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The sesquiterpene lactone polymatin B from *Smallanthus sonchifolius* induces different cell death mechanisms in three cancer cell lines

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ABSTRACT

A 8β-angeloyloxy-9α-hydroxy-14-oxo-acanthospermolide and five known melampolide sesquiterpene lactones (uvedalin, enhydrin, polymatin B, sonchifolin, and fluctuanin) were isolated from the leaves of *Smallanthus sonchifolius*. The compounds were identified by 1D-, 2D-NMR, HRMS, IR and UV analyses. *In vitro* cytotoxicity assays (MTT) showed that these sesquiterpene lactones display poor cytotoxic effects on peripheral blood mononuclear cells (PBMC) of healthy human subjects, whereas a strong cytotoxicity was observed in leukemia and pancreas cancer cells. For the mechanism of action of polymatin B, oxidative stress seems to be involved. Interestingly, reactive oxygen species (ROS) formation mainly induced different effects: apoptosis in CCRF-CEM cells, necroptosis in CEM-ADR5000 cells through induction of RIP1K, neither apoptosis nor necroptosis in MIA-PaCa-2 cells. Additionally, cells also died partly by necrosis.

1. Introduction

Smallanthus sonchifolius (Poepp. & Endl.) H. Rob. (Asteraceae), commonly known as "yacon", is an herbaceous perennial plant native of the Andes and mostly known for its large edible tuberous roots (Lachman et al., 2003). Previous biological studies have demonstrated anti-inflammatory, antifungal, and antibacterial activities (Inoue et al., 1995; Lin et al., 2003). A wide variety of compounds including different phenolic acids (protocatechuic, chlorogenic, caffeic and ferulic acids), essential oil, ent-kaurenoic acid and related diterpenoid substances are known from leaves of "yacon" (Mercado et al., 2010; Ojansivu et al., 2011). In addition, phytochemical studies have shown the presence of bioactive sesquiterpene lactones (SLs) of the melampolide-type (Frank et al., 2013; Genta et al., 2010).

Sesquiterpene lactones are a large group of secondary plant metabolites mostly known from the Asteraceae family (Zhang et al., 2005). They have been described as the active constituents from several medicinal plants traditionally used to treat inflammatory diseases. However, they possess not just anti-inflammatory activity, but a rather broad spectrum of biological activities, including antibacterial, anthelmintic, uterus contracting, and antimalarial activities (Merfort, 2011). In recent years, the anticancer properties of these compounds have attracted a great interest and extensive research has been carried out to characterize the molecular mechanisms, and their potential use as chemopreventive and chemotherapeutic agents (Kreuger et al., 2012).

Some SL-derived drugs, for example those from thapsigargin, artemisinin, and parthenolide, have reached cancer clinical trials (Ghantous et al., 2010). In the case of parthenolide, the derivatization to a more hydrophilic form, diaminomethylparthenolide (DMAPT or LC-1), has enhanced its therapeutical potential. Furthermore, the ability of DMAPT to selectively eradicate acute myeloid leukemia stem cells led to the initiation of an ongoing phase I clinical trial for its use in hematologic malignancies in the UK (Ghantous et al., 2013).

The aim of this work was to evaluate the *in vitro* cytotoxicity of the SLs enhydrin (**1**), uvedalin (**2**), polymatin B (**3**), sonchifolin (**4**), 8β-angeloyloxy-9α-hydroxy-14-oxo-acanthospermolide (**5**), and fluctuanin (**6**) on three cancer cell lines – the T-cell acute lymphoblastic leukemia cell line (CCRF-CEM), the doxorubicin

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resistant T-cell leukemia cell line (CEM-ADR5000), the pancreatic carcinoma cell line (MIA PaCa-2)–, and on peripheral blood mononuclear cells (PBMC) from healthy human subjects. The isolation of compounds (**1**–**3**) has previously been published (Frank et al., 2013), compounds **4** and **6** have been re-isolated from *S. sonchifolius* and **5** is reported for the first time as a natural product. Polymatin B (**3**) was selected for further investigations on its possible mode of action.

2. Results and discussion

The leaves of *S. sonchifolius* were extracted by maceration with CH_2Cl_2 to obtain the organic extract (OE). This extract was fractionated by column chromatography (CC) on silica gel and afforded compounds **4** and **5**. Compound **4** was identified on the basis of spectroscopic data (HRMS, 1D- and 2D-NMR) as sonchifolin (Inoue et al., 1995).

