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Sesquiterpene lactones from *Ambrosia* spp. are active against a murine lymphoma cell line by inducing apoptosis and cell cycle arrest

Renzo Martino [a,b,*,1], María Florencia Beer [a,c,1], Orlando Elso [d], Osvaldo Donadel [c], Valeria Sülsen [a,d], Claudia Anesini [a,d]

[a] Instituto de Química y Metabolismo del Fármaco – IQUIMEFA (UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 2nd floor – (ZC: 1113), Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina
[b] Cátedra de Inmunología, Facultad de Farmacia y Bioquímica, Junín 956 4th floor – (ZC: 1113), Buenos Aires, Argentina
[c] Instituto de Tecnología Química INTEQUI (CONICET), Universidad Nacional de San Luis, Chacabuco y Pedernera, (ZC: 5700), San Luis, Argentina
[d] Cátedra de Farmacognosia, Facultad de Farmacia y Bioquímica, Junín 956 2nd floor – (ZC: 1113), Buenos Aires, Argentina

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ABSTRACT

Sesquiterpene lactones (STLs) are natural terpenoid compounds. They have been recognized as antitumor agents. The purpose of this investigation was to explore the antiproliferative effects of psilostachyin, psilostachyin C, peruvin and cumanin on the murine lymphoma cell line BW5147. Cells were treated with the STLs at different concentrations. Tritiated thymidine uptake was employed to determine cell proliferation. MTT assay was used to analyze cell viability. Flow cytometry assay with annexin V-FITC and propidium iodide was employed to evaluate cell death. Reactive oxygen species (ROS), mitochondrial membrane potential and cell cycle analysis were also evaluated by flow cytometry. Antioxidant enzymes activities were determined spectrophotometrically by kinetic assays. Results showed that these STLs inhibited cell proliferation in a concentration-dependent manner by exerting cytotoxicity through apoptosis. Psilostachyin C was the most active and the less toxic compound. This STL induced apoptosis with an impairment in mitochondrial membrane potential. Psilostachyin C was able to induce ROS generation, related to a modulation of the antioxidant enzymes activity. In addition, it induced cell cycle arrest in S phase. In conclusion, psilostachyin C was found to be active against lymphoma cells exerting both cytostatic and cytotoxic effects. These findings may provide a novel approach for lymphoma treatment.

1. Introduction

Cancer is the leading cause of death worldwide, and according to the World Health Organization (WHO), this disease produced 8.2 million deaths in 2012 (Ferlay et al., 2012). Among cancer, leukemia and lymphoma are a group of heterogeneous neoplastic disorders of white blood cells characterized by the uncontrolled proliferation and the blockage in the differentiation process of hematopoietic cells. Lymphoma is characterized by abnormal lymphocyte proliferation and appears as a solid tumor, most commonly in the lymph nodes of the neck, chest, armpit or groin (Jaffe, 2009). According to the WHO, leukemia and lymphoma stand at the fifth place of cancer-related deaths over the world (Armitage, 2012). The treatment of these pathologies includes radiotherapy, chemotherapy, hormonal therapy, immune therapy and symptomatic and supportive therapy. These conventional treatments may be inefficient and also produce severe adverse effects on different organs (Hopfinger et al., 2012). Natural products have played an important role in drug discovery processes. According to Newman and Cragg (2012), over 60% of the current anticancer drugs derive from natural sources. The vinca alkaloids vinblastine and vincristine, the semisynthetic derivatives etoposide and teniposide, within others, are currently used for cancer treatment (Newman and Cragg, 2013). Therefore, the search of new antitumor agents of natural origin may be a good approach.

Sesquiterpene lactones (STLs) are one of the most relevant secondary metabolites in the Asteraceae family. These compounds display a wide range of biological activities such as antitumor,

Abbreviations: STLs, sesquiterpene lactones; [^{3}H]TdR, tritiated thymidine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; H2DCF-DA, 2–7 dichlorofluorescin diacetate; NAC, N-acetyl-L-cysteine; EC_{50}, effective concentration 50%; PI, propidium iodide; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; Px, peroxidase; CC_{50}, cytotoxic concentration 50%; SI, selectivity index.

* Corresponding author at: IQUIMEFA (UBA-CONICET), Junín 956 2nd floor – (ZC: 1113), Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina.
E-mail address: renzo16martino@hotmail.com (R. Martino).
[1] These two authors contributed equally to this work.

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anti-inflammatory and antiparasitic. These metabolites are C15-terpenoid compounds that commonly contain an α-methylene-γ-lactone group in its structure which is considered essential for their activity (Chaturvedi, 2011). During the last decades, the potential of STLs as anticancer agents has been pointed out by different researchers (Gach and Janecka, 2014). Many STLs have proved to have a significant cytotoxic activity and selectivity against different tumor cell lines that make them attractive for cancer therapy. In this sense, artemisinin, thapsigargin, parthenolide and many of their synthetic derivatives are currently being tested in clinical trials (Ghantous et al., 2010).

In previous reports we have described the antiprotozoal activity of four STLs of the pseudoguaianolide type (psilostachyin, psilostachyin C, peruvin and cumanin) isolated from *Ambrosia* spp. (Asteraceae) (Sülsen et al., 2008a, 2011a, 2011b, 2013). Taking into account the limitations of lymphoma chemotherapy currently available, the need of less toxic and effective drugs and the potential of STLs as anticancer agents, the aim of the present work was to evaluate the effect of four STLs: psilostachyin, psilostachyin C, peruvin and cumanin on a lymphoma cell line. The effect of the compounds on the proliferation and viability on tumoral and normal lymphocytes was assessed. The most active compound was selected to study its mechanism of action.

