Purified Salograviolide A isolated from *Centaurea ainetensis* causes growth inhibition and apoptosis in neoplastic epidermal cells

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Abstract. Many of the best-selling anticancer drugs are plant-derived. We tested for the anticancer properties of extracts isolated from *Centaurea ainetensis*, a plant species endemic to Lebanon and which is often used in folk medicine. We performed bioassay-guided fractionation of *Centaurea ainetensis* extracts using a panel of normal and neoplastic murine cells to identify a component that is associated with antitumor activities. Among several compounds that were fractionated, the sesquiterpene lactone, Salograviolide A, was identified and found to exert the most significant growth inhibitory effects on neoplastic cells. At concentrations that were non-cytotoxic to primary keratinocytes, *Centaurea ainetensis* crude extract and Salograviolide A preferentially inhibited the proliferation of papilloma and squamous cell carcinoma (SCC) cell lines without significantly affecting the growth of normal cells. Flow cytometric analysis of DNA content indicated that the inhibition of cell proliferation by *Centaurea ainetensis* crude extract and Salograviolide A was due to G_0/G_1 cell cycle arrest and increased pre-G_0/G_1, respectively. The increase in pre-G_0/G_1, and presumably apoptosis induction, in Salograviolide A-treated keratinocytes was confirmed by DNA Hoechst staining. Western blot analysis and electrophoretic mobility shift assay showed that both the crude extract and the isolated molecule differentially modulated key cell cycle and apoptotic regulators as well as NF-κB signaling. Salograviolide A-induced growth inhibition in neoplastic cells was mediated by the accumulation of reactive oxygen species (ROS) highlighting a potent oxidant role of this molecule. These studies suggest the potential therapeutic effects of *Centaurea ainetensis*, and its component, Salograviolide A, against epidermal squamous cell carcinogenesis.

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Abbreviations: ROS, reactive oxygen species; PMK(s), primary mouse keratinocyte(s); SCC, squamous cell carcinoma; FBS, fetal bovine serum; K, keratin; H_2DCFDA, 2'-7'-dichlorofluorescin diacetate; Vit C, vitamin C; DTT, dithiothreitol; CDKI, cyclin-dependent kinase inhibitor

Key words: plant extract, Salograviolide A, skin cancer

Introduction

Most cancers are of epithelial origin and epidermal squamous cell carcinoma (SCC) is the most frequent (1,2). A wide range of naturally occurring compounds has been identified as skin chemotherapeutic agents (3), and many, as Paclitaxel (Taxol), have been derived from plants and are among the best-selling anticancer drugs today.

Plants contain many secondary metabolites that interact in a complex manner to exert their biological activities (4). Four major groups of plant secondary metabolites have been identified: terpenes, phenolic compounds, acetogenins and alkaloids.

While screening for anti-cancer activities of Middle Eastern plant extracts, we identified the Lebanese indigenous plant, *Centaurea ainetensis*, to possess potent and selective activities against cancer cells.

The genus Centaurea (Asteraceae) comprises 500 species predominantly distributed around the Mediterranean area and in West Asia (5), many of which have been used for millennia in folk medicine. Several studies have revealed that the Centaurea genus is a source of many secondary metabolites that have antimicrobial, anti-inflammatory, cytotoxic and antitumor properties (6-9). Among these metabolites are sesquiterpene lactones which are the most widely investigated metabolites in these plants and which contribute significantly to their anticancer potential (7). Structure-activity relationship studies of several sesquiterpene lactones have indicated that their biological activities are mainly due to the presence of α-, ß- unsaturated carbonyl groups (10,11).

Through bioassay-guided fractionation of *Centaurea ainetensis* crude extract using a panel of murine skin tumor cells, we isolated a guaianolide sesquiterpene lactone, Salograviolide A, which showed a potent anti-carcinogenic potential. Both *Centaurea ainetensis* crude extract and

Salograviolide A preferentially inhibited the growth of murine squamous cell lines vs. primary keratinocytes. Both treatments modulated key cell cycle and apoptotic regulators as well as NF-κB signaling. The anticancer properties of *Centaurea ainetensis* have not yet been fully investigated and our study highlights the therapeutic potential of this plant against skin cancer and a potent oxidant role of Salograviolide A.

Materials and methods

Cell culture and plant extract treatments. Primary mouse keratinocytes (PMKs) were prepared from one- to two-day-old BALB/c mice as described according to established protocols (12,13) and was approved by the Institutional Animal Care and Use Committee of the American University of Beirut. The neoplastic SP-1 and 308 papilloma cell lines were produced in SENCAR and BALB/c mice, respectively, as described (14,15). PAM212 is a SCC cell line that spontaneously transformed *in vitro* (16). The I7 is a spindle cell line derived from a skin carcinoma formed from PMKs infected with the v-*ras*^{Ha} and v-*fos* oncogenes and grafted to nude mice (16). All cell lines were generously provided by Dr Stuart H. Yuspa (NIH, Bethesda, MD).

All cells were cultured in fresh Eagle Minimum Essential Medium (Bio Whittaker, Cambrex Co., MD) containing 9% chelated fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomysin antibiotics (Gibco-BRL Life Technologies, Carlsbad, CA). Except for the I7 cell line, the complete medium for all other keratinocytes contained no more than 0.05 mM Ca^{2+} to maintain a basal proliferating cell phenotype (17). Keratinocytes were grown in a humidified incubator which was set at 95% air and 5% CO_2 for neoplastic cell lines or 93% air and 7% CO_2 for PMKs. Cell culture medium was replenished every other day.

