Differentiating and Apoptotic Dose-Dependent Effects in (−)-α-Bisabolol-Treated Human Endothelial Cells

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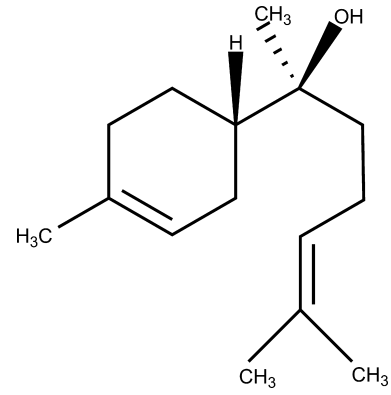
The effect on angiogenesis of (−)-α-bisabolol [(−)-6-methyl-2-(4-methyl-3-cyclohexen-1-yl)-5-hepten-2-ol] (**1**), a widely distributed plant sesquiterpene alcohol, was investigated for the first time. Human endothelial cells treated with **1** were analyzed for their ability to differentiate and organize in microvessels and for their sensitivity to this compound in terms of cytotoxicity and cell growth inhibition. Within 24 h of the treatment with 5 μM **1**, cells underwent massive death. Apoptosis induction was responsible for cytotoxicity triggered by **1** as revealed by the release of cytochrome *c* from the mitochondria, reduction of the Bcl-2/Bax ratio, and caspase 3 activation. At a lower, non-apoptotic concentration (0.25 μM), **1** showed a differentiating effect resulting in growth inhibition, invasiveness reduction, and tubule stabilization.

Plants have been used for thousands of years to treat a number of illnesses. Many plant-derived natural compounds possess a broad variety of therapeutic properties. In the past decade, several anticarcinogenic compounds from plants have been identified that promise safer approaches to cancer therapy and prevention.[1,2] (−)-α-Bisabolol (**1**), a sesquiterpene alcohol with a molecular mass of 222.37 Da, may be isolated from the essential oils of a variety of plants,[3] such as *Matricaria chamomilla* L. (Asteraceae) and the Peruvian medicinal plant *Peperomia galioides* Kunth (Piperaceae). It has been reported that **1** reduces inflammation in arthritis, prevents the development of gastric ulcers, acts against the growth of bacteria and fungi, and promotes wound healing.[4−10] Recently, a selective cytotoxic activity of **1** was demonstrated against human and murine malignant glioblastoma[11] and human pancreatic carcinoma cell lines.[12] Angiogenesis, the process of new blood vessel formation, is a crucial component for the development and progression of pathophysiological changes associated with a variety of disorders: cancer, retinopathy, rheumatoid arthritis, ischemic disease, and wound healing.[13,14] Angiogenesis is a multistep process triggered by a number of mediators, growth factors, and cytokines that target endothelial cells and stimulate proliferation, migration, and assembly, as well as recruitment of perivascular cells and extracellular matrix remodelling. Inhibition of these events severely affects angiogenesis. Many endogenous factors and pharmacological agents that regulate cancer progression and inflammation can also affect angiogenesis.[15] To date, antiangiogenic therapy is considered one of the most promising approaches to control cancer, and the identification of nontoxic agents endowed with direct or indirect effects on pathological angiogenesis is an important area of research.

In the present study, the effect of **1** on endothelial cell proliferation, migration, and survival in vitro has been investigated. The data obtained indicated that **1** may be considered a promising inducer of apoptosis at high concentrations with antiangiogenic properties. In contrast, at low concentrations, this compound inhibits proliferation, inhibits Matrigel invasion and migration, and stimulates capillary tube formation.



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Results and Discussion

Since **1** is a highly lipophilic molecule, the dose-dependent solubilization in the culture medium was evaluated initially by gas chromatography, as previously described.[11] In the present study, the indicated concentrations of **1** are referred to as the soluble fraction of **1**.

(−)-α-Bisabolol (**1**), at a concentration of 5 μM, has been previously characterized for its toxicity toward cancer cell lines with no toxic effects evident on normal glial cells and normal fibroblasts.[11,12] However, reports about its effects on endothelial cells are found lacking. We have thus investigated the activities of **1** on the angiogenic process enacted by human umbilical endothelial cells (HUVEC), focusing attention on separate steps of this process: proliferation and viability, chemoinvasion, and capillary morphogenesis.

