

Acidic Triterpenes Compromise Growth and Survival of Astrocytoma Cell Lines by Regulating Reactive Oxygen Species Accumulation

Rubén Martín,¹ Juliana Carvalho,¹ Elvira Ibeas,¹ Marita Hernández,¹ Valentina Ruiz-Gutierrez,² and María Luisa Nieto¹

¹Instituto de Biología y Genética Molecular, Consejo Superior de Investigaciones Científicas-Universidad de Valladolid, Valladolid, Spain and ²Instituto de la Grasa de Sevilla, Consejo Superior de Investigaciones Científicas, Seville, Spain

Abstract

Several studies have shown how pentacyclic triterpenes can inhibit proliferation and induce apoptosis of some tumor cell lines; however, its effect on astrocytic tumors, one of the most malignant forms of cancer, has rarely been reported. The aim of this study was to examine how the pentacyclic triterpenes, oleanolic acid and maslinic acid, isolated from olive juice, affected astrocytoma cell morphology and survival. Cell proliferation was inhibited in 1321N1 astrocytoma cells by using 1 to 50 $\mu\text{mol/L}$ of either oleanolic acid or maslinic acid, with an average IC_{50} of 25 $\mu\text{mol/L}$. Growth inhibition led to morphologic and cytoskeletal alterations associated with the loss of stellate morphology and characterized by a retraction of the cytoplasm and collapse of actin stress fibers. Using 4',6-diamidino-2-phenylindole and Annexin V, we showed that astrocytoma cell death induced by oleanolic acid or maslinic acid were mainly due to apoptotic events. Furthermore, we showed that caspase-3 is activated as a consequence of triterpene treatment. Finally, we found that exposure of the cells to oleanolic acid or maslinic acid resulted in a significant increase of intracellular reactive oxygen species, followed by loss of mitochondrial membrane integrity. Importantly, enzymatic scavengers, such as catalase, or phenolic antioxidants, such as butylated hydroxytoluene, rescued cells from the triterpene-mediated apoptosis, suggesting that the potential therapeutic effect of these acidic triterpenes is dependent on oxidative stress. Our data show that acidic triterpenes play a major role in 1321N1 astrocytoma morphology and viability and support the conclusion that oleanolic acid and maslinic acid may thus be promising new agents in the management of astrocytomas. [Cancer Res 2007;67(8):3741–51]

Introduction

Malignant astrocytic tumors are the most common primary brain tumors; their growth is rapid and they are associated with a poor prognosis, despite the use of multimodal treatment regimens. The lack of progress in the treatment of this cancer has urged the identification of novel agents.

The growth rate of tumors is dependent both on the rate of proliferation and on the rate of cell loss (1). Cell loss may occur either because of apoptosis or necrosis. Necrotic cell death is characterized by membrane damage, and it generates an inflam-

matory response (2). Apoptosis, the intrinsic suicide program of the cell, is a process associated with nuclear and cytosolic condensation and the formation of apoptotic bodies (3). Mammalian cell apoptosis requires the triggering of complex regulatory pathways that include effectors, inhibitors, and activators (4, 5). This process often involves a family of proteases known as caspases, which are activated in a proteolytic cascade to cleave specific substrates (6–8). The intrinsic pathway is generated by multiple signals, including heat shock, lymphokine deprivation, radiation, and chemotherapy (9, 10). Even oxidative stress has also been suggested to play a role as a common mediator of apoptosis (11). This apoptotic route involves the mitochondria-dependent activation of initiator caspases, which in turn activate downstream executive caspases, such as caspase-3 (12, 13). The extrinsic death receptor pathway is triggered by death ligands through the formation of the death-inducing signaling complex. This complex results in caspase-8 activation, which initiates the downstream apoptotic signaling (14–16).

The aggressive cancer cell phenotype is the result of a variety of alterations leading to deregulation of these intracellular signaling pathways (17, 18). Thus, current attempts to improve cancer survival will have to include strategies that specifically target tumor cell resistance to apoptosis. In recent years, the development of more effective and safer agents has been intensively required for chemoprevention of human cancer, and natural products from plants and their synthetic derivatives have been expected to play an important role in creating new and better chemopreventive and therapeutic agents.

Herbal medicines derived from plant extracts are being increasingly used to treat a wide variety of clinical diseases, with relative little knowledge of their modes of action. The triterpenoids, biosynthesized in plants by the cyclization of squalene, are found widely distributed throughout the vegetable kingdom and are the major components of many medicinal plants used in Asian countries. There is a growing interest in the elucidation of the biological and pharmacologic roles of the plant-derived triterpenoid compounds, in terms of hepatoprotective, analgesic, anti-tumor, antiinflammatory, and immunomodulatory effects (19, 20).

In this study, we focus on two pentacyclic triterpenes, oleanolic acid (3 β -hydroxy-12-oleanen-28-oic acid) and maslinic acid (2 α ,3 β -dihydroxy-12-oleanen-28-oic acid), which are present in plants, such as *Olea europaea*, and can be isolated in appreciable amounts from the nonglyceride fraction of pomace olive oil. To our knowledge, the potential therapeutic significance of this olive subproduct has not yet been completely studied. It has been reported that oleanolic acid produces a wide variety of excellent antitumor-promoting activity in the *in vivo* carcinogenesis test, including decrease of the incidence of induced intestinal tumor in a rat model (21), inhibition of angiogenesis, which is important for the progressive growth of solid tumors (22), tumor promotion (23),

Requests for reprints: María Luisa Nieto, Instituto de Biología y Genética Molecular, Consejo Superior de Investigaciones Científicas-Universidad de Valladolid, C/Sanz y Forés s/n, 47003 Valladolid, Spain. Phone: 34-983-184836; Fax: 34-983-184800; E-mail: mlnieto@ibgm.uva.es.

©2007 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-06-4759

and implanted tumor growth in mice (24). However, in spite of these pleiotropic effects, the exact mechanisms by which natural oleananes promote such cellular outcomes are not well understood and the biochemical basis of these actions remains to be determined.

Our interest in both oleanolic and maslinic acid as anticarcinogenic agents arises from their cytotoxic properties on different human tumor cell lines and from their ability to inhibit tumor promotion as mentioned earlier in experiments *in vivo* and *in vitro*. Although diverse mechanisms have been proposed for the anti-tumor effects of these oleanane triterpenoids, information on the molecular effect in brain cancer cells is not available. Therefore, the purpose of this study was to determine the effect of the triterpenoids found in pomace olive oil, oleanolic acid, and maslinic acid on proliferation and apoptosis in the human astrocytoma cellular line 1321N1. We now report for the first time a study showing that both oleanolic acid and maslinic acid are powerful molecules that inhibit the proliferative capacity of human 1321N1 cells in culture. Moreover, both triterpenes at a range of 5 to 25 $\mu\text{mol/L}$ cause apoptosis in a dose-dependent manner. Hence, these natural agents may represent an effective therapeutic strategy in the treatment of human brain cancer. The underlying mechanism implicated in their apoptotic responses was also preliminarily investigated, by using different experimental protocols.

Materials and Methods

Reagents and antibodies. 4',6-Diamidino-2-phenylindole (DAPI), propidium iodide, *N*-acetylcysteine (NAC), butylated hydroxytoluene (BHT), catalase, DTT, 1,3-dimethyl-2-thiourea (DMTU), rhodamine 123, and chemicals were obtained from Sigma (St. Louis, MO). 2',7'-Dichlorofluorescein diacetate (DCFH-DA), 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM), and JC-1 were purchased from Molecular Probes (Eugene, OR), and human thrombin was obtained from Preprotech (Rocky Hill, NJ). [^3H]thymidine was from Amersham Biosciences (Little Chalfont, United Kingdom).

Cell lines and cultures. The human astrocytoma cell lines 1321N1, U87 MG (U87), U181 MG (U181), and U373 MG (U373) are four well-characterized permanent astrocytoma cell lines derived from patients with malignant astrocytomas and were kindly supplied by Dr. J.H. Brown (University of California San Diego, San Diego, CA), Dr. M. Guzman (Universidad Complutense de Madrid, Madrid, Spain), and Dr. M. Izquierdo (Universidad Autonoma de Madrid, Madrid, Spain).

The astrocytoma cell lines were cultured in DMEM supplemented with 10% FCS, 100 units/mL penicillin, and 100 mg/mL streptomycin at 37°C in 5% CO_2 .

Extraction of pentacyclic triterpene acids from olives. Oleanolic acid and maslinic acid, natural pentacyclic triterpenes, derivative from pressed olive (*O. europaea*) fruits, were obtained by using the method described by Juan et al. (25). The crude extract was chromatographed on a silica gel column to separate maslinic and oleanolic acids, which were eluted with CHCl_3 . The chloroform fraction was evaporated in a rotary evaporator and the extract was kept as a powder. Triterpenes were dissolved at 25 mmol/L in DMSO.