Methanolic extracts of *Centaurea ainetensis* were prepared, filter-sterilized, dissolved in distilled water and diluted in culture medium at concentrations ranging from 0.2 to 1.1 mg/ml. Using bioassay-guided fractionation, Salograviolide A was purified with 0.021% yield from the *Centaurea ainetensis* dry plant material and was used at concentrations ranging from 4 to 16 μg/ml in ethanol. The concentration of ethanol in culture medium did not exceed 0.1%, as such a concentration had no effect on the proliferation of all cells (data not shown).

Cytotoxicity and proliferation assays. Keratinocytes were seeded into 96-well plates at a density of 5,000 cells per well. At 50-60% confluency, cells were treated with crude plant extract or Salograviolide A. Cytotoxicity was assayed at 6 h using the CytotoTox 96® non-radioactive cell cytotoxicity assay kit according to manufacturer's instructions (Promega Corp., Madison, WI). The CytoTox 96 assay quantitatively measures the activity of lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatants is measured with a coupled enzymatic assay which results in the conversion of a tetrazolium salt into a red formazan product, the absorbance of which is recorded at 490 nm using an ELISA microplate reader. Proliferation was assayed at 24 h using the CellTiter 96® non-radioactive cell proliferation assay kit according to manufacturer's instructions (Promega Corp.). The proliferation assay is an MTT-based method which measures the ability of metabolically active cells to convert tetrazolium salt into a blue formazan product, the absorbance of which is recorded at 570 nm using an ELISA microplate reader. Cytotoxicity and proliferation results were expressed as percentage of control and were derived from the mean of quadruplicate wells.

Cell cycle analysis. Asynchronously growing keratinocytes plated in 100 mm-tissue culture dishes were treated for 24 or 48 h with crude plant extract or Salograviolide A. Attached keratinocytes were trypsinized, pooled with the detached population, fixed in 80% ethanol and stained with 50 μg/ml propidium iodide (Sigma Chemicals Co., St. Louis, MO), as previously described (12).

Hoechst nuclear staining. Keratinocytes were grown on uncoated glass coverslips in 6-well Falcon tissue culture trays and treated with 0.1% ethanol (control) or 16 μg/ml of Salograviolide A. Following 24 or 48 h, attached and detached cells were separately stained with 2 μg/ml Hoechst 33342 nuclear stain (Molecular Probes Inc., Eugene, OR) and fixed in 2% formaldehyde/5% glycerol solution. Stained keratinocytes were then suspended in Prolong antifade (Molecular Probes Inc.), mounted on slides and analyzed separately.

Western blot analysis. Total cellular protein extracts were prepared from cultured keratinocytes, washed twice with PBS, and scraped into SDS-lysis buffer (0.25 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.002% bromophenol blue, 10% ß-mercaptoethanol). Protein concentrations were determined using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol. Equal amounts of total cellular proteins (up to 50 μg) were resolved by 10-12% SDS-polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes (Amersham, Arlington, IL), and then probed with primary antibodies against keratin (K)14, Bcl_2 (N-19), p53 (FL-393), p21 [(c-19)-G)], cyclin B_1 (H-344), Bax, Bcl-x_L, cyclin D_1, p16, and IκBα followed by secondary antibodies conjugated with horseradish peroxidase [all antibodies, except for p53 and K14, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); p53 was obtained from Novocastra (Newcastle, UK) and K14 was kindly provided by Dr Stuart H. Yuspa]. K14 was used to ensure equal loading. The immunocomplexes were visualized using enhanced chemiluminescent kits obtained from Santa Cruz (ECL system). Bands were quantified using ImageQuant software and the Molecular Dynamics 860 System (Molecular Dynamics, Sunnyvale, CA).

Electrophoretic mobility shift assay. Nuclear protein extracts were prepared and NF-κB electrophoresis mobility shift assay was performed, as previously described (18). Briefly, NF-κB consensus oligonucleotides (Santa Cruz) were end-labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase. Nuclear proteins (10 μg) and labeled probe (0.4 ng, >30,000 cpm) were run on a 4% non-denaturing polyacrylamide gel. The gels were dried and processed for autoradiography at -80˚C. Specificity of NF-κB binding was assessed by competition

experiments using 10-fold excess unlabeled or mutant NF-κB oligonucleotides.

Measurement of reactive oxygen species. Generation of intracellular ROS was measured using 2'-7'-dichlorofluorescin diacetate (H_2DCFDA) upon oxidation to the fluorescent derivative 2'-7'-dichlorofluorescin by reactions with ROS. We used the ROS kit: CM-H_2DCFDA (Molecular Probes Inc.) according to manufacturer's instructions. Keratinocytes were continuously exposed to 16 μg/ml of Salograviolide A for 24 h. The anti-oxidants vitamin C (ascorbic acid sodium salt; Sigma Chemicals) and dithiothreitol (DTT) (Fisher-Biotech, Wembley, Western Australia) were added 2 h prior to Salograviolide A at a final concentration of 1000 μM. Following indicated treatments, cells were spun down and resuspended in 500 μl RPMI containing 2% FBS and 10 μM H_2DCFDA for 20 min at 37˚C. Subsequently, cells were washed twice with PBS and then analyzed with a FACS scan flow cytometer (Becton-Dickinson, San Jose, CA) with excitation set at 488 nm and emission at 530 nm.

Results

Centaurea ainetensis crude extract and its isolated pure molecule, Salograviolide A, inhibit cell proliferation of murine neoplastic keratinocytes. Using an *in vitro* murine model of squamous cell carcinogenesis, we tested for the cytotoxicity and anti-proliferative effects of *Centaurea ainetensis* crude methanolic extract on cells representative of the normal and progressing stages of epidermal carcinogenesis. We used the PMKs as representatives of normal epithelial cells, the SP-1 and 308 murine papilloma cell lines as benign tumor cells, the PAM212 cell line as SCC and the spindle I7 cell line as representative of the highly malignant and metastatic tumor cells.