Compound **5** was obtained as a white powder. Its molecular formula $C_{20}H_{24}O_6$ accounted for nine degrees of unsaturation followed from its HRMS spectrum. The IR spectrum showed absorptions at 3530 cm^{-1} (OH), 1782 cm^{-1} (lactone), 2725, 1668 and 1635 cm^{-1} (unsaturated aldehyde), 1750 and 1656 cm^{-1} (unsaturated ester). The ^1H NMR spectrum exhibited signals attributed to a germacranolide-type compound bearing an angelate ester, because it was similar to the spectra of related melampolides (Cartagena et al., 2000; Macías and Fischer, 1992; Macías et al., 1993). The presence of a 1,10-*cis*-double bond with an aldehyde group at C-10 followed from the chemical shifts of H-1 (δ 6.66,dd) and H-14 (δ 9.50, d) (Herz and Kalyanaraman, 1975). The *trans*-configuration of the 4,5-double bond was deduced from the typical chemical shifts of H-5 (δ 4.94, br d) and H-6 (δ 5.09, t), as well as a large coupling constant for $J_{6,7}$ of 10 Hz (Bohlmann et al., 1979) which agrees with a dihedral angle of approximately 180° between H-6 and H-7. In heliangolides with a 6,12-*trans*-lactone moiety and a *cis*-configured 4,5-double bond, a dihedral angle of about 120° can be calculated between H-6 and H-7 which is in accordance with a coupling constant of $J_{6,7}$ (1.5–3.5 Hz) (Castro et al., 1989; de Hernández et al., 1997, 1999; de Gutiérrez et al., 2001; de Heluani et al., 1989). This small coupling constant is also reported for *cis*,*cis*-germacranolides (Stokes et al., 1992).

The two exo-methylene-γ-lactone hydrogens H-13a and H-13b appeared as doublets at 6.28 ppm and 5.70 ppm with J = 3.2 Hz and 3.0 Hz respectively, clearly indicating the occurrence of a *trans* lactonized melampolide (Diaz et al., 1992; Herz and Sharma,1975; Samek, 1970). The signal at δ 6.52 (dd) is typical for a proton attached to a carbon supporting an ester group (angeloyloxy) and was assigned to H-8. The small coupling constant between H-7 and H-8 (1.5 Hz) indicated that the ester residue at C-8 is β-oriented. The chemical shift of H-9 at 3.99 ppm (dd) is typical for an α-oriented hydroxyl group at C-9 (de Pedro et al., 2003; Le Van and Fischer, 1979). Moreover, the α-configuration at C-9 is confirmed by the coupling constant $J_{8,9}$ (8.5 Hz) and a W-type coupling between H-9β and H-14 (J = 2.0 Hz) (Cartagena et al., 2000). The ^{13}C NMR spectrum showed 20 carbons whose assignments were achieved with the aid of DEPT, HSQC, and HMBC experiments, consisting of one conjugated aldehyde (δ 195.2), two carbonyl carbons (δ 169.2, 167.7), three methylene carbons (δ 121.6, 36.8, 26.4), three olefinic methine carbons (δ 155.2, 139.9, 126.9), four methine carbons including three oxygenated signals (δ 75.3, 71.2, 70.7, 51.3), four quaternary olefinic carbons (δ 144.6, 137.6, 134.5, 126.8) and three methyl carbons (δ 20.5, 16.9, 15.9). Comparison of the ^1H and ^{13}C NMR spectra of compound **5** with those of polymatin A (de Pedro et al., 2003; Le Van and Fischer 1979; Serra-Barcellona et al., 2014) clearly shows that compound **5** is the aldehyde analog at C-14. A C-9 epimer of **5** has been isolated from *Grazielia intermedia* (Bohlmann et al., 1981; López Pérez et al., 2007) as evidenced by the large difference in chemical shift for H-9 (3.99 ppm vs. 5.06 ppm), the smaller value for $J_{8,9}$ (8.5 Hz vs. 5.0 Hz) and the missing W-coupling with H-14. A C-9 O-ethyl derivative of **5** has been isolated from *S. sonchifolius* cultivated in Korea (Hong et al., 2008). Based on the above spectroscopic data, the structure of **5** was therefore deduced to be 8β-angeloyloxy-9α-hydroxy-14-oxo-1(10)E,4E,11(13)-germacratrien-

Table 1
Cytotoxic activity expressed as IC_{50} values (μM) of sesquiterpene lactones **1**–**6** isolated from *Smallanthus sonchifolius* against three tumor cell lines and PBMCs after 24 h of incubation using the MTT assay.

Compound	CCRF-CEM[a]	CEM-ADR5000[a]	MIA-PaCa-2[a]	PBMC[a]	tPSA (Å^2)	*C*log*P*
1	3.6 ± 0.3	26.6 ± 1.3	12.6 ± 0.2	>20	117.7	3.8
2	9.2 ± 2.2	20.1 ± 1.4	21.2 ± 2.0	>20	130.3	2. 6
3	0.8 ± 0.02	1.3 ± 0.2	3.7 ± 0.2	>20	105.2	3.8
4	3.1 ± 0.1	3.1 ± 0.2	7.4 ± 0.6	>20	78.9	4.1
5	2.2 ± 0.2	6.7 ± 0.3	8.9 ± 0.4	>20	89.9	2.4
6	0.6 ± 0.1	1.4 ± 0.09	4.4 ± 0.5	>20	117.7	2.4
CPT[b]	0.08 ± 0.02	0.3 ± 0.1	>10	>10	n.d.	n.d.
Doxo[b]	0.5 ± 0.03	>10	4.8 ± 0.6	>10	n.d.	n.d.

[a] The data represent the IC_{50} values (μM) ± s.d. obtained from the non-linear regression of three independent experiments.
[b] Camptothecin (CPT) and doxorubicin (Doxo) were used as positive controls. tPSA: topological polar surface area. *C*log*P*: partition coefficient. n.d.: not determined.