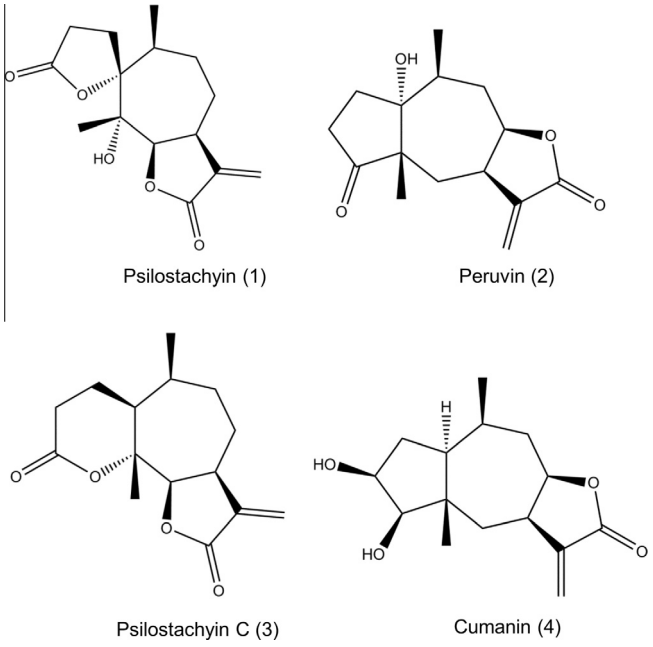

Fig. 1. Chemical structures of the sesquiterpene lactones psilostachyin, peruvin, psilostachyin C and cumanin.

2. Methods

2.1. Chemicals

The four STLs tested in this work had previously been isolated and identified: psilostachyin (1) and peruvin (2) from *Ambrosia tenuifolia* (Sülsen et al., 2008a); psilostachyin C (3) from *Ambrosia scabra* (Sülsen et al., 2011a); and cumanin (4) from *Ambrosia elatior* (Sülsen et al., 2013) (Fig. 1). The purity of compounds 1–4 (>95%) was confirmed by high-performance liquid chromatography (HPLC) analysis.

Tritiated thymidine ([^{3}H]TdR) was purchased from Perkin Elmer (Boston, USA). Annexin V-FITC, propidium iodide, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2–7 dichlorofluorescin diacetate (H2DCF-DA), vincristine and rhodamine 123 were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell line and culture

The tumor cell line BW5147 (ATCC) is a T cell lymphoma cell line expressing the H-2k haplotype, it is CD3$^+$ and bears a TCRαβ, as determined by flow cytometric analysis. Cells were cultured at optimal concentrations of 2×10^5 cells/ml in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and penicillin/streptomycin. Lymphoid cell suspensions from the lymph nodes of C3H (H-2d) inbred male mice (2–4 months old) were obtained aseptically by nylon wool purification of T cells as described previously (Davicino et al., 2011). Animals were humanly handled according to the National Institutes of Health guide for the care and use of Laboratory animals (NIH 8023, 1978). The protocols used in this work were approved by the ethics committee of the Facultad de Farmacia y Bioquímica, UBA (Number: 220612-1). Normal lymph node cells, at a concentration of 1×10^6 cells/ml, were cultured in the same medium as tumor cells. Cells were cultured at a final volume of 0.2 ml in 96-well flat-bottom microtiter plates.

2.3. Cell proliferation

The effects of different concentrations of psilostachyin, psilostachyin C, peruvin and cumanin (0.1–100 μg/ml) on the proliferation of tumoral lymphocytes were studied. Vincristine (0.01–10 μg/ml) was employed as positive control. The proliferation of normal lymphocytes in the presence of psilostachyin C (0.1–50 μg/ml) was also tested. Cell proliferation was evaluated by the [^{3}H]TdR uptake technique. Briefly, cells were cultured during 24 h and then pulsed with [^{3}H]TdR (20 Ci/mmol) for the last 6 h as previously described (Davicino et al., 2011). In the experiments involving N-acetyl-L-cysteine (NAC) treatment, BW5147 cells were pre-incubated with NAC for 18 h, prior psilostachyin C treatment (Di Rosso et al., 2013). Cell proliferation was evaluated as described previously. Data represents the Mean ± SEM of three experiments performed in triplicate. The effective concentration 50% (EC_{50}) for each compound was calculated by the Alexander's method (Alexander et al., 1999).

2.4. MTT colorimetric survival assay

The cell viability of tumoral and normal lymphocytes was determined by the reduction of MTT as previously described (Martino et al., 2013). Briefly, normal and tumoral lymphocytes were incubated alone or in presence of different concentrations of the STLs (0.1–100 μg/ml) in 100 μl of RPMI 1640 containing 10 μl of a 5 mg/ml solution of MTT during 24 h. Additional incubation times (48 and 72 h) on normal lymphocytes were also tested for psilostachyin C. The purple formazan formed was solubilized by the addition of acidic isopropanol. The absorbance was measured using a microplate reader at 570 nm. Untreated cells were used as 100% viability control. Results were expressed as percentage of viability relative to the control.