At 6 h-treatment, *Centaurea ainetensis* crude extract was non-cytotoxic to PMKs with concentrations ranging from 0.2 to 0.6 mg/ml but was mildly cytotoxic at 1.1 mg/ml causing a 22% cell death (data not shown). Treatment for 24 h at 0.6 mg/ml inhibited the growth of PAM212 and SP-1 cell lines by 67±1% and 87±2%, respectively, while decreasing the proliferation of PMKs by only 40±3% (Fig. 1A). The proliferation of 308 keratinocytes and of the aggressive I7 spindle cells decreased by 43±7% and 45±3%, respectively (Fig. 1A). The inhibitory effect of the crude extract at 0.6 mg/ml on the growth of SP-1 and PAM212 cells was only 1.7-2.2-fold stronger than that on PMKs. Therefore, using bioassay-guided fractionation, a purified bioactive molecule was isolated from the crude methanolic extract. The sesquiterpene lactone, Salograviolide A (Fig. 2), was contained in one of four subfractions and was found to exert the most significant growth inhibitory effects on neoplastic cells (data not shown).

Salograviolide A (up to 16 μg/ml) was non-cytotoxic to PMKs at 6 h-treatment (data not shown). Non-cytotoxic concentrations of Salograviolide A ranging from 4 to 16 μg/ml were found to inhibit in a dose-dependent manner the proliferation of SP-1, PAM212 and 308 cells at 24 h to a larger extent than PMKs and I7 cells (Fig. 1B). Salograviolide A at 8 μg/ml inhibited the growth of SP-1, PAM212 and 308 cells

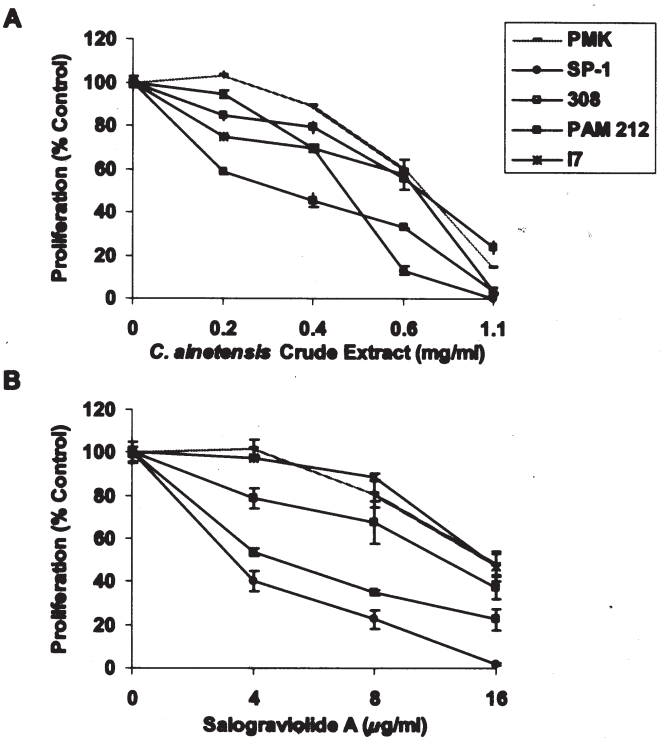

Figure 1. Effects of *Centaurea ainetensis* crude extract and Salograviolide A on primary and neoplastic keratinocyte cell growth. Neoplastic cells were plated in 96-well plates at a density of $1x10^5$ cells/ml; PMKs were seeded as three mouse equivalents per plate. At 50-60% confluency, cells were treated with different concentrations of (A) *Centaurea ainetensis* crude extract (0.2 to 1.1 mg/ml) or (B) Salograviolide A (4 to 16 μg/ml). Cell proliferation was determined at 24 h using the CellTiter 96 non-radioactive cell proliferation kit, as described in Materials and methods. Cell viability is expressed as percentage of control-treated cells for each group and are representative of at least two independent experiments. Each value represents the mean of quadruplicate measurements ± SD.

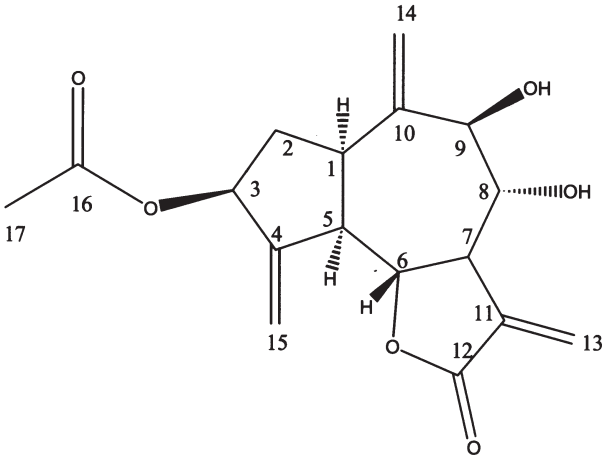

Figure 2. Chemical structure of Salograviolide A.

by 77±4%, 65±1% and 33±10%, respectively (Fig. 1B). This concentration decreased the growth of PMKs and I7 cells by only 19±6% and 11±2%, respectively. The inhibitory effect of Salograviolide A at 8 μg/ml on the growth of SP-1 and PAM212 cells was 3.4-4.1-fold stronger than that on PMKs. Compared to 0.6 mg/ml of the crude plant extract which

contains only 0.1 μg/ml Salograviolide A, 8 μg/ml of Salograviolide A showed a greater selectivity towards SP-1, 308 and PAM212 cell lines vs. PMKs. The IC_{50} values of Salograviolide A on PMKs and I7 cells is 16 μg/ml, while that on SP-1, PAM212 and 308 is 3.5, 4.8 and 12.7 μg/ml, respectively.

