Maslinic acid, a natural triterpene from *Olea europaea* L., induces apoptosis in HT29 human colon-cancer cells via the mitochondrial apoptotic pathway

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**Abstract**

We have investigated the mechanisms of maslinic acid with regard to its inhibitory effects on the growth of HT29 colon-cancer cells. High concentrations of maslinic acid are present in the protective wax-like coating of olives. Our results show that treatment with maslinic acid results in a significant inhibition of cell proliferation in a dose-dependent manner and causes apoptotic death in colon-cancer cells. We found that it inhibits considerably the expression of Bcl-2 whilst increasing that of Bax; it also stimulates the release of mitochondrial cytochrome-c and activates caspase-9 and caspase-3. All these results point clearly to the activation of the mitochondrial apoptotic pathway in response to the treatment of HT29 colon-cancer cells with maslinic acid. Our results suggest that maslinic acid has the potential to provide significant natural defence against colon-cancer.

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1. Introduction

In recent years intensive efforts have been made to identify new natural antitumoral agents. Triterpenoids are compounds present in a wide range of plants used in traditional medicine and known to have antitumoral properties [1–3]. Low concentrations of maslinic acid (Fig. 1) are to be found in plants with medicinal properties, such as *Cornus kousa*, *Junilla aspera*, *Malus pumila*, and *Ulmus pumila* [4–7] but its concentration in the waxy skin of *Olea europaea* L. may be as high as 80%. It is known for its important effects as an anti-oxidant [4,8,9], an anti-inflammatory [10] and its anti-microbial and anti-viral activities [11–13]. The identification of new cytotoxic agents that enhance or restore the capability of malignant tumour cells to undergo apoptosis may be crucial for more effective anticancer therapies. Triterpene compounds have been reported to be effective against some drug-resistant cells [14–16]. Nevertheless, the mechanism for the antitumoral activity of maslinic acid has not yet been explored. The normal process of apoptosis is inactive in several cancer cells lines but in many cases natural compounds can restore this process through two major pathways: the intrinsic and the extrinsic [17–19].

One key mechanism in the process involved in the function of antitumoral drugs is the activation of the mitochondrial apoptotic pathway. The mitochondrial function via the intrinsic apoptotic pathway (mitochondrial pathway) appears to be controlled by the Bcl-2 family of proteins [19,20]. The inhibition of Bcl-2, an anti-apoptotic protein of the Bcl-2 family, and the activation of Bax, a pro-apoptotic protein of the Bcl-2 family, result in mitochondrial disruption and the release of pro-apoptotic mitochondrial factors such as cytochrome-c. When released cytochrome-c
interacts with Apaf-1 and activates caspase-9, which in turn proteolytically activates caspase-3 downstream; this caspase is the principal protease in the induction phase of apoptosis. We show here that maslinic acid induces cell death through the direct activation of the mitochondrial apoptotic pathway.

Bcl-2 protein is over-expressed in many types of cancer cell. It blocks apoptotic cell death and protects the disruption of mitochondrial cells. Other triterpenoids such as betulinic acid have been shown to induce apoptosis by activating this pathway, although the over-expression of Bcl-2 tends to block the apoptosis produced by this compound. Bcl-2 inhibition has also been observed in the CDDO and CDDO-Me, in which case a loss of mitochondrial membrane potential and Bax activation has been found [15,21]. Finally, it has been reported that other oleanane derivatives such as oleanolic acid are responsible for a wide variety of antitumoral effects in vivo, including the inhibition of angiogenesis [22] and tumour promotion [15], but the molecular mechanisms by which natural oleananes promote these processes have not yet been fully identified.

Anti-tumoral effects of maslinic acid have been reported previously in CaCo2 and HT29 colon-cancer cell lines [23,24] and in the astrocytome cell line [25]. A loss of mitochondrial membrane potential has also been described in response to maslinic-acid treatment [23,25]. Kim et al. have reported the cytotoxic effect of maslinic acid on the following tumor cell lines: A549 (non-small lung cells), SK-OV-3 (ovary), SK-MEL-2 (melanoma), XF-498 (central nervous system), and HCT-15 (colon) [26]. The detailed molecular mechanism by which maslinic acid induces its apoptotic and cytotoxic effects has not, however, been reported before this present study.

