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Lauroside B, a Megastigmane Glycoside from *Laurus Nobilis* (Bay Laurel) Leaves, Induces Apoptosis in Human Melanoma Cell Lines by Inhibiting NF-κB Activation

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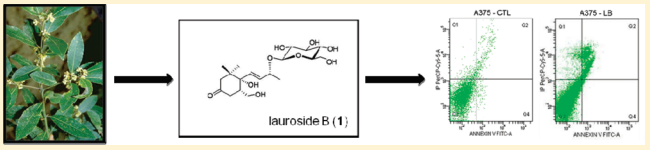
ABSTRACT: Malignant melanoma is a highly aggressive tumor that frequently resists chemotherapy, so the search for new agents for its treatment is of great importance. In the present study, the antiproliferative propensity against human melanoma cell lines of lauroside B (**1**), a megastigmane glycoside isolated from *Laurus nobilis* (bay laurel) leaves, was investigated. This compound suppressed the proliferation of three human melanoma cell lines, namely, A375, WM115, and SK-Mel-28. The **1**-induced inhibition of human melanoma cell proliferation was due to the induction of apoptosis, as demonstrated by FACS analysis with annexin V/PI staining and confirmed by activation of caspase-3 and by the cleavage of poly(ADP-ribose) polymerase (PARP). Growing evidence implicates NF-κB as an important contributor to metastasis and increased chemoresistance of melanoma. Thus, it was hypothesized that **1**-induced apoptosis could be associated with suppression of NF-κB activation. The results showed that exposure of human melanoma cells to **1** inhibited IκB-α degradation and constitutive NF-κB DNA-binding activity as well as the expression, regulated by NF-κB, of two antiapoptotic genes, XIAP and c-FLIP. Induction of apoptosis by **1** in human aggressive melanoma cell lines has a potential high biological value.



Malignant melanoma is the most deadly form of skin cancer, and its incidence is rising faster than that of any other cancer. The prognosis for patients with metastatic disease is poor, and even the most effective therapies produce an overall response rate of only 10−15%. Melanocytes, the natural photoprotectors of the skin, secrete melanin and protect neighboring epidermal cells from DNA-damaging agents, such as UV light.[1] Therefore, it is not surprising that the very poor survival seen in melanoma patients is due mainly to the notorious resistance of melanoma to radiation or chemotherapy. In fact, due to the complex nature of the disease, metastatic melanoma has been proven to be typically resistant to radiation, immunotherapy, and biochemotherapy (combining conventional chemotherapies such as dacarbazine and IL-2 or IFN-α), suggesting that some melanomas maintain an antiapoptotic phenotype, thus achieving both advanced survival capacity and resistance to chemotherapeutic agents.[2,3] Therefore, novel approaches for treating this disease are needed urgently.

Growing evidence has implicated NF-κB as an important contributor to metastasis and increased chemoresistance of melanoma. The NF-κB proteins constitute a family of proteins with homology to the chick oncogene, *rel*. There are five known mammalian NF-κB subunits, p65 (Rel A), Rel B, c-Rel, p50/p105, and p52/p100, which can form a variety of homodimers and heterodimers to differently control gene expression.[4] NF-κB proteins are normally sequestered in the cytoplasm, due to the binding of IκB to the nuclear localization sequence of the NF-κB complex. A wide range of stimuli, including cytokines, and viral and bacterial products, activate NF-κB, mostly through IκB kinase (IKK)-dependent phosphorylation and subsequent degradation of IκB, allowing the NF-κB complex to translocate into the nucleus and modulate transcription of many genes encoding cytokines, growth factors, cell adhesion molecules, and antiapoptotic proteins.[4] Recently, NF-κB activation has been connected with multiple aspects of oncogenesis, including the control of apoptosis, cell migration, cell cycle progression, and cell differentiation. Several reports have shown that in melanoma the constitutive activation of NF-κB, often due to constitutive IKK activity,[5,6] confers tumor survival capacity and the avoidance of apoptosis.[7] Inhibition of NF-κB correlates with a decrease in the expression of known antiapoptotic NF-κB target genes. These genes include BCLXL (also known as BCL2L1), X-linked inhibitor of apoptosis protein (XIAP), FLICE-inhibitory protein (c-FLIP), cIAP1 (also known as BIRC2), and cIAP2 (also known as BIRC3), as well as proliferative genes, such as IL-6 and cyclin D1.[8] In this perspective, inhibition of NF-κB is expected to be an ideal therapeutic target in those tumors where NF-κB appears to play an important survival role. In fact, our group has shown