Cell cycle analysis. Cells were seeded at the density of 5×10^5 in 25-cm² plastic tissue culture flasks. After 24 h, the cells were treated with or without different doses of triterpenes for 18 h. They were then washed in PBS and resuspended in cold 70% ethanol. Cells were subjected to flow cytometric analysis on an EPICS XL cytofluorimeter (Beckman Coulter, Spain) after propidium iodide labeling, and the results were analyzed using CELL QuestV1 software.

Cell proliferation. Cell proliferation was evaluated by a [^3H]thymidine incorporation assay. Cells were plated onto six-well cell culture plates at 5×10^6 /well. Before treatment, the cells were switched to serum-free medium for a 24-h starvation period. Then, cells were treated with 5% FCS or

0.5 unit/mL thrombin in the presence of 0, 1, 5, 25, or 50 $\mu\text{mol/L}$ oleanolic acid or maslinic acid. After an overnight incubation, the proliferative response was measured by supplementing the cultures with 1 μCi /well of [^3H]thymidine for 4 additional hours. Then, cells were washed and DNA was precipitated and analyzed by liquid scintillation counting. Results are expressed as the percentage of [^3H]thymidine uptake by either FCS- or Th-stimulated 1321N1 cells, respectively, in the absence of triterpenes. Numerical data are mean \pm SD of four different experiments, each done in triplicate.

Assays for reactive oxygen species and reactive nitrogen species production, and mitochondrial inner transmembrane potential ($\Delta\Psi_m$). To monitor the intracellular production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), we used the cell-permeable probes DCFH-DA and DAF-FM diacetate. Briefly, cells in 25-cm² plastic tissue culture flasks were treated for 30 min with 1 $\mu\text{mol/L}$ DCFH-DA or DAF-FM and stimulated 30 and 60 min with 25 $\mu\text{mol/L}$ oleanolic acid, maslinic acid, or Me_2SO . Then, cells were harvested, washed, resuspended in 1 mL of PBS at 37°C, and analyzed immediately by flow cytometry (EPICS XL cytofluorimeter, Beckman Coulter). In some experiments, cells were treated for 30 min with the indicated antioxidant before incubation with the triterpenes.

The cationic dyes JC-1 and rhodamine 123 were used as probes to evaluate mitochondrial transmembrane potential ($\Delta\Psi_m$). Cells in 25-cm² plastic tissue culture flasks were treated with 25 $\mu\text{mol/L}$ oleanolic acid, maslinic acid, or Me_2SO for 6 or 18 h. Then, the cells were harvested, and JC-1 or rhodamine 123 were added directly to the culture medium to a final concentration of 60 and 150 nmol/L, respectively. Cells were harvested, resuspended in 1 mL of PBS at 37°C, and analyzed immediately for fluorescence intensity by flow cytometry or fluorescence microscopy.

Fluorescence microscopy. Astrocytes growing on 16-mm glass coverslips were used for immunofluorescence analysis. After triterpenoid treatment, cells were fixed with 3.7% formaldehyde in PBS and then permeabilized in PBS containing 0.1% Triton X-100. For filamentous (F)-actin labeling, cells were incubated with FITC-phalloidin (1:200) at 37°C in the dark for 30 min. Intermediate filaments were stained with antivimentin (1:100) followed by incubation with Alexa 546-conjugated goat anti-mouse IgG antibody (1:200). Cellular nuclei were detected by incubation with 1 mg/mL DAPI in the dark for 5 min. Apoptosis was also analyzed by using the Annexin V binding assay. Cells were labeled with R-phycoerythrin-conjugated Annexin V according to the manufacturer's protocol. After these treatments, cells were washed with PBS and images were captured with either a Nikon Eclipse TS100 or a Nikon Eclipse 80i inverted fluorescence microscope using $\times 20$, $\times 40$, or $\times 60$ objective lens.

Western blots. To measure actin by Western blot, cell lysates were subjected to electrophoresis in SDS-PAGE 10% polyacrylamide gels; proteins were transferred to a membrane and probed with a mouse monoclonal antiactin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by incubation with an HRP-linked secondary antibody. Signals were detected using the enhanced chemiluminescence system (Amersham Pharmacia Biotech). The membrane was stained with Ponceau S to confirm equal loading and transfer of protein.

Flow cytometric analysis of apoptotic cells. After exposure to the indicated doses of oleanolic acid or maslinic acid for 18 h, the percentage of apoptotic cell death was determined using an Annexin V-phycoerythrin Apoptosis Detection Assay. Briefly, cells (1×10^5 /0.5 mL binding buffer) were incubated with R-phycoerythrin-conjugated Annexin V for 5 min, followed by flow cytometric analysis using an EPICS XL cytofluorimeter (Beckman Coulter). In some experiments, cells were treated for 30 min with the indicated antioxidant before incubation with the triterpenes.

The presence of processed caspase-3 was used as marker of apoptosis. The active form of caspase-3 was measured by flow cytometry using FITC-conjugated polyclonal antihuman-active caspase-3 antibody (C92-605, BD PharMingen, San Diego, CA), which specifically recognizes cleaved activated caspase-3 but not inactive pro-caspase-3. Astrocytoma cells were washed, fixed, and permeabilized using 4% paraformaldehyde-0.1% Tween 20. Thereafter, cells were incubated with 20 μL of FITC-conjugated polyclonal anti-active caspase-3 antibody or the isotype control antibody, and analyzed by flow cytometry.

Results

Antiproliferative effects of the oleanolic and maslinic acid.

To observe the effect of the pentacyclic triterpenes, oleanolic acid and maslinic acid, on astrocytoma cells, we used various concentrations of the triterpenes for stimulation. The 1321N1 cells were incubated with different concentrations of oleanolic acid or maslinic acid (1, 5, 25, and 50 $\mu\text{mol/L}$) for 24 h in the presence of 5% FCS or 0.5 units/mL Th, and cell growth was studied by measuring [^3H]thymidine incorporation. We found that both triterpenes inhibited cell proliferation in a concentration-dependent manner (Fig. 1A and B). The extent of oleanolic acid- and maslinic acid-induced growth inhibition was approximately equal in the FCS- and in the Th-stimulated cells, and the dose of triterpenes required for growth inhibition did not differ significantly between the proliferative agents tested. The IC_{50} of these compounds obtained after 24 h of incubation were around 25 $\mu\text{mol/L}$.

In addition, cell morphology was also examined using a phase-contrast microscope. Microscopic observations revealed that triterpenes affect not only cell proliferation but also cell appearance and attachment (Fig. 1C). Exposure of 1321N1 cells to 25 $\mu\text{mol/L}$ oleanolic acid or maslinic acid provoked dramatic morphologic alterations in the cells: from flat, polygonal shape to a stellate morphology. Mild morphologic changes were already apparent at 30-min incubation with 25 $\mu\text{mol/L}$ oleanolic acid or maslinic acid (data not shown). At 2 h, shape change was almost completed: many astrocytoma-treated cells acquired a round shape with long cell processes and spindle-like structures. These morphologic changes occurred as a result of retraction of the cytoplasm around cytoskeletal elements as well as elongation of processes. At 6 h of incubation with the triterpenes, the number of cells attached to the coverslip had dramatically decreased, mostly cells rounded up and individual processes continued to elongate.

The tendency of 1321N1 astrocytoma-treated cells to retreat from and lose contact with culture plaques suggested that both acidic triterpenes may cause defects in cell adhesion and spreading. In fact, long treatment with 25 $\mu\text{mol/L}$ oleanolic acid or maslinic acid caused substantial cell detachment from the culture surface.

Cytoskeleton alterations following acidic triterpene exposure. To examine the molecular basis of this phenomenon, the organization of the F-actin and the distribution of the vimentin were analyzed because these two types of cytoskeletal elements frequently work together to enhance both structural integrity and cell shape. Therefore, 1321N1 cells were stained to visualize cytoplasmic F-actin and vimentin. Treatment of quiescent cells with 25 $\mu\text{mol/L}$ oleanolic acid or maslinic acid causes dramatic changes in the F-actin pattern. As shown in Fig. 2, untreated astrocytoma cells showed normal morphology with actin spread out throughout the cell and concentrated mainly in the cell cortex (Fig. 2A). Upon treatment with either oleanolic acid or maslinic acid, cells rounded up and the net of actin fibers, observed in control cells, disappeared. The effect on F-actin distribution could already be observed after 3 h treatment, where the fine linear strands of actin seen in untreated cells were disrupted, and instead astrocytes displayed a spindle-like appearance with actin condensed in the cell body, showing a compact F-actin network. Immunostaining for vimentin, an intermediate filament protein, showed high expression in resting 1321N1 astrocytoma cells (Fig. 2A). In untreated cells, vimentin appeared as an elaborate filamentous network surrounding the nucleus throughout the cytoplasm; however, after triterpene treatment, the distribution of

vimentin labeling revealed high density staining on the cytoplasm, extending towards the cell periphery as filament bands.

