Research Article

Erythrodiol, a natural triterpenoid from olives, has antiproliferative and apoptotic activity in HT-29 human adenocarcinoma cells

M. Emília Juan[1], Uwe Wenzel[2], Hannelore Daniel[3] and Joana M. Planas[1]

[1] Department of Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Spain
[2] Molecular Nutrition Research, Interdisciplinary Research Center, Justus-Liebig-University of Giessen, Germany
[3] Molecular Nutrition Unit, Department of Food and Nutrition, Technical University of Munich, Freising, Germany

Erythrodiol is the precursor of pentacyclic triterpenic acids present in *Olea Europaea*. Although olive oil and some of its constituents are reported to have anticarcinogenic activities, erythrodiol has not been assessed in its cell biological functions in detail. We therefore determined its effects on cell growth and apoptosis in human colorectal carcinoma HT-29 cells. Proliferation, cytotoxicity, and apoptosis were measured by fluorescence-based techniques. Erythrodiol inhibited cell growth with an EC_{50} value of 48.8 ± 3.7 μM without any cytotoxic effects in a concentration range up to 100 μM. However, exposure of cells for 24 h to 50, 100, and 150 μM erythrodiol increased caspase-3-like activity by 3.2-, 4.8-, and 5.2-fold over that in control cells. We here demonstrate for the first time that, in colon adenocarcinoma cells, erythrodiol exerts antiproliferative and proapoptotic activity.

Keywords: Apoptosis / Erythrodiol / HT-29 cells / Olive oil / Proliferation

Received: July 31, 2007; revised: October 8, 2007; accepted: October 9, 2007

1 Introduction

Olea europaea L. has been known for a long time to contain a variety of triterpenoids including erythrodiol, oleanolic acid, and maslinic acid [1, 2]. Very little attention has been paid so far to these pentacyclic triterpenes, although they have been used for medicinal purposes in many Asian countries [3, 4]. In support of the hypothesis that constituents of olives may contribute to the cancer protective activity of the Mediterranean diet, we have recently reported an antitumor activity of an olive fruit extract composed of maslinic acid and oleanolic acid in HT-29 human colon cancer cells [5]. These pentacyclic triterpenes are present in the skin of olive fruits and in olive oil [1, 6, 7].

The rising interest in the biological properties for human health of pentacyclic triterpenic acids [5, 8–10] led us investigate compounds arising from the nonsteroidal triterpenoids biosynthetic pathway from *O. europaea* L. Erythrodiol (Fig. 1) is the intermediate from which oleanolic acid and its isomer maslinic acid are formed [11]. The concentration of this pentacyclic triterpenic alcohol in olive oil is around 90 mg/kg [12]. It has been described to exert anti-inflammatory activities on different experimental models [13, 14] and vasorelaxant effects on rat aorta [15] have been reported. Antioxidant properties are proposed as well as it was shown to protect PUFAs in rat-liver microsomal membranes from oxidation [16]. Moreover, the modulation of cytokine secretion by erythrodiol in human mononuclear cells has been recently described [17].

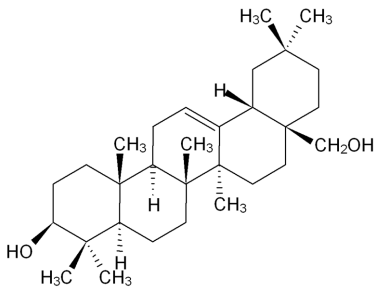

Figure 1. Chemical structure of erythrodiol.

Correspondence: Dr. M. Emília Juan, Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, E-08028 Barcelona, Spain
E-mail: mejuan@ub.edu
Fax: +34-93-403-59-01

The aim of the present work was to investigate whether erythrodiol can inhibit cell proliferation and induce apoptosis in HT-29 human colon adenocarcinoma cells. This cell line was selected since it has been widely used for assessing bioactive compounds that affect intestinal tumor cell growth.

2 Materials and methods

2.1 Chemicals

Erythrodiol was purchased from Extrasynthese (Genay, France). Media and supplements for cell culture were from Invitrogen (Karlsruhe, Germany). Cell culture plates were from Renner (Dannstadt, Germany). The fluorophore SYTOX-Green was from Bioprobes (Leiden, The Netherlands). The fluorogenic caspase-3 substrate acetyl-aspartyl-glutamyl-valyl-aspartyl-amino-4-methyl-coumarine (Ac-DEVD-AMC) was obtained from Calbiochem (Bad Soden, Germany).

