

# Potent induction of apoptosis by germacranolide sesquiterpene lactones on human myeloid leukemia cells

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## Abstract

This paper studies the cytotoxic effect induced by four known natural sesquiterpene lactones (tatridin A, tamirin, reynosin, ineupatorolide A) and one synthetic derivative (tatridin A diacetate) on the myeloid leukemia cell lines HL-60 and U937. Tatridin A diacetate and ineupatorolide A were found to be the most cytotoxic compounds with growth inhibition caused by induction of apoptosis as determined by flow cytometry and microscopy of nuclear changes. The results reported here support the conclusion that apoptosis was accompanied by both the activation of caspase-3 and the fragmentation of poly(ADP-ribose) polymerase-1 and was also associated with an early release of cytochrome *c* from the mitochondria.

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## 1. Introduction

Sesquiterpene lactones are natural products isolated from many plant families and most widely distributed within the Compositae. These compounds are known for their various biological activities, including cytotoxicity to tumor cells (Beekman et al., 1997). Covalent binding of sesquiterpene lactones to free sulfhydryl groups in proteins and interference with the functions of these macromolecules have been described (Lee et al., 1997; Schmidt, 1997). For example, sesquiterpene lactones inhibit many enzymes involved in biological processes, such as DNA–RNA–protein synthesis (Schmidt, 1999). Although the precise mechanism of action of sesquiterpene lactones on growth inhibition is not quite clear, it is believed that  $\alpha,\beta$ -unsaturated carbonyl compounds, in particular  $\alpha$ -methylene lactones, exert their biological effect by acting as alkylating agents. These

compounds react with nucleophiles, especially cysteine sulphhydryl groups, by a Michael-type addition (Lyss et al., 1998). Thus, compounds with two  $\alpha,\beta$ -unsaturated carbonyl groups may show considerable cytotoxic properties. The introduction of a more lipophilic residue (e.g. esterification of hydroxyl groups) should yield a compound with higher cytotoxic activity, although bulky groups near the reaction center could reduce the activity, i.e. there must be a balance between lipophilicity and steric hindrance. Moreover, conformational flexibility seems to be important in binding to the molecule target: a rather rigid molecule can lead cause important steric hindrance. In this study, all compounds present at least one  $\alpha,\beta$ -unsaturated carbonyl group, the alkylating agent.

Physiological cell death occurs through an evolutionary conserved suicide process, termed apoptosis, which plays a considerable role in early development and homeostasis of adult tissues (Ameisen, 2002). Previous studies have demonstrated that apoptosis of human leukaemic cells can be induced with anticancer agents (Makin and Dive, 2001, 2003). The main aim of this work was to investigate the

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cytotoxic effects of five sesquiterpene lactones (**1** to **5**) with wide structural variation in order to determine the structure–activity relationship (Fig. 1): the germacranolides tatrudin A (**1**), diacetyl tatrudin A (**2**), tamirin (desacetylchrysanolide, **3**), and ineupatorolide A (**4**), and the eudesmanolide reynosin (**5**). We were also interested in investigating the possible mechanisms of action of these compounds on the growth inhibition/cell viability of human myeloid leukemia cells.

The present report demonstrates that exposure to sesquiterpene lactones of the germacranolide type elicits apoptosis on HL-60 and U937 cells as assessed by flow cytometry, induction of double-stranded DNA damage and appearance of apoptotic morphology. Also cleavage of poly(ADP-ribose) polymerase-1 (PARP-1) and procaspase-3 processing occurred after treatment with sesquiterpene lactones. We further investigate the mechanism underlying tumor cell growth inhibition by these compounds and find mitochondria to be one of the primary targets of the sesquiterpenes' proapoptotic function.