When HUVEC cells were exposed to a concentration of 5 μM **1**, a death-inducing effect was observed. (−)-α-Bisabolol (**1**) turned out to be cytotoxic, stimulating massive cell death of the whole cell population; within 24 h of treatment (Figure 1A) the cumulative cell deaths were more than 60% at 6 h. Using phase-contrast microscopy, dead cells were seen as nonadherent, round, and refracting. A strong correlation between the early collapse of the mitochondrial membrane potential and apoptosis has been demonstrated for some nutritional supplements and extracts of herbs.[16] Cytochrome *c* release from mitochondria to cytosol, a main event in the activation of the apoptotic pathway, was investigated to confirm that **1**-induced death in endothelial cells is an apoptotic event. Cytochrome *c* release was detected by western immunob-

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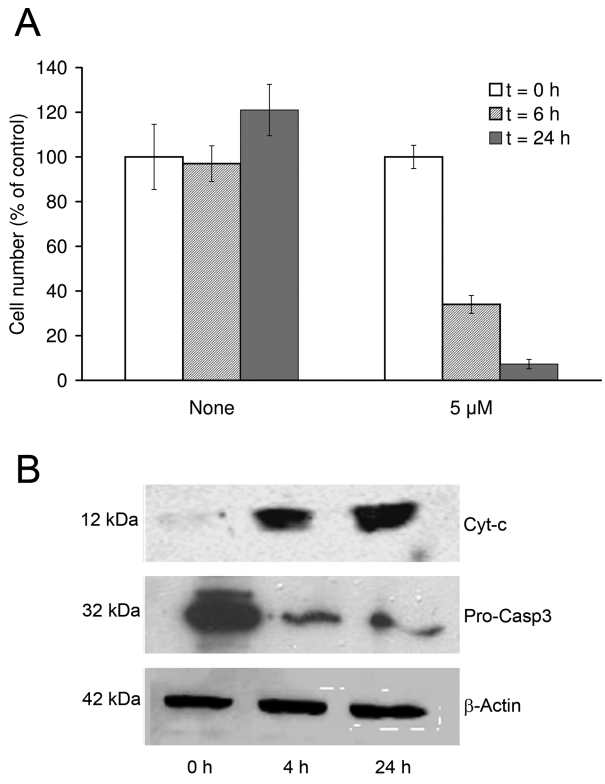

Figure 1. Cytotoxic effect of 5 μM (−)-α-bisabolol (**1**) on HUVEC cells. (A) Effect of **1** on cell proliferation. Cells were seeded in complete medium and treated the next day with 5 μM **1**. Cells were harvested and counted after 6 and 24 h of treatment. Values are means ± SEM of three separate experiments. (B) Western blot analysis for cytochrome *c* and procaspase 3 in cells treated with **1** for 4 and 24 h. β-Actin was detected for loading correction.

lotting, using a specific primary antibody (Figure 1B). A barely detectable staining pattern of cytochrome *c* was observed in control cells, while in **1**-treated cells, an intense and marked staining pattern was observed, after both 4 and 24 h, suggestive of cytochrome *c* release from the mitochondria into the cytosol. To further demonstrate the involvement of the apoptotic pathway, the cleavage of the inactive procaspase 3 to active effector caspase 3[17] was examined following 4 and 24 h of 5 μM **1** treatment (Figure 1B). (−)-α-Bisabolol (**1**) caused a prominent decrease in the intensity of the staining pattern of inactive procaspase 3.