We have evidence to show that maslinic acid exerts anti-proliferative and pro-apoptotic effects in the HT29 colon adenocarcinoma cell line. It also induces morphological changes that are characteristic of apoptosis, such as chromatin condensation and fragmentation, as well as cell shrinkage [23]. We describe in this paper the morphological apoptotic changes and percentage of apoptotic cells as determined by fluorescence microscopy, and propose a possible molecular mechanism to explain these processes. Our results suggest that maslinic acid acts by directly inhibiting the expression of Bcl-2, increasing that of Bax, releasing cytochrome-c from the mitochondria and activating caspase-9 and then caspase-3. We report for the first time the precise molecular mechanism by which maslinic acid produces its apoptotic effects in HT29 colon carcinoma cells. Natural agents such as maslinic acid that are able to activate the intrinsic process of programmed cell death may prove to be a useful approach both to the chemoprevention and chemotherapy of cancer. Thus, maslinic acid isolated from olive pomace may provide a useful new therapeutic strategy for the treatment of colon carcinoma.

2. Materials and methods

2.1. Materials

Dulbecco’s modified Eagle’s medium (DMEM, Sigma, St. Louis, MO, USA), foetal calf serum (FCS, Gibco-BRL, Eggenstein, Germany), penicillin/streptomycin (Gibco-BRL, Eggenstein, Germany), phosphate buffered saline (PBS, Sigma, St. Louis, MO, USA), mowiol (Calbiochem, Merck, Darmstadt, Germany), annexin V-FITC (Bender Med-Systems, Vienna, Austria), propidium iodide (PI, Sigma, St. Louis, MO, USA), culture flasks, and well-plates (Techno Plastic Products, Trasadingen, Switzerland).

2.2. Drugs

Maslinic acid, a natural hydroxyl pentacyclic derivative from pressed olives (Olea europaea L.), was obtained from olive pomace using the method described by Garcia-Granados et al. [27]. The extract used was a white powder comprising 98% maslinic acid and 2% of oleanolic acid. This extract was stable when stored at 4°C. It was dissolved before use at 10 mg/ml in 25% DMSO and 75% PBS. A stock solution was frozen and stored at −20°C. Prior to experiments this solution was diluted in cell-culture medium. All experiments were made at IC50 = 28.8 ± 0.9 μg/ml and IC80 = 37.5 ± 0.2 μg/ml, the values of maslinic-acid concentrations required for 50% and 80% growth inhibition after 72 h treatment.

2.3. Cell culture

Human colorectal adenocarcinoma cell line HT29 (ECACC no. 91072201, provided by the cell bank of the University of Granada, Spain) was cultured in DMEM supplemented with 2 mM glutamine, 10% heat-inactivated FCS, 10,000 units/ml of penicillin and 10 mg/ml of streptomycin. Subconfluent monolayers of cells were used in all experiments.

2.4. Cell proliferation activity assay

The effect of treatment with maslinic acid upon proliferation in HT29 colon-cancer cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
IC50 and IC80 concentrations. After treatment the cells were washed with ice-cold PBS and resuspended in ice-cold PBS and resuspended in 200 μL of lysis buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM ortovanadate, 1 mM sodium glycerophosphate, 5 mM sodium fluoride, 1 mM sodium pyrophosphate, 5 mM β-mercaptoethanol, 1 mM bezamidine, 35 μg/mL PMSF, and 5 μg/mL leupeptine). The samples were homogenized ultrasonically and incubated on ice for 20 min before being centrifuged at 12,000 g for 15 min. The supernatants were assayed for protein concentration using a BCA assay kit (Pierce Biotechnology, Rockford, USA).

For Western blot analyses 25–50 μg proteins were loaded onto 15% SDS–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Richmond, California, USA). The membranes were blocked by incubation in TBS buffer containing 0.1% Tween 20 and 5% dry milk for 1 h at room temperature and washed 3 times with TBS buffer containing 0.1% Tween 20. The membranes were then blotted overnight at 4 °C with primary antibodies [rabbit polyclonal anti-caspase 3 and anti-caspase-9, 1/1000 dilution (Cell Signalling Technology, Danvers, USA), mouse monoclonal anti-Bcl-2 1/500 and rabbit polyclonal anti-Bax, 1/500 dilution (Santa Cruz Biotechnology, Santa Cruz, California, USA)]. For cytochrome-c determination, membranes were blotted for 1 h at 25 °C with a mouse monoclonal primary antibody anti-cytochrome-c, 1/3000 (Santa Cruz Biotechnology, Santa Cruz, California, USA). All blots were developed by ECL Western Blotting Detection Kit Reagent (Amersham Biosciences, Freiburg, Germany) and detected using an LAS-3000.
imaging system (Fuji Photo Film Europe, TK Tilburg, The Netherlands).