## 2. Materials and methods

### 2.1. Drugs and reagents

All sesquiterpene lactones were obtained from Canary Island endemic plants and were isolated according to published methods with minor modifications. Structural identities of sesquiterpene lactones were determined spectroscopically (proton nuclear magnetic resonance, infrared spectroscopy, mass spectrometry) as described previously: **1** (tatrudin A), **2** (diacetyl tatrudin A), **3** (tamirin), **4** (ineupatorolide A), **5** (reynosin). Tatrudin A (**1**) was isolated from the aerial parts of *Tanacetum ptarmicaeflorum* (web) Sch. Bip (González et al., 1992a). Diacetyl tatrudin A (**2**) was obtained by acetylation of compound **1** (González et al., 1992b). Tamirin (**3**) was isolated from the aerial parts of

*Lugoa revoluta* according to a published method (Triana et al., 2001). Ineupatorolide A (**4**) was isolated from the aerial parts of *Allagopappus viscosissimus* as described (González et al., 1992a). Reynosin (**5**) was isolated from the aerial parts of *Gonospermum canariense* (Triana et al., 2000). Stock solutions of 10 mM sesquiterpene lactones were made in dimethylsulfoxide (DMSO), and aliquots were frozen at  $-20^{\circ}\text{C}$ .

Tissue culture media were obtained from Invitrogen. Standard analytical grade laboratory reagents were obtained from Sigma and Applichem. Antibodies for poly(ADP-ribose) polymerase-1, caspase 3 and cytochrome *c* were purchased from BD PharMingen and BD Transduction Laboratories. Secondary antibodies were from Amersham Pharmacia Biotech.

### 2.2. Cell culture

Human U-937 and HL-60 myeloid leukemia cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen) and 100 units/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin at  $37^{\circ}\text{C}$  in a humidified atmosphere containing 5%  $\text{CO}_2$ . The cultures were passed twice weekly exhibiting characteristic doubling times of  $\sim 24$  h. The cell numbers were counted by a hemacytometer, and the viability was always greater than 95% in all experiments as assayed by the 0.025% trypan blue exclusion method. Further dilutions of stock solutions of sesquiterpene lactones were made in culture media just before use. In all experiments, the final concentration of DMSO did not exceed 0.5% (v/v), a concentration which is nontoxic to the cells.

### 2.3. Assay for growth inhibition and cell viability

The cytotoxicity of sesquiterpene lactones was assessed using 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay (Mosmann, 1983). Briefly,  $2 \times 10^4$  exponentially growing cells were seeded in 96-well microculture plates with various sesquiterpene lactones concentrations (0.3–50  $\mu\text{M}$ ) in a volume of 100  $\mu\text{l}$  for 24 h. Then, surviving cells were detected based on their ability to metabolize 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (Applichem) into formazan crystals. Optical density at 570 nm was used as a measure of cell viability. Cell survival was calculated as the fraction of cells alive relative to control for each point: cell survival (%) = mean absorbance in treated cells/mean absorbance in control wells  $\times 100$ . Concentrations inducing a 50% inhibition of cell growth ( $\text{IC}_{50}$ ) were determined graphically for each experiment. Parameters describing the concentration–response curves ( $\text{IC}_{50}$ ) were determined using the curve-fitting routine of the computer software Prism™ (GraphPad) and the equation derived by DeLean et al. (1978).

The effects on cell growth and cytotoxicity of sesquiterpene lactones were also assessed using the Hoechst 33258

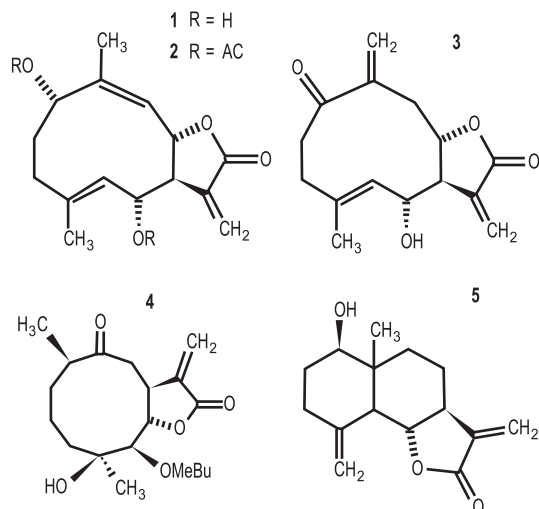


Fig. 1. Structures of the investigated sesquiterpene lactones.

fluorescence assay of cellular DNA in 96-well microtitre plates reading in fluorescent plate reader at 350/460 nm excitation/emission wavelengths as described (Rago et al., 1990).