These results indicate that oleanolic acid and maslinic acid affect the cytoskeleton arrangement of cultured human astrocytoma cells. To provide a better quantitative approach to changes in cellular F-actin, cells stained with phalloidin-FITC were analyzed by flow cytometry. Acidic triterpenes decreased the amount of F-actin, as we found a reduction in the number of cells with high phalloidin-FITC fluorescence and there was an increase in the number of cells with low F-actin staining (Fig. 2B). To determine whether acidic triterpenes also affect cellular actin, lysates from treated cells were subjected to electrophoresis and Western blot analysis. As shown in Fig. 2C, there was no change in total cellular actin after oleanolic acid or maslinic acid incubation. Thus, the effect of triterpenes was presumably associated with a disruption of actin filaments, as there was no modification in total cellular actin with oleanolic acid or maslinic acid treatment.

Induction of apoptosis by oleanolic and maslinic acid. To investigate whether these alterations in cell morphology were an effect due to apoptosis, in cells treated with different times and doses of oleanolic acid and maslinic acid, we analyzed various apoptotic hallmarks, including DNA status, phosphatidylserine exposure, and caspase-3 activation.

Flow cytometry assay after propidium iodide staining allows cell cycle analysis, and cells undergoing apoptosis can be detected as a subdiploid peak. The results observed using this technique revealed that after 18 h of 5, 25, and 50 $\mu\text{mol/L}$ triterpene treatment, the population of 1321N1 astrocytes detected in the sub- G_0 - G_1 peak increased substantially (Fig. 3A). At the IC_{50} value, 25 $\mu\text{mol/L}$ for oleanolic acid or 25 $\mu\text{mol/L}$ for maslinic acid, the hypodiploid region included $\sim 32\%$ and 20% , respectively, of the total events. Consistently, nuclear staining with DAPI revealed chromatin condensation and nuclear fragmentation in the triterpene-treated cells at the same concentrations that inhibited cell growth (Fig. 3B).

In addition, Annexin V assay revealed that cells stained positive with Annexin V-phycoerythrin after triterpene treatment. This effect was observed already with a relatively low dose of triterpenes and in a dose-dependent manner. The treated cells exhibited signs of phosphatidylserine exposure and the percentages of apoptotic cells are shown in Fig. 3C. Significant increase in Annexin V-positive cells was also observed by fluorescence microscopy. Astrocytes incubated with oleanolic acid or maslinic acid showed a clear membrane staining, whereas control cells do not yield positive labeling.

These results were in agreement with the presence of caspase-3 proteolytically processed, measured by flow cytometry in permeabilized cells using an antibody against the active fragment (Fig. 3D). In fact, although untreated cells were primarily negative for the presence of active caspase-3, the cells incubated with these triterpenes at their IC_{50} values for 6 and 18 h had detectable levels of the active form. In the 1321N1-treated cells, a modest increase in active caspase-3 was observed at 6 h, whereas after 18 h the number of cells expressing processed or active caspase-3, compared with untreated control cells, was significantly higher (control cells, $3.5 \pm 1.5\%$; oleanolic acid-treated cells, $28.5 \pm 7.2\%$; maslinic acid-treated cells, $14 \pm 4\%$). The magnitude of active caspase-3-positive cells was similar to that induced by Fas ($19 \pm 5\%$; not shown), a classic inducer of apoptosis and caspase-3 activator.

These results indicate that, despite the chemical differences between these two triterpenes, both showed similar efficacy in inhibiting the viability of astrocytoma cells.

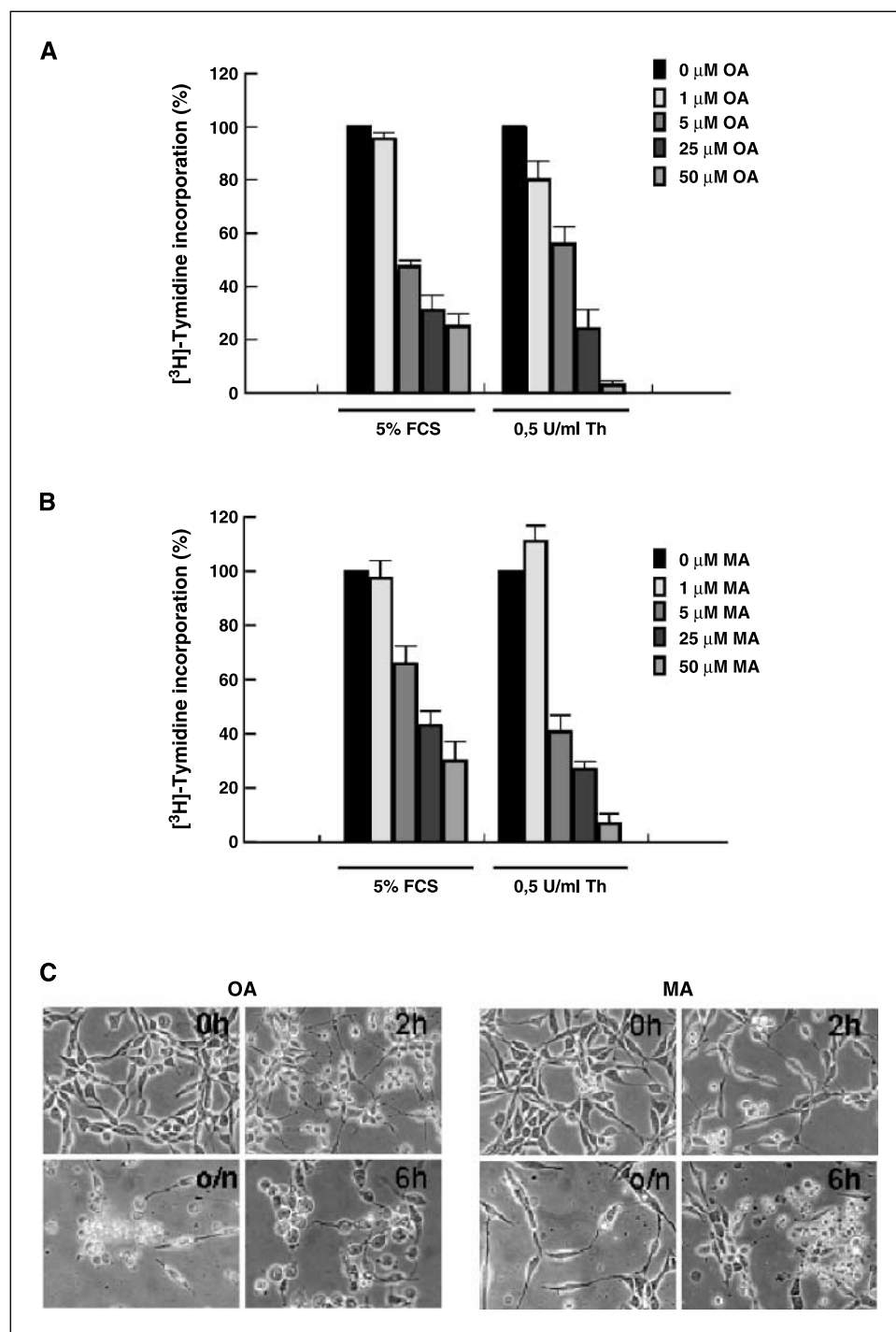


Figure 1. Effect of oleanolic acid and maslinic acid on cellular proliferation. 1321N1 cells were grown in triplicate with the indicated doses of oleanolic acid (OA; A) or maslinic acid (MA; B) for 24 h and assayed for proliferation as mentioned in Materials and Methods. *Columns*, means of three independent experiments; *bars*, SE. Data are expressed as percentage of [³H]thymidine uptake elicited by the proliferative stimuli in the absence of triterpenes (FCS, 278.515 ± 15.611 dpm; Th, 120.558 ± 5.048 dpm). *C*, growth inhibition and morphologic changes of 1321N1 cells treated with 25 μmol/L oleanolic acid or maslinic acid for 2 h, 6 h, or o/n, compared with untreated cells (control). Cells were photographed using a Nikon Eclipse TS100 microscope (×40).

Triterpenes modulate ROS production and mitochondrial membrane potential ($\Delta\Psi_m$) collapse in 1321N1 cells. Several studies have shown that certain apoptotic drugs trigger a rapid production of intracellular ROS or RNS, which might be responsible for their cytotoxic actions (26, 27). To determine whether oxidative stress was possibly associated with oleanolic acid- or maslinic acid-induced apoptosis, we exposed 1321N1 cells to 25 μmol/L oleanolic acid or maslinic acid for 30 min and 1 h and examined them for evidence of ROS/NOS production using the oxidative stress-sensitive dyes DCFH-DA and DAF-FM. The results obtained

indicated that cell exposure to the acidic triterpenes, oleanolic acid or maslinic acid, led to a remarkable increase in DCF fluorescence at 30 min, whereas DAF-FM oxidation was not affected by any compound (Fig. 4A). The shift to the right of the curve due to increased fluorescence indicates an increase in the intracellular levels of ROS. No additional increase in ROS buildup was found at 1 h of triterpene incubation (data not shown).

