Sclareol induces apoptosis in human HCT116 colon cancer cells *in vitro* and suppression of HCT116 tumor growth in immunodeficient mice

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Abstract Labd-14-ene-8, 13-diol (sclareol) is a labdane-type diterpene, which has demonstrated significant cytotoxic activity against human leukemic cell lines, but its effect on solid tumor-derived cells is uknown. Here, we demonstrate that addition of sclareol to cultures of human colon cancer HCT116 cells results in inhibition of DNA synthesis, arrest of cells at the G_1 phase of the cell cycle, activation of caspases-8, -9, PARP degradation, and DNA fragmentation, events characteristic of induction of apoptosis. Intraperitoneal (ip) administration of sclareol alone, at the maximum tolerated dose, was unable to induce suppression of growth of HCT116 tumors established as xenografts in immunodeficient SCID mice. In contrast, ip administration of liposome-encapsulated sclareol, following a specific schedule, induced suppression of tumor growth by arresting tumor cell proliferation as assessed by detecting the presence of the cell proliferation-associated nuclear protein, Ki67, in thin tumor sections. These findings suggest that sclareol incorporated into liposomes may possess chemotherapeutic potential for the treatment of colorectal and other types of human cancer.

Keywords Plant product · Labdane diterpene · Sclareol · Apoptosis · Tumor suppression

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Introduction

A large number of the phytochemicals, diterpenoids, abundantly occur in nature [1]. Labdane diterpenes abundant in several families of plants and algae possess a labdane skeleton consisting of a decalin system and a C-6 ring, which may be open or closed with an oxygen atom, as in manoyl oxide and its derivatives [2, 3]. Of special interest among these compounds that exhibit a variety of activities [4] is forskolin, a manoyl oxide derivative that can activate adenylate cyclase by directly interacting with the catalytic subunit or a closely associated protein of the adenylate cyclase system [5]. Because of its unique biochemical properties, forskolin is an invaluable tool for investigations of the role of c-AMP in biological processes. Further, several labdane diterpenes are cytotoxic and cytostatic for human leukemia [6–11], breast [12], and colon [13] cancer cell lines.

The ditertiary alcohol, labd-14-ene-8, 13-diol (sclareol), which was first isolated from the plant *Salvia sclarea* [14], is abundant in nature, and is used as a fragrance in cosmetics, flavoring additive in food and beverage industry, and folk medicine. Sclareol induces apoptosis in human leukemia cell lines, via an interesting mechanism that down-regulates

c-*myc* without affecting the expression of the anti-apoptotic protein, Bcl-2 [9], and in cells derived from solid tumors by a mechanism that appears to be independent on p53-expression [12]. Further, in a preliminary study we have shown that sclareol possesses a modest anticancer activity in a human colon cancer xenograft/mouse model, when this compound is administered in an encapsulated (liposomal) form, as this was concluded calculating the doubling time of tumors from 200 to 400 mm^3 [13]. Our choice of liposomes as lipid-based drug carriers to deliver sclareol was motivated by their well documented ability to deliver bioactive compounds in a targeted manner, and their favourable physicochemical properties that typically result in improvement of the pharmacokinetic properties and pharmacological response of the encapsulated drugs, while reducing drug-associated side effects (for recent reviews, see refs. [20–24]).

In most cases, anticancer drug treatment results in the activation of caspases, enzymes which effectively execute various forms of cell death by apoptosis. The death receptor-dependent apoptotic pathway is triggered at the cell surface and requires activation of caspase-8, whereas the mitochondrion-dependent pathway is initiated with release of mitochondrial cytochrome c into the cytoplasm and requires activation of caspase-9. Subsequently, caspase-8 or -9 can activate caspase-3, which in turn targets and degrades specific and vital cellular proteins, ultimately resulting in nuclear DNA degradation and apoptotic death of the cells (for reviews on caspases, see refs. [15–17]). However, it is not unusual for the activated pathway to display only some of the characteristics of the "classical" apoptotic pathways [18, 19].

Our initial published observations and results [13] prompted us to investigate more extensively the effect of sclareol on the cell cycle of human colon cancer cells *in vitro*. Furthermore, and since the apoptotic pathway activated by sclareol was still unknown, we sought to assess whether sclareol's death inducing activity is mediated via an established apoptosis pathway. Finally, we wanted to investigate conditions that could induce significant suppression or regression of HCT116 tumors.

In this report, we describe experimental findings to demonstrate for the first time that (i) sclareol can induce G$_1$-phase arrest and apoptosis in HCT116 colon cancer cells *in vitro* associated with activation of caspases-8, -9 and -3, and, (ii) sclareol alone at the maximum tolerated dose (MTD) is inactive, whereas liposome-encapsulated sclareol, at doses much higher than the MTD of sclareol alone, can suppress colon tumor growth presumably by inhibiting tumor cell proliferation.

Materials and methods

Chemicals/biological reagents

Trichloroacetic acid (TCA), sulphorhodamine B (SRB), Trizma base, acetic acid, sclareol, rabbit polyclonal antibodies to caspase-8 (GD-13), caspase-3 (CPP32), mouse monoclonal antibody to PARP (clone C-2-10), the secondary antibodies, peroxidase-conjugated anti-mouse and anti-rabbit IgG, and the chromogen, 3,3′-diaminobenzidine (DAB), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal antibody to caspase-9 (p35/H170), and monoclonal antibody to β-actin (C-2) were from Santa Cruz (Santa Cruz, CA, USA). For immunohistochemistry studies, the monoclonal antibody to Ki67/MIB1 (catalog No. M7240) was purchased from Dako (Denmark). All antibodies were diluted in TBS-T (0.1% Tween-40 in 100 mM Tris-Cl, pH 7.4, 0.8% NaCl). Anti-BrdUdr (FITC; catalog No. 347583) was purchased from Becton Dickinson (NJ, USA); propidium iodide (PI) was from Roche (Basel, Switzerland), and all other chemicals were from Sigma-Aldrich. All cell culture reagents were purchased from Euroclone Life Sciences Division (Milano, Italy).