Bcl-2 family members play an important role in apoptosis induction through the mitochondrial pathway.[18] Pro- and antiapoptotic Bcl-2 protein family members act upstream of the release of cytochrome *c* by inducing or preventing permeabilization of the outer membrane. It was reported that **1** rapidly induced apoptosis in malignant cells via a mitochondrial pathway, interacting with the Bcl-2 family member Bid in an in vitro reconstituted system.[12] The next aim was to examine the involvement of treatment with **1** on Bcl-2 and Bax protein levels, for which the ratio is often critical for apoptosis initiation. Western blot analysis showed no great effect on Bax, but a rapid (1 h) reduction in Bcl-2 level, thus shifting the Bcl-2/Bax balance in favor of mitochondria-mediated apoptosis in HUVEC cells treated with 5 μM **1** for 30 min, 1 h, 2 h, and 4 h (Figure S1A, Supporting Information). Band intensity was measured using the Image J software and reported as a Bcl-2/Bax ratio (Figure S1B, Supporting Information). Bcl-2 downregulation occurs very early in **1**-treated HUVEC cells, according to the very potent effect of a 5 μM concentration on cell viability (Figure 1). Such an early Bcl-2 downregulation has been documented.[19−21] In particular, our data are consistent with those of Zehiler and co-workers,[22] who observed a 50% Bcl-2 reduction in HUVEC cells following TNF-α treatment at a time when DNA fragmentation is not yet detectable.

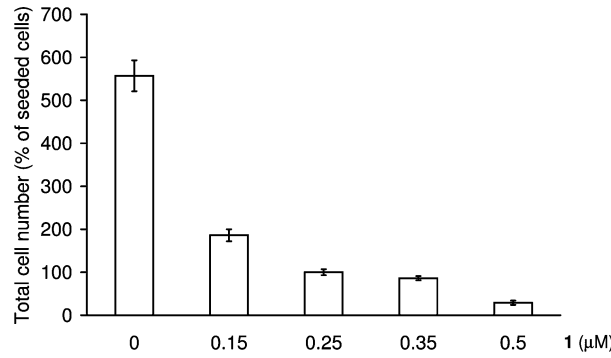

Figure 2. Effect of (−)-α-bisabolol (**1**) on HUVEC cell proliferation. Cells were seeded in complete medium and treated the next day with different doses of **1**, as indicated. After 72 h of treatment, cells were harvested and counted. Values, expressed as % of seeded cells, are means ± SEM of three separate experiments.

Endothelial cell proliferation modulation is a prerequisite for angiogenesis hallmarks such as invasion and migration for new vessel sprouting.[15] Angiogenesis could be regarded as a multiphase process constituted by dynamic phases beginning with cell commitment by angiogenic factors, leading to extracellular matrix degradation, migration, and proliferation. The process ends in a static phase in which cells align, forming vascular loops, with the activation of a differentiation program, leading to mature vessel production. Angiogenesis has been studied using a variety of approaches, with both in vivo and in vitro models developed. In particular, in vitro approaches allow the separation of otherwise undistinguishable angiogenesis steps in in vivo models. In order to establish a cytostatic, sublethal concentration allowing the performance of angiogenic assays, HUVEC cells were cultured for up to 72 h in the presence of various concentrations of **1**, as indicated in Figure 2. (−)-α-Bisabolol (**1**) treatment of HUVEC cells at 0.15, 0.25, 0.35, and 0.5 μM resulted, respectively, in 65%, 78%, 82%, and 90% decreases in the total cell number as compared to the control. Among these concentrations, that at 0.25 μM appeared cytostatic with no change in cell number at 72 h nor in procaspase 3 activation within 24 h of treatment (Figure S2, Supporting Information). Only concentrations of **1** greater than 0.25 μM appeared cytotoxic both by cell number reduction (Figure 2) and by the trypan blue exclusion test (data not shown).

To study the effect of cytostatic concentration of **1** on invasion and migration of endothelial cells, HUVEC cells were first treated with 0.25 μM **1** in a low-serum medium for 24 h in order to wash out any residual chemoattractant, then seeded on Matrigel-coated filters. FGF2, as a unique chemoattractant and angiogenic factor (positive control), was added to the companion (lower) Boyden chamber. After 4 h of treatment, on the lower side of the PET membrane, a significant decrease in migrated cells was observed (Figure 3A), with respect to the positive control (−24%, $p < 0.05$).