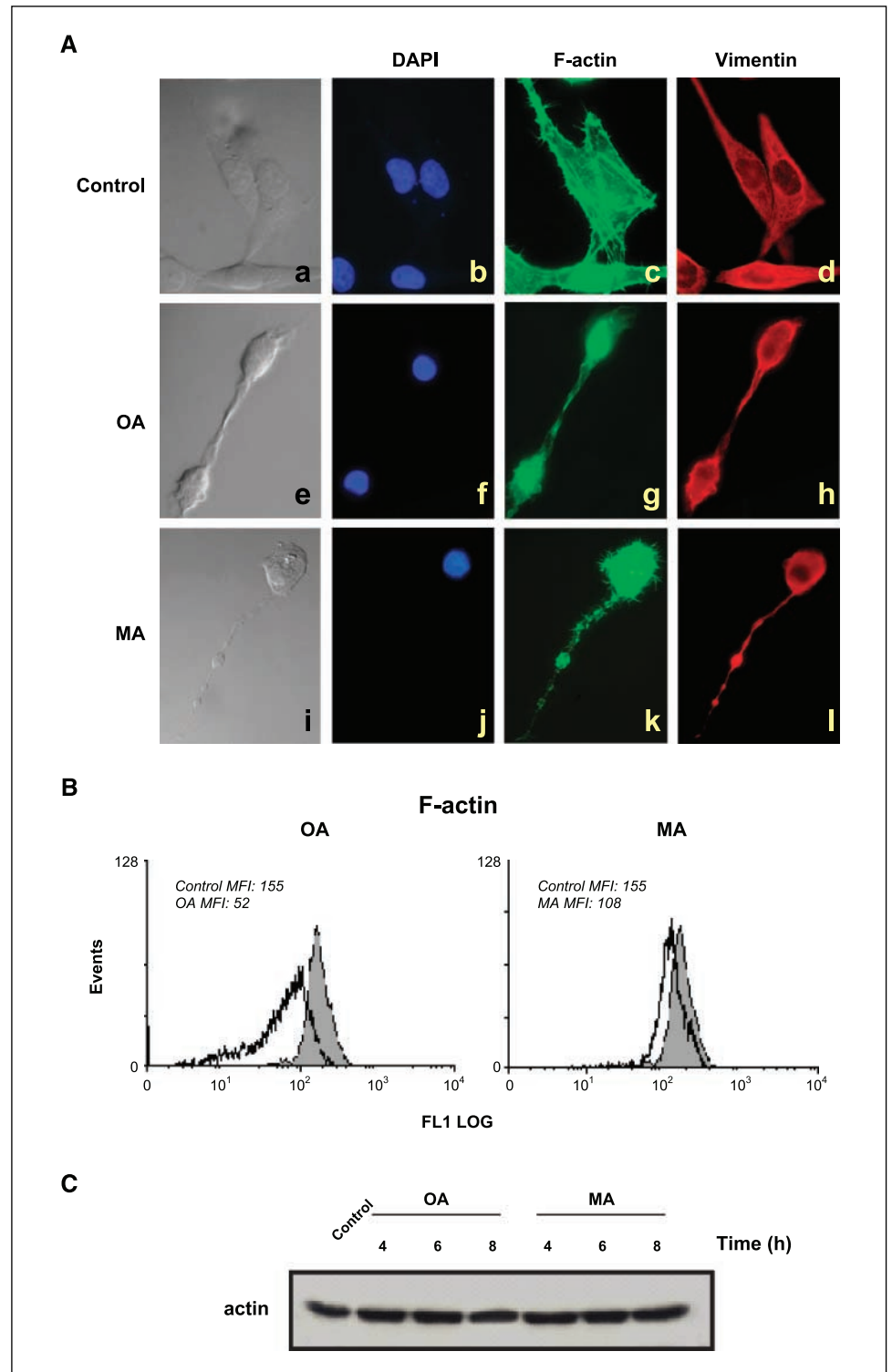
It has been suggested that ROS overproduction leads to mitochondrial damage that results in the loss of mitochondrial membrane potential and dysfunction. Moreover, alterations in

mitochondrial function have been shown to play a crucial role in apoptosis. Therefore, the ability of mitochondria to maintain membrane potential after incubation with acidic triterpenes was measured in 1321N1 cells using the fluorescent dyes rhodamine 123 and JC-1.

Rhodamine 123 is recognized as a specific fluorescent probe to monitor active mitochondria. Its uptake and retention into the

mitochondrial matrix depends on the $\Delta\Psi_m$. The fluorescence of Rh 123 in 1321N1 cells after 6 h treatment with 25 $\mu\text{mol/L}$ oleanolic acid or maslinic acid compared with untreated control was found to be substantially reduced. Figure 4B shows that both oleanolic acid and maslinic acid treatments caused a significant decrease on fluorescence; it dropped to 116.5 ± 13.5 and 349 ± 33 , respectively, compared with 698 ± 31 in untreated control cells. Under

Figure 2. Rearrangement of the cytoskeleton in oleanolic acid- or maslinic acid-treated 1321N1 cells. **A**, cells were grown on coverslips; stimulated with 25 $\mu\text{mol/L}$ oleanolic acid or maslinic acid for 3 h; fixed, permeabilized, and incubated with DAPI (*b, f, and j*), FITC-phalloidin (F-actin; *c, g, and k*), or antivimentin (*d, h, and l*; as described in Materials and Methods). *a, e, and i*, Nomarski images. Untreated 1321N1 astrocytoma cells showed actin filaments distributed as stress fibers through the cytoplasm (*c*), and vimentin in typical networks across the cytosol, with evidence of perinuclear localization (*d*). Maslinic acid- or oleanolic acid-treated cells show actin filament disruption as small punctuated microfilaments (*g* and *k*) with vimentin remaining in the perinuclear region extending into long cytoplasmic processes (*h* and *l*). Cells were visualized using a Nikon Eclipse 80i fluorescent microscope ($\times 60$). **B**, FACS analysis of 1321N1 cells treated as mentioned above. Representative histograms show F-actin expression: *solid gray curves*, cells cultured in the absence of treatment (control); *empty black curves*, triterpene-treated cells. *MFI*, mean fluorescence intensity. **C**, 1321N1 lysates from cells treated with 25 $\mu\text{mol/L}$ oleanolic acid or maslinic acid for different times were analyzed by immunoblots with antiactin antibody.



Downloaded from <http://aacrjournals.org/cancerres/article-pdf/67/8/3741/2581689/3741.pdf> by guest on 23 May 2024