Cell cultures

The human colon cancer HCT116 cell line was obtained from the National Cancer Institute, NIH (Bethesda, MD, USA), and was adapted to propagate in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum, 2 mM L-glutamine and antibiotics. The cultures were grown in a humidified 37°C-incubator with 5% CO$_2$ atmosphere.

In vitro cytotoxic activity of sclareol

Cell viability was assessed at the beginning of each experiment by the trypan blue dye exclusion method, and was always greater than 95%. Cells were seeded into 96-well microtiter plates in 100 μL of medium at a density of 5000 cells per well, and subsequently, the plates were incubated at standard conditions for 24 h to allow the cells to resume exponential growth prior to addition of sclareol. Then, in order to measure the cell population, cells in one plate were fixed *in situ* with TCA followed by SRB staining, as described elsewhere [12]. To determine the sclareol activity, the compound dissolved in DMSO was added at 10-fold dilutions (from 100 to 0.01 μM) and incubation continued for an additional period of 48 h. The assay was terminated by addition of cold TCA followed by SRB staining and absorbance measurement at 530 nm, in an EL-311 BIOTEK microelisa reader (BioTek, Winooski, VT, USA), to determine the GI$_{50}$, that

is, the sclareol concentration required in the cell culture to inhibit cell growth by 50% as described [25].

Flow cytometry analysis of cell cycle and assessment of apoptosis

Untreated and sclareol-treated cells were collected at the desired periods of time, and then prepared and subjected to flow cytometry analysis to monitor perturbations in the cell cycle and apoptosis as described [18, 19, 26, 27]. In addition, apoptosis was assessed with the aid of the ssDNA Apoptosis, and the Cellular DNA Fragmentation ELISA kits. The ssDNA Apoptosis kit (Chemicon International, CA, USA) is used to detect formamide-denatured DNA in apoptotic cells with a monoclonal antibody that recognizes single-stranded DNA (ssDNA). In our studies, 5000 cells were seeded onto microplates and then treated with various sclareol concentrations for the desired periods of time, fixed into the wells, treated with formamide and stained with the antibody mixture as described in the assay protocol provided by the manufacturer. Further, the Cellular DNA Fragmentation ELISA kit (Roche, Switzerland) can quantify DNA fragments, after DNA damage, released in the cytoplasm upon induction of apoptosis. The experiments were carried out, as described by the manufacturer, in microtiter plates (containing 1×10^6 cells/ml) using various sclareol concentrations after BrdU-incorporation. At selected periods of scareol treatment, the supernatant was removed and kept at $-20°C$ until use, and cell lysates and supernatants were assayed for presence of DNA fragments associated with apoptosis.

Protein extraction and Western blotting

Cells were washed twice in ice-cold PBS, detached with a latex cell-remover, collected by gentle centrifugation, and whole cell protein extracts were prepared as previously described [26–28]. Protein concentrations were determined with the Bradford assay, and subsequently, aliquots containing 30 μg of protein were subjected to gel electrophoresis on 8% or 10% polyacylamide SDS-gels, and then transferred to PVDF membranes (Millipore Immobilon). The membranes were stained with Ponseau S solution (0.1% Ponseau in 5% acetic acid; AlterChem, Athens, Greece), and proteins were visualized using an enhanced chemoluminescence detection system (ECL or ECL Plus, Amersham, Arlington Heights, IL) according to the manufacture's instructions.

Preparation of liposomes and liposomal sclareol

Liposomes and liposome-encapsulated sclareol (termed liposomal sclareol, hereafter) were prepared as described [13]. The encapsulated sclareol to lipid molar ratio was 0.43 suggesting an incorporation efficiency of nearly 80% [13]. Both,

empty liposome (control) and liposomal sclareol suspensions were freeze-dried and stored at 4°C until used.

In vivo antitumor activity

Male SCID (NOD.CB17 Prkdc[scid]) mice were purchased from Jackson Laboratories/Charles River Laboratories (L'Arbresle, France). The mouse colony was maintained under restricted flora conditions in a pathogen-free environment in type IIL-cages. Male mice, 7–9 weeks old, were injected subcutaneously according to the British practice of bilateral trocar implants at the axillary region. Each inoculum contained 10^6 cells exponentially growing at the time of harvesting. The two tumor cell inoculums were added together for each mouse, thus producing a tumor burden per mouse value for data analysis. The advantage of the British system is reduced mouse-to-mouse variability and, thus, the ability to reduce the number of animals per group, with five being sufficient for accurate data analysis of tumor response and evaluation of toxicities. The virtues of the British system of mouse xenografting over the "traditional" single implantation are described in ref. [29]. The mice were randomly divided into groups of 6 to 8 animals per group. Treatments started when the average tumor volume had reached about 100 mm^3. Tumor volume was calculated as described in ref. [29]. All administrations were intraperitoneal. Treated animals received a single injection daily for 5 days per week throughout the experiment. Tumor volume was measured with a calliper twice per week. In addition to tumor volume, we calculated the parameter, $\%\Delta T/\Delta C$, where $\Delta T = T - D_o$ and $\Delta C = C - D_o$ (D_o is the average tumor volume at the beginning of the treatment; T and C are the volumes of treated and untreated tumors, respectively, at a specified day). Concurrently, we scored the number of tumor-free animals, number of drug-related deaths, and average number of days required to reach a defined tumor volume. Optimal $\Delta T/\Delta C$ value was used as a measure of drug activity. Losses of weight, neurological disorders and behavioral and dietary changes were also recorded as indicators of toxicity (side effects). The experiment was terminated when tumor size in untreated animals reached a volume of about 1000 mm^3. All animals were treated according to Greek laws (2015/92), guidelines of the European Union and the European council (86/609 and ETS123, respectively), and Compliance with Standards for Human Care and Use of Laboratory Animals, NIH, USA (Assurance No. A5736-01).