2.2 Cell culture

HT-29 cells (passage 106) were provided by American Type Culture Collections and were used between passages 150 and 200. Cells were cultured and passaged in RPMI 1640 supplemented with 10% fetal calf serum and 2 mM glutamine. Antibiotics added to the media were 100 U/mL penicillin and 100 μg/mL streptomycin. The cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Fresh medium was given every second day and on the day before the experiments were done. Cells were passaged at preconfluent densities by the use of a solution containing 0.05% trypsin and 0.5 mM EDTA. Erythrodiol was applied to the cell cultures dissolved in DMSO to a final concentration of 2% v/v in all the experiments. Controls were always treated with the same amount of DMSO.

2.3 Necrosis

The potential nonspecific toxicity of erythrodiol was assessed by exposing HT-29 cells seeded at a density of 5×10^4 cells/well onto 24-well cell culture plates and allowed to adhere for 4 h. Subsequently, the medium was replaced by a fresh one and cells were exposed for 3 h to increasing concentrations of erythrodiol. Necrotic cell death was determined with SYTOX-Green, which becomes fluorescent after DNA binding. The percentage of dead cells was derived from SYTOX-fluorescence prior to cell lysis in relation to the fluorescence measured after the solubilization of cells. Cell numbers were determined based on a calibration curve measured using cell numbers between 1×10^3 and 1.5×10^5 cells, which had been adjusted after counting the cells in a Neubauer chamber. Fluorescence was measured at 538 nm after excitation at 485 nm using a fluorescence multiwell-plate reader (Fluoroskan Ascent, Thermo Electron, Dreieich, Germany).

2.4 Cell proliferation

In the proliferation assay HT-29 cells were seeded at a density of 5×10^3 cells/well onto 24-well cell culture plates and allowed to adhere for 24 h. Thereafter, medium was substituted by a fresh culture medium containing increasing concentrations of erythrodiol. Cells were allowed to grow for another 72 h and total cell counts were determined afterwards. Cells were lysed with 1% Triton X-100 in isotonic NaCl and DNA was stained with SYTOX-Green. Cell numbers were counted using the fluorescence multiwell-plate reader.

2.5 Apoptosis

Caspase-3-like activity serving as an apoptosis marker was measured based on the method of Nicholson *et al*. [18]. Briefly, cells were seeded at a density of 5×10^5 per well onto six-well plates and allowed to adhere for 24 h. Afterwards, the medium was removed and cells were exposed to erythrodiol in final concentrations of 10, 25, 50, 100, and 150 μM for 24 h. Afterwards, cells were trypsinized, cell numbers were determined, and the cells were centrifuged at $2500 \times g$ for 10 min. Cytosolic extracts were prepared by adding 750 μL of a buffer containing 2 mM EDTA, 0.1% 3-((cholamidopropyl)-dimethyl-ammonium)-1-propane-sulfonate, 5 mM dithiotreitol, 1 mM phenyl-methyl-sulfonyl-fluoride, 10 mg/L pepstatin A, 20 mg/L leupeptin, 10 mg/L aprotinin, and 10 mM HEPES/KOH (pH 7.4) to each pellet and homogenized with ten strokes. The homogenate was centrifuged at $100\,000 \times g$ at 4°C, and the cytosolic supernatant was incubated with the fluorogenic caspase-3 tetrapeptide-substrate Ac-DEVD-AMC at a final concentration of 20 μM. Cleavage of the caspase-3 substrate was followed by determination of emission at 460 nm after excitation at 390 nm using the fluorescence plate reader.

2.6 Statistics

Results are expressed as means with their standard errors. To derive the EC_{50} values for growth inhibition, a nonlinear approximation model by the least square method based on a competition curve using one component was applied. Results were tested for normal distribution and for homogeneity of variance by standardized residual plot. When necessary, logarithmic transformations were performed. Normally distributed results were analyzed by one-way ANOVA. If the result was found to be significant ($p < 0.05$) the Tukey's Multiple Comparison test was used to determine specific differences between results. All statistics analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA).