The effect of **1** on in vitro capillary morphogenesis was evaluated in HUVEC cells seeded in 96-well culture plates precoated with Matrigel, and cellular morphology was observed over a time interval. A representative experiment, showing the dose-dependent effect of **1** on in vitro tube capillary formation after 4 h of treatment, was conducted (Figure 3B). Untreated cells showed no organization (Figure 3B, left upper panel). HUVEC cells treated with **1**, at both a 0.1 μM and a 0.25 μM concentration, were able to organize in a capillary-like structure; cells became elongated and formed thin cords of interconnecting cells (Figure 3B, left and right central panel) mimicking FGF2-treated cells (Figure 3B, right upper panel). However, a concentration of 0.5 μM (Figure 3B, lower panel) inhibited capillary tube organization, resulting in aggregation in cell cumuli of rounded cells and producing a few distorted cord-like structures.

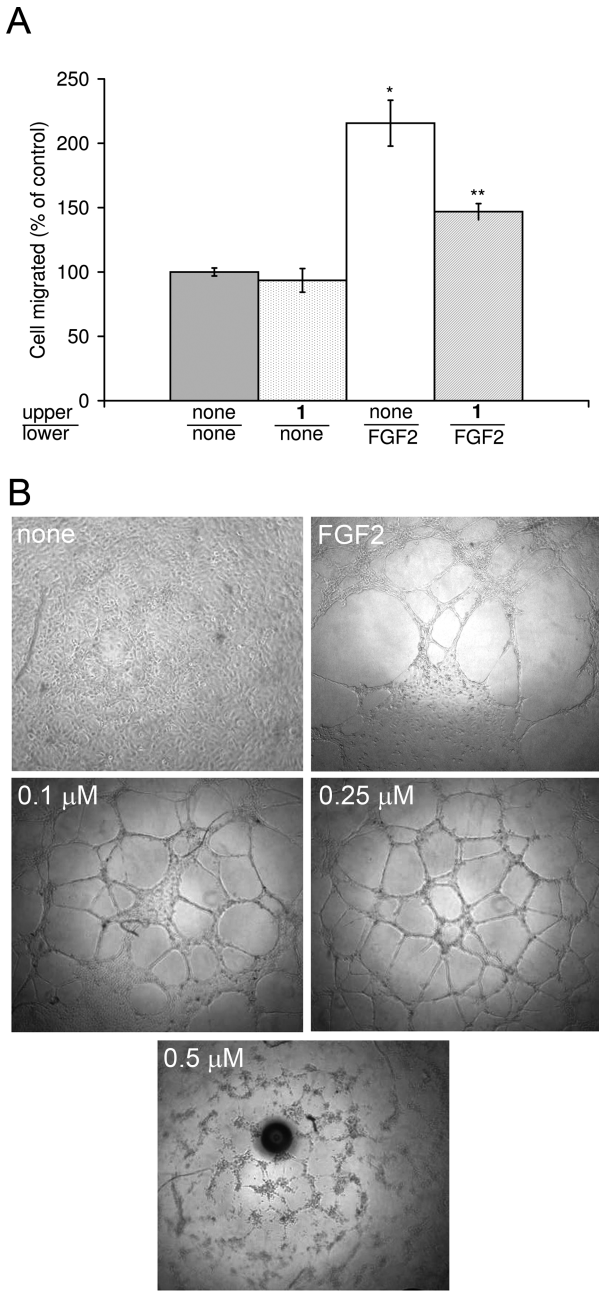

Figure 3. Effect of (−)-α-bisabolol (**1**) on chemoinvasion and capillary morphogenesis. (A) Cells, treated with 0.25 μM **1**, were plated in Boyden chambers onto a 8 μm pore size PET membrane precoated with Matrigel (upper chamber) and exposed to 10 ng/mL FGF2 (lower chamber) as a chemoattractant. Data expressed as number of cells migrated through pores (% of control, i.e., 500 ± 47 cells) are means ± SEM of three separate experiments. *p < 0.05 compared to control; **p < 0.05 compared to FGF2. (B) Cells were seeded with different doses of **1**, as indicated, in 96-well culture plates precoated with Matrigel. Tube formation was observed over time up to 24 h under a phase-contrast microscope; representative pictures, taken at 40× magnification after 4 h of treatment, are reported.