Effects of Centaurea ainetensis crude extract and Salograviolide A on cell cycle distribution and apoptosis of neoplastic keratinocytes. Further investigations of the mechanisms of inhibition of cell proliferation were performed on SP-1 and PAM212, as these cell lines were the most sensitive to drug treatment. To determine whether the crude extract or purified molecule induce cell cycle arrest and/or apoptosis, flow cytometric analysis of propidium iodide-stained DNA content and DNA Hoechst staining by fluorescence microscopy were performed. Treatment with crude extract for 24 h caused a dose-dependent G_0/G_1 cell cycle arrest in SP-1 (24% increase at 0.6 mg/ml; Fig. 3) and PAM212 cells (14% increase at 0.6 mg/ml; data not shown), while no changes were observed in the pre-G_0/G_1 population for up to 48 h of treatment (Fig. 3; data not shown). In contrast, an increase in the pre-G_0/G_1 population of SP-1 and PAM212 cells was observed in response to incubation for 24 h with 16 μg/ml of Salograviolide A. The pre-G_0/G_1 regions of the control vs. the 24-h treated neoplastic cells increased from 3 to 20% in SP-1 cells (Fig. 3) and from 1 to 42% in PAM212 cells (data not shown). Treatment of SP-1 and PAM212 with Salograviolide A for 48 h further increased the pre-G_0/G_1 regions to 62 and 46%, respectively (data not shown). Up to 48-h treatment of SP-1 or PAM212 cell lines, Salograviolide A did not induce G_0/G_1, S or G_2/M cell cycle arrest (Fig. 3; data not shown).

To confirm whether the increase in pre-G_0/G_1 regions in the Salograviolide A-treated keratinocytes was due to apoptosis induction, cells were stained with Hoechst. Incubation with 16 μg/ml Salograviolide A for 24 h induced slight chromatin condensation in attached SP-1 cells (data not shown) and did not induce sufficient cell detachment to observe chromatin condensation in shed cells. However, following 48 h-treatment with 16 μg/ml of Salograviolide A, the attached SP-1 cells exhibited partial chromatin condensation (Fig. 4, arrows) and the detached keratinocytes showed complete chromatin condensation (Fig. 4, arrowheads). This is supported by our previous results which showed that 16 μg/ml of Salograviolide A caused in SP-1 cells an increase in the pre-G_0/G_1 regions up to 20% after 24 h-treatment vs. 62% 48 h-post treatment.

Centaurea ainetensis crude extract and Salograviolide A modulate key cell cycle and apoptotic regulators. To investigate the effects of crude extract (0.4 mg/ml) and purified molecule (4, 8 and 16 μg/ml) on key mediators of cell cycle and apoptosis, we examined changes in the protein expression levels of cyclin-dependent kinase inhibitors (CDKIs p16, p21), cyclins (D_1 and B_1), the tumor suppressor protein p53 and Bcl_2 family members (Bax, Bcl_2 and Bcl-x_L).

After treatment of SP-1 cells with crude extract or Salograviolide A, cyclin D_1 protein levels were decreased or rendered undetectable; whereas, cyclin B_1 and p53 proteins levels were unchanged (Fig. 5). Similar results were obtained in crude extract-treated PAM212 cells (data not shown). In contrast, p16 protein levels were increased as early as 2 h following both treatment conditions and were then decreased or rendered undetectable at later time points (Fig. 5). Similarly, p21 protein levels increased in crude extract or purified molecule treated-keratinocytes (Fig. 5).

	Treatment Conditions	% Total Cell Number	
		Pre-G_0/G_1	G_0/G_1
C. ainetensis Crude Extract (mg/ml)	Control	3	59
	0.2	3	62
	0.4	3	69
	0.6	1	73
Salograviolide A (μg/ml)	Control	3	54
	4	1	64
	8	4	56
	16	20	54

Figure 3. Effects of *Centaurea ainetensis* crude extract and Salograviolide A on the cell cycle distribution of SP-1 papilloma cells. SP-1 cells were plated in 100 mm-culture dishes at a density of 50,000 cells/ml. At 50-60% confluency, keratinocytes were treated for 24 h with corresponding vehicle control or with different concentrations of *Centaurea ainetensis* crude extract (0.2 to 0.6 mg/ml) or Salograviolide A (4 to 16 μg/ml). Attached and detached cells were pooled and stained with propidium iodide. DNA content was quantified by flow cytometry. The distribution of cells in the pre-G_0/G_1 and the different phases of the cell cycle was determined using the CellQuest histogram analysis program. Results are representative of two independent experiments.

Then, changes in the pro-apoptotic Bax and anti-apoptotic Bcl_2 proteins were monitored upon treatment with the crude plant extract or with Salograviolide A. An increase in the ratio of pro-apoptotic over anti-apoptotic proteins leads to an increase in mitochondrial permeability and subsequent release of cytochrome c, an event central to apoptosis signaling (19). Bax protein levels are undetectable in control SP-1 cells. Treatment of SP-1 cells with crude extract resulted in time-dependent increases in Bax protein levels starting 8 h-post-treatment (Fig. 5A), and a gradual decrease in Bcl_2 protein levels as early as 2 h to reach undetectable levels by 12 h. However, Salograviolide A induced an early and sharp increase in Bax proteins with undetectable changes in the protein levels of Bcl_2 or of its anti-apoptotic relative, Bcl-x_L (Fig. 5B; data not shown). Treatment of SP-1 cells with the other concentrations of Salograviolide A (4 and 8 μg/ml) resulted in similar trends of the aforementioned cell cycle and apoptotic regulator proteins as 16 μg/ml (Fig. 5B; data not shown).