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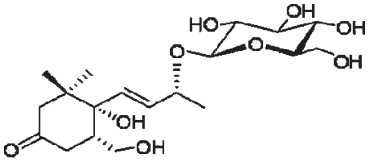
recently that the NEMO-binding domain peptide inhibits proliferation of human melanoma cells by inhibiting NF-κB activation.[9]

Natural products have proven to be a rich source of agents of value to medicine. More than half of currently available drugs are natural compounds or are related to them structurally, and, in the case of cancer, this proportion surpasses 60%.[10] Additionally, many new natural compounds of diverse structures, isolated from plant and other sources, have been considered lead compounds, and their later structural modification has afforded substances with enhanced therapeutic possibilities.[11]

Moreover, it has been recently shown that impairing sodium pump alpha-1 activity by means of selective ligands, which are cardiotonic steroids (cardenolides), markedly impairs cell migration and kills apoptosis-resistant cancer cells, an effect probably related to the inhibition of the NF-κB pathway.[3,12]

Recently, it has been shown that an extract from *Laurus nobilis* L. (Lauraceae) (bay laurel) inhibits proliferation of human mammary and renal adenocarcinoma cells.[13,14] While seldom used in pharmaceutical applications, the bay leaf has utilization in digestive disorders and is used traditionally to treat symptoms such as epigastric bloating, impaired digestion, and flatulence.[15,16] We have previously shown that megastigmane and phenolic components as well as sesquiterpene lactones isolated from *L. nobilis* leaves inhibit NO production from murine macrophages J774 stimulated with lypopolysaccharide.[17,18] The major aim of the current study was to investigate the potential anticancer effect of lauroside B (**1**), a megastigmane glycoside isolated from *L. nobilis* leaves, and to clarify the mechanisms involved in its cytotoxic properties. Three different human melanoma cell lines were used in this investigation, namely, the highly aggressive A375 cell line and the primary cutaneous melanoma cell lines, WM115 and SK-Mel-28, all with constitutive NF-κB activity. It was demonstrated that **1** suppressed the proliferation of human melanoma cell lines by inducing apoptosis. Moreover, the results showed that apoptosis induced by **1** is associated with the inhibition of NF-κB activation.



lauroside B (**1**)

RESULTS AND DISCUSSION

In preliminary experiments, individual constituents of *L. nobilis* leaves, previously isolated by our group, were assessed for their antiproliferative effect on several human melanoma cell lines (data not shown). After a preliminary screening, the megastigmane glycoside lauroside B (**1**) was selected, and its cytotoxic potency against human melanoma cell lines investigated. The antiproliferative effect of **1** on human melanoma A375, WM115, and SK-Mel-28 cells was evaluated using the MTT assay. As shown in Figure 1, treatment with **1** (3−10−30 μM) for 24−48 and 72 h caused inhibition of human melanoma cell growth in a time- and concentration-dependent manner. In particular, the effect of **1** (30 μM) on cell growth, evaluated at 72 h, resulted in almost 65% ($p < 0.001$, $n = 3$) inhibition for A375 cells, 50% ($p < 0.001$, $n = 3$) for WM115 cells, and 33% ($p < 0.001$, $n = 3$) for SK-Mel-28 cells. Normal human epidermal