#### 2.4. Quantitative fluorescent microscopy

Cells were harvested and fixed in 3% paraformaldehyde and incubated at room temperature for 10 min. The fixative was removed and the cells were washed with phosphate-buffered saline (PBS), resuspended in 30–50  $\mu$ l of PBS containing 16  $\mu$ g/ml bis-benzimide trihydrochloride (Hoechst 33258) (Kaufmann et al., 1993), and incubated at room temperature for 15 min. Ten-microliter aliquots of the cells were placed on glass slides, and triplicate samples of 500 cells each were counted and scored for the incidence of apoptotic chromatin condensation using a Zeiss fluorescent microscopy. Stained nuclei with condensed chromatin (supercondensed chromatin at the nuclear periphery), or nuclei that were fragmented into multiple smaller dense bodies were considered as apoptotic. Nuclei with uncondensed and dispersed chromatin were considered as not apoptotic.

#### 2.5. Flow cytometry analysis

To study changes in the cell DNA content, histogram measurements of hypodiploid DNA formation was performed by flow cytometry using a Coulter EPICS<sup>TM</sup> cytometer (Beckman Coulter). Histograms were analyzed with the Expo 32 ADC Software<sup>TM</sup> (Beckman Coulter). Briefly, cells ( $\sim 1 \times 10^6$ ) were centrifuged for 10 min at  $500 \times g$ , washed with 1 ml PBS and resuspended in 50  $\mu$ l of PBS. Following dropwise addition of 1 ml of ice-cold 75% ethanol, fixed cells were stored at  $-20^\circ\text{C}$  for 1 h. Samples were then centrifuged at  $500 \times g$  for 10 min at  $4^\circ\text{C}$  and washed with PBS before resuspension in 1 ml of PBS containing 50  $\mu$ g/ml propidium iodide and 100  $\mu$ g/ml RNase A and incubation for 1 h at  $37^\circ\text{C}$  in the dark. The percentage of cells with decreased DNA staining, composed of apoptotic cells resulting from either fragmentation or decreased chromatin, of a minimum of 10,000 cells per experimental condition was counted. Cell debris was excluded from analysis by selective gating based on anterior and right angle scattering.

#### 2.6. DNA fragmentation assay

HL-60 cells ( $1-2 \times 10^6$ ) were washed once with ice-cold PBS, pelleted by centrifugation ( $500 \times g$ , 10 min at  $4^\circ\text{C}$ ) and resuspended with 150  $\mu$ l of cold TTE buffer (containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% Triton X-100). After incubation for 30 min at  $4^\circ\text{C}$ , cellular debris was eliminated by centrifugation ( $14,000 \times g$  for 10 min at  $4^\circ\text{C}$ ) and the supernatants containing low molecular weight fragmented DNA were collected. Five microliters of RNase A (10  $\mu$ g/ $\mu$ l, from Sigma) was then added and the mixture was incubated for 1 h at  $37^\circ\text{C}$ . The addition of 5  $\mu$ l of

proteinase K (10  $\mu$ g/ $\mu$ l, from Sigma) was followed by an additional 1 h incubation at  $37^\circ\text{C}$ . DNA was extracted twice with 150  $\mu$ l of phenol (pH 7.4) and the aqueous phase was mixed with 10  $\mu$ l of 3 M sodium acetate (pH 5.5), 5  $\mu$ l of tRNA (1  $\mu$ g/ $\mu$ l, from Sigma) as a carrier and 250  $\mu$ l of ethanol. Samples were incubated overnight at  $-20^\circ\text{C}$  and DNA was precipitated by centrifugation ( $14,000 \times g$  for 15 min at  $4^\circ\text{C}$ ). After a washing step with 1 ml of 70% ethanol, the pellet was resuspended in 20  $\mu$ l of 10 mM Tris-HCl (pH 7.4). Aliquots (10  $\mu$ l) were analyzed by electrophoresis on 2% agarose gel containing ethidium bromide (0.5  $\mu$ g/ $\mu$ l) for 4 h at 40 V in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The gel was exposed to UV illumination and the image was captured (DC290 Zoom Digital Camera, Kodak) and analyzed (Quantity-One software, Bio-Rad).

#### 2.7. Immunoblotting of caspase-3 (CPP32)

HL-60 cells ( $1 \times 10^6$ ) were treated with sesquiterpene lactones at the indicated concentrations in RPMI 1640 medium. Cell pellets were lysed in lysis buffer containing 125 mM Tris-HCl pH 6.8, 2% SDS, 5% glycerol, and 1%  $\beta$ -mercaptoethanol, and boiled for 5 min. The samples were separated on 15% SDS-polyacrylamide gel, electrotransferred to a nitrocellulose membrane, immunoblotted with anti-CPP32 polyclonal antibody (BD PharMingen) (1:1000 dilution in TBST supplemented with 3% nonfat milk) overnight. After washing and incubation with horseradish peroxidase-conjugated anti-rabbit (Amersham Pharmacia Biotech), the antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) using the manufacturer's protocol.