Centaurea ainetensis crude extract and Salograviolide A regulate NF-κB signaling. NF-κB is a ubiquitous transcription factor that mediates growth and apoptotic effects of

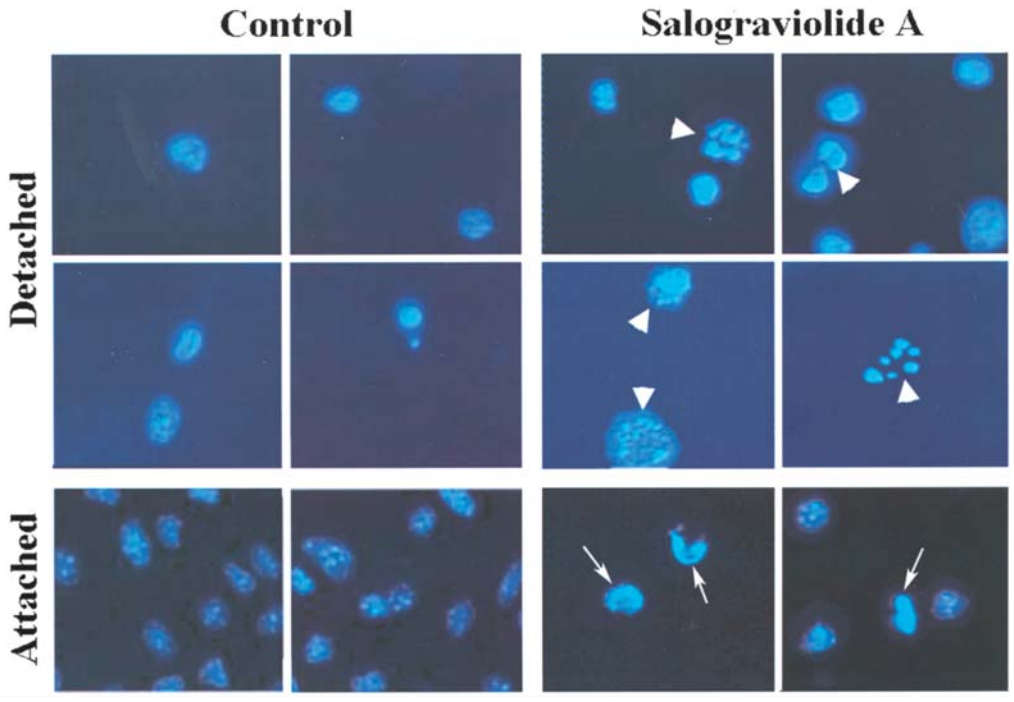

Figure 4. Salograviolide A induces apoptosis in SP-1 papilloma cells. SP-1 cells were grown on uncoated glass coverslips and treated, when 50-60% confluent, either with ethanol (0.1%) or with 16 μg/ml of Salograviolide A. After 48 h-treatment, detached and attached cells were stained with Hoechst and mounted separately in antifade, as described in Materials and methods. Hoechst staining reveals chromatin condensation characteristic of apoptosis. Arrowheads indicate apoptotic cells which display a pattern of condensed and fragmented nuclei; whereas, arrows indicate partial chromatin condensation. Results are representative of two independent experiments.