2.5. Analysis of apoptotic cells by flow cytometry

To determine whether psilostachyin C induced apoptosis in tumoral lymphocytes, 3×10^5 tumoral cells were incubated in the presence or absence of different concentrations of the compound (5 and 10 μg/ml) during 24 h. Cells were then washed twice with PBS, re-suspended in binding buffer at a concentration of 1×10^6 cells. Aliquots of 1×10^5 cells were incubated with annexin

V-FITC and propidium iodide (PI) for 15 min at room temperature in the dark. Samples were shaken gently, diluted with binding buffer and analyzed by flow cytometry FACSCalibur flow cytometer (Becton Dickinson Biosciences) within 1 h. Data were analyzed with the FCS Express 4 software, and expressed as the percentage of viable cells (annexin-V −, PI −), early apoptotic cells (annexin-V +, PI −), late apoptotic cells (annexin-V +, PI +), and necrotic cells (annexin-V −, PI +) (Martino et al., 2013).

2.6. Mitochondrial membrane potential (MMP) assay

BW5147 cells were cultured in the absence or presence of psilostachyin C (5 and 10 μg/ml) for 24 h. Aliquots of cell cultures (1×10^6 cells/ml) were incubated with 1 μM rhodamine 123 for 30 min. Cells were then centrifuged and resuspended in PBS. Changes in the MMP were detected by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson Biosciences). Data were analyzed with the FCS Express 4 software and expressed as the percentage of rhodamine 123 negative cells (Di Rosso et al., 2013).

2.7. Assay for reactive oxygen species (ROS) production

The intracellular level of ROS was determined by using the fluorescent probe H2DCF-DA. Briefly, BW5147 cells were cultured in the absence or presence of psilostachyin C (5 and 10 μg/ml). After 24 h, 1×10^6 cells were washed and resuspended in 1 ml of PBS. Cells were incubated with 10 μM H2DCF-DA for 20 min at 37 °C and the fluorescence was quantified using a FACSCalibur flow cytometer at 480 nm. Data were analyzed with the FCS Express 4 software and expressed as the mean of the fluorescence intensity (MFI) (Di Rosso et al., 2013).

2.8. Superoxide dismutase (SOD), catalase (CAT) and peroxidase (Px) activity assays

Tumoral cells (2×10^6 cells/ml) were incubated 24 h in absence or presence of psilostachyin C (5 and 10 μg/ml). After the addition of PMSF (Sigma) and Triton X 100 (Sigma) the cells were disrupted by pippetting and then centrifuged at 1500*g* during 15 min. After centrifugation the supernatant was used for the estimation of enzymatic activity.

The activities of the three enzymes were determined spectrophotometrically by a kinetic assay as described previously (Martino et al., 2013).

2.9. Determination of cell cycle arrest

Lymphoma cells were treated with psilostachyin C (5 and 10 μg/ml) for 3, 6 and 24 h. Untreated cells (control) were also included. After incubation, cells were harvested and fixed with ice-cooled 70% ethanol (1 ml) at −20 °C for 2 h. Prior to analysis, cells were washed with cold PBS and re-suspended in sodium citrate buffer, containing PI and RNase A. The DNA content was determined by a FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed with the FCS Express 4 software and were expressed as percentage of cells in each stage (G0/G1, S and G2/M) (Manuele et al., 2009).

2.10. Statistical analysis

Results are presented as Mean ± SEM. The level of statistical significance was determined by a one-way analysis of variance (ANOVA and Dunnett's test) employing the GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). Comparisons were referred to the control group. *P* values <0.05 were considered significant.

3. Results

In order to determinate the effect of the STLs on the proliferation of BW5147 cells, a [^{3}H]TdR uptake assay was performed. Fig. 2 shows the proliferation curves of BW5147 cells treated with increasing concentrations of psilostachyin, psilostachyin C, peruvin and cumanin for 24 h. A significant concentration-dependent inhibitory effect was observed for all compounds. Psilostachyin C was the most active compound with an EC_{50} value of 4.89 μg/ml. The other three STLs showed EC_{50} values higher than 15 μg/ml (Table 1). Although all compounds were able to induce a decrease in cell viability also in a concentration-dependent manner, the decrease induced by psilostachyin C was more significant. At a concentration of 10 μg/ml, this compound reduced cell viability to 15.40%. On the other hand, cumanin was able to decrease cell viability at lower concentrations than the other compounds (0.1 and 1 μg/ml) (Fig. 3).

To evaluate if the compounds affected normal cells, the effect on the viability of normal T lymphocyte was assayed. Psilostachyin presented the lowest cytotoxic concentration 50% (CC_{50}) value (25.70 μg/ml) while psilostachyin C did not affect cell viability up to a concentration of 50 μg/ml (Fig. 4 and Table 1).

The relationship between CC_{50} and EC_{50} values for the four STLs was calculated and expressed as a selectivity index (SI). Psilostachyin C showed the highest SI value; whereas cumanin presented the lowest (Table 1). To confirm that psilostachyin C was

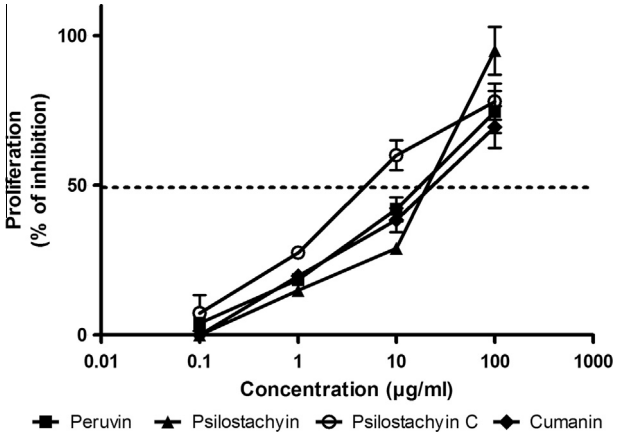

Fig. 2. Effect of STLs on tumoral cell proliferation. BW5147 cells were cultured during 24 h in the absence or presence of increasing concentrations of STLs (psilostachyin, peruvin, psilostachyin C and cumanin). Cell proliferation was determined by [^{3}H]TdR incorporation. Results were expressed as proliferation (% of inhibition) and represent Mean ± SEM of three independent experiments, performed by triplicate.