Many molecules inhibit separate steps of angiogenesis: plasminogen activator inhibitors block endothelial cell invasion, intracellular signaling inhibitors block endothelial cell migration and proliferation, and matrix metalloproteinase inhibitors block capillary morphogenesis.[23,24] In the past decade, the concept of angiogenic therapy has been developed with the aim to obtain new modulators able to inhibit angiogenesis. Unfortunately, such molecules tend to exhibit side effects such as hypertension, nausea, vomiting, fatigue, and proteinuria during clinical trials.[25−27] The present study might open a promising perspective with regard to future investigations of **1**-dependent modulation of angiogenesis, considering it is a natural oily compound used for hundreds of years as a component of natural medicines. The present study has indicated that low and high concentrations of **1** have respectively pro- and antiangiogenic properties. At low concentrations, **1** slowed down proliferation and invasion, promoting the late phase of angiogenesis, which is *the conditio sine qua non* to sustain the organization of endothelial cells into primitive capillaries, enacting a differentiating program. On the other hand, at high doses, **1** showed antiangiogenic properties mainly related to its direct proapoptotic effect on endothelial cells. Dose-efficacy is generally a linear function for chemotherapy. In contrast, several angiogenesis inhibitors have been reported to produce a biphasic dose-efficacy curve,[28] a paradigm being represented by statins that are antiangiogenic at high doses and proangiogenic at low doses.[29] Another plant-derived compound, safrole oxide, offers a good example of a compound with dual concentration-dependent effects on endothelial cells by promoting Fas-dependent apoptosis at high doses and inducing differentiation at low doses by lowering the intracellular reactive oxygen species (ROS) levels.[30] In fact, ROS were involved in the retrograde signaling from mitochondria, responsible for cell differentiation.[31,32] Since **1** has been demonstrated to destabilize cell membranes,[12] we speculate that it may promote the angiogenic differentiation final step at low doses by altering retrograde mitochondrial signaling, while it could induce apoptosis at high doses by promoting cytochrome *c* release.

The properties described of high doses of **1** offer important perspectives for the treatment and prevention of diseases where angiogenesis excess is involved, including some ophthalmologic diseases,[33] and cancers that rely on neoangiogenesis for nutrient supplies. At low doses, **1** could be suitable for the treatment of some diseases, such as postischemic revascularization, in which promotion of angiogenesis is needed, and wound healing,[34] where a number of cellular events are involved. However, the angiogenic process in vivo is a much more intricate process, and further studies will be necessary to establish the outcome of administration of low doses of **1** in vivo.

Experimental Section

Cell Cultures. Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords and identified as HUVEC by labeling with anti-CD31 antibody (DACO, Glostrup, Denmark). Cells were grown on gelatin-coated plastic, in endothelial cell basal medium (EBM-2, Clonetics, Cambrex, Walkersville, MD) supplemented with 4% FCS and EGM-2 SingleQuots (Clonetics, Cambrex), at 37 °C in a 5% CO_2 humidified atmosphere. The primary cultured cells used were between passage 2 and 5. All assays were performed on subconfluent cell monolayers. Where indicated, cells were preincubated for 12 h in starvation medium (MCDB-131 medium pH 7.2, containing 1% FCS, 10 μg/mL hydrocortisone, 15 U/mL, and heparin; Sigma, Milan, Italy). Appropriate chemicals were added to the medium at various times as indicated for individual experiments: 10 ng/mL FGF2 (Boehringer, Mannheim, Germany) and (−)-α-bisabolol (Sigma Chemical, St. Louis, MO; purity ≥95%) from a freshly prepared 22.5 mM solution in absolute ethanol. The final ethanol concentration had no effect on cells (less than 0.1%, data not shown). Prior to cell administration, (−)-α-bisabolol (**1**) was allowed to solubilize in each incubation medium at 37 °C for 24 h, in order to reach both the highest free concentration[11] and sedimentation of unsolubilized **1**. For cell lysate preparation, both adherent and floating cells were collected.