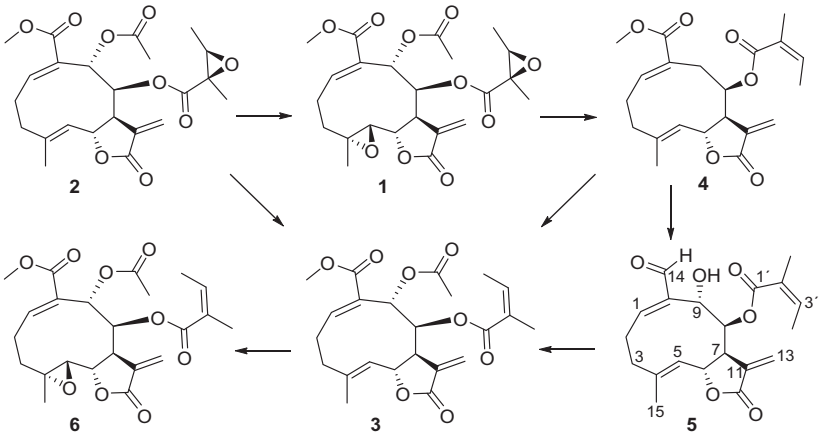

Fig. 1. Structures of the SLs from *S. sonchifolius*. Each arrow represents a structural modification that enhances the cytotoxic activity in CCRF-CEM and MIA-PaCa-2 cells.

12,6α-olide named as 8β-angeloyloxy-9α-hydroxy-14-oxo-acanthospermolide. The absolute configuration of this melampolide, while not determined, should be the same as reported for enhydrin (Kartha and Tee Go, 1976).

Compound **6** was isolated from another cultivar of *S. sonchifolius* (clone LIEY 97-1) (isolation is given in Supplementary information) and identified as fluctuanin based on its NMR and MS data (Lin et al., 2003).

Different biological activities of some of the SLs isolated from *S. sonchifolius* have been previously reported, that include antibacterial, antifungal, cytotoxic, anti-inflammatory, hypoglycemic, and the inhibition of nitric oxide production (Choi et al., 2010; Genta et al., 2010; Schorr et al., 2007; Siriwan et al., 2011a). As part of previous work with SLs isolated from *S. sonchifolius*, the antitrypanosomal activity of enhydrin (**1**), uvedalin (**2**) and polymatin B (**3**) on *Trypanosoma cruzi* was reported (Frank et al., 2013). Previous studies have demonstrated that enhydrin (**1**), uvedalin (**2**), and sonchifolin (**4**) have chemopreventive activity against TPA-induced Raji cells deformation (Siriwan et al., 2011a). These compounds, in doses in the range of 0.22–10 μM, inhibited cell proliferation in cervical cancer cells and induced apoptosis, associated with caspase-3/7 activation and NF-κB inhibition, both in a dose- and time-dependent manner (Siriwan et al., 2011b).

When SLs **4**, **5**, and **6** together with enhydrin (**1**), uvedalin (**2**) and polymatin B (**3**), were assayed for their cytotoxicity on PBMC cells from healthy human subjects, they were shown to be barely

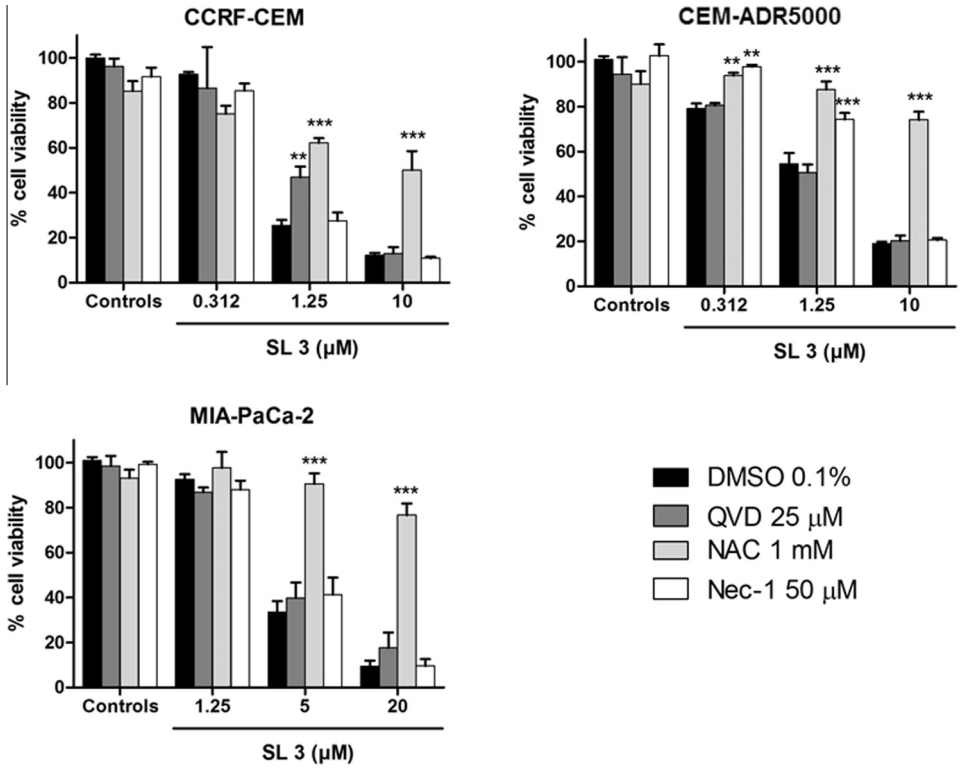

Fig. 2. Effect of SL **3** on the cell viability of cancer cells in the presence and absence of inhibitors. Data represent mean ± s.d. of two independent experiments. QVD-OPh: pan-caspase inhibitor; NAC: N-acetylcysteine, ROS scavenger; Nec-1: necrostatin-1, RIP1K inhibitor. $^{**}p < 0.01$; $^{***}p < 0.001$, **3** with inhibitor vs. **3** without inhibitor (black bars).