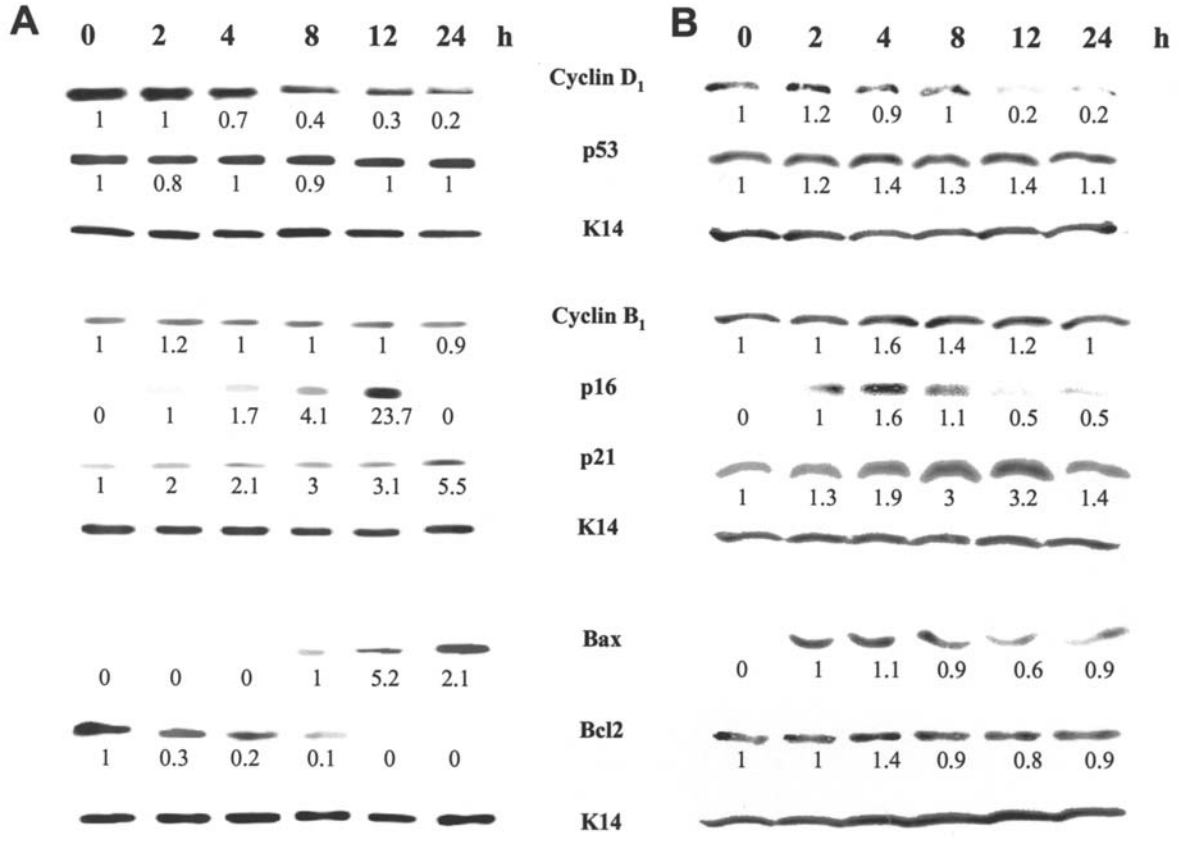

Figure 5. *Centaurea ainetensis* crude extract and Salograviolide A differentially modulate cell cycle and apoptotic regulators in SP-1 papilloma cells. SP-1 cells were plated in 100 mm-culture dishes at a density of 50,000 cells/ml. At 50-60% confluency, cells were treated with 0.4 mg/ml of *Centaurea ainetensis* crude extract (A) or Salograviolide A (16 μg/ml) (B) for the indicated time points. Whole cell protein lysates were prepared and immunoblotted with either cyclin D_1, p53, cyclin B_1, p16, p21, Bax, or Bcl_2. Blots were stripped and reprobed with K14 to ensure equal protein loading. Bands were quantified and expressed as fold of control cells, set as one, or as indicated. Results are representative of at least two independent experiments.

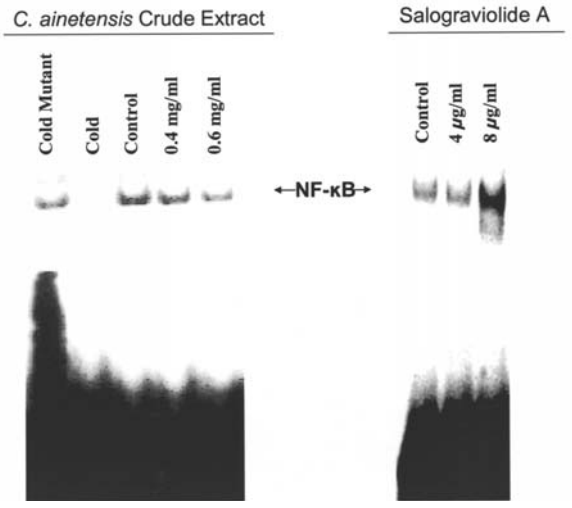

Figure 6. *Centaurea ainetensis* crude extract and Salograviolide A differentially regulate NF-κB DNA-binding activities in SP-1 papilloma cells. SP-1 cells were plated in 100 mm-culture dishes at a density of 50,000 cells/ml. At 50-60% confluency, cells were treated for 24 h either with corresponding vehicle control or with the indicated concentrations of *Centaurea ainetensis* crude extract or Salograviolide A. Electrophoretic mobility shift assay was determined, as described in Materials and methods. Competition of binding was observed upon the addition of 10-fold excess cold probe, but not with the addition of 10-fold excess cold mutant probe. Results are representative of two independent experiments.

both external and internal stimuli and is a prime target of several plants with antitumor activities (20). Therefore, we investigated whether *Centaurea ainetensis* crude extract and Salograviolide A modulate NF-κB activity.

NF-κB activity is tightly regulated by many inhibitors including IκBα. Several signaling cascades lead to the phosphorylation of this inhibitor, its subsequent degradation, and the translocation of NF-κB into the nucleus where it binds its consensus sequence and activates many genes. Hence, we monitored IκBα protein levels at several time points after treatment. SP-1 cells treated with either the crude extract or Salograviolide A did not show any changes in IκBα protein levels (data not shown). However, the activity of NF-κB is not solely mediated by IκBα. Therefore, we performed a gel shift mobility assay using an NF-κB consensus oligonucleotide to test for NF-κB DNA-binding activity. A dose-dependent decrease in NF-κB binding activity was observed upon treatment of SP-1 and PAM212 with 0.4 or 0.6 mg/ml of the crude plant extract for 24 h (Fig. 6; data not shown). When Salograviolide A was administered to SP-1 cells, NF-κB DNA-binding activity was unaltered at 4 μg/ml but potently increased at 8 μg/ml (Fig. 6). The NF-κB DNA binding to its labeled consensus sequence is specific since it disappeared upon the addition of excess cold probe and was sustained