Morphology and immunohistochemistry of tumors

Tumors were excised, fixed in buffered formalin (4%), embedded in paraffin, and 4-μm sections were prepared with the aid of a Leica, model RM2125, microtome. Subsequently, sections were stained with hematoxylin-eosin and

examined under a microscope to assess the histological phenotype of the tumor, the type and degree of differentiation, and the presence of regressive changes. Other sections were de-paraffinized and subjected to standard two-stage indirect immunohistochemical technique (ABC method) using the polyclonal Ki67-antibody at 1:150 dilution and incubated overnight at room temperature. For antigen retrieval, the sections were pretreated (boiled in microwave oven) for 20 min in citrate buffer, pH 6 [30], and stained with the chromogen, DAB. Unstained and positively-stained nuclei were scored in 10 fields, from two independently-removed mouse biopsies (that is four tumors per group), at high power, and the percentage of the positively-stained cells was estimated. Sections stained with hematoxylin-eosin or examined for presence of Ki67 antigen were photographed under a Nikon Eclipse 80i equipped with Digital Sight DS-2Mv.

Statistical analysis

Significant difference in tumor volume was determined by the Student's t-test using the SPSS for Windows (release 11.0.0, SPSS Inc., USA) software package. A difference was considered significant if $p < 0.05$.

Results

Sclareol induces cell cycle arrest at G_1-phase

The ability of sclareol to induce perturbations in the cell cycle was studied after we determined the GI_{50} to be 34 μM for HCT116 cells treated with various sclareol concentrations for 48 h. Subsequently, identical cultures of exponentially grown cells were treated with 100 μM sclareol for various periods of time for the flow cytometry studies. The results (histograms) of this study and the cell fractions (%) in each cell cycle phase and apoptosis are shown in Fig. 1 and Table 1. There were no significant changes in the cell cycle

Table 1 Flow cytometry analysis of exponentially grown cells treated with sclareol

	$G_{/1}$	S	G_{2+M}	Ap
ut	34 (35)	38 (39)	26 (26)	2
8 h	36 (37)	35 (37)	25 (26)	4
16 h	42 (51)	22 (27)	19 (22)	17
24 h	35 (47)	24 (32)	16 (21)	25

HCT116 cells were treated for various time points with 100 μM of sclareol. Results are expressed as the mean of three independent experiments. Numbers in parentheses denote percentages calculated only on gated live cells. CV $\leq$ 17%; ut, Untreated cells; h, hours; Ap, apoptotic cells.
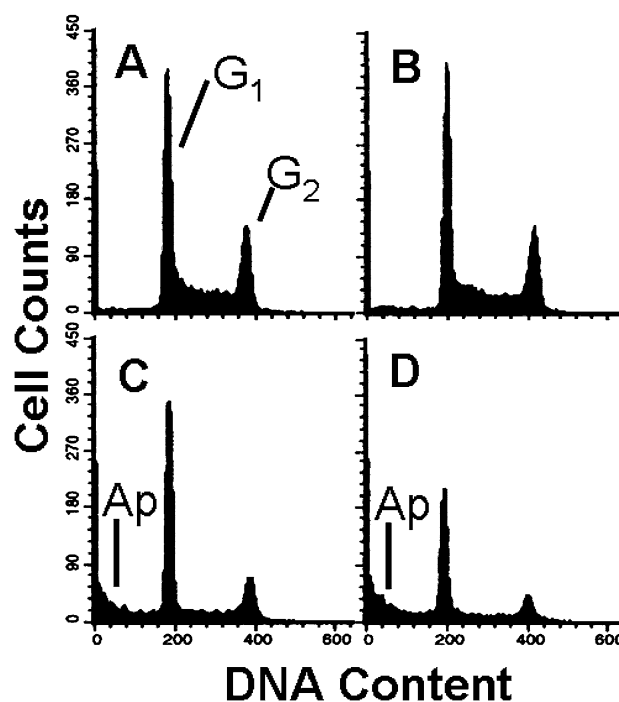