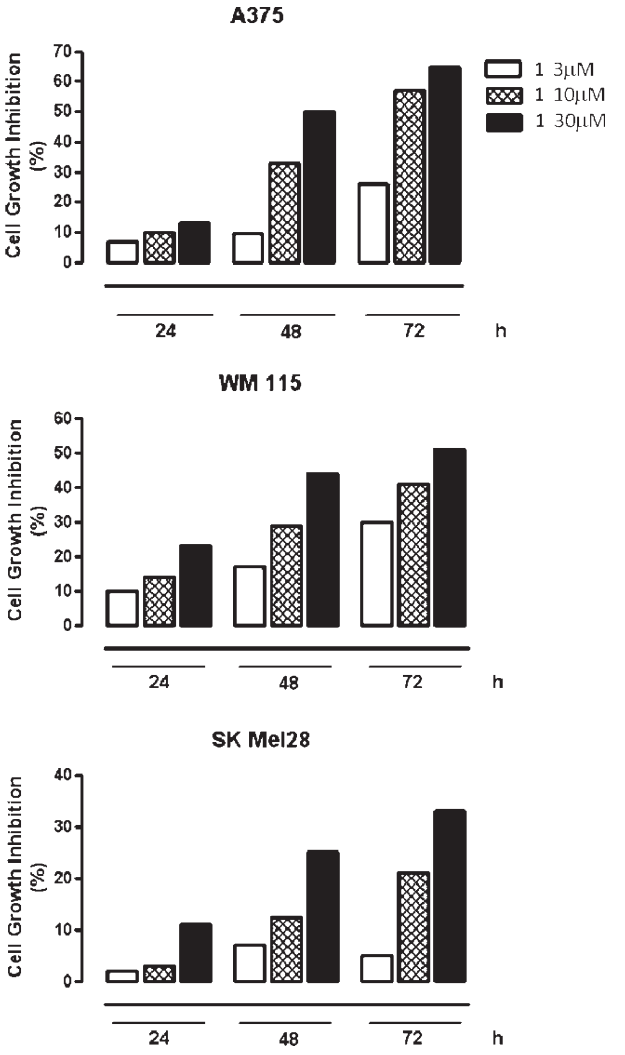


Figure 1. Treatment with lauroside B (**1**) inhibits human melanoma cell line proliferation in a time- and concentration-dependent fashion.

melanocyte (NHEM) growth was not affected by any of the concentrations of **1** tested at all the time points considered (data not shown). To further analyze the mechanism of **1**-induced cell death, induction of apoptosis was measured by annexin V/propidium iodide (PI) staining, which detects the externalization of phosphatidylserine (PS), a characteristic feature of cells entering apoptosis. The phospholipid-binding protein, annexin V, binds to cells with externally exposed PS, while PI staining occurs only after loss of membrane integrity.[19] This dual staining distinguishes between unaffected cells (unlabeled; quadrant 3, Figure 2), early apoptotic cells (annexin V positive; quadrant 4, Figure 2), late apoptotic cells (annexin V positive, PI positive; quadrant 2, Figure 2), and necrotic (PI positive; quadrant 1, Figure 2). Treatment of A375 cells for 48 h with **1** (30 μM) resulted in 35% of the cells exhibiting markers of late apoptosis (Figure 2). Curcumin, a natural compound, for which the proapoptotic activity is well-known, has been used as a positive control.[20] Treatment of cells with curcumin (20 μM) resulted in 80% of the cells exhibiting markers of late apoptosis (Figure 2).

To evaluate the involvement of caspases in cell death induced by **1**, western blot experiments were performed using whole-cell A375 lysates collected at different time points (0, 1, 2, 4, 8 h) after

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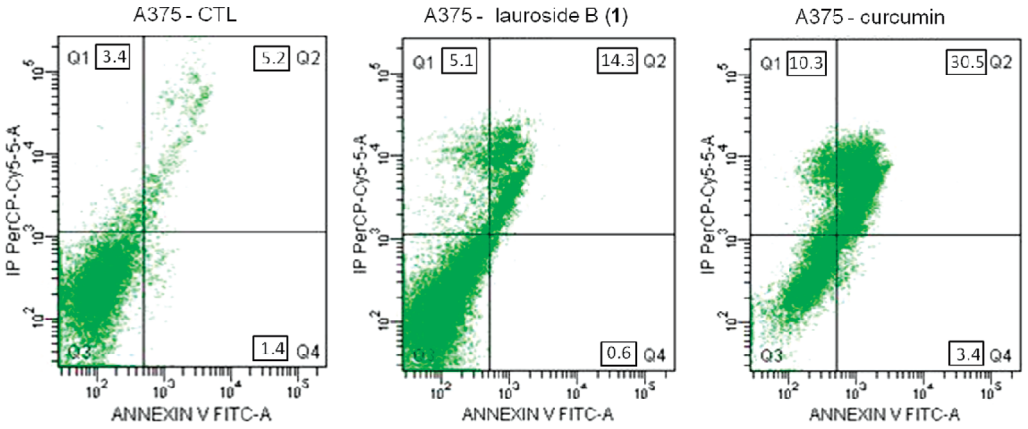

Figure 2. A375 melanoma cells were double-stained with annexin V/PI and analyzed by flow cytometry. Treatment with lauroside B (**1**) (30 μM) for 48 h induced apoptosis of A375 melanoma cells. CTL: untreated cells. Similar results were obtained in three separate experiments.