#### 2.8. Immunoblot analysis of poly(ADP-ribose) polymerase-1 degradation

Induction of apoptosis was also examined by proteolytic cleavage of poly(ADP-ribose) polymerase-1. Briefly,  $7 \times 10^5$  exponentially growing HL-60 cells were treated with sesquiterpene lactones at the indicated concentrations for 6, 12 or 24 h at  $37^\circ\text{C}$ . Cells were pelleted by centrifugation, and resuspended in lysis buffer containing 25 mM PBS, 0.1 mM phenylmethylsulfonylfluoride and protease inhibitors leupeptin, aprotinin and pepstatin A (5  $\mu$ g/ml each). After centrifugation, the pellet was resuspended in the loading buffer containing 125 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 5% glycerol, and 1%  $\beta$ -mercaptoethanol. The mixture was sonicated for 30 s at  $4^\circ\text{C}$  and then boiled to  $100^\circ\text{C}$  for 3 min. For Western blotting, the cell lysates were fractionated on a 7.5% polyacrylamide gel containing 0.1% SDS. The gel and the nitrocellulose membrane (Amersham Pharmacia Biotech) were equilibrated for 20 min in transfer buffer (25 mM Tris, 192 mM glycine, 3.5 mM SDS, 20% methanol) and transferred to nitrocellulose by semi-dry

electrophoretic transfer system (Bio-Rad). The loading and transfer of equal amounts of protein was confirmed by staining the nitrocellulose membrane with Ponceau S. Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h, followed by incubation with anti-poly(ADP-ribose) polymerase-1 monoclonal antibody (BD PharMingen; 1:1000) and developed as above. The appearance of an 85-kDa cleavage product was used as a measure of apoptosis.

### 2.9. Detection of cytochrome *c* release from mitochondria

Release of cytochrome *c* from mitochondria was detected by Western blot analysis. HL-60 cells ( $1 \times 10^7$ ) were treated with sesquiterpene lactones at 37 °C. Cells were washed twice with PBS and then suspended in ice-cold buffer [20 mM HEPES (pH 7.5), 1.5 mM  $MgCl_2$ , 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, and 5 µg/ml leupeptin, aprotinin, and pepstatin A] containing 250 mM sucrose. After 15 min incubation on ice, cells were lysed by pushing them several times through a 22-gauge needle and the lysate spun down at  $1000 \times g$  for 5 min at 4 °C. The supernatant fraction was centrifuged at  $105,000 \times g$  for 45 min at 4 °C and the resulting supernatant was used as the soluble cytosolic fraction. Cytosolic proteins (50 µg) were resolved on an SDS/15% polyacrylamide gel and electrotransferred onto a nitrocellulose membrane. The membrane was probed with monoclonal anti-cytochrome *c* (BD Transduction Laboratories) (1:250 dilution) antibody conjugated to horseradish peroxidase. Protein bands were detected by chemiluminescence (ECL, Amersham Pharmacia Biotech) as described above.

## 3. Results

### 3.1. Sesquiterpene lactones induce apoptosis in human myeloid leukemia cells

All sesquiterpene lactones tested (Fig. 1) were found to inhibit the growth and cell viability of HL-60 and U937 cells in culture as determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) dye-reduction assay (Table 1). Compounds **2** and **4** were found to be the most potent, while **1** and **5** were least potent. The potency of these sesquiterpene lactones on inhibition of proliferation in HL-60 cells were as follows: **2** > **4** ~ **3** > **1** ~ **5**; meanwhile in U937 cells, the potency of these sesquiterpene lactones on inhibition of proliferation were as follows: **4** > **2** > **3** ~ **1** > **5**, as determined by two independent experiments. The same order of potency was obtained measuring DNA content using the Hoechst 33258 fluorescence assay although the  $IC_{50}$  values were slightly higher (results not shown).