2.9. Statistics

Data are given as the means ± SEM. For each assay Student’s t-test was used for statistical comparison with the untreated control cells.

3. Results

3.1. Effects of maslinic acid on HT29 colon-cancer cell proliferation

In a previous work [23], we evaluated the differentiation-inducing activity and the selective antitumoral effect of maslinic acid, a newly discovered triterpenoid isolated as the main compound from olive-skin pomace. In this study we have evaluated the effects of maslinic acid on cell proliferation (Fig. 2) and apoptosis in HT29 colon-cancer cells. In this

![Fig. 2. Right, diagram of annexin V/propidium iodide flow cytometry. HT29 cells were labelled with annexin V-FITC/IP as described in Section 2. The upper quadrants of each diagram (annexin+/IP− and annexin+/IP+) represent apoptotic cells for each treatment. Left, optical-microscope images of HT29 cells, untreated cells (CT) and cells incubated with maslinic acid at the IC_{50} and IC_{80} concentrations for 72 h.]
cell line the concentration of maslinic acid required for 50% growth inhibition (IC50) was 28.8 ± 0.9 μg/mL and the concentration required for 80% growth inhibition (IC80) was 37.5 ± 0.2 μg/mL.

3.2. Maslinic acid induces apoptosis in HT29 colon adenocarcinoma cells

Annexin V-FITC staining and propidium iodide (PI) accumulation were used to determine the percentage of apoptotic cells as well as the morphological changes characteristic of this process. Early events in the apoptotic process are loss of plasma membrane asymmetry accompanied by translocation of phosphatidylserine (PS) from the inner to the outer membrane leaflet, thereby exposing PS to the external environment [28]. The phospholipid-binding protein annexin V has a high affinity for PS and binds to cells fluorescently labelled with FITC (fluorescein isothiocyanate). The percentages of apoptosis, as determined with annexin V-FITC/PI flow cytometric analysis (Fig. 3), were 25% and 38% annexin V-FITC positive cells at the IC50 and IC80 concentrations, respectively. These results show that the number of apoptotic cells increased concomitantly with dosage and that annexin V/PI double-positive cells also increased at IC80.

We went on to verify these results by fluorescence microscopy. In comparison with untreated controls, maslinic acid treatment of HT29 cells for 72 h induced apoptosis in 31% of cells at the IC50 concentration, 16% of the cells being stained with eosin. At the IC80 concentration, the percentage of apoptosis was 46%, 23% of the cells being stained with eosin. Fluorescence microscopic observations showed that a significant number of cells treated with maslinic acid acquired apoptotic features (Fig. 4). As far as morphological changes are concerned, the cells appeared clearly stained with annexin V-FITC and showed typical apoptotic features, including cell shrinkage, chromatin condensation and a loss of normal nuclear architecture. At IC80 the number of apoptotic cells with disruption of cell-membrane integrity was more evident.

3.3. Maslinic acid induces apoptosis via Bax activation and Bcl-2 inhibition

Mitochondrial integrity is regulated by pro-apoptotic and anti-apoptotic members of the Bcl-2 group of proteins such as Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic). Bcl-2 protects cells from the induction of apoptosis by interacting with pro-apoptotic members of the Bcl-2 group, thus blocking the release of cytochrome-c from the mitochondria. To determine the effects of maslinic acid on the expression of the Bcl-2 protein group, cells were incubated with maslinic acid for 4, 6, 12, and 24 h at the IC50 and IC80 concentrations. We examined Bcl-2 and Bax expression in the HT29 colon-cancer cell line using Western blotting analysis. As shown in Figs. 5 and 6, Bcl-2 inhibition and an increase in Bax were confirmed in response to treatment with maslinic acid. Bax expression increased concomitantly with dosage and incubation time, beginning after 6 h treatment at IC80 (35% with respect to
untreated cells) and 12 h treatment at IC80 (ninefold increase). The maximum level of Bax expression was reached after 24 h treatment at IC80 (ninefold increase). This Bax induction goes hand in hand with the inhibition of Bcl-2. An increase in the Bax/Bcl-2 ratio has often been described in association with activation of the mitochondrial apoptotic route. Our results showed a marked decrease in Bcl-2 expression from 6 h onward after treatment at IC50 (55% with respect to untreated cells) and from 12 h at IC80 (80%), reaching a minimum (90%) at this latter concentration after 24 h.

