(2α,3β)-2,3-Dihydroxyolean-12-en-28-oic acid, a new natural triterpene from *Olea europea*, induces caspase dependent apoptosis selectively in colon adenocarcinoma cells

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Abstract Triterpenoids are known to induce apoptosis and to be anti-tumoural. Maslinic acid, a pentacyclic triterpene, is present in high concentrations in olive pomace. This study examines the response of HT29 and Caco-2 colon-cancer cell lines to maslinic-acid treatment. At concentrations inhibiting cell growth by 50–80% (IC\textsubscript{50}HT29 = 61 ± 1 µM, IC\textsubscript{50}Caco-2 = 76 ± 1 µM and IC\textsubscript{50}Caco-2 = 85 ± 5 µM, IC\textsubscript{80}Caco-2 = 116 ± 5 µM), maslinic acid induced strong G0/G1 phase arrest and DNA fragmentation, and increased caspase-3 activity. However, maslinic acid did not alter the cell cycle or induce apoptosis in the non-tumoural intestine cell lines IEC-6 and IEC-18. Moreover, maslinic acid induced cell differentiation in colon adenocarcinoma cells. These findings support a role for maslinic acid as a tumour suppressant and as a possible new therapeutic tool for aberrant cell proliferation in the colon. In this report, we demonstrate for the first time that, in tumoural cancer cells, maslinic acid exerts a significant anti-proliferation effect by inducing an apoptotic process characterized by caspase-3 activation by a p53-independent mechanism, which occurs via mitochondrial disturbances and cytochrome c release.

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1. Introduction

Currently, about one-fourth of all medications contain an active ingredient derived from plants. Pentacyclic triterpenes, biosynthesised in plants by squalene cycling, have been used for more than 2000 years in traditional Asiatic medicine as anti-inflammatory and anti-carcinogenic agents. Moreover, their beneficial effects have been analysed by extensive preclinical and epidemiological studies [1–4]. Some natural triterpenoids such as oleanolic, betulinic and ursolic acids have shown notable effects in suppressing tumourigenesis as well as inhibiting tumours [1,5–9]. Also, a range of newly developed synthetic triterpenoids are more potent than the naturally occurring parent structure [10,11]. In addition, several of these compounds induce apoptosis in a wide variety of cancer cells, including breast carcinoma, melanoma, hepatoma, prostate carcinoma and acute myelogenous leukaemia [7]. To identify potential new anticancer compounds, several pharmaceutical companies are currently evaluating extracts from plants, among these new natural triterpenoids and their derivatives [12–14].

Olives are a rich source of pentacyclic triterpenes such as oleanolic and maslinic acids. Maslinic acid is also the main component of protective wax-like coatings of olives. Researchers have recently sought to characterize oleanolic acid and its derivatives, which present strong anti-tumoural properties [10,11,13,14]. The natural triterpenoid maslinic acid (Fig. 1), which accounts for 80% of the wax in the olive skin, has also been isolated and found to be an antioxidant [15], although its properties as an anti-tumour agent have not yet been described. In this paper, we assess the anticancer properties of maslinic acid isolated from olive pomace. Maslinic acid has potent differentiating and anti-proliferation properties in colon-cancer lines, inducing cell-cycle arrest in the G0 phase and apoptosis, through caspase activation, in these cells, but not in normal intestinal cell lines. Apoptosis or programmed cell death is defined as an active physiological process of cell self-destruction, with specific morphological and biochemical changes in the nucleus and cytoplasm. Apoptotic death is known to involve a cascade of proteolytic events driven mainly by family of cysteine proteases such as caspase-3, the major executioner of apoptosis. Agents that suppress the proliferation of malignant cells, by inducing apoptosis, may represent a useful mechanistic approach to both chemoprevention and chemotherapy of cancer. Due to these effects, maslinic acid isolated from olive pelt may provide a useful new therapeutic strategy for colon carcinoma.