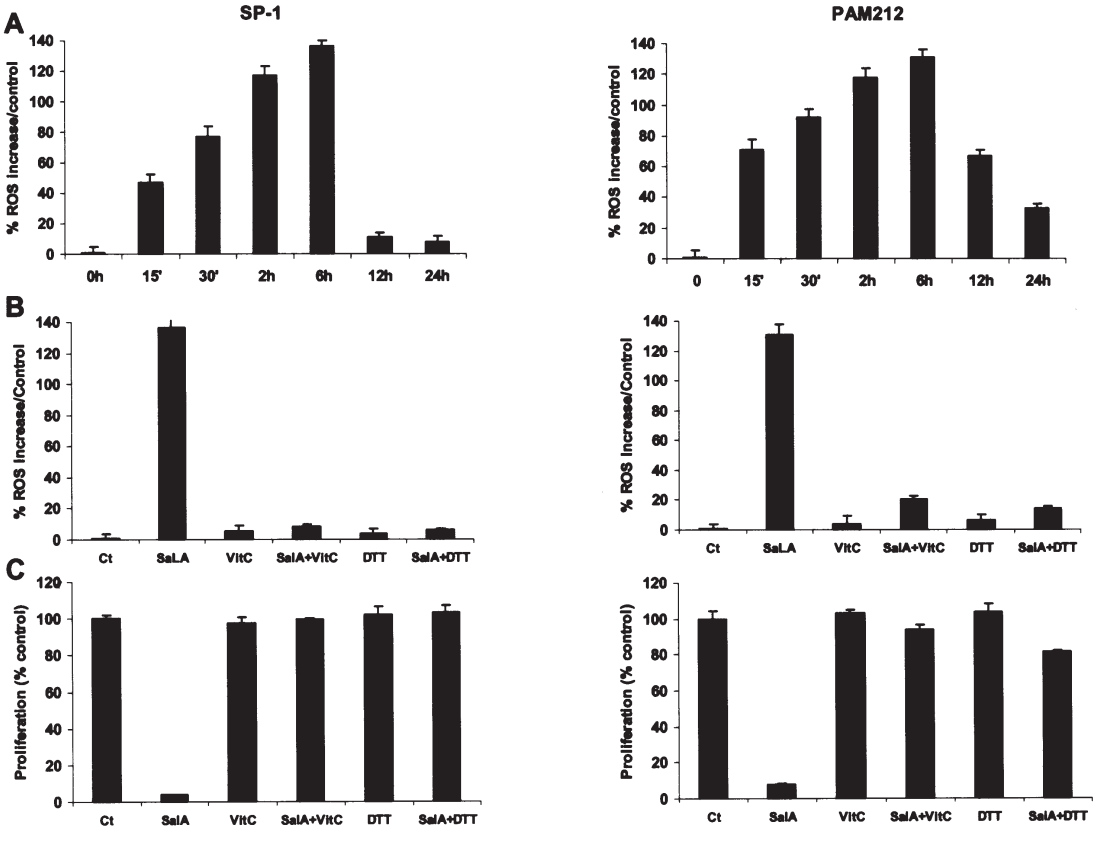

Figure 7. Reactive oxygen species accumulation mediates Salograviolide A-induced growth inhibition in neoplastic keratinocytes. SP-1 and PAM212 cells were continuously exposed to 16 μg/ml of Salograviolide A and ROS levels were determined by the H_2DCFDA assay for the indicated time points. (A) ROS levels, calculated from duplicate measurements, are expressed as percentage increase over control, set as one, and represent the mean ± range (n=2). SP-1 and PAM212 cells were treated either with 0.1% ethanol (as control), 16 μg/ml of Salograviolide A (SalA), 1000 μM vitamin C (VitC) or 1000 μM DTT or for 2 h with vitamin C or DTT prior to Salograviolide A treatment. (B) ROS levels and (C) cell growth were then calculated at 6 and 24 h of treatment, respectively. ROS levels were calculated from duplicate measurements and are expressed as percentage increase over control (Ct) set as one. Each bar represents the mean ± range (n=2). Cell proliferation was assayed in quadruplicate wells with the CellTiter 96 non-radioactive cell proliferation kit. Cell proliferation is expressed as percentage of control (Ct) and represents the mean of quadruplicate measurements ± SD. Results are representative of two independent experiments.

upon the addition of excess mutant cold probe which lacks affinity to the NF-κB transcription factor.

Reactive oxygen species accumulation mediates Salograviolide A-induced growth inhibition. Several recent studies have shown that sesquiterpene lactones sensitize human cancer cells to chemotherapeutic drugs as a result of ROS generation and regulation of the NF-κB signaling pathway. Moreover, oxidative stress was shown to induce apoptosis in cancer through NF-κB activation (21).

To investigate the involvement of ROS in Salograviolide A-mediated growth inhibition, we measured ROS levels at different time points following treatment of SP-1 and PAM212 cells with the most potent tested concentration of Salograviolide A (16 μg/ml). Salograviolide A-induced growth inhibition was preceded by an early and progressive accumulation of ROS in both cell lines (Fig. 7). ROS levels increased in Salograviolide A-treated SP-1 and PAM212 cell lines, as early as 15 min, by 47 and 71% of control cells, respectively (Fig. 7A). These oxidant levels peaked at 6 h reaching 136 and 131% of control in SP-1- and PAM212-treated cells, respectively. Altogether, these results indicate that Salograviolide A-induced growth inhibition is associated with early ROS accumulation in SP-1 and PAM212 cells.

To explore the direct role of the observed ROS accumulation in Salograviolide A-induced growth inhibition, SP-1 and PAM212 cells were pre-treated for 2 h with the anti-oxidant agents, vitamin C and DTT and then exposed to 16 μg/ml Salograviolide A for 24 h. Both vitamin C and DTT completely abrogated Salograviolide A-induced ROS generation in both cell lines (Fig. 7B). This was accompanied by a reversal of Salograviolide A-induced growth inhibition in treated cells (Fig. 7C). These results indicate that ROS accumulation directly mediate Salograviolide A-induced growth inhibition in neoplastic epidermal cells, suggesting that this molecule is a potent oxidant.