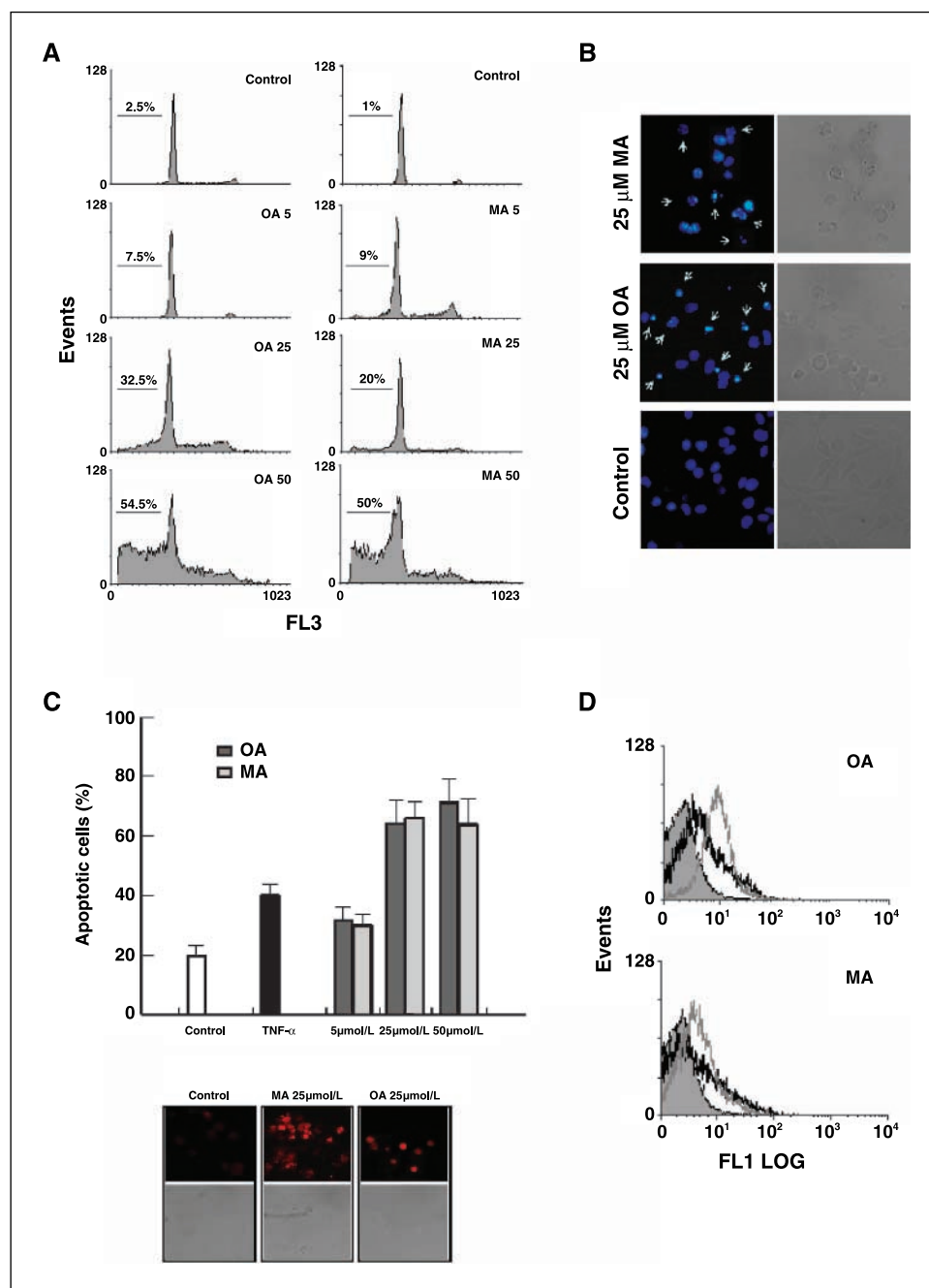


Figure 3. Analysis of apoptotic events induced by oleanolic acid or maslinic acid treatment on 1321N1 cells. *A*, representative histograms show cell cycle analysis of cells stimulated for 18 h with the indicated doses of oleanolic acid or maslinic acid. Percentages indicate the number of cells in the sub-G₀-G₁ phase of the cell cycle, delimited by the line in the graphics. *B*, nuclear condensation in response to 25 μ mol/L oleanolic acid or maslinic acid treatment for 18 h, detected with DAPI staining and fluorescent microscopy. *Arrows*, condensed nuclei in apoptotic cells. Cells were visualized using a Nikon Eclipse TS100 microscope ($\times 40$). *C*, percentage of Annexin V-positive cells after 18 h treatment, related to the concentration of triterpenes used. *Columns*, mean of three independent experiments; *bars*, SE. *Bottom*, representative images of 1321N1 cells treated with or without 25 μ mol/L triterpene for 18 h and stained with Annexin V-phycoerythrin. Cells were photographed using a Nikon Eclipse TS100 microscope ($\times 20$). *D*, representative histograms showing processed caspase-3 in quiescent and triterpene-stimulated cells.

fluorescence microscopy, all the control cells were stained extensively with rhodamine 123, whereas the triterpene-treated cells were less stained or not stained at all (Fig. 4B).

JC-1 is a mitochondrial-selective sensor and aggregates in normal and highly polarized mitochondria, resulting in a green-orange emission of 590 nm after excitation at 490 nm. Upon depolarization of the mitochondrial membrane, JC-1 forms green fluorescent monomers; therefore, the loss of JC-1 aggregates directly correlates to changes of $\Delta\Psi_m$. Figure 4C shows a clear decrease in the percentage of cells that emitted orange fluorescence after triterpene treatment compared with untreated cells.

These results reflected the collapse of mitochondrial membrane potential in 1321N1 cells after triterpene treatment.

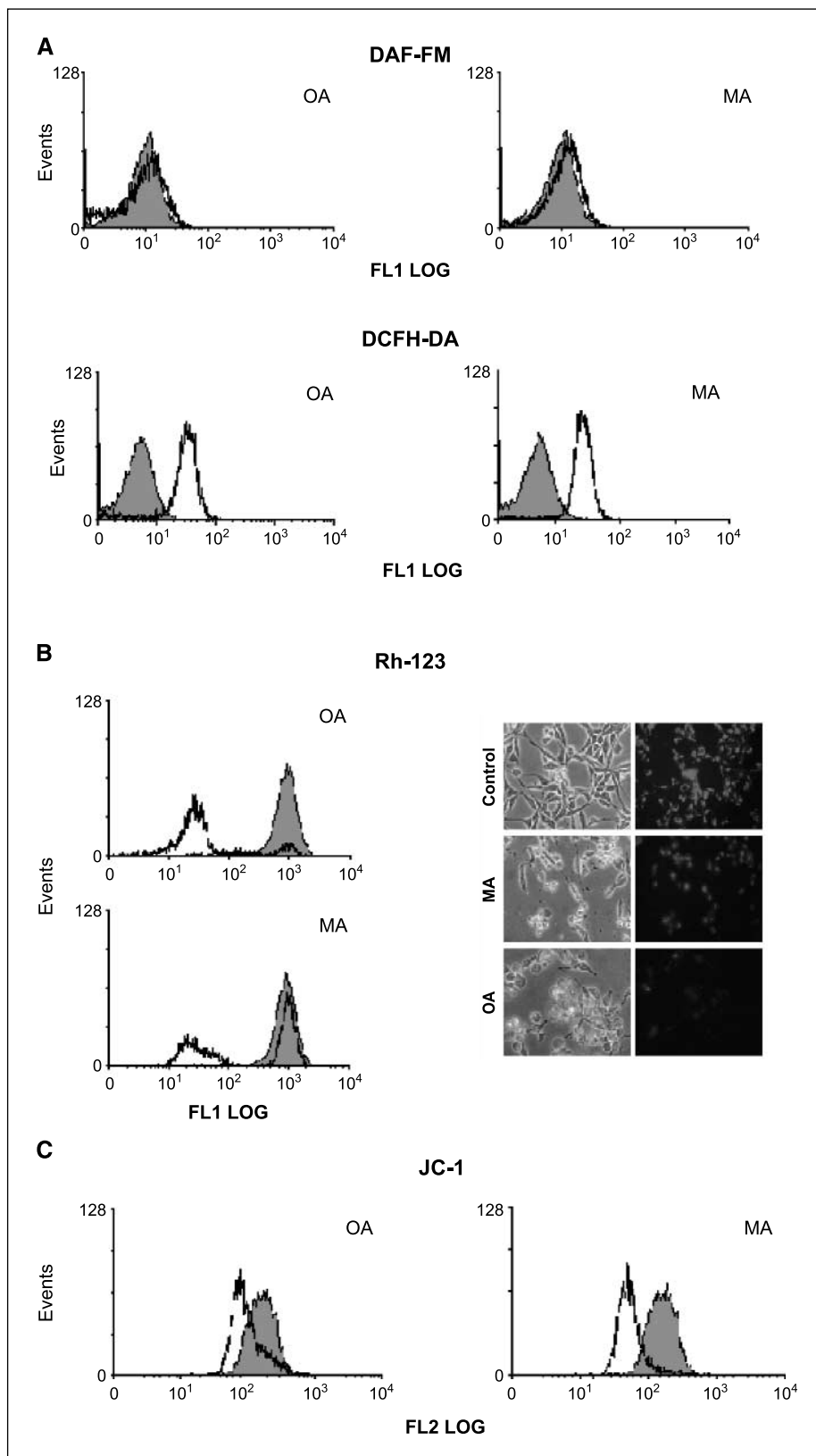
Triterpene-induced apoptosis involves ROS formation in 1321N1 cells.

To elucidate whether oxidative stress participates in the oleanolic acid- or maslinic acid-induced apoptosis, 1321N1 cells were pretreated with different antioxidants, on the basis of their scavenging capabilities. We examined the effect of scavenger enzymes, such as catalase, disulfide-reducing agents, such as NAC, and hydroxyl radical scavengers, such as and DMTU. First, we determined the ability of these antioxidants to reduce the quantifiable DCF fluorescence of triterpene-treated cells. For this purpose, 1321N1 cells were incubated for 30 min with 100 or 500 units/mL of catalase, or 5 or 10 mmol/L of NAC, or 3 or 30 μ mol/L of DMTU before the addition of triterpenes. Under these conditions, all the antioxidants significantly attenuated triterpene-induced ROS up-regulation (Fig. 5).

Next, to evaluate whether these hydrophilic antioxidants or the lipophilic scavenger BHT—characterized to inhibit lipid peroxides formation—were effective in preventing apoptotic cell death, Annexin V staining analysis was done in oleanolic acid- and

maslinic acid-treated cells, at their IC₅₀ value, in the presence or absence of the antioxidants. We found that none of them by themselves were toxic at the concentrations used. Overnight treatment of cells with 25 μmol/L oleanolic acid or 25 μmol/L

Figure 4. Effect of oleanolic acid and maslinic acid on ROS/RNS production and on mitochondrial membrane potential ($\Delta\Psi_m$). **A**, 1321N1 cells were treated with 25 μmol/L oleanolic acid or maslinic acid for 30 min. ROS levels were measured by FACS analysis after staining with the fluorescent probes DCFH-DA and DAF-FM, respectively. The mitochondrial membrane potential was analyzed using the selective dyes rhodamine 123 (**B**) or JC-1 (**C**). 1321N1 cells were treated with 25 μmol/L oleanolic acid or maslinic acid for 6 h, and mitochondrial dysfunction was determined by FACS and fluorescence microscopy. **B**, representative images and histograms showing $\Delta\Psi_m$ as measured on the basis of rhodamine 123 fluorescence intensity in resting and triterpene-stimulated cells. Cells were photographed using a Nikon Eclipse TS100 microscope ($\times 40$). **C**, representative histograms of triterpene-treated cells stained with JC-1. *Solid gray curves*, cells cultured in the absence of treatment (control); *empty black curves*, triterpene-treated cells.