Table 1
Effective concentration 50% (EC_{50}) on tumoral cells, cytotoxic concentration 50% (CC_{50}) on normal lymphocytes and selectivity indexes (SI) for peruvin, psilostachyin, psilostachyin C and cumanin.

	EC_{50} (24 h) BW5147 cell line (μg/ml)	CC_{50} (24 h) normal T lymphocytes (μg/ml)	Selectivity index (SI)[a]
Peruvin	17.62	35.00	1.99
Psilostachyin	16.40	25.70	1.57
Psilostachyin C	4.89	>50	>10.22
Cumanin	24.54	36.60	1.49
Vincristine[b]	0.7	–	–

[a] The SI was calculated as the ratio between CC_{50} and EC_{50}.
[b] Positive control.

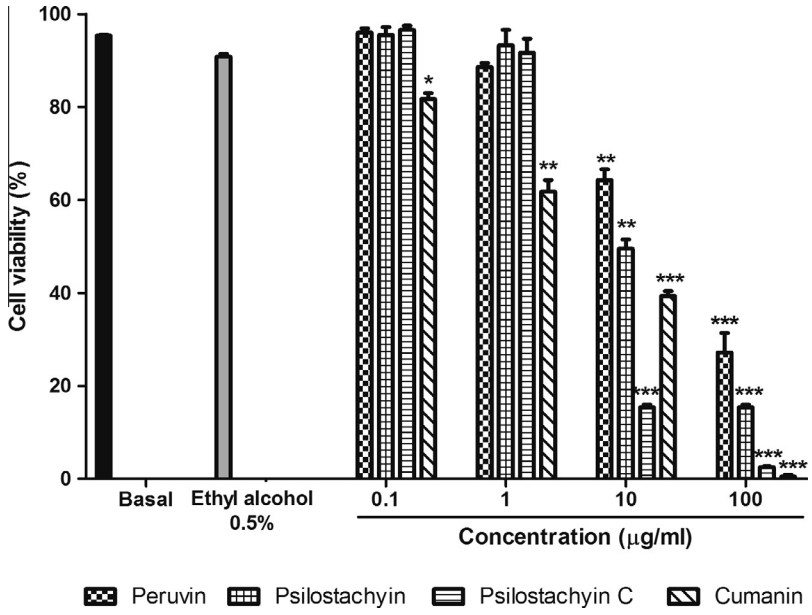

Fig. 3. Effect of STLs on tumoral cell viability. BW5147 cells were cultured during 24 h in the absence or presence of increasing concentrations of STLs (psilostachyin, peruvin, psilostachyin C and cumanin). Cell viability was determined by the MTT colorimetric method. Results were expressed as cell viability (%) related to control and represent Mean ± SEM of three independent experiments performed by triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$ significant differences with respect to basal value (ANOVA and Dunnett's test).

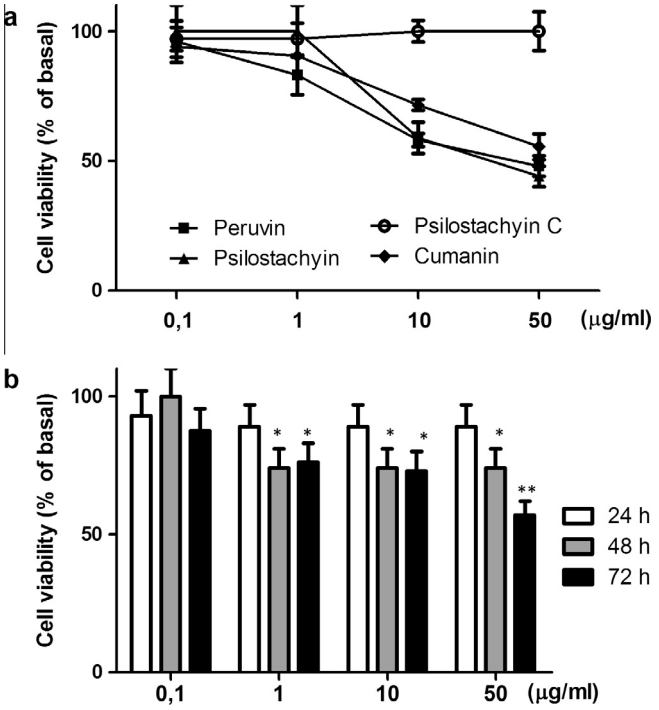

Fig. 4. Effect of STLs on normal lymphocyte viability. (a) Cells were cultured with or without treatments with the STLs during 24 h. After that, cell viability was assessed by the MTT method. (b) Effect of psilostachyin C on normal lymphocyte viability during 24, 48 and 72 h of incubation. Results were expressed as cell viability (% of basal) and represent Mean ± SEM of three independent experiments performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significant differences respect to basal value (ANOVA and Dunnett's test).