Fig. 1 Sclareol-induced cell cycle perturbations. Identical asynchronous HCT116 cell cultures were treated with 100 μM sclareol for 8 h (histogram B), 16 h (histogram C) and 24 h (histogram D), and attached and detached cells were harvested and subjected to flow cytometry analysis of the relative DNA content (i.e., cell cycle perturbations and apoptosis). Control culture received carrier alone (histogram A). G_1, $G_0 + G_1$ cells; G_2, $G_2 + M$ cells; and, Ap, apoptotic cells (sub-G_1 cell population). The estimate of each fraction expressed as % of the total cell population treated and as % of the gated live cells is shown in Table 1. Ten thousand events were scored per sample (histogram)

of the cells treated with sclareol for 8 h (see histograms A and B), but an increase in the G_1- and Ap-fractions, at expense of the S-fraction, was observed at 16 h of treatment (histogram C) with a further increase in the Ap fraction after 24 h of treatment (histogram D). These results showed an increase in apoptosis but did not address whether the cells were at a specific cell cycle phase when entering apoptosis. Therefore to identify the cell cycle phase targeted by sclareol we studied the effect of this compound on cells already arrested at G_1. This was accomplished by incubating several identical cell cultures for 72 h in media without serum (i.e., serumless media). At the end of the 72 h-incubation period the attached cells were gently washed with medium and afterwards received fresh medium containing 5% serum without or with 100 μM of sclareol. Exponentially growing HCT116 cells exhibited a typical distribution in various fractions (i.e., phases) of the cell cycle as shown in Fig. 2 (histogram A). This histogram indicates that approximately 34%, 39%, and 25% of the cells were in the G_1-, S-, and G_2-phase, repectively. However, after exposure of the cells for 72 h in serumless media, more than 80% of the cells were arrested in G_1, while a small number of cells were

Table 2 Cell cycle analysis of serum starved cells treated with sclareol

	ut	scl-tr	ut	scl-tr	ut	scl-tr
0 h	81		9		9	
8 h	77	83	16	10	7	7
10 h	66	82	26	10	8	8
12 h	49	81	44	12	6	7
24 h	46	80	31	8	21	7
	G_1		S		G_{2+M}	

HCT116 cells serum starved for 72 h were subsequently released in RPMI supplemented with 5% FCS for various periods of time. Results are expressed as the mean of three independent experiments. CV $\leq$ 10%; ut, Untreated cells; scl-tr, cells treated with 100 μM of sclareol. Sub $G_{/1}$ cells were less than 5% in all cases.

still traversing the S- and G_2-phases (Fig. 2, histogram B and Table 2). Subsequently, after the G_1-arrested cells were transferred to media containing 5% serum for 12 h, about 50% of these cells had re-entered cycling as indicated by the decrease in the number of cells in G_1, and an increase in the number of cells in the S-phase (see histogram C and Table 2). In contrast, the cells remained arrested in the G_1-phase in the presence of sclareol in the serum supplemented media (histogram D). Further, at 24 h, cells in media containing serum without sclareol had progressed from the S- to the G_2-phase (histogram E), whereas the presence of sclareol in the serum supplemented media continued to block progression of the cells from the G_1- to S-phase (histogram F). Taken together, these results unequivocally demonstrated that the presence of sclareol in cultures of HCT116 cells led to arrest of these cells in the G_1-phase of the cell cycle (Fig. 2, compare C to D and E to F).

Sclareol induces DNA damage in a time-dependent manner

The results of the flow cytometry studies demonstrated that in the presence of sclareol the HCT116 cells were arrested at the G_1-phase of the cell cycle, whereas cells in other phases of the cell cycle died by apoptosis (Figs. 1 and 2). These results prompted us to further elucidate how early sclareol alters the conformation and integrity of the chromatin DNA. First, we monitored sclareol-induced conformational changes in the DNA using the ssDNA Apoptosis kit as described in Materials and Methods. The results of this study demonstrated that addition of 100 μM sclareol to HCT116 cell cultures resulted in presence of DNA breaks as early as 4 h after sclareol addition (Fig. 3, graph A). Further, chromatin DNA breaks continued to accumulate as the cells continued to be exposed to sclareol.

We also assessed the timing of appearance of DNA fragments in the cytoplasm and cell-conditioned media, after addition of sclareol to the cell culture, events that have been associated with late and irreversible stages of apoptosis.
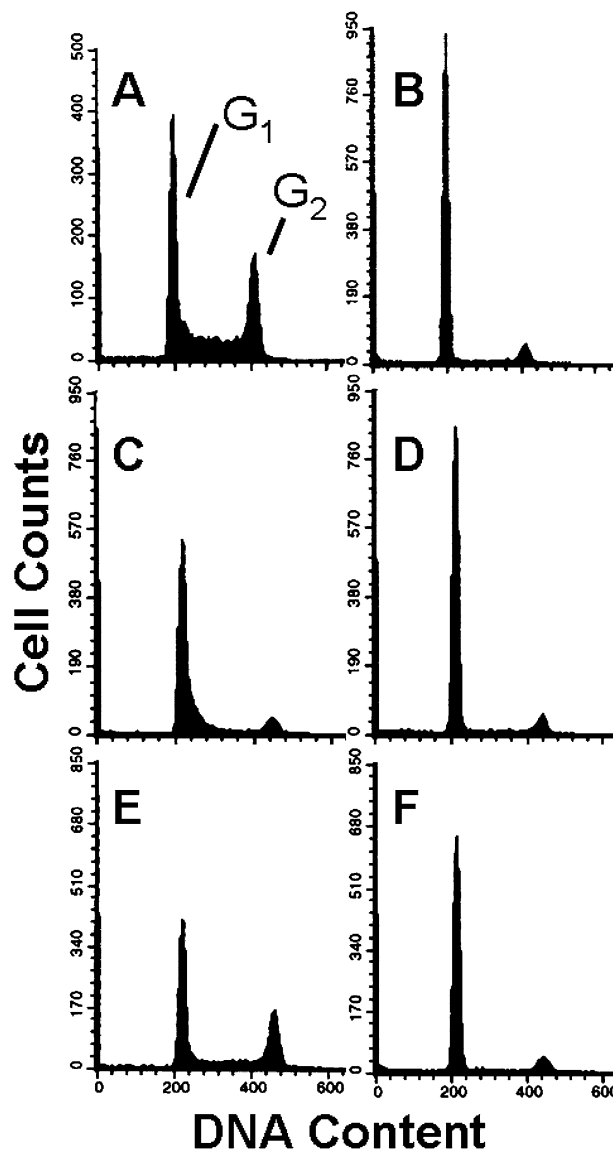