Downloaded from <http://aacrjournals.org/cancerres/article-pdf/67/8/3741/2581689/3741.pdf> by guest on 23 May 2024

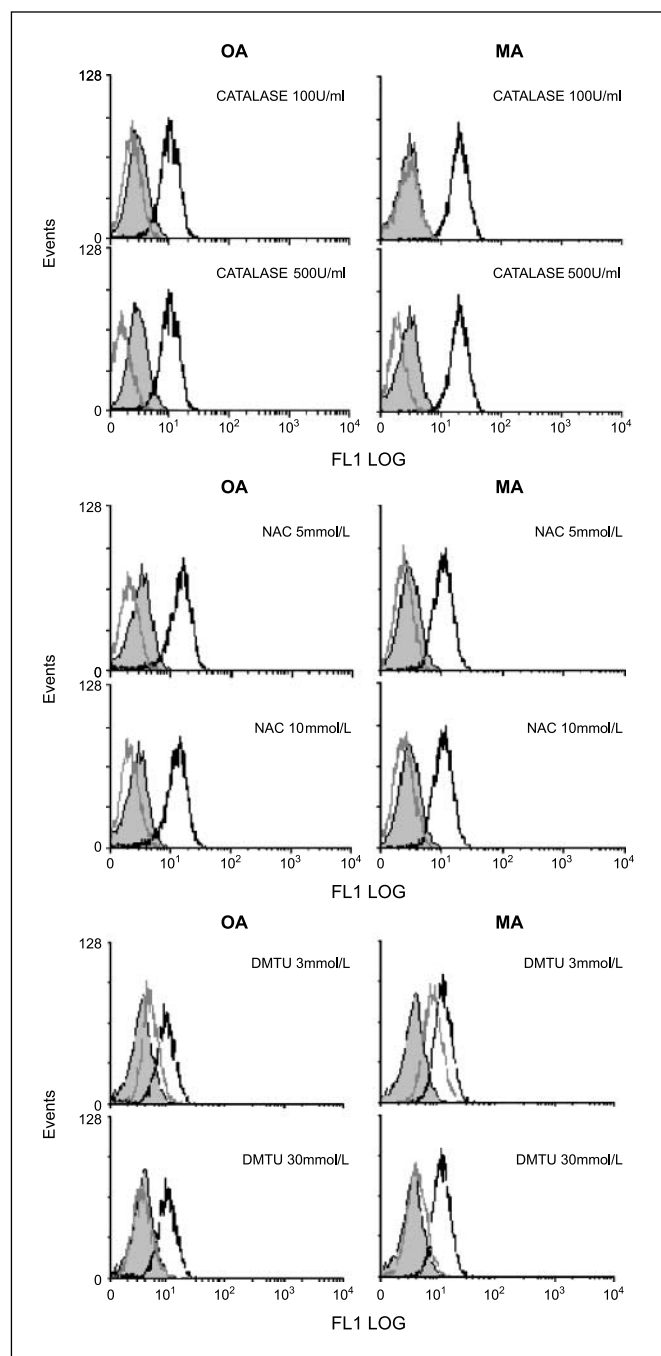


Figure 5. Effects of antioxidants on intracellular ROS production in 1321N1 cells. Cells were stimulated for 30 min with 25 $\mu\text{mol/L}$ oleanolic acid or maslinic acid in the absence or presence of different antioxidants. *Solid gray curves*, cells cultured in the absence of any treatment (control); *empty gray curves*, triterpene-treated cells in the absence of the antioxidant; *empty black curves*, triterpene-treated cells in the presence of the antioxidant.

maslinic acid increased Annexin V binding by $\sim 74\%$ and 62% , respectively, compared with cells that were not exposed to triterpenes. When the antioxidant was added to the cell culture medium, the extent of apoptosis was significantly reduced in a concentration-dependent manner, except for NAC that was almost ineffective. The representative effect of antioxidants on the triterpene-induced astrocytic death was determined morphologically under a phase-contrast microscope (Fig. 6A) and by

fluorescence-activated cell sorting (FACS) analysis after staining with Annexin V-phycoerythrin (Fig. 6B). Cells were treated for 30 min with 500 units/mL catalase, 30 mmol/L DMTU, or 10 $\mu\text{mol/L}$ BHT, before exposure to 25 $\mu\text{mol/L}$ of the acidic triterpenes for 18 h, and as shown in Fig. 6A and B, the antioxidants at the concentration used, protected cells from oleanolic acid- or maslinic acid-induced cell death/damage.

The results showed that these antioxidants could significantly reduce both DCF and Annexin V fluorescence in a dose-dependent manner, attenuating the cell injury and apoptotic effect of oleanolic acid or maslinic acid treatment. Moreover, the results suggest that enhanced ROS production was directly associated with acidic triterpene-induced programmed cell death on 1321N1 cells.

Triterpene-associated apoptosis is not restricted to 1321N1 cells. To rule out the possibility of a cell line-specific phenomenon, we examined the ability of oleanolic acid and maslinic acid to promote apoptosis in three additional human astrocytoma cell lines (U373, U118, and U87). After exposure to 5 or 25 $\mu\text{mol/L}$ oleanolic acid or maslinic acid for 18 h, the cell lines were assayed for Annexin V. Although the extent of the effects observed varied from cell to cell, all cell types under study were found to bind Annexin V to a significantly higher degree than untreated cells, indicating that all of them undergo programmed cell death (Fig. 6C and D).

Discussion

The present study is the first to evaluate the potential antitumoral effects of oleanolic acid and maslinic acid on human astrocytoma cell lines. Oleanolic acid and maslinic acid have a similar steroidal structure, but present small differences in the A loop: with the addition of a hydroxyl group in C2, oleanolic acid becomes maslinic acid. Nevertheless, both triterpenes showed similar activity, shutting down the runaway proliferation of 1321N1 cells. Although we obtained them from pressed olive (*O. europaea*) fruits, they are widely distributed in plants, and because of its steroid-like structure it is not surprising that plants with triterpenoids have anti-inflammatory properties.

We report here the novel finding that oleanolic acid and maslinic acid inhibit DNA synthesis and induce apoptosis in human 1321N1 astrocytoma cells in a dose-dependent manner, fast and efficiently (18 h). This conclusion is based on the following evidences: appearance of typical apoptotic morphologic features, redistribution of cells to the subdiploid phase of the cell cycle, translocation of plasma membrane phosphatidylserine, and generation of processed caspase-3. Indeed, these hallmarks are accompanied of ROS accumulation and disruption of $\Delta\Psi_m$. The morphologic changes, such as chromatin condensation and fragmentation, cytoskeletal rearrangements, as well as cell shrinkage and detachment, were shown by both phase-contrast optical and fluorescence microscopy. Treatment with triterpenes resulted in cells being more rounded and phase bright, showing a significant reduction in the ability to both adhere and spread. Immunofluorescence showed that F-actin-containing stress fibers were reduced in triterpene-treated cells, with a concomitant gross change in the intermediate filament organization.

Our observation, that the inhibition of proliferation induced by triterpenes in astrocytoma cell lines is mediated via apoptosis, is consistent with earlier reports that show how these active triterpenes produce a wide variety of inhibitory effects on tumor promotion (21–24, 28), as well as antiproliferative and proapoptotic effects on human cancer cells of different lineage such as leukemia,