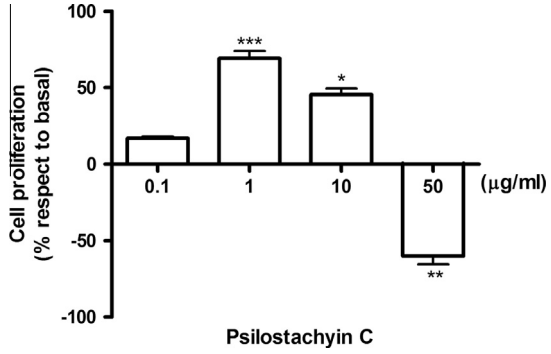

Fig. 5. Effect of psilostachyin C on normal lymphocytes proliferation. Cells were incubated with or without different concentrations of psilostachyin C (0.01–50 μg/ml) during 24 h. Cell proliferation was determined by the incorporation of [^{3}H]TdR. Results were expressed as proliferation (% respect to basal) and represent Mean ± SEM of three independent experiments performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significant differences respect to basal value (ANOVA and Dunnett's test).

the less toxic compound on normal cells, the effect on the viability of normal lymphocytes was studied over the time, specifically at 24, 48 and 72 h. It was observed that this STL maintained CC_{50} values that were above 50 μg/ml over time (Fig. 4b). Moreover, psilostachyin C was capable of inducing the proliferation of normal lymphocytes, mainly at low concentrations (Fig. 5).

Taking into account these results, psilostachyin C was selected for further studies. In order to assess if the growth inhibitory effect observed for this compound was related to apoptosis, this mechanism was evaluated by flow cytometry. As shown in Fig. 6a, psilostachyin C induced cell apoptosis in a concentration-dependent manner. It was observed that this drug induced mainly late apoptosis at 24 h of incubation. To analyse the mechanism involved in apoptosis, the ability of psilostachyin C to interfere with the mitochondrial functioning was assessed using the fluorescent dye rhodamine 123. A significant decrease in the MMP was observed in BW5147 cells treated with psilostachyin C (5 and 10 μg/ml, Fig. 6b). The effect of psilostachyin C on the production of intracellular ROS was also analyzed. Psilostachyin C increased significantly the production of ROS at the concentrations assayed (Fig. 7a). This compound was also able to increase 6.5-fold SOD activity at a concentration of 5 μg/ml. Meanwhile a significant decrease in CAT and Px activities was observed after treatment with this compound (Fig. 7b–d). To further evaluate the role of ROS in the

a

	Viable	Early apoptosis	Late apoptosis	Necrosis
Basal	90.7 ± 2.7	2.7 ± 0.7	3.9 ± 0.4	3.6 ± 0.2
Psilostachyin C 5µg/ml	65.9 ± 0.8 *	11.5 ± 1.3 **	21.4 ± 1.2 ***	1.2 ± 0.4
Psilostachyin C 10µg/ml	36.4 ± 1.2 **	16.0 ± 1.1 **	40.5 ± 2.5 ***	7.1 ± 0.9 *

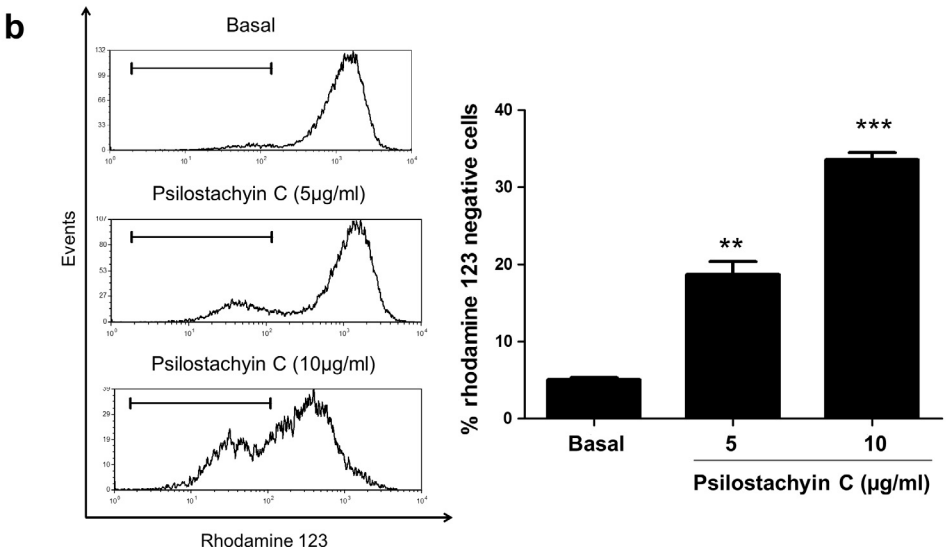

Fig. 6. Effect of psilostachyin C on apoptosis and mitochondrial membrane potential in tumoral cells. Cells were incubated in the presence or absence of 5 and 10 µg/ml of psilostachyin C during 24 h. (a) Flow cytometry analysis of cells in apoptosis. Data represent percentage of cells in each stage of apoptosis, according to their stain with annexin-V and/or propidium iodide. (b) Effect of psilostachyin C on mitochondrial membrane potential (MMP). Changes in MMP were detected by flow cytometry. The histograms (events *vs.* Rhodamine 123 fluorescence intensity) are representative of three independent experiments performed in triplicate. The bar graph indicates the percentage of rhodamine 123-negative cells. Results represent Mean ± SEM of three independent experiments performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significant differences respect to basal value (ANOVA and Dunnett's test).

antiproliferative effect of psilostachyin C, BW5147 cells were incubated with the ROS scavenger NAC (5, 10 or 15 mM) for 18 h, before treatment. Pre-treatment with NAC was able to reverse the growth-inhibitory effect of psilostachyin C, and this result was observed even at the lowest concentration of NAC (Fig. 7e).