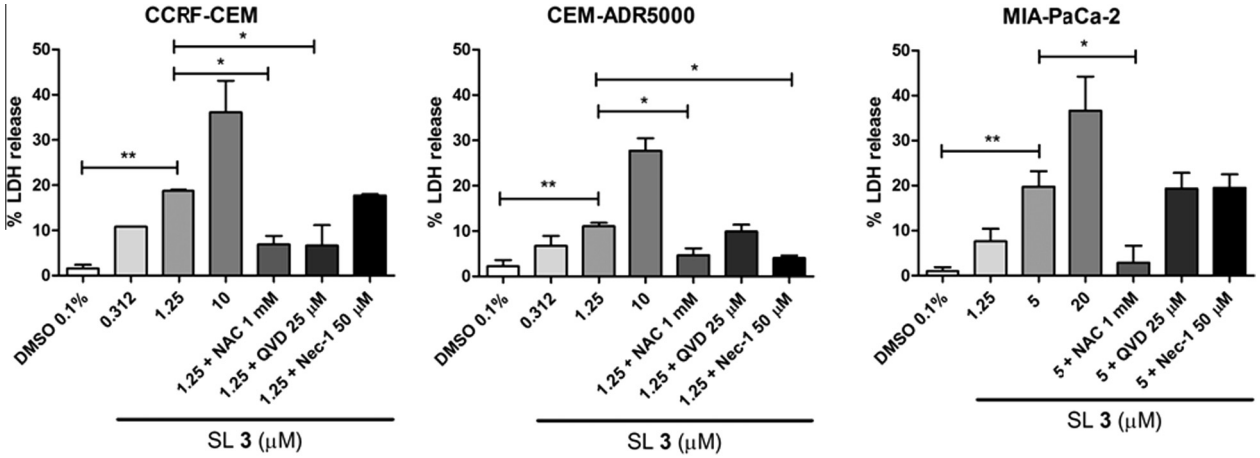

Fig. 3. Effect of SL **3** on the release of cytosolic LDH in cancer cell lines after 24 h treatment. Data represent mean ± s.d. of two independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, **3** with inhibitor vs. **3** without inhibitor.

cytotoxic (IC_{50} values >20 μM) (Table 1). This encouraged a study of their cytotoxicity against further tumor cell lines using the MTT assay. As leukemia and pancreas carcinoma are still cancers with a bad survival prognosis, human T-cell acute lymphoblastic leukemia (CCRF-CEM and CEM-ADR5000) and human pancreatic carcinoma cell lines (MIA PaCa-2) were chosen for study. The IC_{50} values indicated that SLs **3** and **6** are the most cytotoxic compounds in all the cell lines tested, whereas compound **2** is the least cytotoxic in CCRF-CEM and MIA-PaCa-2 cells (see Table 1).

All six SLs belong to the germacranolides subgroup melampolides. They only differ by their functional groups attached to the skeleton. Fig. 1 and Table 1 show the structural formula and how the activity improves gradually. Comparison of the IC_{50} values from SLs **2** and **1** clearly shows that the double bond between C4-C5 decreases the cytotoxicity in CCRF-CEM and MIA-PaCa-2 cells, whereas the presence of an epoxide enhances it. However, only a marginal increase can be observed if the compound already possesses a high cytotoxicity (compare SL **3** and **6**). In contrast, displacement of the epoxide in the side-chain at C-8 seems to boost the activity significantly (compare **2** and **3**). Moreover, the presence of an angeloyl moiety at C-8 is well-known as an important structural feature for cytotoxicity (Kupchan et al., 1971). In addition, this conjugated side-chain represents a further extracyclic Michael acceptor that favors adduct formation with thiol groups. It has been repeatedly reported that Michael type structural elements, such as α,β-unsaturated carbonyl groups, mediate a variety of biological activities of SLs including their cytotoxicity (Schmidt, 2006). Therefore, the different number of alkylating structural