To understand the mechanism of growth inhibition, HL-60 cells treated with sesquiterpene lactones were analyzed

Table 1

Effects of sesquiterpene lactones on the growth of HL-60 and U937 cells cultured for 24 h

Sesquiterpene lactone	$IC_{50}$ (µM)	
	HL-60	U937
<b>1</b>	$9.8 \pm 2.4$	$15.6 \pm 2.3$
<b>2</b>	$0.7 \pm 0.1$	$4.3 \pm 0.9$
<b>3</b>	$5.1 \pm 0.8$	$12.1 \pm 2.1$
<b>4</b>	$2.8 \pm 1.2$	$2.6 \pm 0.7$
<b>5</b>	$14.9 \pm 4.5$	$33.4 \pm 0.9$

The data shown represent the mean ( $\pm$  S.E.M.) of two independent experiments with three determinations in each.

and quantified by fluorescent microscopy of nuclear changes. The results show that these compounds induced morphological changes that are characteristic of apoptotic cells (Fig. 2A and B). Whereas untreated cells exhibit a typically nonadherent, fairly round morphology (Fig. 2B, upper panel), cells exposed to 5 µM sesquiterpene lactones for 12 h display condensation of chromatin and appearance of apoptotic bodies (Fig. 2B, lower panel). The germacranolide **2** was the most potent apoptosis inducer among them (Fig. 2A).

Similar results as above were obtained by measurement of the number of hypodiploid cells (apoptotic cells) by flow cytometry in both cell lines (HL-60 and U937 cells) after 12 h of treatment with 5 or 30 µM of sesquiterpene lactones (Fig. 2C and D). Furthermore, when cells were incubated with 5 µM sesquiterpene lactones, the DNA showed the typical fragmentation pattern formed by internucleosomal hydrolysis of chromatin thus confirming the apoptosis-inducing effects (Fig. 3). DNA fragmentation by sesquiterpene lactones was dose-dependent (results not shown).

### 3.2. Sesquiterpene lactone-induced apoptosis involves activation of the caspase cascade

To determine whether germacranolide-induced apoptosis involves activation of caspase-3, we assessed cleavage of procaspase-3 by immunoblot analysis with the most potent germacranolide, tatridin A diacetate **2**. The results demonstrate caspase-3 activation is detected at 6 h by diacetyl tatridin A treatment (Fig. 4A). In concert with this result, the caspase-3 substrate poly(ADP-ribose) polymerase-1 was cleaved with similar kinetics (Fig. 4B). These results demonstrate that tatridin A diacetate-induced apoptosis involves activation of the caspase-3 cascade.

One of the downstream targets of caspase-3 is poly(ADP-ribose) polymerase-1. This enzyme catalyzes the transfer of the ADP ribose moiety from its substrate,  $NAD^+$ , to a limited number of protein acceptors involved in chromatin architecture or in DNA metabolism, including topoisomerase I (Smith, 2001). Poly(ADP-ribose) polymerase-1 has been implicated in DNA repair and maintenance of genomic integrity (Shall and de Murcia, 2000). The cleavage of poly(ADP-ribose) polymerase-1 inactivates the