2. Materials and methods

Dulbecco’s modified Eagle’s medium (DMEM, Sigma, St. Louis, MO, USA), foetal bovine serum (FCS, Gibco-BRL, Egggenstein, Germany), penicillin/streptomycin (Gibco-BRL, Egggenstein, Germany), phosphate buffer saline (PBS, Sigma, St. Louis, MO, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO, USA), dimethylsulfoxide (DMSO, Sigma, St. Louis, MO, USA), Hoechst (Sigma, St. Louis, MO, USA), mowiol (Calbiochem, Merck, Darmstadt, Germany), annexin V-FICT (Bender Med-
IEC-18 cells, a cell line derived from the rat intestinal ileum, have normal cells’ characteristics, since they have a contact inhibited cell growth, do not form colonies in soft agar and are not tumorigenic when injected in nude mice. IEC-18 has normal rat diploid karyotype, a similar growth rate and surface antigens to intestinal epithelial cell in vivo [19,23]. IEC-6 and IEC-18 cells express characterised antigens from intestinal epithelia, these cells line have been qualified as normal intestinal cells or non-transformed intestinal cells in numerous papers [22,24–28].

Human colorectal adenocarcinoma cell lines HT29 (ECACC no. 91072201) and Caco-2, (ECACC no. 86010202), as well as normal mammalian intestinal cell lines IEC-6 (ECACC no. 88071401) and IEC-18 (ECACC no. 88011801) (all cell lines used were provided by the cell bank of the University of Granada, Spain) were cultured in DMEM, supplemented with 2 mM glutamine, 10% heat-inactivated FCS, 10000 units/ml of penicillin, and 10 mg/ml of streptomycin. Subconfluent monolayers of cells were used in all experiments.

2.3. MTT assay

Cell viability was determined by measuring the absorbance of MTT dye staining of living cells, as described elsewhere [29]. For this assay, 6 x 10⁴ HT29 cells/well and 15 x 10⁴ Caco-2 cells/well were cultured on 96 well plates.

Concentrations that inhibited cell growth by 50% (IC₅₀) and 80% (IC₈₀) after 72 h of treatment were calculated based on the survival rate compared with untreated cells. Relative cell viability was measured by the absorbance on an ELISA plate reader (Tecan Sunrise MR20-301, TECAN, Austria) at 550 nm.

2.4. Hoechst-stained

The morphological changes were analysed by fluorescent microscopy using Hoechst staining. For this, 175 x 10⁴ HT29 cells/well and 4 x 10⁴ Caco-2 cells/well were plated in 6 well plates. After 24 h maslinic acid was added and the cells were incubated for 24, 48 and 72 h at their respective IC₅₀ values obtained at 72 h. Then the cells were washed twice with PBS and harvested by tripinization. The cells were resuspended in 100% MetOH cold for 3 min. Afterwards, cells were washed in PBS and incubated in 500 µL of Hoechst solution 50 ng/mL in PBS for 15 min in darkness. The samples were prepared with mounting medium mowiol and visualized by fluorescent microscopy (DMRB, Leica Microsystems, Wetzlar, Germany) with a DAPI filter.

2.5. Cell-cycle analysis

The cell cycle was analysed with flow cytometry by using a fluorescence-activated cell sorter (FACS) at 488 nm in an Epics XL flow cytometer (Coulter Corporation, Hialeah, FL, USA), as described previously [30]. For this assay 175 x 10⁴ HT29 cells/well, 4 x 10⁴ Caco-2 cells/well, 150 x 10⁴ IEC-6 cells/well and 50 x 10⁴ IEC-18 cells/well were plated in 6 well plates with 2 ml of medium. After 24 h, maslinic acid was added and the cells were incubated for 72 h with maslinic acid. Maslinic acid doses used were the respective IC₅₀ and IC₈₀ values with the tumoral cell lines. IEC-6 and IEC-8 cells were incubated with maslinic acid concentrations corresponding to the IC₅₀ and IC₈₀ determined for HT29 cells at 72 h. In addition, HT29 cells were incubated with their IC₅₀ and IC₈₀ concentrations for 96 h to observe the appearance of subG₀ peaks. All experiments were performed five times with two replicates per experiment.

2.6 Annexin V-FITC binding assay

The same numbers of HT29, Caco-2, IEC-6 and IEC-18 cells as in the cell-cycle assay were treated with maslinic acid, as described above. Cells were collected and resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). Annexin V-FITC conjugate (1 µg/ml) was added and incubated for 30 min at room temperature in darkness. Just before FACS analysis, cells were stained with 20 µL of 1 mg/ml PI solution. In each experiment, approximately 20 x 10⁵ cells were analysed and the experiment was performed five times.