Both Bax activation and Bcl-2 inhibition are required for the release of cytochrome-c from mitochondria [29]. Following the treatment of HT29 cells with maslinic acid we observed a rapid decrease in Bcl-2 in cell lysates accompanied by an increase in Bax protein levels. To discover whether the inhibition of Bcl-2 and the concomitant increase in Bax expression enhanced the activation of mitochondria-mediated apoptosis we determined the levels of cytochrome-c released.

3.4. Maslinic acid induces cytochrome-c release

A key step in the intrinsic apoptotic pathway is the release of cytochrome-c from the mitochondria to activate Apaf-1, which turns on the caspase cascade. After 24 h treatment of HT29 cells with maslinic acid at IC50 and IC80 concentrations cytochrome-c was detectable in the cytosolic fractions and reached a peak with IC80 (Fig. 7), proving that maslinic acid induces the release of cytochrome-c from the mitochondria in HT29 colon-cancer cells. The release of cytochrome-c from mitochondria is usually preceded or accompanied by a reduction in mitochondrial membrane potential. Once cytochrome-c is released from the mitochondria it binds to Apaf-1 to activate caspase-9, the primary caspase involved in the intrinsic mitochondrial pathway. Caspase-9 then activates caspase-3, which plays a central role in the caspase cascade responsible for the apoptotic process.

3.5. Maslinic acid induces apoptosis by triggering the activation of caspase-9 and caspase-3

The activation of the caspase pathway is known to be an important mechanism in apoptotic cell death in most cell systems [30]. In our experiments treatment with maslinic acid resulted in a significant increase in apoptotic cell death in the HT29 cell line but did not activate caspase-8 at the different times and concentrations used (data not shown) and so the extrinsic apoptotic pathway is probably not involved in this process. Maslinic-acid treatment did however result in a strong dose- and time-dependent cleavage of caspase-9 (Fig. 8) and caspase-3 (Fig. 9).

According to our results, the treatment of HT29 cells with maslinic acid at these doses resulted in the cleavage of caspase-9 as well as downstream caspase-3 after only 6 h treatment, increasing to a maximum effect towards the end of the treatment. As far as caspase-9 is concerned, an increase was observed after 6 h treatment both at IC50 (39% compared to untreated cells) and IC80 (28%). The maximum was reached after 24 h treatment (twofold at IC50 and threefold at IC80). Caspase-3 activation reached a maximum after 6 h treatment (119% at IC50 and 120% at IC80), at which level it remained until 24 h treatment.

In summary, our data clearly indicate that maslinic acid activates the intrinsic caspase cascade via the release of cytochrome-c into the cytosol from the mitochondria. Bcl-2 inhibition and Bax activation, leading to the activation of caspase-9 followed by that of caspase-3, are possibly involved in all the apoptotic phenomena observed.

Fig. 5. Western blotting and Bcl-2 levels. HT29 cells were treated at the IC50 and IC80 concentrations for 4, 6, 12, and 24 h. The levels of protein expression are expressed as arbitrary intensity units of each band compared to arbitrary intensity units of actin. In the variation of protein expression throughout incubation with maslinic acid the dots represent relative percentage compared to the untreated cells for each time and concentration. The values represent means ± SE of three independent experiments.
4. Discussion

Chemopreventive agents of a natural origin, often a part of our daily diet, may provide a cheap, effective way of controlling such diseases as cancer of the colon. A wide range of studies in recent years has shown that triterpenoids hinder carcinogenesis by intervening in pathways such as carcinogen activation, DNA repair, cell cycle arrest, cell differentiation and the induction of apoptosis in cancer cells. This study is the first to investigate the molecular mechanisms of the anti-tumoral and pro-apoptotic effects of maslinic acid, a triterpenoid compound isolated from olive-skin pomace.

We report here on the activity of maslinic acid against colon-cancer using HT29 cells as a model for malignant colon-cancer cells. We observed by fluorescent microscopy and FACS analysis with annexin V-FITC/IP staining that maslinic acid-induced morphological changes characteristic of apoptosis, e.g., the translocation of PS and loss of cell-membrane asymmetry, and was thus responsible for
a significant cytotoxic effect by inducing apoptosis in the cells in question (Fig. 4). The percentage of apoptosis quantified by FACS analysis showed that after 72 h treatment 25% of the HT29 cells shown signs of cell death at IC\textsubscript{50} concentration and 38% at IC\textsubscript{80} concentration. To determine the sequential activation or inhibition of the proteins involved in this signalling pathway, assays of apoptotic protein expression by Western blotting were made after shorter incubation times: 4, 6, 12, and 24 h.