Fig. 2 Flow cytometry analysis of the cell cycle of serum-starved cells subsequently treated with sclareol. The histograms were derived from untreated exponentiall grown cells (A); cells grown in serumless medium for 72 h (B); cells grown in serumless media for 72 h, then receiving 5% serum for 12 h without (C) or with (D) 100 μM of sclareol; and cells grown in serumless media for 72 h, then receiving 5% serum for 24 h without (E) or with (F) 100 μM of sclareol. Ten thousand events were scored per sample (histogram) and estimate of each cell cycle phase is presented as percentage of the total cells treated in Table 2

For this study, we utilized the Cellular DNA Fragmentation ELISA kit as described in Materials and Methods. The results indicated the absence of DNA fragments in cells treated for 4 h with 100 μM sclareol, but the presence of DNA fragments was readily detected at 8 h of sclareol treatment (Fig. 3, graph B). Further, the relative amount of DNA fragments continued to increase within the treated cells, whereas no DNA fragments were detected in the cell-conditioned media of the treated cells even after 24 h of treatment.
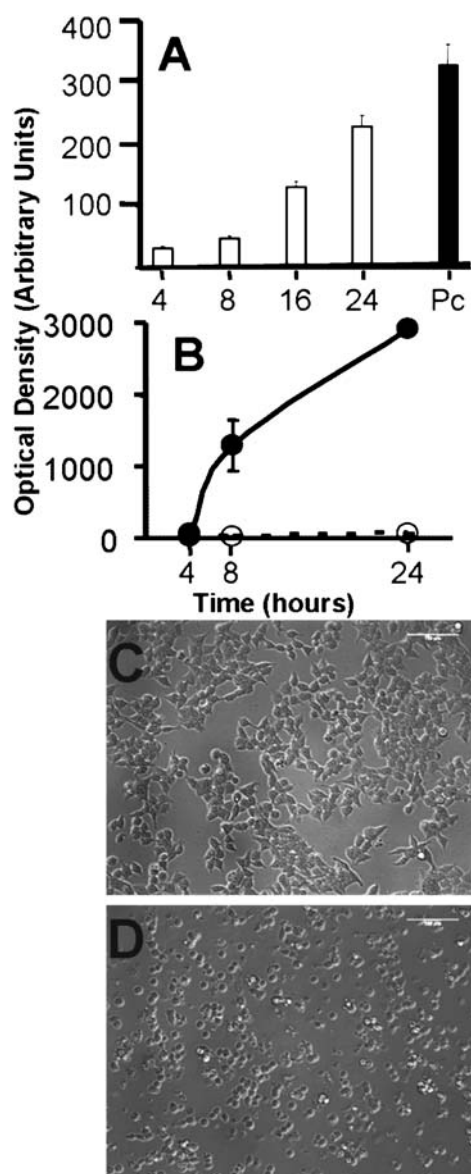
Fig. 3 Sclareol-induced DNA damage is time-dependent. HCT116 cells were treated with 100 μM sclareol for various periods of time, and then, cells (cell lysates) were assayed for DNA unwinding (A) and presence of DNA fragments (B). Also, cell-conditioned media (supernatant) was assayed for presence of cell released DNA fragments (A). DIC microscopy (magnification 200 $\times$) of HCT116 cells treated for 24 h with carrier alone (C) and carrier with sclareol (D). Pc, positive control provided by the kit manufacturer; $p \leq 0.05$
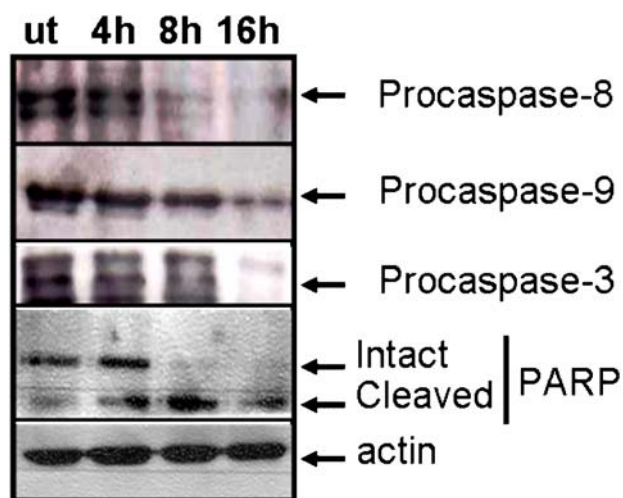
Fig. 4 Sclareol induces caspase activation and PARP cleavage. HCT116 cells were untreated (ut) or treated with 100 μM of sclareol for 4 h, 8 h, and 16 h and then, whole cell protein extracts were prepared and subjected to Western blot analysis for presence of procaspase-8, procaspase-9, procaspase-3, PARP and the control (housekeeping) protein, β-actin using specific antibodies

Activation of caspases in sclareol-treated HCT116 cells

Having established that sclareol induces DNA damage in a time-dependent manner, we sought to investigate whether sclareol treatment of HCT116 cells induces activation of caspase-8 and caspase-9 (Fig. 4). The results indicated that procaspase-8 was extensively processed at 8 h of sclareol treatment and almost completely processed at 16 h of treatment. On the other hand, procaspase-9 was also processed, although not as extensively as procaspase-8, at 8 h of treatment, but a nearly complete process had occurred at 16 h of treatment (Fig. 4).