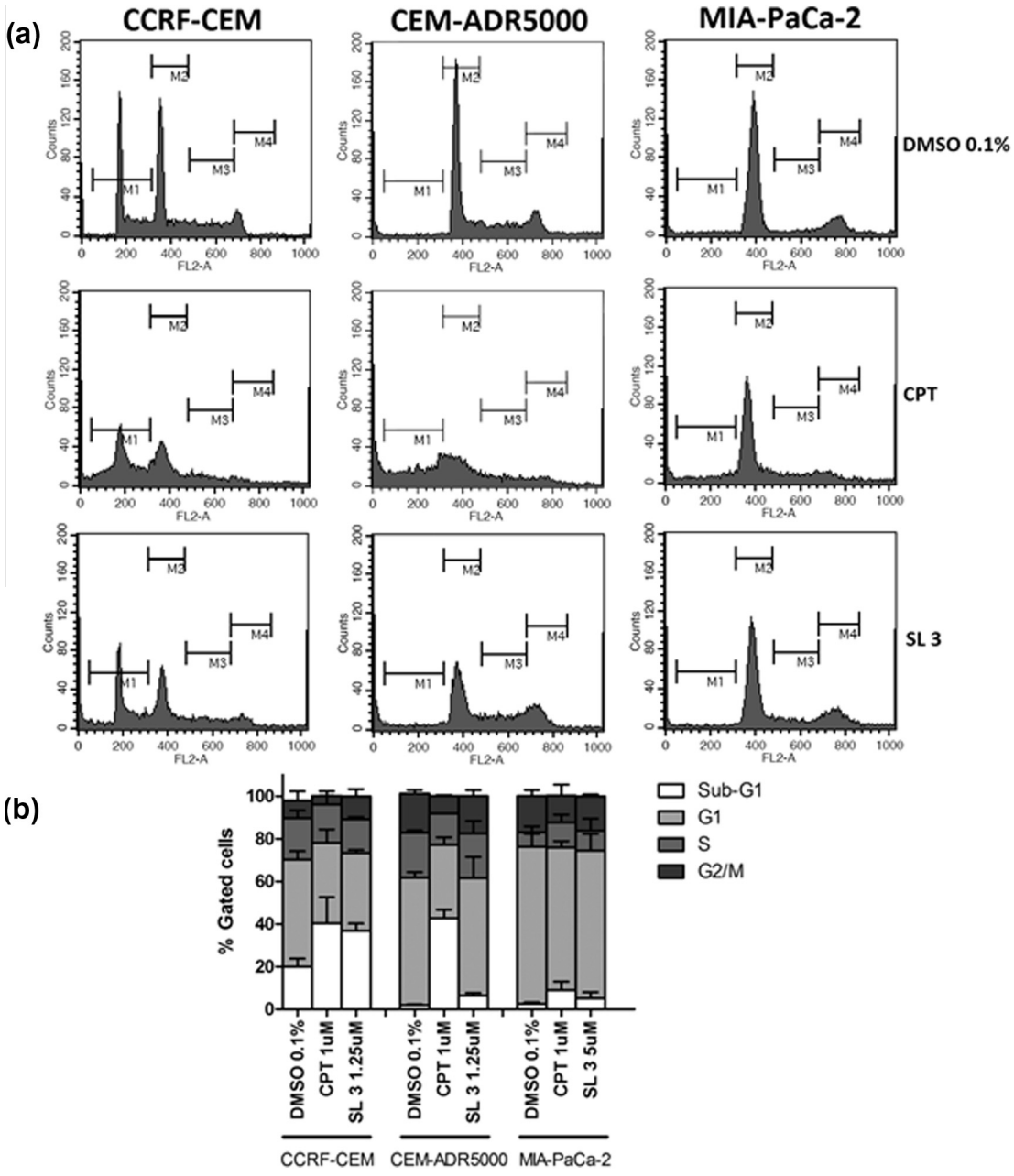

Fig. 4. Effect of SL **3** on cell cycle distributions. The cell cycle was analyzed according to Nicoletti et al. (1991). Cells were cultured for 24 h with and without SL **3** at a concentration of 1.25 or 5 μM. Subsequently the cells were washed with PBS, fixed in cold 70% ethanol and stained with propidium iodide. The cell suspensions were incubated for 30 min at 37 °C and analyzed by FACS. (a) Representative histograms of one cell cycle analysis. (b) DNA content of the gated cells ± s.d. of two independent experiments. CPT: camptothecin.

elements in SLs **1**–**6** may explain the observed differences in their cytotoxic activity. Besides, the type of these reactive elements and their surrounding may also be important. Thus, an acetoxy group at C-9 would increase the reactivity of the α,β-unsaturated ester or aldehyde group at C-10 and hence the activity (compare SL **3** with **4**). A structural feature that is essential for the cytotoxic activity, and that is present in all of these SLs, is the α-methylene-γ-lactone. In contrast, hydrophobic descriptors, such as the partition coefficient (log*P*), are not important for classifying SLs according to their cytotoxicity (Arantes et al., 2011). This is the same with the topological polar surface area (tPSA), another hydrophobicity descriptor (see Table 1).

To gain further insights on how these natural compounds exert their cytotoxic activity, mechanistic studies were performed with polymatin B (SL **3**). The MTT assay was carried out with three different concentrations of this SL, either alone or after addition of different inhibitor molecules (Fig. 2). The reactive oxygen species (ROS) scavenger, N-acetylcysteine (NAC), was able to restore the cell viability of all the cancer cell lines tested after treatment with SL **3** in a statistically significant manner. Cell death of MIA-PaCa-2 cells seems to be more sensitive to ROS since the cell viability was recovered almost to negative control values after treatment with NAC.

A carboxy terminal phenoxy group conjugated to the amino acids valine and aspartate (QVD-OPh), a pan-caspase inhibitor, was not able to recover MIA-PaCa-2 and CEM-ADR5000 from SL **3**-induced cell death in any of the tested concentrations. Nevertheless, a slight increase was observed in CCRF-CEM cells after pretreatment with QVD-OPh at a 1.25 μM concentration of SL **3**. Therefore, it can be assumed that in MIA-PaCa-2 and CEM-ADR5000 cells, the induced cell death by SL **3** may be caspase-3 independent.