Cell Growth Assay. HUVEC cells were seeded (10 000/well) in 24 multiwell culture dishes, in complete growing medium. After 24 h, cells were treated with different doses of (−)-α-bisabolol (**1**) (0.10, 0.25, 0.50, 5.0 μM doses) and with ethanol alone (control) for 30 min, 24 h, 48 h, and 72 h. At the end of each treatment, total cells were collected by a brief trypsinization and counted with a hemocytometer. Trypan blue dye was used for assessing dead cells.

Western Blot Analysis. For cytochrome *c* analysis, cells were permeabilized with the following lysis buffer: 350 μg/mL digitonin,

75 mM NaCl, 8 mM Na_2PO_4, 1 mM NaH_2PO_4, 5 mM EDTA, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 0.2 U/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin. Following 5 min incubation on ice, cells were centrifuged at 13 000 rpm for 5 min to detect mitochondria-released cytochrome *c* in the supernatant fraction. For procaspase 3, Bax, and Bcl-2 detection, cells were lysed for 30 min on ice in 20 mM Tris-HCl pH 7.4, 1% Nonidet P-40, and protease inhibitors, as indicated above, and then centrifuged at 13 000 rpm to remove debris. Then, 80 μg of proteins (Bradford protein assay) was fractionated by 12.5% SDS-PAGE gels and transferred to a nitrocellulose membrane using an electroblotting apparatus (Biorad, Hercules, CA). Membranes were blocked with 5% nonfat milk in T-PBS (PBS containing 0.1% Tween) for 1 h and then incubated under constant agitation overnight at 4 °C with specific monoclonal antibodies (E-8 for caspase 3, C-2 for Bcl-2, N-20 for Bax, 7H-8 for cytochrome *c*; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Membranes were then incubated with the proper secondary antibody (Sigma-Aldrich Co., St. Louis, MO) for 60 min at room temperature; the signal was revealed by the enhanced chemiluminescence system (ECL, Amersham, Amersham Place, UK). The membrane was stripped and reprobed with anti-β-actin antibody for loading correction.

Migration Assay. Migration assays on HUVEC cells were performed using the Boyden chamber technique to assess the effect of (−)-α-bisabolol (**1**) on the cellular migration potential in response to serum and growth supplements. PET filters (polyvinylpyrrolidone-free polycarbonate filters; Neuro Probe Inc., Gaithersburg, MD) (8 μm pore size) were coated with 50 μg of Matrigel in water for 30 min at 37 °C, dried overnight, equilibrated in MCDB-131 supplemented with 1% BSA, and placed between the upper and lower chamber. HUVEC cells were resuspended in 200 μL of MCDB-131, supplemented with 1% FCS, and seeded in the upper compartment (1×10^4 cells/mL) in the presence or absence (control) of 0.25 μM **1** at 37 °C. The lower chamber was filled with the same medium with or without 10 ng/mL FGF2 as chemoattractant. After 4 h of incubation, the filter was removed, and following methanol fixation, cells on the upper side were scraped by a rubber policeman. After staining with Giemsa solution (Diff-Quik, Baxter Diagnostics, Rome), filters were mounted onto microscope slides. Migrated cells were counted in 10 different fields for each filter. Experiments were performed in triplicate. Migration values were expressed as % of control.

In Vitro Angiogenesis. Because Matrigel can induce spontaneous in vitro angiogenesis, we tested preparations of Low Growth Factor Matrigel (Becton Dickinson, Frankin Lake, NJ), selecting a batch with the lowest activity. In brief, 50 μL of 10 mg/mL Matrigel was added per well in 96-well tissue culture plates and allowed to gel at 37 °C for 30 min. To examine the effect of **1** on in vitro angiogenesis, HUVEC cells (10 000 cells/well) were seeded in the starvation medium containing growing concentrations of **1**, and tube formation was observed periodically under a phase-contrast microscope. The plates were monitored for up to 24 h and photographed with a Nikon microscope (Nikon Co., Shinjuku, Japan). Each experiment was repeated at least three times with identical results.

Statistical Analysis. Data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and a p value of <0.05 was considered statistically significant. All data are presented as the mean ± SEM of three experiments.

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Supporting Information Available: Effects of (−)-α-bisabolol on Bcl-2, Bax, and procaspase 3 activation in HUVEC cells. This information is available free of charge via the Internet at http://pubs.acs.org.

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