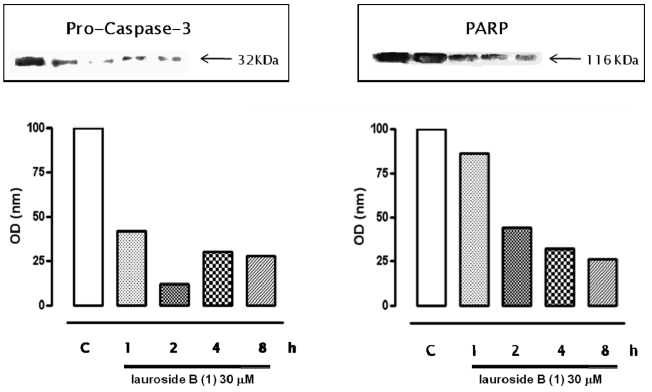

Figure 3. Treatment of A375 melanoma cells with lauroside B (**1**) (30 μM) led to a time-dependent reduction of pro-caspase-3, the precursor form of caspase-3, and to the cleavage of the caspase-3 substrate PARP. Similar results were obtained in three separate experiments.

treatment of cells with 30 μM **1**. Caspases are specific cysteine proteases, which play an important role in executing apoptotic cell death. They act in a cascade initiated by "regulator" caspases, ultimately leading to the cleavage of substrates by "effector" caspases that produce the characteristic features of apoptosis.[21] Thus, it was investigated whether caspase-3, which is the main "effector" caspase in the apoptotic pathway, was activated following treatment of A375 cells with **1**. Activation of caspases was suggested by the time-dependent reduction of pro-caspase-3, the precursor form of caspase-3, and confirmed by the cleavage of the well-known caspase-3 substrate PARP (Figure 3). NF-κB, a transcription factor involved in the control of apoptosis, cell cycle progression, and cell differentiation, has been shown to be constitutively activated in several types of neoplastic cells. NF-κB is normally retained in an inactive form in the cytoplasm tightly associated to the inhibitory protein IκB-α. Western blot analysis carried out on the cytosolic extracts obtained from A375 cells treated with 30 μM **1** for 1, 2, 4, and 8 h showed a time-dependent inhibition of IκB-α degradation (Figure 4 A). In order to investigate the effect of lauroside B (**1**) on NF-κB activity, A375 cells were treated with this compound (30 μM), and EMSA analysis was carried out on whole-cell extracts obtained at different time points (0, 1, 2, 4, 8, and 24 h). The levels of NF-κB DNA-binding activity were quantified as shown in Figure 4 B. The A375 cell line was found to display a constitutively high NF-κB DNA binding activity that was significantly ($p < 0.001$) reduced in a time-dependent manner by **1**. In fact, treatment with **1** (30 μM) for 0, 1, 2, 4, 8, and 24 h caused inhibition of NF-κB DNA-binding activity by 6%, 20%, 60%, 86%, and 100%, respectively (Figure 4 B). The major NF-κB band in A375 cells consisted of the p50 and p65 subunits, as determined by supershift analysis (data not shown). The reduced basal constitutive degradation of IKB-α shown in Figure 4A accounts for the decreased constitutive NF-κB DNA-binding activity (Figure 4 B), as has been recently shown.[22]

Since induction of apoptosis by **1** was found to be associated with NF-κB inhibition in A375 cells, the effect of this compound was evaluated on the levels of two proteins that inhibit apoptosis, XIAP and c-FLIP, for which their expression is known to be modulated by the transcriptional activity of NF-κB. The effect of **1** was compared with that of curcumin. Thus, A375 cells were treated with 30 μM **1** or 20 μM curcumin for 8 h. Western blot experiments showed that treatment with **1** greatly decreased the expression of XIAP, while only slightly reduced the expression of c-FLIP. Similar effects were shown by curcumin.