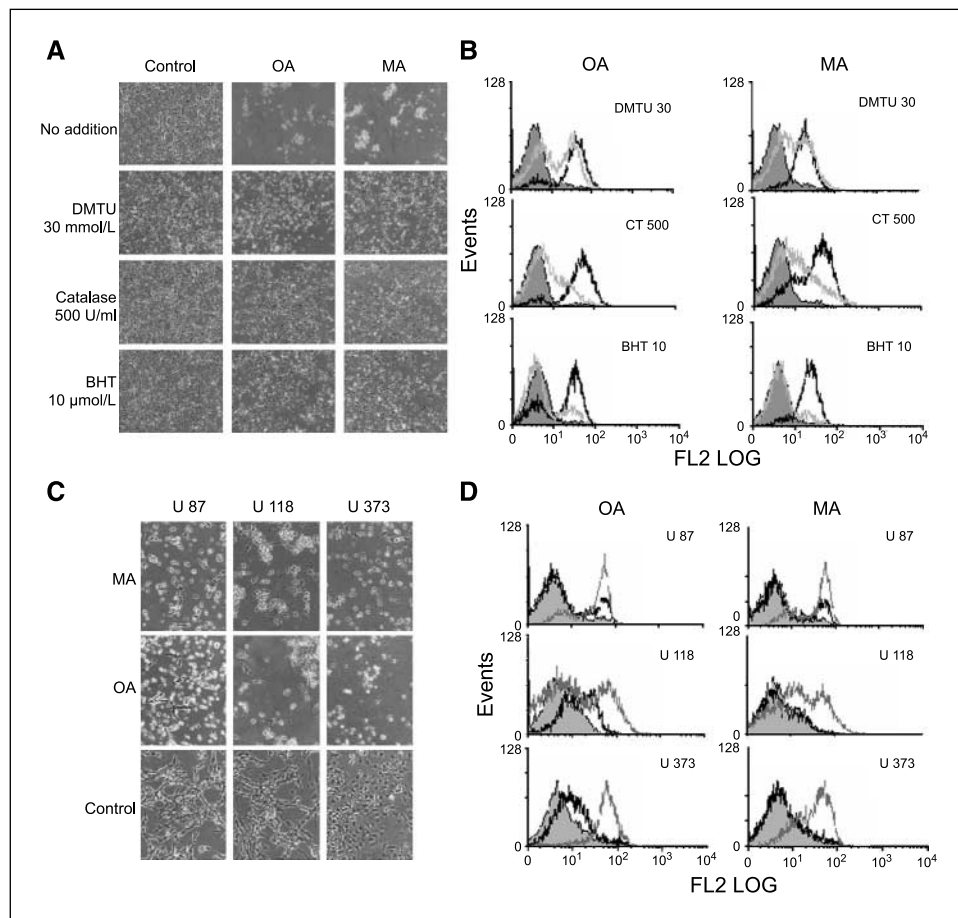
mamarian and colon adenocarcinoma, or melanoma cells (25, 29–33). However, the precise biochemical basis of these antitumor activities remains to be determined. Some preliminary studies on their mechanism of action have shown nuclear factor- κ B (NF- κ B) transactivation in RAW 264 macrophages (34) and caspase-3 activation in human colorectal adenocarcinoma cell lines (25, 35). As reported for human cancer cell lines by other investigators, modulation of the NF- κ B signal transduction pathway is a key mechanism for the antitumoral activity of various drugs, from bioactive triterpenes: natural, such as celastrol (36), or synthetic, such as CDDO (37) and CDDO-imidazolide (38), to nonsteroidal anti-inflammatory drugs, such as aspirin (39). In addition, because triterpenes have a common steroid-like structure, it would not be surprising that they acted like some steroid-related molecules, modulating similar transduction pathways, including NF- κ B (40). However, in 1321N1 astrocytoma cells, no evidence of NF- κ B regulation was observed after oleanolic acid or maslinic acid treatment, at any time or dose tested (data not shown). In fact, NF- κ B activation induced by tumor necrosis factor α in these cells is neither potentiated nor attenuated by the presence of the triterpenes (data not shown), indicating that the oleanolic acid and maslinic acid antineoplastic activity involves a complex set of biochemical pathways, depending on cell type and context.

Our data suggest that oleanolic acid and maslinic acid act as stressors to which these cells respond by generating ROS and decreasing the mitochondrial membrane potential, indications of heightened oxidative stress, which may promote a cascade of molecular events that prompts a cell to commit suicide. Significant

and persistent increases in intracellular ROS causes severe oxidative stress within cells, through the formation of oxidized cellular macromolecules, including lipids, proteins, and DNA. Finally, their cleavage may generate enough structural and functional damage to trigger cell death. We found in our study that triterpene-induced ROS accumulation preceded triterpene-induced apoptosis, being detected as early as 30 min after treatment. In addition, pretreatment with antioxidants inhibited triterpene-induced ROS generation and subsequent cell death. Taken together, these findings suggest that among the pleiotropic effects of oleanolic acid and maslinic acid on cancer cells, in 1321N1 they act as compounds to which the cell responds by accumulating ROS and causing perturbation of mitochondrial functions, ultimately leading to apoptosis. However, the redox status leading to cell death is not a general mechanism of action of pentacyclic triterpenes. Although this also occurs in HT-29 human colon adenocarcinoma cells (25), some studies in HL-60 cells have reported that the natural echinocystic acid induces apoptosis-mediated mitochondria dysfunction, independently of ROS generation (41). Moreover, the novel synthetic analogue of the oleanolic acid, methyl-2-cyano-3,12-dioxoleana-1,9-dien-28-oate, promotes apoptosis without inducing oxidative stress in multiple myeloma cells (42); even ursolic acid prevents the PC12 cell from reactive oxygen species toxicity.

However, a general process in cells in response to a plethora of apoptosis inducers is the rearrangement of cytoskeleton organization. In this process, the components of the cytoskeleton network, which extend throughout the entire cytoplasm of a cell, acquire

Figure 6. Triterpene-induced cell damage: role of antioxidants and role in different human astrocytoma cells lines. 1321N1 cells were stimulated for 18 h with 25 μ mol/L oleanolic acid or maslinic acid in the absence or presence of the indicated antioxidants. Then, the cells were analyzed by phase-contrast microscopy using a Nikon Eclipse TS100 microscope ($\times 20$; A) or labeled with Annexin V-phycoerythrin and analyzed by flow cytometry (B). In the histograms, cells obtained after triterpene treatment (*open black curves*) in the absence of antioxidants are compared with those of cells treated in the presence of antioxidants (*open gray curves*). *Gray solid curves*, resting control cells. C and D, the indicated cell lines were treated with or without 25 μ mol/L of oleanolic acid or maslinic acid for 18 h. Then, cells were analyzed by phase-contrast microscopy using a Nikon Eclipse TS100 microscope ($\times 20$; C) or labeled with Annexin V-phycoerythrin and analyzed by flow cytometry (D). *Solid gray curves*, untreated control cells; *open black and gray curves*, 5 and 25 μ mol/L triterpene-treated cells, respectively.



Downloaded from <http://aacrjournals.org/cancerres/article-pdf/67/8/3741/2581689/3741.pdf> by guest on 23 May 2024

special relevance. Thus, actin, an element of microfilaments, tubulin, a constituent of microtubules, and vimentin, one of the proteins of intermediate filaments, apart from structural support, exert important functions in cell motility, mitosis, and intracellular trafficking; moreover, changes in the distribution/dynamics of these cytoskeletal proteins seem to be consistent with features of apoptosis (43). Although the mechanism is not well understood, the involvement of cytoskeletal actin in apoptosis has been suggested in some morphologic studies, which show actin cleavage during morphologic apoptosis (44). In addition, it has also been shown that caspase proteolysis of vimentin filaments plays an active role in the execution of apoptosis and serves to amplify the cell death signaling (45).

In our study, triterpene-treated cells showed impaired adherence, disruption of the actin filaments, stronger F-actin labeling in the center of the cell, and altered distribution of vimentin. Similar actions have been reported for other triterpenes and cytostatic drugs in prostate carcinoma cells (46), human lung cancer (47), or leukemia cells (48). It seems likely that the proteolytic disassembly of the rigid cytoskeleton network may facilitate many of the morphologic manifestations of apoptosis, including cellular rounding.

It is noteworthy that the effect of maslinic acid and oleanolic acid on apoptosis induction is not selective for 1321N1 cell line, because they induce apoptosis in other astrocytoma cell lines, such as U87, U373, or U118, suggesting a broad spectrum of action and, therefore, a potential interest of these compounds in brain cancer patients.

In summary, we found that oleanolic acid and maslinic acid inhibit DNA synthesis and induce apoptotic events in human astrocytoma cells. Our findings show that the pentacyclic triterpenes oleanolic acid and maslinic acid induce an oxidative apoptotic pathway, causing cell cycle and cytoskeleton alterations. Moreover, because recent evidences suggest that the failure of cells to undergo apoptotic cell death may be involved not only in cancer but also in the pathogenesis of a variety of human diseases, including autoimmune diseases, this opens new expectations to design more specific therapies directed to remove cells that have lost growth control by modifying their ability to activate the intrinsic suicide program. Therefore, these results support the hypothesis that oleanolic acid and maslinic acid may be useful molecules for the development of better antiproliferative agents.

Acknowledgments

Received 12/27/2006; accepted 2/12/2007.

Grant support: Plan Nacional de Salud y Farmacia (SAF2005-01242), CICYT (AGL-2005-0572), and Junta de Andalucía (FS/FN/ARM:AGR163). M. Hernández is under the Ramon y Cajal Program (co-funded by F.S.E.) and E. Ibeas, R. Martín, and J. Carvahlo are supported by a fellowship from the Spanish Ministerio de Educación y Ciencia.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank P. Maeso and M. García (supported by the Instituto de Salud Carlos III, Red de Centros RECAVA, C03/01) for their expert technical support.