Firstly we analysed caspase expression. Maslinic acid activated caspase-3, the major effector caspase. Our results showed considerable caspase-9 and caspase-3 activity after 6 h treatment but no caspase-8 activity. These results agree with the current notions regarding the activation of the apoptotic intrinsic pathway. Caspase-3 and caspase-9 play an important role in the apoptotic cell program and they were both activated following maslinic acid treatment in HT29 colon-cancer cells. To confirm this mechanism we next analysed the cytosolic levels of cytochrome-c after 24 h, when caspase activity was at its maximum. The release of mitochondrial cytochrome-c is a signal event in the intrinsic apoptotic activation pathway.

Our results show that the treatment of cancerous cells with maslinic acid released cytochrome-c from the mitochondria into the cytosol, suggesting an activation of the intrinsic apoptotic pathway. Mitochondria have been shown to play a central role in the apoptotic process because both the intrinsic and extrinsic pathways can con-
verge at the mitochondrial level and trigger mitochondrial membrane permeabilization [31,32]. After apoptotic-stimulated mitochondrial membrane permeabilization, cytochrome-c and other mitochondrial pro-apoptotic factors are released into the cytosol. The cytochrome-c thus released subsequently triggers the activation of caspases, the induction of the apoptotic process and subsequent cell death. Cytochrome-c binds to Apaf-1 and pro-caspase-9 to form the apoptosome, activating caspase-9, the primary caspase involved in the mitochondrial apoptotic pathway.

Mitochondrial disorganisation is usually mediated by the participation of the Bcl-2 protein group. Bcl-2 and Bax have been identified as major regulators in controlling the release of mitochondrial cytochrome-c [33,34]. Bcl-2 binds to the mitochondrial outer membrane, thus blocking cytochrome-c efflux. In contrast, Bax translocates from the cytosol to the mitochondria where it enhances the release of cytochrome-c and encourages apoptosis. Many anticancer agents or apoptotic stimuli can trigger the release of cytochrome-c through either the down-regulation of Bcl-2 and/or up-regulation of Bax. Treatment with maslinic acid produced these effects in HT29 cells, minimum level of Bcl-2 coincided with the Bax and caspases 3 and 9 maximum levels.

According to these results we propose an intrinsic mechanism for the induction of apoptosis by maslinic acid in HT29 colon-cancer cells (Fig. 10). This mechanism is regulated via the inhibition of Bcl-2 and a concomitant

Fig. 9. Western blotting and pro-caspase-9/caspase-9 levels. HT29 cells were treated at the IC50 and IC80 concentrations for 4, 6, 12, and 24 h. The levels of protein expression are expressed as arbitrary intensity units of each band compared to arbitrary intensity units of actin. In the variation of protein expression throughout incubation with maslinic acid the dots represent relative percentage compared to the untreated cells for each time and concentration. The values represent means ± SE of three independent experiments.
stimulation of Bax protein expression, producing mitochondrial disruption and the release of cytochrome-c, which leads finally to the activation of caspases 9 and 3. Triterpenoids such as betulinic acid, ursolic acid, and CDDO (2-cyano-3,12-dioxooolean-1,9-dien-28-oic acid) have been reported to produce apoptosis by similar mechanisms. The following events have been proposed in the apoptosis induced by pentacyclic triterpenoid: (a) participation of the Bcl-2 protein family; (b) increase in mitochondrial membrane permeability; (c) cytochrome-c release and (d) caspase-9 and -3 activation [35–38].

In conclusion, maslinic acid is a novel natural compound and it is able to induce caspase-dependent apoptosis in human colon-cancer cells via the intrinsic mitochondrial pathway. The precise signalling pathway by which maslinic acid triggers Bax activation and Bcl-2 inhibition remains to be identified. Nevertheless, our data could contribute to the development of maslinic acid and related drugs for use as cancer chemotherapeutic or chemopreventive agents.

Conflicts of interest statement

I, José Antonio Lupiáñez Cara, declare that: I have no proprietary, financial, professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in the manuscript entitled.

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