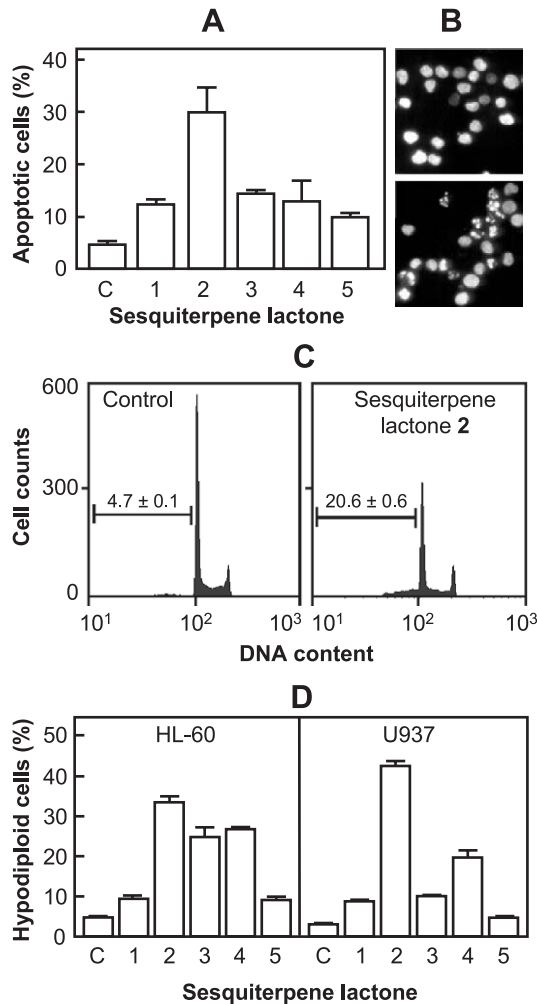


Fig. 2. Apoptosis induction by sesquiterpene lactones on HL-60 and U937 cells. (A) HL-60 cells were stained with bisbenzimidazole trihydrochloride after treatment of the indicated compounds (5  $\mu$ M, 12 h) and apoptotic cells were determined by fluorescence microscopy. (B) Photomicrographs of representative fields of HL-60 cells (upper panel, control cells) stained with bisbenzimidazole trihydrochloride to evaluate nuclear chromatin condensation (i.e. apoptosis) after treatment for 12 h with 5  $\mu$ M tatrindin A diacetate (lower panel). (C) HL-60 cells were treated with 5  $\mu$ M of **2** (tatrindin A diacetate), harvested at 12 h and subjected to DNA flow cytometry. Hypodiploid cells (apoptotic cells) are shown in region marked with bar. (D) HL-60 and U937 cells were treated with 30  $\mu$ M of the indicated compounds, harvested at 12 h and subjected to DNA flow cytometry. The percentage of hypodiploid cells is expressed as the mean ( $\pm$  S.E.M.) of two independent experiments each performed in duplicate.

enzyme, thereby making DNA repair impossible. The Western blot analysis in Fig. 4B (upper and lower panel) demonstrates that the 116-kDa poly(ADP-ribose) polymerase-1 protein was cleaved into its characteristic 85-kDa fragment after treatment of the cells with the germacranolides **1**, **2**, **3** and **4**, but not with the eudesmanolide **5** at doses and times assayed (10  $\mu$ M, 12 h). Some sesquiterpene lactones (**1**, **3** and **4**) induced faint cleavage of poly(ADP-ribose) polymerase-1 detected at 6 h posttreatment (Fig. 4B upper panel), while cleavage induced by **2** was completed at 6 h. Longer incubation time (12 h) was

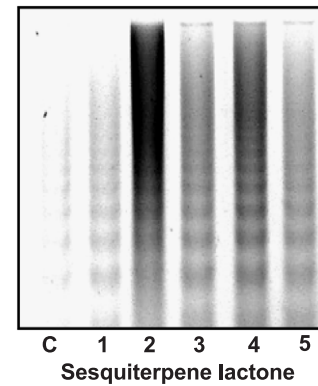


Fig. 3. Qualitative assessment of apoptotic DNA damage. HL-60 human cells were incubated in absence (C, Control) or presence of 5  $\mu$ M of the indicated sesquiterpene lactone (**1**, **2**, **3**, **4**, **5**) for 12 h. Laddered electrophoretic patterns of oligonucleosomal DNA fragments were resolved by conventional agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light.

necessary to obtain complete cleavage by the germacranolides **1**, **3** and **4** (Fig. 4B, lower panel). No hydrolysis of poly(ADP-ribose) polymerase-1 was detected at the concentration (up to 30  $\mu$ M) and within the time frame of the study by the eudesmanolide reynosin **5**.