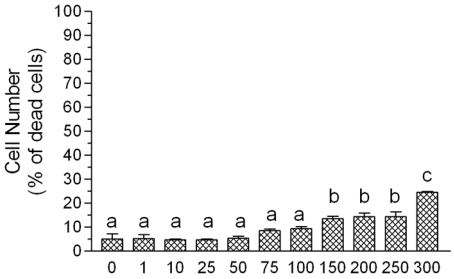

Figure 2. Effects of erythrodiol on necrosis in HT-29 cells. Necrosis was assessed after incubating the cells for 3 h with medium alone (control) or containing different concentrations of each compound. Cell numbers were determined subsequently using SYTOX-Green nucleic acid stain. Values are means ± SEM, $n = 13$. Means without a common letter differ, $p < 0.05$.

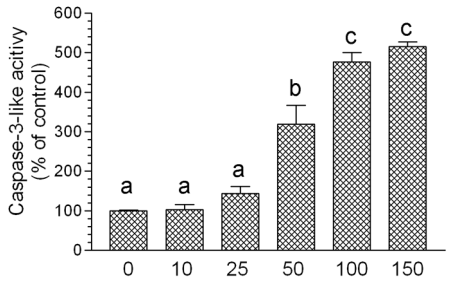

Figure 4. Dose-dependent caspase-3 activity in HT-29 cells. Induction of caspase-3-like activity was determined at 24 h based on the cleavage of the fluorogenic substrate Ac-DEVD-AMC. Values are means + SEM, $n = 3$. Means without a common letter differ, $p < 0.05$.

3 Results

3.1 Necrosis

The effect of erythrodiol on cell viability was assessed *via* SYTOX-Green as a dead cell indicator. SYTOX-Green stain is a high-affinity nucleic acid stain that does not cross the membranes of live cells but easily penetrates cells with compromised plasma membranes.

Erythrodiol did not induce any signs of nonspecific cytotoxicity up to 100 μM with 94.5 ± 3.0% of the cells still viable (Fig. 2). However, concentrations of >150 μM decreased cells number to 86.3 ± 0.6% ($p < 0.05$) and at 300 μM to 75.6 ± 1.4% ($p < 0.01$).

3.2 Proliferation

The growth inhibitory activity of erythrodiol as a reference compound was determined in preconfluent HT-29 cells after 72 h of exposure to different concentrations. Cell growth was completely inhibited by erythrodiol at concentrations of >100 μM (Fig. 3) while at 25 and 75 μM cell numbers were reduced by 18 and 68%, respectively. The concentration that caused 50% inhibition of cell growth was determined with 48.8 ± 3.7 μM.

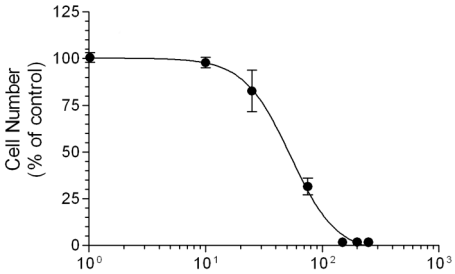

Figure 3. Effects of erythrodiol on proliferation in HT-29 cells. Proliferation was measured over 72 h in the absence (control) or presence of each compound at different concentrations. Cell numbers were determined subsequently using SYTOX-Green nucleic acid stain. Values are means ± SEM, $n = 3$.

3.3 Apoptosis

A fluorogenic assay was used to determine whether the decrease in cell growth observed after the treatment with erythrodiol was the result of an enhanced apoptosis in HT-29 cells. Caspase-3-like activity was assessed as the prime downstream target enzyme in the apoptotic pathway and is considered to serve as a valid marker of programmed cell death [19]. Erythrodiol exerted a dose-dependent pro-apoptotic activity in HT-29 cells (Fig. 4) with increments of 3.2-, 4.8-, and 5.2-fold over that in the control cells at concentrations of 50, 100, and 150 μM, respectively.

4 Discussion

The waxy coating of olives constitutes a rich source of pentacyclic triterpenes such as oleanolic acid, maslinic acid, and erythrodiol. Pentacyclic triterpenes, biosynthesized in plants by squalene cycling, have been used for more than 2000 years in traditional Asiatic medicine as anti-inflammatory and anticarcinogenic agents [3, 4]. The present study is the first to assess the potential antitumor activity of erythrodiol in cancer cells and provides evidence that this triterpene exerts antiproliferative and pro-apoptotic effects.