Finally, the effect on cell cycle was analyzed. Psilostachyin C, at a concentration of 5 µg/ml, arrested cells in S phase, during 3, 6 and 24 h of cell culture, whereas at 10 µg/ml this compound arrested cells in S phase during short time treatments (3 and 6 h), but induced G1/G0 arrest with a longer incubation time. A decrease in G2/M was observed at both concentrations and all times tested (Fig. 8).

4. Discussion

In this work, the antiproliferative activity of four sesquiterpene lactones: psilostachyin, psilostachyin C, peruvin and cumanin, isolated from *Ambrosia* spp., was demonstrated. All compounds exerted a concentration-dependent antiproliferative activity on tumor cells with psilostachyin C presenting the lowest EC_{50} value (4.89 µg/ml).

It is known that STLs possess anti-tumor, anti-inflammatory, ameobicidal and trypanocidal activities (Chaturvedi, 2011). In fact, psilostachyin, psilostachyin C, peruvin and cumanin exerted trypanocidal and leishmanicidal activities by inhibiting cell growth (Sülsen et al., 2008a, 2011a, 2013). Psilostachyin and psilostachyin C are considered novel checkpoint inhibitors by arresting breast cancer cells (MCF-7) in G2 phase (Sturgeon et al., 2005). These compounds are also known to block mitosis by causing the formation of aberrant microtubule spindles. However, these STLs have not been found to interfere with microtubule polymerization *in vitro* (Csupor-Löffler et al., 2009).

Other STLs such as costunolide and dehydrocostus lactone, exert antiproliferative action on soft tissue sarcoma lines (SW-872, SW-982 and TE-671), by inhibiting cell proliferation in a time- and dose-dependent manner and by arresting cells at the G2/M phase through the decrease in the expression of cyclin-dependent kinase CDK2 (Lohberger et al., 2013).

Even though the four STLs assayed, psilostachyin, psilostachyin C, peruvin and cumanin, proved to have antiproliferative activity on BW5147 cells, important differences in EC_{50} values were obtained. Chemically, these compounds are sesquiterpene lactones which differ in the chemical group associated to the lactone. It has been reported that the elimination of the α, β-unsaturated carbonyl group in this kind of compounds causes a loss of the antiproliferative activity, suggesting that the compounds can bind covalently to target proteins through the Michael's addition (Csupor-Löffler et al., 2009). The alquilation of cellular thiol groups could affect key biological processes leading to cell death (Gach and Janecka, 2014). Moreover, the irreversible binding may be an important factor against drug resistance especially in cancer therapy (Amslinger, 2010).

The four STLs tested in this investigation psilostachyin, peruvin, psilostachyin C and cumanin, possess an α-methylene-γ-lactone

