proliferation, PBMC were incubated with 100 ng/ml of each compound for 90 h. Cells incubated under the same conditions in complete medium (non-exposed culture), vincristine (1 μg/ml) or IL-2 (500 IU/ml) were used as for controls. Eighteen hour before culture termination, 1 μCi of [^{3}H]-thymidine/well (The Radiochemical Center, Amersham, UK) was added and cells were harvested in a semi-automatic cell harvester (Skatron Inc., Tranby, Norway). The amount of radioactivity incorporated into DNA was measured in a liquid scintillation counter (Wallac, Turku, Finland) and expressed as cpm. Stimulation index (SI) was calculated according to the formula: cpm of exposed to the compound culture/cpm of non-exposed culture. For the proliferation assays two experiments were conducted using PBMC from two different healthy donors.

Acknowledgements

The authors are grateful to Dr. Theophanis Constantinidis (Institute of Systematic Botany, Agricultural University of Athens) for the identification of the plant material. We thank Mr. J. Kiermaier (Zentrale Analytik der Naturwissenschaftlichen Fakultät IV, Universität Regensburg) for recording the HR ESIMS spectra.

References

Dimas, K., Demetzos, C., Vaos, B., Marselos, M., Kokkinopoulos, D., 1999. Cytotoxic and antiproliferative effects of heptaacetyltiliroside on human leukemic cell lines. Leuk. Res. 23, 1021–1033.

Dimas, K., Demetzos, C., Angelopoulou, D., Kolokouris, A., Mavromoustakos, T., 2000. Biological activity of myricetin and its derivatives against human leukemic cell lines *in vitro*. Pharmacol. Res. 42, 475–478.

Ellery, J.M., Nicholls, P.J., 2002. Alternate signalling pathways from the interleukin-2 receptor. Cytokine Growth Factor Rev. 13, 27–40.

Grace, M.H., Jin, Y., Wilson, G.R., Coates, R.M., 2006. Structure, biogenetic relationships, and cytotoxicity of pimarane-derived diterpenes from *Petalostigma pubescens*. Phytochemistry 67, 1708–1715.

Henry, G.E., Adams, L.S., Rosales, J.C., Jacobs, H., Heber, D., Seeram, N.P., 2006. Kaurene diterpenes from *Laetia thamnia* inhibit the growth of human cancer cells *in vitro*. Cancer Lett. 244, 190–194.

Iliopoulou, D., Mihopoulos, N., Vagias, C., Papazafiri, P., Roussis, V., 2003. Novel cytotoxic brominated diterpenes from the red alga *Laurencia obtusa*. J. Org. Chem. 68, 7667–7674.

Karioti, A., Skaltsa, H., Heilmann, J., Sticher, O., 2003. Acylated flavonoid and phenylethanoid glycosides from *Marrubium velutinum*. Phytochemistry 64, 655–660.

Karioti, A., Heilmann, J., Skaltsa, H., 2005a. Labdane diterpenes from *Marrubium velutinum* and *Marrubium cylleneum*. Phytochemistry 66, 1060–1066.

Karioti, A., Heilmann, J., Skaltsa, H., 2005b. Secondary metabolites from *Marrubium velutinum*, growing wild in Greece. Zeitschr. Naturforsch. 60b, 1–5.

Mabberley, D.J., 1997. The Plant Book, second ed. Cambridge University Press, Cambridge.

Papazafiri, P., Avlonitis, N., Angelou, P., Calogeropoulou, T., Koufaki, M., Scoulica, E., Fragiadaki, I., 2005. Structure–activity relationships of antineoplastic ring-substituted ether phospholipid derivatives. Cancer Chemother. Pharmacol. 56, 261–270.

Rasikari, H.L., Leach, D.N., Waterman, P.G., Spooner-Hart, R.N., Basta, A.H., Banbury, L.K., Winter, K.M., Forster, P.I., 2005. Cytotoxic clerodane diterpenes from *Glossocarya calcicola*. Phytochemistry 66, 2844–2850.

Rigano, D., Grassia, A., Bruno, M., Rosselli, M., Piozzi, F., Formisano, C., Apostolides-Arnold, N., Senatore, F., 2006. Labdane diterpenoids from *Marrubium globosum* ssp. *libanoticum*. J. Nat. Prod. 69, 836–838.

Skopeliti, M., Voutsas, I.F., Klimentzou, P., Tsiatas, M.L., Beck, A., Bamias, A., Moraki, M., Livaniou, E., Neagu, M., Voelter, W., Tsitsilonis, O.E., 2006. The immunologically active site of prothymosin a is located at the carboxy-terminus of the polypeptide. Evaluation of its *in vitro* effects in cancer patients. Cancer Immunol. Immunother. 55, 1247–1257.

Tsavaris, N.B., Katsoulas, H.L., Kosmas, C., Papalambros, E., Gouveris, P., Papantoniou, N., Rokana, S., Kosmas, N., Skopeliti, M., Tsitsilonis, O.E., 2004. The effect of edrecolomab (Mo17-1A) or fluorouracil-based chemotherapy on specific immune parameters in patients with colorectal cancer. A comparative study. Oncology 67, 403–410.