Likewise, the cells were pre-treated with necrostatin-1 (Nec-1) in order to determine if SL **3** could induce necroptosis. Only in CEM-ADR5000 cells, Nec-1 promoted a statistically significant increase in cell viability after stimulation with low concentrations of SL **3**.

Cell death studies with SL **3** have shown that the mechanism of cell death induction is cell dependent, but that not only one type of cell death is involved. To study whether cells also partly die by necrosis, the LDH release assay was performed. This assay indicates cell death by necrosis which is accompanied by irreversible membrane damage, cell lysis, and release of lactate dehydrogenase in the extracellular medium (Decker and Lohmann-Matthes, 1988; Korzeniewski and Callewaert, 1983;Fotakis and Timbrell, 2006). All concentrations of SL **3** induce LDH release in the three cell lines (Fig. 3). Moreover, pretreatment of the cells with NAC (1 h prior to treatment with **3**) was able to restore the cell membrane integrity in the three cell lines which confirms again that SL-**3** mediated cell death involves ROS. Furthermore, addition of QVD-OPh only had an effect in CCRF-CEM cells and Nec-1 in CEM-ADR5000 cells, as already observed in the MTT assay.

To determine the effect of SL **3** on the cell cycle, FACS analysis was carried out. Fig. 4 shows the cell cycle distribution of untreated and treated cells after 24 h of incubation. Flow cytometry analysis of untreated cells (DMSO 0.1%) revealed a typical diploid DNA peak corresponding to the majority of the cells which are in the G1 phase of the cell cycle. In addition, a minor peak of doubled diploid cells occurred that corresponds to cells in G2/M phase. No significant change in the sub-G1 population was observed in MIA-PaCa-2 cells after treatment with SL **3**. CEM-ADR5000 cells displayed a very similar behavior. Nonetheless, a 5% increase in the sub-G1 phase and S phase is observed. The missing increase in the sub-G1 DNA content strengthened our findings that caspase-3 dependent apoptosis is not induced by SL **3** in these cell lines.

However, necroptosis seems to be one of the cell death programs induced by SL **3** in CEM-ADR5000 cells since the inhibition of RIP1K with Nec-1 1 h prior to the treatment with the SL recovers partially the cell viability and restores the cell membrane integrity in a statistically significant manner. At high concentrations, no difference is observed which may be due to the induction of uncontrolled necrosis through an overload of ROS in the cells. The reduction of 10% in the G1 phase in CEM-ADR5000 may be explained by an induction of necroptosis at early stages, that means before the complete dismantling of the cell membrane and release of intracellular content into the extracellular space takes place. Nevertheless, further experiments are needed to confirm these assumptions.

Concerning CCRF-CEM cells, SL **3** caused a sub-G1 population augmentation of 17% compared to the negative control (see Fig. 4) suggesting that apoptosis is involved in the cytotoxicity of this leukemic cell line. This is in accordance with our results that the pan-caspase inhibitor QVD-OPh partially restored cell viability and membrane integrity.

3. Conclusions

In summary, these results demonstrate that SLs from *S. sonchifolius* possess a remarkable cytotoxicity, especially fluctuanin (**6**) and polymatin B (**3**) and may have a potential to be developed to therapeutically useful drugs. However, further studies have to be undertaken to gain more insights in the apoptosis and necroptosis mechanism and most importantly their efficacy has to be proven in *in vivo* studies.

4. Experimental

4.1. General experimental procedures

Analytical grade solvents were purchased from Sintorgan (Buenos Aires, Argentina). MeOH (HPLC grade) was from Merck (Darmstadt, Germany). Silica gel (70–230 mesh; Macherey-Nagel, Germany) was used for CC. Analytical TLC was performed on silica gel 60 F_{254} aluminum sheets (Merck KGaA, Germany). Low-pressure liquid chromatography (LPLC) was carried out using a Waters Model 510 pump (Waters Millipore®, USA), an UV detector (Labomatic, Switzerland), a Retriever II sample collector (Teledyne Isco, USA) and a SE 120 chart recorder (BBC Goerz-Metrawatt, Germany). The stationary phase consisted of Lichroprep® RP-8 (25–40 μm; Merck KGaA, Germany). Ultrapure H_2O for HPLC was produced *in situ* with a F.M.4 water distiller (Figmay, Argentina). HPLC equipment included a Waters® 600 chromatographer, equipped with a Waters Delta 600 pump, Waters AF on-line degasser, Waters 2996 photodiode-array detector (PDA) and Empower® software. An Agilent 6890 Series GC system coupled to an Agilent 5973 Network Mass Selective Detector and an Agilent 7683 Series Injector (Agilent Technologies, USA) was used. Mass spectrometry experiments were carried on a ThermoQ Exactive mass spectrometer with Orbitrap-Analyzer (Thermo Scientific, West Palm Beach, FL, USA). IR experiments were performed on solid state using KBr pellets on a Nicolet 380 spectrometer (Thermo Scientific, USA). UV spectral data were acquired from HPLC-PDA. For the ^{1}H NMR and ^{13}C NMR experiments, compounds were dissolved in $CDCl_3$, and TMS was used as internal standard (Sigma, USA). For NMR experiments a Bruker DRX 400 MHz NMR spectrometer at 400 MHz (^{1}H) and 100 MHz (^{13}C) was used. High resolution mass spectrometry (HRMS) analysis was done in the atmospheric pressure chemical ionization (APCI) mode. Inhibitors: quinolyl-valyl-O-methylaspartyl-[2,6-difluorophenoxy]-methyl ketone (QVD-OPh, MP Biomedicals, California,