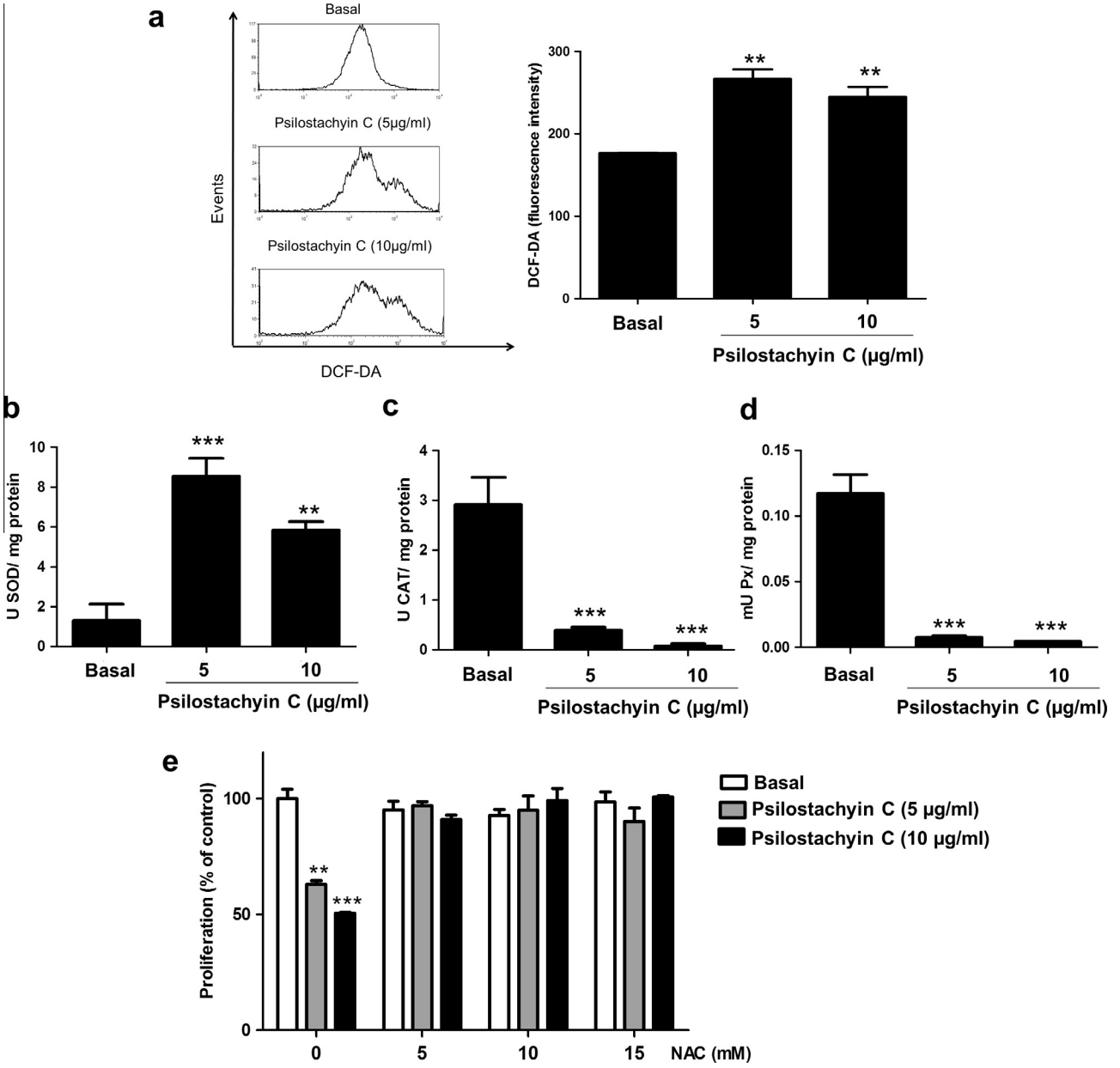

Fig. 7. Effect of psilostachyin C on tumoral oxidative status. Cells were incubated in the presence or absence of 5 and 10 μg/ml of psilostachyin C during 24 h. (a) Flow cytometry analysis of ROS production. After treatment, cells were incubated with the H2DCF-DA dye and the fluorescence intensity was determined by flow cytometry. The histogram (events *vs.* DCF-DA) and the bar graph indicate the mean fluorescence intensity of DCF-DA. (b–d) SOD, CAT and Px activities. Treated and untreated cells were disrupted and supernatant was used to the determination of the ROS metabolism-involving enzymes activity. Briefly, supernatants were incubated with the enzymes' substrates and the changes in absorbance were recorder by a kinetic mode. Results were expressed as units of enzyme (U) per milligram of protein. (e) Effect of NAC on BW5147 cell proliferation. Cells were incubated with NAC for 18 h, followed by 24 h incubation with psilostachyin C (5 and 10 μg/ml). Cell proliferation was determined by [^{3}H]TdR incorporation. Results represent Mean ± SEM of three independent experiments performed in triplicate. $^{**}p < 0.01$, $^{***}p < 0.001$ significant differences respect to basal value (ANOVA and Dunnett's test).

moiety in its structure. These STLs belong to the pseudo-guaianolide type, specifically they are ambrosanolides characterized by the presence of β-methyl groups in C-5 and C-10. Even though the four STLs have these common features, they showed differences in their antiproliferative activity on lymphoma cells. The four compounds presented antiproliferative activity, being psilostachyin C the most active one followed by psilostachyin. Unlike the others, these two STLs, possess an additional lactone function, being γ-lactone and δ-lactone for psilostachyin and psilostachyin C, respectively, that might be contributing to the antiproliferative activity. These results are in accordance with those reported by other authors who have indicated that an α, β-unsaturated carbonyl group is an important but not a sufficient structural requirement for the antiproliferative activity (Sturgeon et al., 2005). Factors such as lipophilicity, molecular geometry and chemical environment could also influence the activity of STLs (Scotti et al., 2007).

To determine if the antiproliferative activity was related to a cytotoxic or a cytostatic effect, the activity of the four STLs on tumor cell viability was assayed. Peruvin, psilostachyin and psilostachyin C exerted a cytostatic effect at 1 μg/ml as they decreased cell proliferation without modifying cell viability. At higher concentrations, a decrease in cell proliferation was accompanied by a decrease in cell viability, demonstrating a cytotoxic effect. On

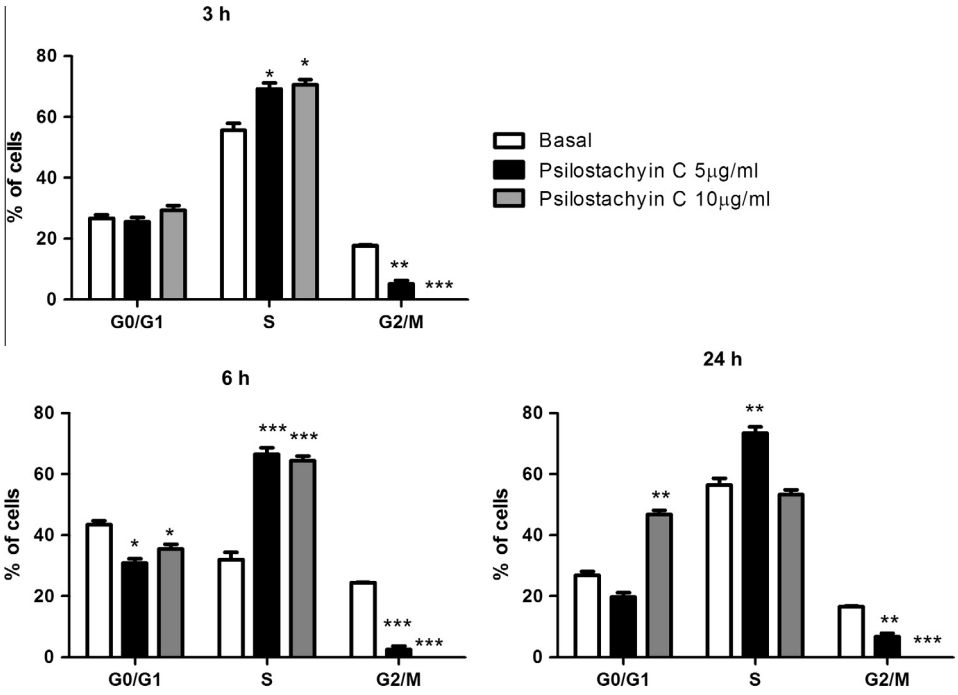

Fig. 8. Effect of psilostachyin C on tumoral cell cycle. Cells were incubated with or without psilostachyin C (5 and 10 μg/ml) during 3, 6 and 24 h. After treatment, the cell DNA content was quantified with the propidium iodide dye by flow cytometry. Results were expressed as% of cells in different cycle phases and represent Mean ± SEM of three independent experiments, performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significant differences respect to basal value (ANOVA and Dunnett's test).