Erythrodiol inhibited cell growth with a half-maximal effect at a concentration of around 50 μM. These results are particularly interesting since it is well known that defects in the regulation of cell cycle progression are the most common feature of transformed cells [20]. Consequently, the antiproliferative actions of this pentacyclic triterpene indicate that it may serve as cancer-protective agent. It is important to mention that the growth inhibition was not a consequence of cytotoxic effects, since even at concentrations of 100 μM which caused complete inhibition of proliferation, less than 5% of cells were found to be nonviable. Therefore,

the inhibition of cell proliferation appeared to result from either inhibition of cell cycle progression or the induction of apoptosis, or both.

These results are in line with recent reports on growth-inhibitory properties of other pentacyclic triterpenic acids in HT-29 cells. We have previously reported the anticarcinogenic effects of olive fruit extracts [5] composed mainly of maslinic (73%) and oleanolic acids (26%). Studies on dose-dependent effects showed antiproliferative activity at an EC50 value of 74.0 ± 3.2 μM for maslinic and 26.6 ± 2.5 μM for oleanolic acid. Moreover, maslinic acid displayed antiproliferative activities with an IC50 of 61 ± 1 μM in the same cell line through a cell cycle arrest in G_0/G_1 [10].

When HT-29 cells were exposed to increasing concentration of erythrodiol, a pro-apoptotic activity became evident. Caspases are specific proteases and it is generally accepted that caspase-3 is the major downstream effector caspase which cleaves major cell components during apoptosis [21]. The pro-apoptotic activity displayed by erythrodiol was associated with a reduction in adherent cell number, and a dose-dependent increase in caspase-3 activity in adherent cells that started at 50 μM of erythrodiol with a maximal activation of 320% as compared to control cells. This pro-apoptotic activity appears to account, at least in part, for the observed inhibition of cell growth since the EC50 for the antiproliferative activity was also around 50 μM. Maslinic acid and oleanolic acid from the olive fruit extract also were shown to induce an activation of caspase-3 and here apoptosis was initiated by the intrinsic pathway as evidenced by increased superoxide anion production in the mitochondria [5].

Apoptosis is frequently impaired during tumorigenesis, presumably through the systematic loss of regulatory control mechanisms, ultimately resulting in the generation of a malignant phenotype. Consequently, compounds such as erythrodiol that can eliminate aberrant cell clones by the induction of apoptosis may have a chemopreventive or an even therapeutic potential [20, 22]. Although erythrodiol is present in the Mediterranean Diet in the form of olives and olive oil [6, 23], the amounts are not sufficient to attain cellular concentrations that displayed the cancer chemopreventive activity shown here. However, a special source of this compound could be "orujo" olive oil which is obtained from the waste of olives, skin, and seeds after cold-press extraction of virgin olive oil. This by-product is richer in pentacyclic triterpenoids and contains erythrodiol in concentrations of up to 690 mg/kg [7, 24].

In conclusion, erythrodiol inhibits cell proliferation without necrosis and also restores apoptosis in HT-29 cells. The present findings strengthen the hypothesis that pentacyclic triterpenic compounds from olives possess chemopreventive activity against colon carcinogenesis.

Supported by grant AGL2005-05728 from the Ministerio de Ciencia y Tecnología and grant 2005-SGR-00632 from the Generalitat de Catalunya, Spain.

The authors have declared no conflict of interest.

5 References

[1] Vioque, E., Maza, M. P., On triterpenic acids from olive and olive pomace oils. *Grasas y Aceites* 1963, *14*, 9 – 11.

[2] Caputo, R., Mangoni, L., Monaco, P., Previtera, L., New triterpenes from leaves of *Olea europaea*. *Phytochemistry* 1974, *13*, 2825 – 2827.

[3] Setzer, W. C., Setzer, M. C., Plant-derived triterpenoids as potential antineoplastic agents. *Mini Rev. Med. Chem*. 2003, *3*, 540 – 556.

[4] Ovesna, Z., Vachalkova, A., Horvathova, K., Tothova, D., Pentacyclic triterpenic acids: New chemoprotective compounds. *Minirev. Neoplasma* 2004, *51*, 327 – 333.