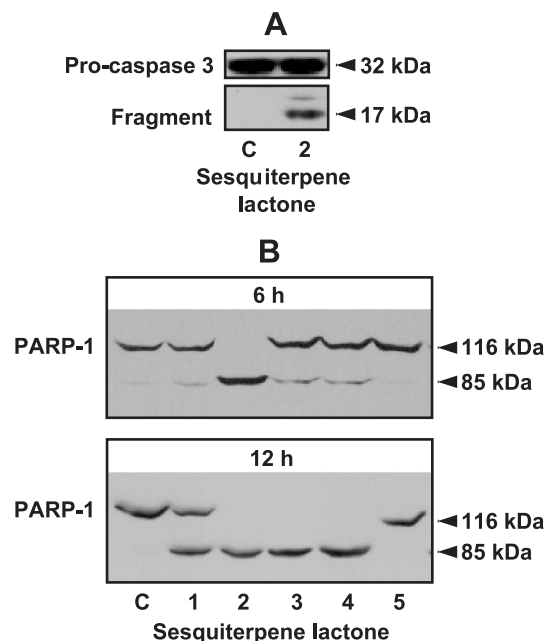


Fig. 4. (A) Western blot analysis for the cleavage of procaspase-3. Control lane refers to untreated cells. In the other lane, the cells were treated with tatrindin A diacetate (10  $\mu$ M) for 6 h. Whole cell lysates were subjected to SDS-PAGE followed by blotting with an anti-procaspase 3 antibody. The molecular weight of each protein is indicated. (B) Western blot analysis for the cleavage of poly(ADP-ribose) polymerase-1 (PARP-1). Control lanes refer to untreated cells. In the other lanes, the cells were treated with 10  $\mu$ M sesquiterpene lactones for 6 h (upper panel) or with 10  $\mu$ M for 12 h (lower panel). Whole cell lysates were subjected to SDS-PAGE followed by blotting with an anti-poly(ADP-ribose) polymerase-1.

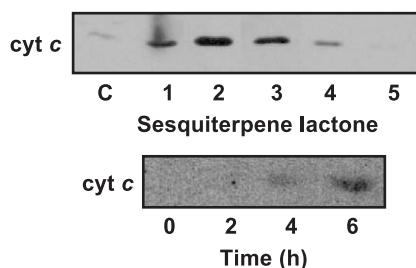


Fig. 5. Analysis of cytochrome *c* release from mitochondria on HL-60 cells. Cells were incubated in absence (C, Control) or presence of 10  $\mu$ M of the indicated sesquiterpene lactone (1, 2, 3, 4, 5) for 6 h (upper panel), or treated with 10  $\mu$ M of tatridin A diacetate for the indicated times (lower panel). After treatment, cytosolic extracts were prepared, separated by SDS-PAGE and cytochrome *c* was detected by immunoblotting.

### 3.3. Sesquiterpene lactone-induced apoptosis involved mitochondrial cytochrome *c* release

To determine whether sesquiterpene lactone-induced apoptosis involves the release of cytochrome *c*, we subjected cytosolic preparations to immunoblot analysis. The exposure of HL-60 cells to these compounds resulted in increased cytosolic cytochrome *c* and this response was observed at 4 h of treatment. As shown in Fig. 5, cytochrome *c* was released into cytoplasm shortly after treatment when cells were fully viable by trypan blue exclusion and MTT assay, suggesting that cytochrome *c* release is an early event in apoptosis of HL-60 cells induced by these compounds. However, the eudesmanolide reynosin **5** was not able to release cytochrome *c* from mitochondria at times and doses assayed. In the cytosol of tatridin A diacetate treated cells, a dramatic increase in cytochrome *c* levels was seen (Fig. 5). However, *in vitro* incubation of purified mitochondria with tatridin A diacetate demonstrated that this compound is not directly able to induce cytochrome *c* release from the intermembrane space (results not shown).

## 4. Discussion

In this study, we have tested HL-60 and U937 cells for their sensitivity to different sesquiterpene lactones. In HL-60 cells, compound **2** was the most cytotoxic compound tested, probably due to its high lipophilicity. However, other factors, such as molecular geometry, the chemical environment of the target sulfhydryl and the presence of two  $\alpha,\beta$ -unsaturated esters (although these groups have a low alkylating potency on their own) may also influence the activity of sesquiterpene lactones. The fact that ester groups can be hydrolyzed by cellular esterases, leading to a hydroxyl in beta position relative to the alkylating center, is also relevant. Several studies have found enhanced activities in compounds bearing an oxygen at this position, suggesting that this oxygen may form a hydrogen bond with amino acid residues that are adjacent to the reactive cysteine of the

target protein. This possibility increases the reactivity to the Michael addition site and its toxicity to some extent. Compound **3** also showed important cytotoxic activity in spite of its low lipophilicity. As mentioned above, the presence of a hydroxyl group in a beta position with respect to the alkylating center probably increases potency. Thus, few molecules can pass through the cell membrane, but these few molecules do produce a great effect. Compound **4** also yielded an important cytotoxic activity, in both cell lines, probably due to its high lipophilicity and the fact that its alkylating center allows binding to the protein target. Furthermore, the ester group can be hydrolyzed in the intracellular medium, yielding a hydroxyl group at C-5, which improves binding to the target protein. The least cytotoxic compounds were found to be **1** and **5** in both cell lines. Compound **1** is a mildly lipophilic molecule which does not easily pass through the cell membrane. This is demonstrated by comparison with compound **2**, a diester of compound **1**, which is the most active compound. Compound **5** possesses a very rigid structure; this may hinder its approach to a target molecule, which might be the reason for its low activity.