the other hand cumanin exerted cytotoxic activity at all the concentrations assayed. Cytotoxic activity of STLs appears to be associated with their ability to act as alkylating agents. This class of compounds could react with biological nucleophiles like cysteine, via the Michael's addition (Merfort, 2011).

On the other hand, psilostachyin C appeared to be the less toxic compound on normal lymphocytes, as it was demonstrated by the high CC_{50} values obtained at all culture times. Moreover, at 24 h it presented a SI index greater than 10 indicating that the concentration that induced a decrease of cell viability (50%) in normal cells was 10 times greater than the concentrations that produced the antiproliferative activity (50%) in tumoral cells. Furthermore, the CC_{50} did not change over the time, demonstrating that psilostachyin C was selective for the inhibition of tumor cells. This compound was demonstrated to be selective on *Trypanosoma cruzi* epimastigotes, trypomastigotes and amastigotes with SI indexes between 25 and 140 (Sülsen et al., 2011a).

Psilostachyin C was not only the most active compound against lymphoma cells, but also the most innocuous one on normal lymphocytes and furthermore induced lymphocytes proliferation. These findings suggest that the compound could exert an immunomodulatory activity allowing immune cells to exert their defense mechanisms against tumor cells. Although vincristine showed an EC_{50} value lower than the obtained for psilostachyin C, this compound has proved to be toxic on normal cells, especially for those that are in proliferation such as immune system cells. Another reported complication is the peripheral neuropathy (Kiguchi et al., 2009; Porrata et al., 2010).

In order to get an inside in the mechanism of action of psilostachyin C, the induction of apoptosis and cell cycle arrest was studied. The antiproliferative activity of this compound on tumoral cells could be related to the induction of the cell cycle arrest and eventually leading to apoptosis, a sequence of events that has already been reported (Sturgeon et al., 2005).

The decrease in the mitochondrial membrane potential (MMP), could also be related to apoptotic events. Psilostachyin C decreased MMP and this phenomenon could occur due to the opening of the mitochondrial permeability transition pore, which allows the release of proteins such as cytochrome C that triggers the apoptotic pathway (Kroemer et al., 2007). In addition, the decrease in the MMP could be related to the increase in ROS levels. It is known that an increase in intracellular ROS may cause damage to DNA, lipids and proteins, leading to apoptosis (Valko et al., 2007). Low levels of ROS are crucial to maintain the proliferation in BW5147 cells. Davicino et al. (2009) have observed that low hydrogen peroxide (H_2O_2) levels were related to cell proliferation, while an increase of H_2O_2 was implicated in the induction of apoptosis via p38 activation. We further have evaluated the effects of psilostachyin C on the activity of antioxidant enzymes that modulate H_2O_2 levels: SOD, CAT and Px. The increase in SOD activity and the decrease in CAT and Px activities, make the intracellular levels of H_2O_2 rise. These findings suggest that the increase in H_2O_2 levels could be involved in BW5147 cell death induced by psilostachyin C. To confirm this hypothesis, cells were pre-treated with NAC, prior incubation with psilostachyin C. NAC protects cells for the oxidative stress, because it acts as a precursor of intracellular glutathione synthesis, leading to restoration of the cell redox status. The addition of NAC fully reversed the effect of psilostachyin C on cell proliferation, confirming that the mechanism of action of this drug is based on the generation of ROS. Alantolactone, other STL, have demonstrated a similar pathway by inducing apoptosis in glioblastoma cell line U87. The alantolactone-induced apoptosis was found to be associated with ROS generation, related to a glutation and CAT depletion (Khan et al., 2012).

In conclusion, these results suggest that psilostachyin C inhibited tumoral cell proliferation through the induction of apoptosis and cell cycle arrest. The induction of apoptosis was related to the increase of ROS which in turn may lead to a decrease in the MMP. It has previously been reported that an extract of *Larrea divaricata* was capable to induce apoptosis in these cell line by generation of ROS (Davicino et al., 2010).

Other STLs with antiprotozoal activity such as eiatol, have the mitochondria as the primary target leading to an increase in the ROS levels via the electron transport chain. This phenomenon

could influence cell membrane and DNA integrity provoking parasite death (Desoti et al., 2012; Saeidnia, 2013).

The evaluation of the effectiveness of drugs against tumor lines and the analysis of the mechanisms of action involved could be useful in the search of novel therapeutic agents for cancer treatment. At the concentrations tested, psilostachyin C proved to be active against lymphoma cells and non-toxic to normal cells. Furthermore, in this work it has been demonstrated that this compound exerted its antiproliferative activity by inducing cell apoptosis and by arresting cell cycle in phase S. In view of these results, we consider that psilostachyin C presents a promising profile for further studies in cancer research.

Conflict of Interest

The authors declare that they have no competing interests.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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