2.7. Assay for Caspase-3 protease

Caspase-3 activity was measured using a colorimetric assay (caspase-3 colorimetric assay kit, Sigma, St. Louis, MO, USA) based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp...
p-Nitroaniline (Ac-DEVD-pNA) by caspase-3, resulting in the release of the p-nitroaniline. In short, 1.2 × 10^6 HT29 cells/well and 2.7 × 10^6 Caco-2 cells/well were cultured in 100-mm plates and were treated for 72 h with maslinic acid at their IC50 and IC80 concentrations. Cells were lysed with lysis buffer (50 mM HEPES, pH 7.4, 5 mM Chaps, 5 mM dithiothreitol, 2 mM dithiothreitol, 2 mM EDTA) containing 0.2 mM of caspase-3 substrate. The p-nitroaniline concentration was determined by the absorbance measured at 405 nm using an ELISA reader.

2.8. DNA fragmentation analysis
The same numbers of HT29 and Caco-2 cells as for the caspase-3 assay were treated with maslinic acid. Chromosomal DNA was isolated using a real pure extraction kit (Real, Durviz, Valencia, Spain) and ladder-formation assays were made as described previously [18,31].

2.9. Preparation of cytosolic extracts for cytochrome c analysis
HT29 cells (1.2 × 10^6) were cultured in 100-mm plates and were treated for 48 and 72 h with maslinic acid at its IC50 concentrations. After treatment, HT29 cells were washed with ice-cold PBS and resuspended in ice-cold lysis buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of pepstatin A, 2 μg/ml of leupeptin and 10 μg/ml of aprotinin). After incubation on ice for 30 min, cells were homogenized with 15 strokes of pre-chilled pestle B homogenizer (Sigma, St Louis, MO, USA), and the homogenates were centrifuged at 25000 g for 30 min at 4 °C. Supernatants were further centrifuged at 250000 × g for 30 min at 4 °C and stored at −80 °C for cytochrome c analysis.

2.10. Western blotting
HT29 cells (1.2 × 10^6) were treated with maslinic acid at their IC50 values of concentration for 48 and 72 h. Non-cancer cells IEC-6 (1.0 × 10^6) and IEC-18 (3.0 × 10^6) were treated at their IC50 value for 6 and 12 h. After treatment, the cells were washed twice with PBS and resuspended in lysis buffer (20 μM Tris/acetate, pH 7.5, 270 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM EDTA, 1 mM EGTA, 1 mM EGTA, 1% Triton X-100, 1 mM orthovanadate, 1 mM sodium glycerophosphate, 5 mM β-mercaptoethanol, 1 mM benzoamide, 34.8 μg/ml PMSF, 5 μg/ml leupeptin). The samples, homogenized by ultrasonic and incubated on ice for 20 min, were then centrifuged at 120000 × g for 15 min, and supernatants were assayed for protein concentration using BCA kit (Pierce Biotechnology, Rockford, USA).

For the Western-blot analysis, 50 μg of proteins were loaded on a 15% SDS–polyacrylamide gel and transferred to a polyvinyl nitrocellulose transfer membrane (Bio-Rad Laboratories, Richmond, C, USA). The membranes were blocked by incubation in TBS buffer containing 0.1% of Tween and 5% of dry milk for 1 h at room temperature and washed 3 times with TBS buffer of 0.1% Tween. Then membranes were blotted overnight at 4 °C with primary antibodies rabbit polyclonal anti-caspase-3 (1/1000, Cell Signalling Technology, Danvers, 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, BD Biosciences, Erembodegem, Belgium). The blots were washed 3 times with TBS-0.1% Tween and developed with peroxidase-labeled secondary antibodies (1/3000, Santa Cruz Biotechnology, California, USA). All blots were developed by ECL Western Blotting Detection Kit Reagent (Amersham Biosciences, Freiburg, Germany) and detected using LAS-3000 imaging system (Fuji Photo Film (Europe), TK Tilburg, The Netherlands).