References

1. Staunton MJ, Gaffney EF. Tumour type is a determinant of susceptibility to apoptosis. *Am J Clin Pathol* 1995;103:300-7.
2. Tannock IF. Cell proliferation. In: Tannock IF, Hill RP, editors. *The basic science of oncology*. 2nd ed. New York: McGraw-Hill, Inc.; 1990. p. 154-77.
3. Corcoran GB, Fix L, Jones DP, et al. Apoptosis: molecular control point in toxicity. *Toxicol Appl Pharmacol* 1994;128:169-81.
4. Green DR. Apoptotic pathways: paper wraps stone blunts scissors. *Cell* 2000;102:1-4.
5. Wang X. The expanding role of mitochondria in apoptosis. *Genes Dev* 2001;15:2922-33.
6. Hengartner MO. The biochemistry of apoptosis. *Nature* 2000;407:770-6.
7. Chinnaiyan AM, Dixit VM. The cell-death machine. *Curr Biol* 1996;6:555-62.
8. Zhivotovsky B, Burgess DH, Orrenius S. Proteases in apoptosis. *Experientia* 1996;52:968-78.
9. Arends MJ, Wyllie AH. Apoptosis: mechanisms and roles in pathology. *Int Rev Exp Pathol* 1991;32:223-54.
10. Cohen JJ, Duke RC, Fadok VA, Sellins KS. Apoptosis and programmed cell death in immunity. *Annu Rev Immunol* 1992;10:267-93.
11. Buttke TM, Sandstrom PA. Oxidative stress as a mediator of apoptosis. *Immunol Today* 1994;15:7-10.
12. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science* 2004;305:626-9.
13. Saelens X, Festjens N, Walle LV, et al. Toxic proteins released from mitochondria in cell death. *Oncogene* 2004;23:2861-74.
14. Muzio M, Chinnaiyan AM, Kischkel FC, et al. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* 1996;85:817-27.
15. Boldin MP, Goncharov TM, Goltsev YV, Wallach D. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* 1996;85:803-15.
16. Ashkenazi A. Targeting death and decoy receptors of the tumor-necrosis factor superfamily. *Nat Rev Cancer* 2002;2:420-30.
17. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 2002;108:153-64.
18. Fulda S, Debatin KM. Targeting apoptosis pathways in cancer therapy. *Curr Cancer Drug Targets* 2004;4:569-76.
19. Huang MT, Ho CT, Wang Z, et al. Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid. *Cancer Res* 1994;54:701-8.
20. Nishino H, Nishino A, Takayasu J, et al. Inhibition of the tumor-promoting action of 12-O-tetradecanoylphorbol-13-acetate by some oleanane-type triterpenoid compounds. *Cancer Res* 1988;48:5210-5.
21. Yoshimi N, Wang A, Morishita Y, et al. Modifying effects of fungal and herb metabolites on azoxymethane-induced intestinal carcinogenesis in rats. *Jpn J Cancer Res* 1992;83:1273-8.
22. Sohn KH, Lee HY, Chung HY, Young HS, Yi SY, Kim KW. Anti-angiogenic activity of triterpene acids. *Cancer Lett* 1995;94:213-8.
23. Tokuda H, Ohigashi H, Koshimizu K, Ito Y. Inhibitory effects of ursolic and oleanolic acid on skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Lett* 1986;33:279-85.
24. Hsu HY, Yang JJ, Lin CC. Effects of oleanolic acid and ursolic acid on inhibiting tumor growth and enhancing the recovery of hematopoietic system postirradiation in mice. *Cancer Lett* 1997;111:7-13.
25. Juan ME, Wenzel U, Ruiz-Gutierrez V, Daniel H, Planas JM. Olive fruit extracts inhibit proliferation and induce apoptosis in HT-29 human colon cancer cells. *J Nutr* 2006;136:2553-7.
26. Tsang WP, Chau SP, Kong SK, Fung KP, Kwok TT. Reactive oxygen species mediate doxorubicin induced-p53-independent apoptosis. *Life Sci* 2003;73:2047-58.
27. Jacobson MD. Reactive oxygen species and programmed cell death. *Trends Biochem Sci* 1996;21:83-6.
28. Liu J. Pharmacology of oleanolic acid and ursolic acid. *J Ethnopharmacol* 1995;49:57-68.
29. Li J, Guo W-J, Yang Q-Y. Effects of ursolic acid and oleanolic acid on human colon carcinoma cell line HCT15. *World J Gastroenterol* 2002;8:493-5.
30. Hata K, Hori Ki, Takahashi S. Differentiation- and apoptosis-inducing activities by pentacyclic triterpenes on a mouse melanoma cell line. *J Nat Prod* 2002;65:645-8.
31. Ko H-H, Yen M-H, Wu R-R, Won S-J, Lin C-N. Cytotoxic isoprenylated flavans of *Broussonetia kazinoki*. *J Nat Prod* 1999;62:164-6.
32. Taniguchi S, Imayoshi Y, Kobayashi E, et al. Production of bioactive triterpenes by *Eriobotrya japonica* calli. *Phytochemistry* 2002;59:315-23.
33. Amico V, Barresi V, Condorelli D, Spatafora C, Tringali C. Antiproliferative terpenoids from almond hulls (*Prunus dulcis*): identification and structure-activity relationships. *J Agric Food Chem* 2006;54:810-4.
34. Choi CY, You HJ, Jeong HG. Nitric oxide and tumor necrosis factor- α production by oleanolic acid via nuclear factor- κ B activation in macrophages. *Biochem Biophys Res Commun* 2001;288:49-55.
35. Reyes F, Centelles J, Lupiáñez J, Cascante M. (2 α ,3 β)-2,3-Dihydroxyolean-12-en-28-oic acid, a new natural triterpene from *Olea europea*, induces caspase dependent apoptosis selectively in colon adenocarcinoma cells. *FEBS Lett* 2006;580:6302-10.
36. Sethi G, Ahn KS, Pandey MK, Aggarwal BB. Celastrol, a novel triterpene, potentiates TNF-induced apoptosis and suppresses invasion of tumor cells by inhibiting NF- κ B-regulated gene products and TAK1-mediated NF- κ B activation. *Blood* 2006. Epub ahead of print.
37. Stadheim TA, Suh N, Ganju N, Sporn MB, Eastman A. The novel triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) potentially enhances apoptosis induced by tumor necrosis factor in human leukemia cells. *J Biol Chem* 2002;277:16448-55.
38. Hans SS, Peng L, Chung ST, et al. CDDO-Imidazole inhibits growth and survival of c-Myc-induced mouse

- B cell and plasma cell neoplasms. *Mol Cancer* 2006;5:22–37.
39. Stark LA, Reid K, Sansom OJ, et al. Aspirin activates the NF- κ B signalling pathway and induces apoptosis in intestinal neoplasia in two *in vivo* models of human colorectal cancer. *Carcinogenesis* 2006. Epub ahead of print.
40. Ramdas J, Harmon JM. Glucocorticoid-induced apoptosis and regulation of NF- κ B activity in human leukemic T cells. *Endocrinology* 1998;139:3813–21.
41. Tong X, Lin S, Fujii M, Hou DX. Echinocystic acid induces apoptosis in HL-60 cells through mitochondria-mediated death pathway. *Cancer Lett* 2004;212:21–32.
42. Yue P, Zhou Z, Khuri FR, Sun SY. Depletion of intracellular glutathione contributes to JNK-mediated death receptor 5 upregulation and apoptosis induction by the novel synthetic triterpenoid methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me). *Cancer Biol Ther* 2006;5:492–7.
43. Ayscough KR, Gourlay CW. The actin cytoskeleton: a key regulator of apoptosis and ageing? *Nat Rev Mol Cell Biol* 2005;6:583–9.
44. Mashima T, Naito M, Fujita N, Noguchi K, Tsuruo T. Identification of actin as a substrate of ICE and an ICE-like protease and involvement of an ICE-like protease but not ICE in Vp-16-induced U937 apoptosis. *Biochem Biophys Res Commun* 1995;217:1185–92.
45. Byun Y, Chen F, Chang R, Trivedi M, Green KJ, Cryns VL. Caspase cleavage of vimentin disrupts intermediate filaments and promotes apoptosis. *Cell Death Differ* 2001;8:443–50.
46. Duncan KL, Duncan MD, Alley MC, Sausville EA. Cucurbitacin E-induced disruption of the actin and vimentin cytoskeleton in prostate carcinoma cells. *Biochem Pharmacol* 1996;52:1553–60.
47. Engeland MV, Kuijpers HJ, Ramaekers FC, Reutelingsperger CP, Schutte B. Plasma membrane alterations and cytoskeletal changes in apoptosis. *Exp Cell Res* 1997;235:421–30.
48. Grzanka A, Grzanka D, Orlikowska M. Cytoskeletal reorganization during process of apoptosis induced by cytostatic drugs in K-562 and HL-60 leukemia cell lines. *Biochem Pharmacol* 2003;66:1611–7.