USA), N-acetylcysteine (NAC, Roth, Karlsruhe, Germany), necrostatin-1 (Nec-1) and camptothecin (CPT, Sigma Aldrich, Missouri, USA). A microplate reader (Bio-Rad, Japan) was used for absorption measurements in the MTT assay. Cell cycle analysis by FACS was performed with a FACScan Cytometer (Beckton Dickinson, USA). Percentage of cell cycle distribution was determined using the MODFIT software (Becton Dickinson, USA).

4.2. Plant material

Leaves of *S. sonchifolius* (Poepp. & Endl.) H. Rob. (Asteraceae), clone LIEY 97-2, were collected in May 2010 from experimental crops located at Centro Universitario "Horco Molle," Universidad Nacional de Tucumán (26°47′S, 65°19′W, 547 m.a.s.l.). A voucher specimen is deposited at the Herbarium of Instituto Miguel Lillo, S. M. de Tucumán, Argentina (LIL 607176). From this cultivar, enhydrin (**1**), uvedalin (**2**), polymatin B (**3**), sonchifolin (**4**) and 8β-angeloyloxy-9α-hydroxy-14-oxo-acanthospermolide (**5**) were isolated as described below. Another cultivar of *S. sonchifolius* (clone LIEY 97-1), that was processed similarly, yielded fluctuanin (**6**) together with **1**, **2**, and **3**.

4.3. Extraction and isolation

Plant material (350 g) was extracted by maceration at room temperature with CH_2Cl_2 (2 × 6.8 L, 30 min) and this extract subjected to silica gel CC yielded 10 fractions (F_{1A}–F_{5B}). Additional chromatography of fraction F_{3A} (1.67%) carried out by silica gel CC, using *n*-hexane with increasing proportions of CH_2Cl_2 (0–100%) and EtOAc with increasing proportions of MeOH (0–100%) yielded 50 fractions (50 ml/each) that were pooled into 18 fractions ($F_{3A\text{-}I}$ through $F_{3A\text{-}XVIII}$) according to their TLC profiles. Fraction $F_{3A\text{-}IV}$ (426 mg) was further subjected to LPLC on a RP-8 column and eluted with MeOH:H_2O (75:25, v/v) to yield compound **4** (9.8 mg).

Fraction $F_{3A\text{-}XVII}$ showed a precipitate that was separated by centrifugation and monitored by TLC [silica gel 60 F_{254}; *n*-hexane:EtOAc (1:1, v/v)]. The precipitate (207 mg) was subjected to silica gel CC using a gradient elution from pure *n*-hexane with increasing amounts of EtOAc to reach a final concentration of 100% EtOAc. From a total of 73 fractions, fractions 35 through 37 were pooled according to their TLC profiles [silica gel 60; *n*-hexane:EtOAc (1:1, v/v)] to afford compound **5** (4.5 mg).

Extraction of air-dried *S. sonchifolius* leaves and isolation of compounds **1**, **2**, and **3** were done as previously reported (Frank et al., 2013).

Purity analysis of compounds **1**, **2**, and **3** was further carried out by HPLC on a RP-18 column [125 × 4 mm] (Merck, Germany); flow: 1.0 ml/min; at room temperature and 210 nm UV-detection. Gradient elution was initiated with H_2O:MeOH (57:43, v/v) from 0 to 32 min; from 47 to 52 min H_2O:MeOH (5:95, v/v) and finally from 55 to 60 min back to initial conditions. Purity was calculated as the ratio of the peak area of the compound and the total area of all the peaks and expressed as percentage. Purity was at least 90% and higher.

Purity of compounds **4** and **5** was analyzed by GC–MS on a cross-bond 100% dimethylsiloxane column [30.0 m × 250 μm] and He as gas carrier (1.8 ml/min). The injector temperature was 250 °C and the initial oven temperature 120 °C reaching a final temperature of 270 °C [temperature rate: 10 °C/min]. Detection was made in a scan mode [40–400 Da] with a transfer line temperature of 280 °C. Purity was calculated as the ratio of the peak area of the compound and the total area, and expressed as percentage and was 90% and higher.