2.11. Alkaline phosphatase activity
Alkaline phosphatase activity was measured by p-nitrophenol formation from p-nitrophenyl phosphate (Sigma, St. Louis, MO, USA). For this assay 1.2 × 10^6 HT29 cells/well and 2.7 × 10^6 Caco-2 cells/well were treated with maslinic acid at IC50 and half IC50 concentrations. After incubation, HT29 and Caco-2 cells were collected by scraping. Cells were broken with lysis buffer (20 μM Tris, pH 7.5, 1 mM DTT, 1 mM EDTA, 0.2 g/l Triton X-100, 0.2 g/l of sodium deoxycholate and 0.2 mM PMSF) and centrifuged at 100000 × g for 1 h at 4 °C. Alkaline phosphatase activity was measured in the supernatants as described previously [31].

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

3. Results
3.1. Maslinic acid inhibits colon adenocarcinoma cell proliferation
We examined the effect of maslinic acid on the proliferation of HT29 and Caco-2 adenocarcinoma cell lines using the MTT assay. HT29 and Caco-2 cells were treated with increasing doses of maslinic acid; their viability was determined by formazan dye uptake and expressed as percent of untreated control cell proliferation. Maslinic acid induced a dose-dependent decrease in viable formazan accumulating cells after 72 h of treatment, ranging from 0 to 120 μM (Fig. 2). Concentrations of maslinic acid required for 50% growth inhibition (IC50) were 61 ± 1 μM for HT29 and 85 ± 5 μM for Caco-2 cells and for 80% growth inhibition (IC80) were 76 ± 1 μM for HT29 and 95 ± 2 μM for Caco-2 cells.
116 ± 5 μM for Caco-2 cells. At the IC50 and IC80, the values of maslinic acid found for HT29 after 72 h of incubation the survival rates of IEC-6 were 78% at IC50 and 63% at IC80, and those of IEC-18 were 68% at IC50 and 62% at IC80.

3.2. Maslinic acid induces morphological changes

The Hoechst procedure stains nuclei that contain nicked DNA, a characteristic exhibited by cells in apoptotic cell death. Morphological analysis of Hoechst-stained cells, in HT29 and Caco-2 cells, indicated that they had undergone remarkable morphological changes (Fig. 3). After 48 h exposure to maslinic acid at IC80 concentration, the cells showed typical apoptotics changes, including cell shrinkage, chromatin condensation, and loss of normal nuclear architecture. At 72 h of incubation the disruption of cell-membrane integrity was more prominent. Fluorescence microscopic observation after Hoechst staining showed that a relevant number of cells treated with maslinic acid acquired apoptotic features, as evident from nuclear fragmentation.

3.3. Maslinic acid induces cell-cycle arrest and apoptosis

HT29 and Caco-2 cells treated with maslinic acid at IC50 and IC80 concentrations showed a significant increase in the population in G0/G1 phases (increases of 13% for HT29 and 13% for Caco-2 at IC50, and of 22% for HT29 and 11% for Caco-2 at IC80) with a concomitant decrease in the percentage of cells in the S phase (10% for HT29 and 12% for Caco-2 at IC80) and G2/M (4% for HT29 and 5% for Caco-2 at IC80) as compared to untreated cells (Table 1).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Maslinic (μM)</th>
<th>Phase of cell cycle (% of cells)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>G0/G1</td>
<td>S</td>
</tr>
<tr>
<td>HT29</td>
<td>0</td>
<td>62 ± 5</td>
</tr>
<tr>
<td></td>
<td>61</td>
<td>75 ± 5**</td>
</tr>
<tr>
<td></td>
<td>76</td>
<td>84 ± 6**</td>
</tr>
<tr>
<td>Caco-2</td>
<td>0</td>
<td>76 ± 12</td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>89 ± 5**</td>
</tr>
<tr>
<td></td>
<td>116</td>
<td>87 ± 3*</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>76</td>
<td>70 ± 2</td>
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<tr>
<td>IEC-18</td>
<td>0</td>
<td>77 ± 9</td>
</tr>
<tr>
<td></td>
<td>61</td>
<td>80 ± 10</td>
</tr>
<tr>
<td></td>
<td>76</td>
<td>82 ± 5</td>
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</table>

HT29 and Caco-2 cells were untreated (first value) or treated with maslinic acid at their IC50 (second value) or IC80 (third value) concentrations. IEC-6 and IEC-18 were treated with concentrations of IC50 and IC80 for HT29 cells. All cell lines were treated for 72 h at 37°C. Cell-cycle analysis was conducted after propidium iodide staining, as described in Section 2. Values represent means ± S.E. of seven independent experiments. Key (*) P < 0.05, (**) P < 0.01, with respect to the untreated cells.