Discussion

We tested and characterized the antitumor properties of *Centaurea ainetensis* crude extract and its purified molecule, Salograviolide A, using an *in vitro* murine model of epidermal squamous cell carcinogenesis. Taking into account that a plant could contain many metabolites acting at many target sites in a given physiological process (4), the assessment of the tumor stage-specific or multi-target effects of a plant extract becomes most suitably applicable to the skin model of carcinogenesis (22).

Treatment of various murine cell lines and primary keratinocytes with *Centaurea ainetensis* crude extract or the isolated guaianolide, Salograviolide A, preferentially inhibited the proliferation of neoplastic epidermal cells. The most aggressive I7 spindle cells were relatively resistant. The crude plant extract and Salograviolide A display little or no cytotoxicity at the tested concentrations against primary keratinocytes.

Whereas the crude plant extract induced G_0/G_1 cell cycle arrest without apoptosis induction, Salograviolide A induced apoptosis in SP-1 and PAM212 cells. All the murine neoplastic cell lines employed in our study contain a characteristic oncogenic mutation of the ras^{Ha} gene and overexpress cyclin D_1. It is well established that cyclin D_1 is a critical target for oncogenic ras in mouse skin and that the ras^{Ha} oncogene signals through cyclin D_1 to promote tumorigenesis (23). Both crude extract and Salograviolide A caused a down-regulation of cyclin D_1 protein levels in neoplastic keratinocytes.

This observed cyclin D_1 down-regulation was concomitant with an earlier up-regulation of the CDKIs p16 and p21. There is compelling evidence that the induction of p16 proteins increases the sensitivity of tumors to chemotherapeutic drugs (24). There is also evidence that p21 promotes Ca^{2+}-induced keratinocyte differentiation. However, sustained expression of p21 renders normal keratinocytes unable to complete their differentiation program in response to high Ca^{2+} levels. Therefore, the rapid subsequent destruction of p21 proteins is necessary for the progression of differentiation (25), a process intimately connected with cell death and shedding of epidermal cells (22). Indeed, Salograviolide A-treated papilloma cell lines transiently up-regulate p21 protein levels in a curvilinear manner consistent with apoptosis induction and keratinocyte shedding. However, crude extract-treated SP-1 cells up-regulate p21 proteins without causing their subsequent degradation consistent with the associated G_0/G_1 cell cycle arrest.

NF-κB is a transcription factor pleiotropically involved in apoptosis and cell cycle control. Treatment of neoplastic keratinocytes with *Centaurea ainetensis* crude extract caused a dose-dependent decrease in both of NF-κB DNA binding activity and cyclin D1 protein levels. Several anticancer dietary agents have been found to be potent inhibitors of NF-κB (26). Furthermore, it was shown that inactivating NF-κB in PAM-LY-2 cells, an aggressive murine SCC cell line, inhibits its malignant phenotype (27). In contrast, Salograviolide A-treated neoplastic keratinocytes increases NF-κB DNA binding activity.

There are conflicting results as to the role of NF-κB signaling in skin cancer. Studies of human and murine models of epidermal squamous cell carcinogenesis show frequent NF-κB activation (27,28) and a likely pro-tumorigenic role of NF-κB in skin carcinogenesis (29-31). In addition, NF-κB activation can lead to increased transcription of target genes such as cyclin D_1 and anti-apoptotic Bcl_2 family proteins (32). Surprisingly, in some mouse models, NF-κB inhibition induces cancer (33-38). Studies have shown that inhibiting NF-κB in initiated keratinocytes actually promotes squamous cell carcinogenesis by reducing 'terminal differentiation', an essential process for keratinocyte cell death (39). Thus, the up-regulation of NF-κB DNA-binding activity at 8 μg/ml of Salograviolide A in neoplastic cells could be, in fact, promoting epidermal differentiation that is preceding cell death.

Several recent studies have shown that sesquiterpene lactones generally possess an α-methylene-ß-lactone moiety essential for their apoptogenic and oxidizing activities, which in turn regulate the NF-κB signaling pathway (8,21,40). We were able to show that ROS accumulation directly mediates Salograviolide A-induced growth inhibition in neoplastic epidermal cells.

Many studies have also reported that sesquiterpene lactones can induce apoptosis by enhancing the pro-apoptotic

regulators, but without being able to alter the levels of anti-apoptotic proteins Bcl_2 and Bcl-x_L (41,42). In fact, our results show that Salograviolide A does not modulate the protein levels of Bcl_2 or Bcl-x_L in neoplastic cells but increases the Bax/Bcl_2 ratio relative to the control by up-regulating the pro-apoptotic Bax protein levels. On the other hand, *Centaurea ainetensis* crude extract not only increases Bax protein levels in neoplastic keratinocytes but also concomitantly decreases Bcl_2 protein levels. It is hence very likely that, in the crude extract, compounds other than Salograviolide A are acting in concert with this pure molecule in order to decrease Bcl_2 levels in neoplastic keratinocytes.

Our results are the first to describe and characterize the antitumorigenic properties of *Centaurea ainetensis* crude extracts and of its purified component, Salograviolide A, in neoplastic epidermal cells. Several studies have suggested that the genus Centaurea comprises many secondary metabolites exhibiting several therapeutic values (7). However, only two reports addressed the biological activities of Salograviolide A, highlighting its antifungal (43) and anti-colon cancer effects (44). In this study, we showed that, in a murine *in vitro* model, the papilloma and carcinoma cell lines are more sensitive to treatment with *Centaurea ainetensis* crude extract or Salograviolide A than the normal keratinocytes or spindle cells. Moreover, the fact that neither the crude extract nor the purified molecule, at the tested concentrations, are cytotoxic to primary keratinocytes necessitates in-depth analysis of the antitumor properties of *Centaurea ainetensis* and Salograviolide A on human squamous cell carcinogenesis.

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