Consistent with the timing of activation of procaspases-8 and -9, processing of procaspase-3 was detectable at 8 h of sclareol treatment, whereas no procaspase-3 was detected at 16 h of sclareol treatment, apparently because it was fully converted, to active caspase-3. These observations were further confirmed upon monitoring the degradation of PARP, which is targeted by active caspase-3 [15–17]. The results again showed that the kinetics of PARP degradation was concurrent or closely followed the kinetics of procaspase-3 activation (Fig. 4). No degradation has been observed with a b-actin control.

Sclareol suppresses growth of HCT116 tumors

The ability of sclareol to inhibit HCT116 cell proliferarion followed by apoptosis prompted us to study the efficacy of this compound to inhibit growth of HCT116 tumors established as xenografts in immunodeficient SCID mice. For this

Microscopy observations of untreated and sclareol-treated HCT116 cells assessed that the treated cells had ceased to proliferate, were shrung and round, but retained their integrity (Fig. 3, panels C and D), that is, chromatin DNA changes and fragmentation preceded or concomitantly occurred with arrest of the cells at G$_1$.

study, we initially determined the MTD of sclareol administered intraperitoneally (ip) to the animals. In our hands, sclareol was found to be insoluble in water-containing solutions, and thus we chose to use a fine suspension of sclareol for ip administration. We tested several carriers (vehicles) in order to obtain a suspension meeting the following criteria: (i) the carrier alone is compatible with the host; (ii) the suspended sclareol does not precipitate after storage for an extensive period of time (nearly 24 h) at room temperature; and, (iii) the suspension can be well-tolerated after ip administration. These criteria were met with the use of a carrier consisting of normal saline (80%, v/v)—absolute ethanol (10%, v/v)—PEG 400 (5%, v/v)—Tween-40 (5%, v/v). We subsequently used this cocktail to determine that the MTD for sclareol alone (free sclareol) administered in SCID mice daily for 5 consecutive days was 50 mg/kg. Alternatively, sclareol was encapsulated into the lipid bilayer of liposomes as described elsewhere [13], and the MTD of the liposomal sclareol administered daily for 5 days was determined to be 275 mg/kg (data not shown).

To study the efficacy of free and liposomal sclareol in suppressing tumor growth, 30 SCID mice were xenografted (2 xenografts per mouse) with HCT116 cells, and when the tumors reached a size of 100 mm^3, animals were divided to 5 groups (6 mice per group). One group of mice received no further treatment (control #1), whereas the other groups received vehicle + free sclareol (50 mg/kg), vehicle alone (control #2), vehicle + empty liposomes (control #3), and vehicle + liposomal sclareol (275 mg/kg). Each treatment consisted of 3 weekly cycles, each cycle consisting of 5 consecutive days of compound administration per week. The study was terminated, when the average tumor size of the untreated animals reached approximately 1000 mm^3 (end-point). Following termination of tumor measurements in untreated and treated mice, we utilized the average tumor measurements to calculate %$\Delta T/\Delta C$ as a function of time (Fig. 5). The first observation was that free sclareol (at 50 mg/kg) had virtually no effect on the tumor size (Fig. 5(A)). Similarly, administration of vehicle + empty liposomes had no effect on tumor size. However, administration of vehicle + liposomal sclareol (at 275 mg/kg) resulted in a dramatic suppression of tumor growth (Fig. 5(A)). In fact, during the initial period of the liposomal sclareol treatment the tumor size suppression was about 50% followed by 40% suppression at later periods of treatment (Fig. 5(A)). No side effects (drug related deaths, loss of weight, neurological disorders etc) have been recorded during the treatment with the MTDs of free and liposomal sclareol. Therefore, taken together the results demonstrate that (a) the MTD of liposomal sclareol is more than 5-fold higher than the MTD of free sclareol; and (b) the antitumor activity of sclareol is exhibited at doses that can be administered only after this compound is encapsulated in liposomes.
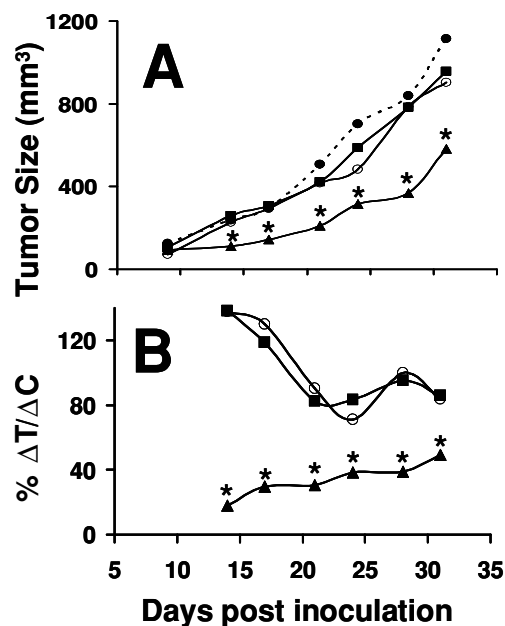

Fig. 5 Growth of HCT116 tumors in mice treated with sclareol and liposomal sclareol. (A), average tumor size (in mm^3) of each mouse group (6 mice per group that is 12 tumors per group) as a function of time (in Days); and, (B), %$\Delta T/\Delta C$ as a function of time (in Days) calculated from the measurements in A for untreated mice (●) and mice receiving free sclareol (50 mg/kg, ■), empty liposomes (○), and liposomal sclareol (275 mg/kg, ▲). Treatment was for three cycles, each cycle consisting of five consecutive days of compound administration followed by two consecutive days with no treatment. Statistical evaluation was assessed with the two-tailed t-test. Points with $p \leq 0.05$ are indicated by a star (*)