IC50, and 18% for HT29 and 15% for Caco-2 at IC80) with respect to untreated cells, suggesting G0/G1 arrest (Table 1).
Moreover, a sub-G0 peak was detected at 96 h of incubation in HT29 cells in response to treatment with maslinic acid, indicating that cells underwent apoptosis. These results suggest that maslinic acid induced cell-cycle arrest in HT29 and Caco-2 cells coinciding with apoptotic cell death. At these concentrations of maslinic acid, no effect was observed on the cell cycle of the normal intestinal cell lines IEC-6 and IEC-18 (Table 1). Assessment of apoptosis in HT29 and Caco-2 cells was performed 72 h after treatment with maslinic acid at the concentrations mentioned above for the analysis of cell cycle.

FACS analysis using Annexin V-FITC staining and PI accumulation was used to differentiate early apoptotic cells (Annexin V+ and PI−) from late apoptotic/necrotic cells (Annexin V+ and PI+). In comparison with untreated controls, maslinic acid treatment of HT29 cells generated apoptosis in 25% of cells at a concentration of IC50 (14% early apoptosis and 11% late apoptosis/necrosis) and in 38% of cells at IC80 (27% early apoptosis plus 11% late apoptosis). In Caco-2 cells the percentage of apoptosis at IC50 was 14% (7% early apoptosis plus 7% late apoptosis/necrosis) with respect to the untreated controls, lower than in HT29, but at IC80 the rate increased dramatically to 60% (15% early apoptosis plus 45% late apoptosis/necrosis), due to a marked increase in the number of late apoptotic cells (Fig. 4). At these concentrations of maslinic acid, no effect on apoptosis induction was observed in the normal intestinal cell lines IEC-6 and IEC-18. Annexin-V binding assay thus confirmed the observation that maslinic acid induced both cell-cycle arrest and apoptosis.

3.4. Involvement of caspase-3 in maslinic acid induced apoptosis

For a direct examination of the involvement of caspase-3 in maslinic-acid-induced apoptosis, caspase-3 activity was determined in cells treated with maslinic acid for 72 h (at IC50 and IC80) using a colorimetric assay based on the hydrolytic rupture of the peptide Ac-DEVD-pNA. As shown in Fig. 5, maslinic acid caused a concentration-dependent activation of caspase-3 in both HT29 and Caco-2 cells.

3.5. Maslinic acid induces DNA fragmentation

Internucleosomal DNA fragmentation in HT29 and Caco-2 cells induced by maslinic acid was detected by DNA ladder on agarose gels. DNA extracted from HT29 and Caco-2 cells treated with maslinic acid for 72 h displayed a characteristic ladder pattern of discontinuous DNA fragments on agarose-gel electrophoresis at both IC50 and IC80, while untreated controls did not (Fig. 6). The effect of maslinic acid on DNA fragmentation was similar in HT29 and Caco-2 cells.

3.6. Western blotting

To begin to address the mechanism by which maslinic acid causes apoptosis, we first examined whether caspase-3 protease is involved in the cell death response in HT29 and Caco-2, and in non-tumoural intestinal cells IEC-6 and IEC-18, for different incubation times. Caspase-3 activity significantly increased after 72 h of treatment (Fig. 5). Then we investigated whether the apparent promotion of apoptosis by maslinic acid could be
linked to a variation in caspase-3 expression at the different incubation times of 6, 12, 48 and 72 h (Fig. 7). At 6 or 12 h of treatment, caspase-3 was activated in Caco-2 cells but not in HT29 cells. In HT29 cells the caspase-3 activation at IC50 was very late (at 72 h of treatment), whereas in Caco-2 cells this activation occurred early (at 6 h of treatment). In non-tumoural cells IEC-6 and IEC-18, no effect was detected on caspase-3 activation at the maslinic-acid concentration used for any of the times assayed.