It has been shown recently that an extract of *L. nobilis* leaves inhibits the proliferation of human mammary and renal adenocarcinoma cells.[13,14] In the present study, it was shown that **1**, a megastigmane glycoside isolated from *L. nobilis* leaves, induces apoptosis and inhibits cell proliferation of three different human melanoma cell lines, (A375, WM115, and SK-Mel-28), indicating that compound **1** has a broad spectrum of activity. The mechanism of action of **1** was further investigated by using the highly aggressive and metastatic human A375 melanoma cell line as a representative model. Treatment of this cell line with **1** for 48 h induced cell-surface annexin V binding, thus demonstrating the ability of this glycoside to induce apoptosis of a highly aggressive human melanoma cell line. Another apoptotic characteristic is the activation of caspases, which play an important role in executing apoptotic cell death. Therefore, it was investigated whether caspase-3, which is the main "effector" caspase in the apoptotic pathway, was activated following treatment of A375 cells with **1**. A time-dependent degradation of both pro-caspase-3 and PARP following treatment of A375 cells with **1** was observed, thus supporting the data obtained by FACS analysis. When considering the general resistance of melanoma cells to apoptosis,[23,24] these results emphasize the potential of **1** as a possible new agent for the treatment of melanoma. In fact, one of the main problems when treating melanoma is chemoresistance. Many chemotherapeutic agents cause cell-cycle arrest but not apoptosis. Growth arrest allows cells to repair their DNA and

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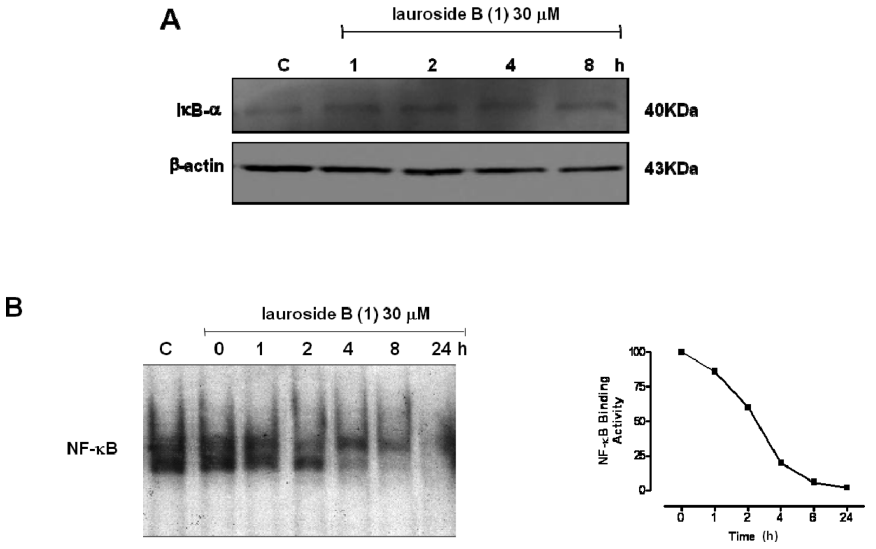

Figure 4. Treatment of A375 human melanoma cells with lauroside B (**1**) (30 μM) inhibits IκB-α degradation (A) and NF-κB DNA-binding activity (B) in a time-dependent fashion. Similar results were obtained in three separate experiments.

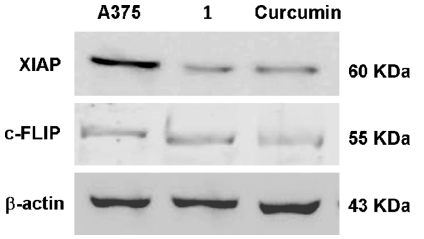

Figure 5. Treatment of A375 human melanoma cells with lauroside B (**1**) (30 μM) inhibits XIAP and c-FLIP expression. Similar results were obtained in three separate experiments.

limits the effectiveness of chemotherapy.[25] Thus, agents that can override tumor resistance toward apoptosis are of great potential value. Since **1** was able to induce apoptosis of a highly malignant melanoma cell line such as A375, a better understanding of the mechanism by which this critical effect is achieved was sought. The results obtained demonstrate that **1** is able to inhibit, in a time-dependent fashion, both IκB-α degradation and constitutive NF-κB DNA-binding activity, suggesting that the apoptotic effect of **1** may be partly mediated via reduction of NF-κB signaling.