[5] Juan, M. E., Wenzel, U., Ruiz-Gutierrez, V., Daniel, H., Planas, J. M., Olive fruit extracts inhibit proliferation and induce apoptosis in HT-29 human colon cancer cells. *J. Nutr.* 2006, *136*, 2553 – 2557.

[6] Bianchi, G., Murelli, C., Vlahov, G., Surface waxes from olive fruits. *Phytochemistry* 1992, *31*, 3503 – 3506.

[7] Perez-Camino, M. C., Cert, A., Quantitative determination of hydroxyl pentacyclic triterpene acids in vegetable oils. *J. Agric. Food Chem*. 1999, *47*, 1558 – 1562.

[8] Liu, J., Oleanolic acid and ursolic acid: Research perspectives. *J. Ethnopharmacol*. 2005, *100*, 92 – 94.

[9] Juan, M. E., Wenzel, U., Ruiz-Gutierrez, V., Planas, J. M., Daniel, H., Maslinic acid, a natural compound from olives, induces apoptosis in HT-29 human colon cancer cell line. *FASEB J.* 2005, *19*, A996.

[10] Reyes, F. J., Centelles, J. J., Lupiáñez, J. A., Cascante, M., (2 alpha,3 beta)-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 – 6310.

[11] Stiti, N., Triki, S., Hartmann, M. A., Formation of triterpenoids throughout *Olea europaea* fruit ontogeny. *Lipids* 2007, *42*, 55 – 67.

[12] Blanch, G. P., Villen, J., Herraiz, M., Rapid analysis of free erythrodiol and uvaol in olive oils by coupled reversed phase liquid chromatography gas chromatography. *J. Agric. Food Chem*. 1998, *46*, 1027 – 1030.

[13] Máñez, S., Recio, M. C., Giner, R. M., Ríos, J. L., Effect of selected triterpenoids on chronic dermal inflammation. *Eur. J. Pharmacol*. 1997, *334*, 103 – 105.

[14] de la Puerta, R., Martinez-Dominguez, E., Ruiz-Gutierrez, V., Effect of minor components of virgin olive oil on topical antiinflammatory assays. *Z. Naturforsch*. 2000, *55c*, 814 – 819.

[15] Rodríguez-Rodríguez, R., Herrera, M. D., Perona, J. S., Ruíz-Gutiérrez, V., Potential vasorelaxant effects of oleanolic acid and erythrodiol, two triterpenoids contained in "orujo" olive oil, on rat aorta. *Brit. J. Nutr*. 2004, *92*, 635 – 642.

[16] Perona, J. S., Arcemis, C., Ruiz-Gutierrez, V., Català, A., Effect of dietary high-oleic-acid oils that are rich in antioxidants on microsomal lipid peroxidation in rats. *J. Agric. Food Chem*. 2005, *53*, 730 – 735.

[17] Marquez-Martin, A., De La Puerta, R., Fernandez-Arche, A., Ruiz-Gutierrez, V., Yaqoob, P., Modulation of cytokine secretion by pentacyclic triterpenes from olive pomace oil in human mononuclear cells. *Cytokine* 2006, *36*, 211 – 217.

[18] Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., *et al*., Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 1995, *376*, 37 – 43.

[19] Hoshi, T., Sasano, H., Kato, K., Yabuki, N., *et al*., Immunohistochemistry of Caspase-3/CPP32 in human stomach and its correlation with cell proliferation and apoptosis. *Anticancer Res*. 1998, *18*, 4347 – 4353.

[20] Watson, A., An overview of apoptosis and the prevention of colorectal cancer. *Crit. Rev. Oncol. Hematol*. 2006, *57*, 107 – 121.

[21] Hengartner, M. O., The biochemistry of apoptosis. *Nature* 2000, *407*, 770 – 760.

[22] Sun, S. Y., Hail, N., Lotan, R., Apoptosis as a novel target for cancer chemoprevention. *J. Natl. Cancer Inst*. 2004, *96*, 662 – 672.

[23] Albi, T., Lanzon, A., Cert, A., Aparicio, R., Erythrodiol values in samples of virgin olive oils from Andalusia. *Grasas y Aceites* 1990, *41*, 167 – 170.

[24] Albi, T., Lanzon, A., Navas, M. A., Quantitative-determination of erythrodiol in oils by gas-chromatography with semicapillary columns (wide bore capillary). *Grasas y Aceites* 1986, *37*, 277 – 281.