In conclusion, we demonstrate that the exposure of HL-60 and U-937 cells to sesquiterpene lactones exerts a strong antiproliferative effect and induces apoptotic cell death. In both cell lines, the germacranolide **2**, tatridin A diacetate, was the most potent apoptosis inducer among the assayed sesquiterpene lactones, while assayed sesquiterpene lactones displayed similar  $IC_{50}$  values. Previous studies have documented the induction of apoptotic cell death and DNA fragmentation by the sesquiterpene lactone of the pseudoguaianolide type helenalin in the human leukemia Jurkat cells (Dirsch et al., 2001). However, sesquiterpene lactones containing a germacranolide skeleton were not assessed. The present findings show the ability of sesquiterpene lactones of the germacranolide type to induce apoptosis in the hematopoietic cell lines HL-60 and U-937. Biochemical characterization of apoptotic DNA damage revealed that sesquiterpene lactones promote the internucleosomal degradation of DNA, resulting in the formation and eventual release of oligonucleosomal DNA fragments. The expression of classically recognized morphological changes associated with apoptosis, including formation of apoptotic bodies, was observed following sesquiterpene lactones exposure, whereas there was no evidence of gross cytolysis or other changes indicative of necrosis. The quantification of apoptosis was also determined by cytometry flow in both cell lines. To examine the mechanisms by which apoptosis is induced, the cleavage of poly(ADP-ribose) polymerase-1 and caspase-3, key enzymes in the apoptosis process (Cain et al., 2002; Virág and Szabó, 2002), was evaluated. Notably, it was found that the induction of apoptosis by these compounds in HL-60 cells correlated with enhanced poly(ADP-ribose) polymerase-1 cleavage. The proteolytic cleavage of poly(ADP-ribose) polymerase-1 was verified by the formation of an 85-kDa fragment from a 116-kDa active poly(ADP-ribose) polymer-

ase-1 enzyme. A clear accumulation of the 85-kDa poly(ADP-ribose) polymerase-1 derived fragment was observable, thus confirming in vivo activation of caspases, the main protease responsible for poly(ADP-ribose) polymerase-1 cleavage (Tewari et al., 1995; Germain et al., 1999).

Previous studies have demonstrated that cytotoxic agents induce the release of mitochondrial cytochrome *c* (Szewczyk and Wojtczak, 2002) and cytochrome *c* triggers a caspase-dependent assembly of the apoptosome (Van Gurp et al., 2003). The present results demonstrate that germacranolides initiated redistribution of cytochrome *c* into the cytosol as early as 4 h after cell treatment. Previous studies have already shown the release of cytochrome *c* in the culture medium of Jurkat T cells on apoptosis induction is triggered by several stimuli including staurosporine, anti-CD95 antibody, etoposide and doxorubicin (Renz et al., 2001). Also, staurosporine causes a release of cytochrome *c* to the extracellular medium of cultured chick embryonic neurons (Ahlemeyer and Klumpp, 2002). To determine whether sesquiterpene lactone-induced apoptosis involves the release of cytochrome *c* to the extracellular medium, we subjected the culture medium of HL-60 cells and U937 cells to immunoblot analysis. The exposure of both cell lines to these compounds resulted in increased extracellular cytochrome *c* and this response was observed at 12 h of treatment. However, we are unable to explain the pharmacologic relevance of this phenomenon because the addition of cytochrome *c* to HL-60 cells (up 125 ng/ml, 24 h) does not induce apoptosis as assessed by fluorescent microscopy (data not shown).

In conclusion, these results together suggest that cytochrome *c* release plays an important role in the mechanism of caspase activation, poly(ADP-ribose) polymerase-1 cleavage, DNA fragmentation and the eventual apoptosis induced by germacranolides in HL-60 cells. We suggest that these findings may have potentially more generalized implications for current efforts to understand the mechanisms underlying pharmacological induction of DNA damage by other agents, including antineoplastic agents.

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