In summary, the present study demonstrates, for the first time, the pro-apoptotic activity of lauroside B (**1**) against human melanoma cell lines and that this effect is associated with the inhibition of IκB-α degradation and constitutive NF-κB activation. Further studies will focus on better elucidating the molecular mechanism of action of this molecule as well as addressing its effects in animal models, in order to promote the potential development of **1** as a possible chemotherapeutic agent to treat human melanoma.

EXPERIMENTAL SECTION

General Experimental Procedures. Electrospray ionization mass spectrometry (ESI-MS) experiments were performed on an Applied Biosystem API 2000 triple-quadrupole mass spectrometer. Optical rotations were determined on a Jasco P-2000 polarimeter. ^{1}H and ^{13}C NMR spectra were determined on a Varian Unity INOVA spectrometer at 500.13 and 125.77 MHz, respectively, equipped with an indirect detection probe. ^{1}H NMR were recorded relative to MeOH-d_4 (δ = 3.30 ppm), whereas ^{13}C NMR spectra were recorded relative to MeOH-d_4 (δ = 49.0 ppm) or XXXX. GC analyses were performed on a Agilent Technologies 6850 Series II gas chromatograph for the capillary column (HP-5, 30 m × 0.25 mm, 180 °C; helium carrier flow 10 mL/min) and a FID detector operated at 260 °C. TLC analysis was performed on precoated silica gel plates (Kieselgel 60 F_{254}, Merck). Spots were detected under UV light at λ_{254} and λ_{366} nm or by using ceric sulfate spray reagent. Droplet counter-current chromatography (DCCC) was performed on a DCC-A apparatus (Tokyo Rikakikai Co., Tokyo-Japan). HPLC was performed using a Waters 510 pump equipped with a Waters U6K injector and a Waters 401 differential refractometer as detector, using a μ-Bondapak C_{18} column (30 cm × 3.9 mm; Waters, Milford, MA, USA). All solvents (analytical, deuterated, and HPLC grade) were purchased from Carlo Erba Reagenti (Milano, Italy).

The human melanoma cell lines A375, WM115, and SK-Mel-28 and the normal human epidermal melanocytes (NHEM) were purchased from PromoCell (Heidelberg, D-69126, Germany). NHEM were grown in melanocyte growth medium 2 (PromoCell). All the other cell lines were grown in complete Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 25 mM HEPES, 100 units/mL penicillin, and 100 μg/mL streptomycin, at 37 °C in a humidified incubator under 5% CO_2.

Plant Material. Leaves of *Laurus nobilis* were collected in the hills of Avellino (Italy) in August 2008 and identified by Prof. Paola Fortini (Dipartimento di Scienze e Tecnologie per l'Ambiente e il Territorio, DiSTAT, University of Molise). A voucher specimen is deposited under No. LN80A-08 in the Herbarium of University of Molise (Pesche, Isernia, Italy). Leaves were kept frozen at −20 °C until analyzed.

Extraction and Isolation. The leaves of *L. nobilis* (404 g) were chopped and soaked in MeOH (3 × 4 L) for 6 h at room temperature. Evaporation of solvent afforded 32.5 g of a glassy material, which was then subjected to a modified Kupchan's partitioning methodology[26] as follows: the MeOH extract was dissolved in 10% aqueous methanol and partitioned against *n*-hexane (3 × 400 mL), yielding 6.3 g of extract. The water content (% v/v) of the MeOH extract was adjusted to 20% and 40% and partitioned against CCl_4 (3 × 400 mL) and $CHCl_3$ (5 × 400 mL), respectively, yielding 0.3 and 5.7 g of each extract; the aqueous residue was concentrated and partitioned against *n*-BuOH (3 × 500 mL) to give 5.2 g of *n*-BuOH extract. The *n*-BuOH extract (5.2 g) was chromatographed by DCCC using *n*-BuOH/Me_2CO/H_2O (3:1:5) in descending mode (the upper phase

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was the stationary phase), flow rate 18 mL/h; 6 mL fractions were collected and monitored by thin-layer chromatography (TLC) on silica gel with *n*-BuOH/HOAc/H_2O (12:3:5) and $CHCl_3$/MeOH/H_2O (80:18:2) as eluents. Six major fractions (A−F) were obtained; fractions A−E were than purified by HPLC on a C_{18} μ-Bondapak column with MeOH/H_2O (2:8). Fraction A gave lauroside B (**1**): yield 1.5 mg; −93.7 (*c* 0.1, MeOH). The identity of **1** (99.9% purity by HPLC) was determined by comparison of its physical and spectroscopic (optical rotation, NMR, MS) data with literature values.[17]