Characteristics of sclareol-treated tumors

To address the ability of sclareol to induce tumor growth suppression *in vivo* we first examined by direct observations the growth of tumors in the mice 30 days post-inoculation of the tumor cells. Advanced and similar tumor growth was readily observed in mice that received no treatment or treatment with sclareol or empty liposomes alone, but tumor growth was substantially slower in mice treated with liposomal sclareol (mice with tumors not shown). We then observed the microscopic morphology of the sclareol-treated tumor in hematoxylin-eosin-stained sections (Fig. 6). A photograph of a representative section shows a highly aggressive undifferentiated carcinoma with extensive foci of necrosis (Fig. 6(A)). Further, we investigated whether sclareol treatment affects proliferation of cancer cells in the section by detecting the presence of the nuclear Ki67/MIB1 protein, a marker indicative of active cell proliferation (reviewed in refs. [30–33]). More than 80% of the cells stained positive for Ki67 in the untreated tumor (Fig. 6(B)) and tumors treated with liposomes (Fig. 6(C)) alone or free-sclareol (not shown) indicating abundant presence of the Ki67 antigen, that is, the cancer cells were actively proliferating. In
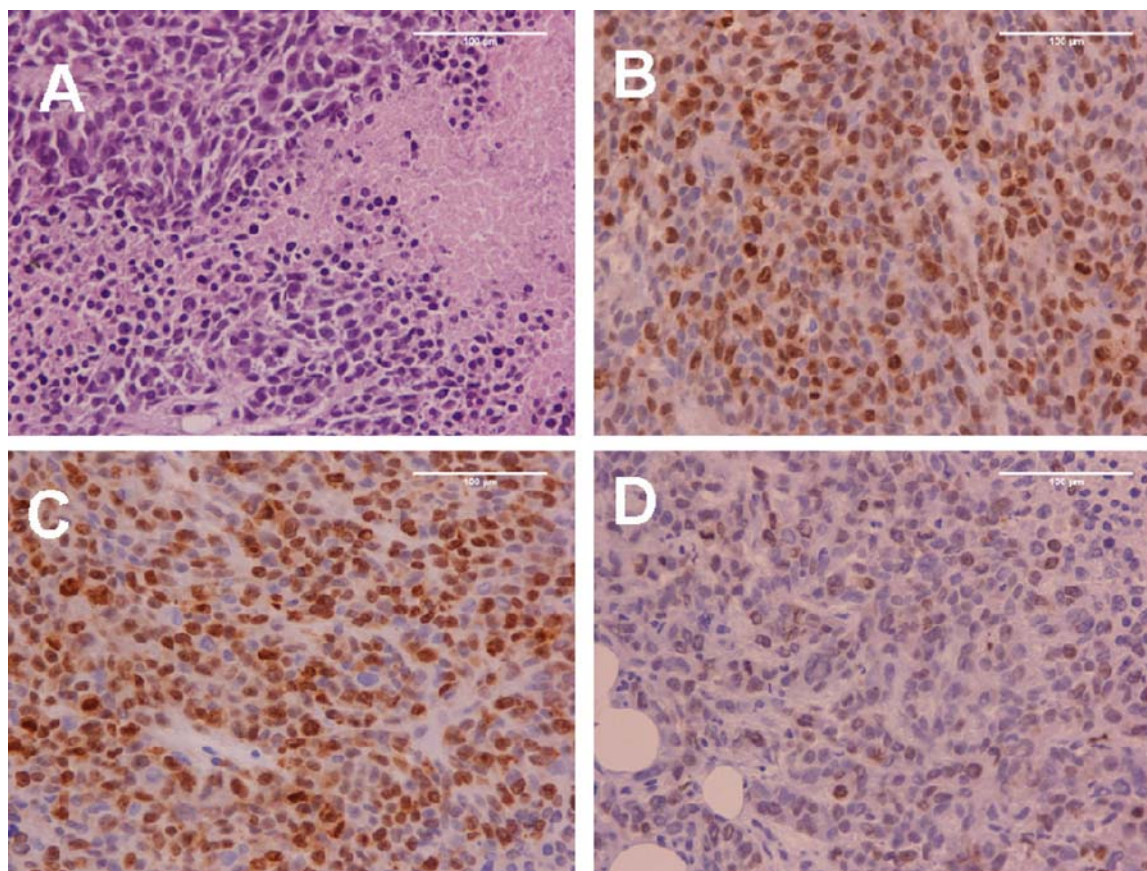
Fig. 6 Detection of expression of the cell proliferation-associated protein, Ki67, in tumor sections. At the end of each treatment, the animals were euthanized, the tumors were surgically removed from the animals and embedded in paraffin and 4-μm sections were observed under a microscope. (A), highly aggressive undifferentiated carcinoma with extensive foci of necrosis (hematoxylin-eosin stain, 400 × magni- fication); (B), intensive nuclear expression of Ki67/MIB1 in untreated tumor [85 ± 11% positive cells; ABC-stain; 400 × magnification), (C), expression of Ki67/MIB1 in tumors treated with empty liposomes (percentage 80 ± 8% positive cells) and (D), loss of Ki67/MIB1 ex- pression after treatment with liposomal sclareol [(50 ± 10% positive cells, $p \leq 0.05$)]

contrast, liposomal sclareol treatment resulted in a 50% re- duction of Ki67 staining in the tumor. These observations taken together demonstrated that treatment with liposomal sclareol had impaired the ability of tumor cells to proliferate.