4.4. 8β-Angeloyloxy-9α-hydroxy-14-oxo-1(10)E,4E,11(13)-germacratrien-12,6α-olide (5)

Amorphous white solid. UV (HPLC-PDA) λ_{max}: 220 nm; IR (KBr) ν cm^{-1}: 3530, 2725, 1782, 1750, 1668, 1656, 1635; ^1H NMR (400 MHz, $CDCl_3$) δ in ppm, mutiplicity (*J* in Hz; assignment): 9.50 d (2 Hz; H-14); 6.66 dd (10 and 7 Hz; H-1); 6.52 dd (8.5 and 1.5; H-8); 6.28 d (3.2; H-13a); 6.16 qq (7.2 and 1.4; H-3′); 5.70 d (3.0; H-13b); 5.09 t (10; H-6); 4.94 broad d (10; H-5); 3.99 dd (8.5 and 2; H-9), 2.67 m (H-2a); 2.62 m (H-7); 2.46 m (H-3b); 2.40 m (H-2b); 2.11 br t (12; H-3a); 2.02 dq (3H; 7.2 and 1.4; H-4′); 1.93 br s (3H; H-15); 1.90 quint (3H; 1.4; H-5′). ^{13}C NMR (100 MHz, $CDCl_3$): δ 195.2 (d, C-14), 169.2 (s, C-12), 167.7 (s, C-1′), 155.2 (d, C-1), 144.6 (s, C-10), 139.9 (d, C-3′), 137.6 (s, C-4), 134.5 (s, C-11), 126.9 (d, C-5), 126.8 (s, C-2′), 121.6 (t, C-13), 75.3 (d, C-6), 71.2 (d, C-9), 70.7 (d, C-8), 51.3 (d, C-7), 36.8 (t, C-3), 26.4 (t, C-2), 20.5 (q, C-5′), 16.9 (q, C-15), 15.9 (q, C-4′). ^{13}C NMR assignments were achieved with the aid of DEPT, HSQC and HMBC experiments. HRMS: pos. APCI (MeOH), (M + H)+ m/z 361.1654 (calculated for $C_{20}H_{25}O_6$: 361.1651). GC–MS m/z (rel. int.): 361 (100) [M + H]+; 261 (19) [M + H-$C_5H_9O_3$]+; 243 (18) [M + H-$C_5H_9O_3$–H_2O]+; 83 (3) [$C_5H_8O_2$]+.

4.5. Cell viability

Cell viability was carried out by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as previously described (Mosmann, 1983). Human tumor cells: CCRF-CEM, CEM-ADR5000 were a gift from Prof. T. Efferth, University of Mainz, and MIA-PaCa-2 from American Type Culture Collection (ATCC number CRL-1420™, Manassas, USA). Normal PBMC cells were isolated from human buffy coats obtained from the University Hospital, Freiburg, Germany (ethical permission number from the ethics commission, University of Freiburg: 356/13:2013). Briefly, cells were plated in 96-well flat-bottomed tissue culture plates followed by overnight incubation at 37 °C and 5% CO_2 to allow cell attachment. Cells were incubated for 24 h in the presence or absence of increasing concentrations of compounds **1**–**6** dissolved in DMSO. Different inhibitors, such as QVD-OPh, NAC and Nec-1 were used and added 1 h prior to the stimulation of the cells with the compounds. CPT was used as positive control. Control cells were treated with the highest concentration of DMSO (0.1%). 25 μl of MTT solution (5 mg/ml in phosphate buffered saline, PBS) were added to each well and the plate incubated for additional 3 h. The plates were centrifuged and the excess MTT was vacuum-aspirated. The formazan crystals were dissolved in DMSO and the absorbance of purple formazan was measured at 595 nm. The sample values were referred to the DMSO control. The results were analyzed using GraphPad 5 computer software (La Jolla, California, USA). The IC_{50} values with 95% confidence interval were obtained by non-linear regression (concentration versus percentage of inhibition).

4.6. LDH release assay

LDH release assay was performed using the Cytotoxicity Detection kit (LDH; Roche Diagnostics) according to the manufacturer's protocol. Briefly, cells were seeded in six-well plates in RPMI medium containing 10% FCS at 37 °C, 5% CO_2. After 24 h of incubation with different concentrations of **3** in the presence or absence of inhibitors (NAC, QVD-OPh or Nec-1) an aliquot of 100 μl/well of medium was removed. The LDH concentration in these samples was assayed allowing the spectrophotometric determination of LDH release into the extracellular space at 490 nm. Controls were performed with 2% (v/v) Triton X-100 and set as

100% LDH release. The relative LDH release is defined by the ratio of LDH released over total LDH in the intact cells.

4.7. Cell cycle analysis by FACS

Cell cycle analysis was performed as previously described (Nicoletti et al., 1991). In brief, cells were incubated with compound **3** for 24 h. After treatment, the cells were harvested and centrifuged (161 g, 4 °C, 5 min). Following washing steps with ice-cold PBS (2×) cells were fixed in cold EtOH–H_2O (70:30, v/v) overnight. After fixation, cells were washed again in ice-cold PBS (2×); pelleted by centrifugation (161 g, 4 °C, 5 min) and resuspended in propidium iodide (PI, 1 ml) assay solution (RNAase 10 μg/ml and PI 40 μg/ml in PBS, Sigma). The cells were incubated for 30 min at 37 °C and 5% CO_2 and the DNA content of cells was measured by a FACScan Cytometer (Beckton Dickinson, San Jose, CA) in the FL2 channel (575 nm). The cell cycle distribution and the correspondent percentages in each of the phases (sub-G1, G0/G1, S, G2/M), were determined using the MODFIT software (Becton Dickinson). Ten thousand events were analyzed for each sample.

4.8. Statistical analysis

Values are expressed as means ± standard deviation (s.d.). Statistical analyses of data sets were performed by using One-way or two-way ANOVA followed by Bonferroni's post-hoc test, p-values were calculated and $p < 0.05$ was considered significant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2015.06.020.

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