In most cases the apoptosis involves a disruption of mitochondrial membrane integrity that is decisive for the cell-death process. We next evaluated the effects of maslinic acid on the release of mitochondrial cytochrome c into the cytosol in HT29 cells. Western-blot analysis, shown in Fig. 8, revealed that the treatment of HT29 cells with its IC50 concentration induce the release of mitochondrial cytochrome c into the cytosol at 48 and 72 h. Taken together, these result lead us to conclude that the mitochondria plays an important role in the activation of caspase-3 and in the induction of apoptosis triggered by maslinic acid in colon carcinoma cells.

3.7. Maslinic acid induce increase in alkaline phosphatase activity

To determine the differentiation effect of the maslinic acid on colon cancer cells we have quantified the alkaline phosphatase activity before and after maslinic acid treatment. The increase
of the activity of this enzyme is used as a differentiation marker in colon cancer cells [32].

The result showed an increase in alkaline phosphatase activity in a dose-dependent way at 72 h, being higher at IC50 than at half IC50 concentrations. In HT29 cells the increment was of fourfold at IC50 concentrations, in Caco-2 cells this increment was twofold respect to the control untreated cells (Fig. 9). This result along with the G0/G1 arrest indicates that the maslinic acid produce cell differentiation effects in colon cancer cells.

4. Discussion

Many modern anti-cancer drugs are secondary metabolites with high bioactivity. Triterpenoids exert various pharmacological effects to control many diseases, including cancer [3,6,33–36]. The present study is the first to evaluate the potential anti-tumoural effects of maslinic acid, a new triterpenoid isolated as a majority compound from olive-pelt pomace. Our data provide evidence that maslinic acid exerts anti-proliferative and pro-apoptotic effects in HT29 and Caco-2 colon adenocarcinoma cell lines. Moreover, maslinic acid induces morphological changes that are characteristic of apoptosis, such as chromatin condensation and fragmentation as well as cell shrinkage, as demonstrated by fluorescence microscopy. Analysis of the mechanisms of maslinic-induced cell death indicated activation of caspase-3 and DNA fragmentation, suggesting apoptotic cell death. Quantification of apoptosis using annexin V-FITC and PI staining by FACS analysis showed that the percentage of apoptotic cells at IC50 was lower in Caco-2 cells 14% (7% early apoptosis plus 7% late apoptosis/necrosis) than in HT29 cells 25% (14% early apoptosis and 11% late apoptosis/necrosis); at IC80, however, it increased dramatically and was even higher in Caco-2 cells 60% (15% early apoptosis plus 45% late apoptosis/necrosis) than in HT29 cells 38% (27% early apoptosis plus 11% late apoptosis), indicating that the activation of the apoptotic pathway may occur more slowly in HT29 cells.

In the process of differentiation, enterocytes acquire structural features of mature cells, express specific gene products such as intestinal alkaline phosphatase and develop specialized cellular functions [31]. This growth inhibition near to the cell cycle arrest and the increase in alkaline phosphatase activity are characteristics markers of enterocyte differentiation [32,37].

The fact that maslinic acid induces an stronger arrest in G0/G1 and a higher increase in alkaline phosphatase in HT29 than in Caco-2 cells indicates that maslinic acid is a more potent inducer of differentiation in HT29 cells than in Caco-2 cells.

It is noteworthy that the effect of maslinic acid on cell cycle and apoptosis induction was selective for malignant cells, since it did not alter the cell cycle or induce apoptosis in normal intestine cell lines (IEC-6 and IEC-18). This effect of maslinic acid on pro-apoptotic pathways specific for malignant cells means that this compound is of potential interest for cancer patients. In conclusion, we show that maslinic acid selectively induces the caspase apoptotic pathway in colon adenocarcinoma cell lines, causing G0/G1 cell-cycle arrest.