Discussion

There is increasing experimental and epidemiological evi- dence that plant-derived chemicals (phytochemicals) possess the potential to serve as chemopreventive agents that arrest or reverse carcinogenesis (for recent reviews see refs. [34– 38]). In this regard, we have previously provided experimen- tal findings to demonstrate that the phytochemical diterpene, sclareol, exhibits anticancer activities *in vitro* [11, 12] and *in vivo* [13]. In the initial reports, we demonstrated that sclareol induced apoptosis in human leukemia cell lines as- sociated with down-regulation of the anti-apoptotic gene, c-*myc*, in absence of quantitative changes in the pro- apoptotic protein, Bcl-2 [9], and that apoptosis was inde- pendent on p53 expression in human breast cancer cell lines [12].

In this report, we have extended our previous studies to show that sclareol can induce arrest at the G_1-phase of the cell cycle in human colon cancer HCT116 cells. Interest- ingly, G_1-arrested cells, following serum starvation, were not killed by sclareol, whereas, the same compound killed cells not arrested at G_1. This finding suggests that sclareol can distinguish between non-proliferating and proliferating cells, that is, sclareol possesses a property highly desirable for an anti-cancer drug. Further, our studies have shown that sclareol-induced apoptosis is associated with activation of caspases-8 and -9, implying that, sclareol-induced cell death is associated with activation of both mitochondrion and death receptor apoptosis pathways, followed by activa- tion of caspase-3 and subsequent degradation of the protein PARP. Activation of caspase-8 or -9 is an early event required to trigger the death receptor-dependent and mitochondrion- dependent apoptosis pathways, respectively, whereas, acti- vation of caspase-3 and subsequent PARP degradation are

late events in the apoptosis pathways [15–17]. These results are consistent with a report that both apoptotic pathways are triggered in human leukemia cell lines treated with other labdane diterpenes [10]. However, activation of caspases-8 and -9 does not exclude the possibility that other caspase-independent mechanisms of cell death are executed by proteases other than caspases [39, 40]. In fact, we have recently shown that such a variant form of apoptosis display only some characteristics typical of the "classical" apoptosis pathways in HCT116 cells treated with the plant-derived product, mastic gum [18, 19]. At any rate, we have concluded that sclareol-induced apoptosis in HCT116 cells is associated with biochemical processing of cellular macromolecules, but we have not investigated the form of apoptosis induced. It should also be noted that treatment of HCT116 cells with sclareol for 8 h irreversibly induced apoptosis even after transferring the cells to media without sclareol (results not shown) indicating that once sclareol initiated the activation of the apoptotic mechanism, continuous exposure of the cells to sclareol was not required for apoptosis to be completed.

Subsequently, we evaluated the antitumor efficacy of sclareol *in vivo* using the human HCT116 tumor xenograft/SCID mouse model. In preliminary experiments, we determined that the high insolubility of the lipophilic sclareol in aqueous solutions (carriers) was limiting us to administer into mice doses lower than 50 mg/kg, applying a schedule of a daily administration for five consecutive days. Even a single administration of a dose higher that 50 mg sclareol per kg was adequate to generate extensive toxicity and eventually death to the animals [13]. However, a dose of 50 mg/kg had no effect on the progression (growth) of the HCT116 tumors grown on the mice. Therefore, we alternatively administered sclareol encapsulated into specifically prepared liposomes [13]. Liposomes can be used as carriers for delivery of a variety of compounds at higher doses, to elicit improved pharmacokinetic properties and pharmacological responses and reduce toxicity observed in systemically administered drugs alone [20–24, 41, 42]. Specifically for anticancer drugs, liposomes tend towards a preferential accumulation in tumor sites and an increased uptake by cells via the enhanced permeability retention (EPR) effect [43].

Our liposomal sclareol preparations allowed us to administer a daily dose of 275 mg sclareol/kg, that is, a dose much higher than 50 mg/kg of the sclareol in aqueous carrier. Administration of a dose of empty (control) liposomes equal to the quantity of the sclareol-containing liposomes indicated absence of apparent toxicity in the mice assuring that the experimental results observed or measured were due to the encapsulated sclareol rather than to the carrier (liposomes). In fact, at the endpoint of the experiment (i.e., when the average tumor size was approximately 1000 mm^3 in the control animals), the average size of tumors in the treated animals was about 50% lower than in control animals.

The dramatic suppression of HCT116-tumor growth after sclareol treatment could be due to sclareol-inhibited proliferation or apoptotic death of the tumor cells as observed in the *in vitro* studies. To investigate this possibility, we stained tumor sections form treated or untreated animals for expression of the nuclear antigen, Ki67/MIB1, a marker selectively expressed in actively proliferating cells [30–33]. Our results clearly show that liposomal sclareol inhibits expression of Ki67 indicating a direct effect on cell proliferation.

In general, other members of the labdane diterpene class have exhibited significant apoptotic activity against human cancer cells *in vitro* [10, 11, 44, 45], but still very little is known about the anticancer properties of these compounds. Moreover, the results described in this report have demonstrated for the first time the ability of a labdane diterpene, sclareol, to suppress growth of human colon cancer in *vivo*. It is our assumption that sclareol, and perhaps other labdane diterpenes, can also suppress or regress the growth of other human tumors, in addition to HCT116 colon tumor, in the human xenograft/mouse model. This assumption is currently tested by our group. In conclusion, the results presented in this report warrant further investigations on the anticancer activity of sclareol and the possibility that this compound can be developed to a novel anticancer drug.

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