Proliferation Assay. Cell proliferation was measured by the 3-[4,5-dimethyltiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. The human melanoma cells (A375, WM115, and SK-Mel-28) and the NHEM cells were seeded on 96-well plates (1×10^4 cells/well) and treated with **1** (3−10−30 μM) for 24−48−72 h before adding 25 μL of MTT (Sigma, Milan, Italy) (5 mg/mL in saline). Cells were thus incubated for an additional 3 h at 37 °C. After this time interval, cells were lysed, and dark blue crystals were solubilized with a solution containing 50% *N*,*N*-dimethyl formamide and 20% sodium dodecylsulfate with an adjusted pH of 4.5. The optical density of each well was measured with a microplate spectrophotometer (TitertekMultiskan MCC/340), equipped with a 620 nm filter.

Electrophoretic Mobility Shift Assay (EMSA). Aliquots of total extracts (12 μg protein/sample) in 0.1% Triton X-100 lysis buffer were incubated with ^{32}P-labeled κB DNA probes in binding buffer for 30 min, as previously described.[8] DNA−protein complexes were analyzed using nondenaturing 4% polyacrylamide gel electrophoresis. Quantitative evaluation of NF-κB-κB complex formation was determined using a Typhoon-8600 imager (Molecular Dynamics Phoshor-Imager, MDP, Amersham Biosciences, Piscataway, NJ) and ImageQuant software (Amersham Biosciences) (MDP analysis). For control of equal loading, NF-κB values were normalized to the level of the nonspecific protein−DNA complex in the same lane.

Flow Cytometry. Apoptosis was detected with an annexin V-FITC kit purchased from BD Pharmingen (San Diego, CA) according to the manufacturer's instructions. A375 cells were seeded in 35 mm culture dishes and allowed to attach overnight. The cells were treated with **1** (30 μM) for 48 h, collected, and washed twice with PBS. To detect early and late apoptosis, both adherent and floating cells were harvested together and resuspended in annexin V binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 10^6 cells/mL. Subsequently, 5 μL of FITC-conjugated annexin V and 5 μL of propidium iodide were added to 100 μL of the cell suspension (10^5 cells). The cells were incubated for 15 min at room temperature in the dark. Finally, 400 μL of annexin V binding buffer was added to each tube, and cells were analyzed using a FACSCalibur (BD Biosciences).

Western Blot Analysis. For western blot analysis, whole-cell extracts of A375 cells were prepared after lysis in extraction buffer (50 mM Tris [tris(hydroxymethyl)aminomethane]/HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 5 mM EDTA [ethylenediaminetetraacetic acid], 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor cocktail tablets, Roche). Cytosolic extracts of A375 cells were prepared as previously described.[27] Equal amounts of protein (20 μg/sample) from whole-cell extracts or (40 μg/sample) from cytosolic extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The filters were first blocked (5% low-fat milk in phosphate-buffered saline [PBS] with 0.1% Tween 20; 1 h at room temperature) and then incubated with the primary antibodies overnight at 4 °C. After three washes, filters were incubated with horseradish peroxidise-conjugated antibodies (1:5000; Amersham, Arlington Heights, IL) for 1 h at room temperature. Detection of immunocomplexes was performed with an enhanced chemiluminescence system (SuperSignal West; Pierce, Rockford, IL) and the Image Quant 400 system (GE Healthcare). Monoclonal antibody to β-actin was purchased from Sigma (Milan, Italy). The following primary antibodies were used: poly(adenosine diphosphate-ribose) polymerase (PARP), pro-caspase-3 and IκB-α (Santa Cruz Biotechnology, Santa Cruz, CA); FLICE-inhibitory protein (c-FLIP) (Upstate Biotechnology, Lake Placid, NY); and X-chromosome-linked inhibitor-of-apoptosis protein (XIAP) (R&D System, Minneapolis, MN).

Statistical Analysis. Values are expressed as the means ± SEM of *n* experiments run in triplicate for in vitro experiments. Comparisons were calculated by one-way analysis of variance and Bonferroni-corrected *p* values for multiple comparisons. The level of statistically significant difference was defined as $p < 0.05$.

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