Because caspases are specific proteases of apoptosis, and among them more particularly caspase-3, we examined whether the possible apoptotic effect or maslinic acid on colon carcinoma cells HT29 and Caco-2 could be linked to induction of caspase-3. From 6 h of treatment, maslinic acid induced caspase-3 activity only in Caco-2 cells. At 72 h of treatment maslinic acid stimulated caspase-3 activity in HT29 cells. As caspase-3 activation is a phenomenon previous to the morphological changes reflected in the FACS analysis, this difference in caspase-3 activation agreed with the results found in FACS analysis, where HT29 cells showed a higher percentage of early apoptosis at 72 h, a circumstance that could be explained by the late activation of caspase-3 (72 h). Moreover, in the FACS analysis, Caco-2 cells presented a very high percentage of late apoptosis at 72 h of treatment, which could be correlated with the early activation of caspase-3 (6 h).

In normal intestinal cells lines IEC-6 and IEC-18, caspase-3 activation was not detected. In addition, caspase-3 activation is apparently time-dependent, this activity occurring prior to the appearance of characteristic apoptotic morphological features. Maslinic acid may thus be effective as a selective apoptogenic agent that exploits endogenous apoptosis-inducing mechanisms as a potential mean of treating or preventing colon adenocarcinoma. However, clinical trials will be required for determining whether maslinic acid can be effectively used in the pharmacology treatment of cancer.

In many cancer cell lines, p53 expression may contribute to therapeutic efficacy of cytotoxic drug. Although it does not seem to be necessary for induction of apoptosis in Caco-2 cells (p53 deficient) by maslinic acid, the cleavage of caspase-3 appears to correlate with maslinic-acid-induced apoptosis in both the HT29 and Caco-2 cell lines. It has been demonstrated that cytochrome c participates in the activation of caspase-3. These data suggest a linear and specific activation of caspase-3 in response to cytochrome c released from the mitochondria. Cytochrome c normally resides in the mitochondrial intermembrane space, where it serves a transducer of electrons in the respiratory chain. Several anti-tumour drugs with diverse intracellular targets have been demonstrated to cause the mitochondrial release and cytosolic accumulation of cytochrome and activation of caspase-3 [38]. Although the mechanism is not yet fully understood, the release of cytochrome c is considered to be a very important event for the induction of apoptosis by maslinic acid.
In conclusion, maslinic acid, a novel triterpene, inhibits proliferation and induces apoptosis of human colon carcinoma cells. Exposure to maslinic acid resulted in the activation of caspase-3 through the release of cytochrome c from the mitochondria. The more precise signalling pathway by which maslinic acid triggers caspase-3 activation, cytochrome c release, and the other apoptotic phenomena described here remain to be identified. Nevertheless, our data should contribute to the development of maslinic acid or related drugs as potential cancer chemotherapeutic or chemopreventive agents.

Although the detailed mechanism by which maslinic acid induces apoptosis remains still to be elucidated, it seems that this compound modulates multiple factors that could increase cellular susceptibility to apoptosis.

For example, the over-expression of a mutated p53 in HT29 cells [39] could explain the cell cycle arrest, differentiation and late caspase-3 induction obtained. In this case, maslinic acid could induce the cell differentiation mediated by p53, previous to the apoptosis activation. On the other hand, the fact that in Caco-2 cells p53 gene has deleted and mutated alleles and no detectable accumulation of the corresponding protein [21,40] could explain the lowest differentiation and the early caspase-3 activation obtained.

By the other hand, apoptosis induction could be due to a receptor mechanism, this mechanism have been proposed by different authors in papers related with colon cancer cells [41,42], a different number of receptors in these cell lines could explain the differential apoptosis induction by maslinic acid. For example, it has been reported that many types of cancer can use signals from multiple growth factor receptor for growth and survival [25,43]. The TGF receptors are intimately related with MAP-kinases mediated apoptotic pathway (JNK, p53/Erk), whereas p53 is activated in the endpoints of the pathway [44-46]. The growth-inhibitory apoptotic potential and growth factor-modulator effects of maslinic acid could be related with MAP-kinases mediated apoptotic pathway [22]. Other MAP-kinases pathways as the TNFf receptor-JNK-JBID pathway could be related with apoptotic activation by maslinic acid [47]. Further studies will be directed in this direction.

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