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Cytotoxic and apoptosis-inducing effect of *ent*-15-oxo-kaur-16-en-19-oic acid, a derivative of grandiflorolic acid from *Espeletia schultzii*

Yarimar Ruiz [a], Juan Rodrígues [b], Francisco Arvelo [c], Alfredo Usubillaga [d], Mariugenia Monsalve [d], Nardy Diez [a], Iván Galindo-Castro [a,*]

[a] *Laboratorio de Genómica y Proteómica, Centro de Biotecnología, Fundación IDEA, Carretera Nacional Hoyo de la Puerta-Baruta, Valle de Sartenejas, Municipio Baruta, Edo. Miranda, Caracas 1015-A, Venezuela*
[b] *Facultad de Farmacia, UCV, Venezuela*
[c] *Laboratorio de Cultivo de Tejidos y Biología de Tumores, Instituto de Biología Experimental, UCV, Venezuela*
[d] *Instituto de Investigaciones, Facultad de Farmacia y Bioanálisis, ULA, Venezuela*

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Abstract

ent-Kaurenic acid and many natural derivatives of this diterpene are known to have interesting biological properties. *ent*-15-Oxo-kaur-16-en-19-oic acid can be easily obtained from grandiflorolic acid which was first isolated from *Espeletia grandiflora*. The present work describes the proapoptotic effect of *ent*-15-oxo-kaur-16-en-19-oic acid on the human prostate carcinoma epithelial cell line PC-3 as evidenced by the changes in the expression level of proteins associated with the execution and regulation of apoptosis. Cell viability was affected upon exposure to the compound, the IC_{50} were determined as 3.7 μg/ml, which is 4 times lower than that corresponding to a primary cell culture of fibroblasts (14.8 μg/mL). Through Western blot analysis, active forms of caspace-3 associated with the specific proteolysis of Poly(ADP-ribose) polymerase (PARP) were detected. Reduced levels of the antiapoptotic protein Bcl-2, as well as the appearance of internucleosomal DNA fragmentation, were also demonstrated. Thus, *ent*-15-oxo-kaur-16-en-19-oic acid may be a promising lead compound for new chemopreventive strategies, alone or in combination with traditional chemotherapy agents to overcome drug resistance in tumoral cells.
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Keywords: *Espeletia schultzii*; *Espeletiinae*; Frailejón; *ent*-Kaurenoids; *ent*-5-Oxo-kaur-16-en-19-oic acid; Apoptosis; PC-3 cell line

1. Introduction

Of the 20,000 plant species described in Venezuela, 1,500 of them are used for medicinal purposes (Taylor et al., 2006). *ent*-Kaurenic acid and many natural derivatives of this diterpene are known to have interesting biological properties (Ghisalberti, 1997). Additionally *ent*-15-oxo-kaur-16-en-19-oic acid (**1c**) (EOKA) can be readily obtained from grandiflorolic acid (**1b**) which was first isolated by Piozzi et al. (1968) from *Espeletia grandiflora*, a Colombian species growing around Bogota city. *E. grandiflora* is one of around 180 species of *Espeletiinae* (Asteracea), resinous plants, popularly known as frailejón, that are characteristic of the high Andean plateaus of Venezuela, Colombia, and Ecuador (Cuatrecasas, 1976). In the present work grandiflorolic acid (**1b**) was obtained by mild hydrolysis of *ent*-15α-acetoxy-kaur-16-en-19-oic acid (**1a**), isolated from *Espeletia schultzii* Wedd, a Venezuelan species (Aristeguieta, 1964). This plant was first studied by Brieskorn and Pöhlmann (1968) who found that its resin contained large amounts of grandiflorolic acid (**1b**), and *ent*-15α-acetoxy-kaur-16-en-19-oic acid (**1a**) was the second

* Corresponding author. Tel.: +58 212 9035087; fax: +58 212 9035086.
E-mail address: igalindo@idea.gob.ve (I. Galindo-Castro).

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most abundant compound. Several *Espeletiinae* have been found to contain *ent*-15α-acetoxy-kaur-16-en-19-oic acid (**1a**) and grandiflorolic acid (**1b**) (Usubillaga et al., 2003), but *E. schultzii* was chosen because it is easily collected.

Asian species pertaining to the genus *Isodon* (Labiatae) have been found to contain an α,β-unsaturated ketone moiety at the D ring of the kaurene skeleton (Xiang et al., 2004; Li et al., 2006). These compounds are poly-hydroxylated and most of them have been found to be cytotoxic against several cancer cell lines. Nagashima et al. (2003) have studied the biological properties of *ent*-11α-hydroxy-kaur-16-en-15-one in a human leukemia cell line and found evidence that this compound was able to induce apoptosis. More recently, Rundle et al. (2006) described the ability of EOKA (**1c**) to irreversibly prolong the mitotic arrest on human epithelial tumoral cell lines, a characteristic effect that sometimes precedes apoptosis (Sasaki et al., 2002; Ling et al., 2002).

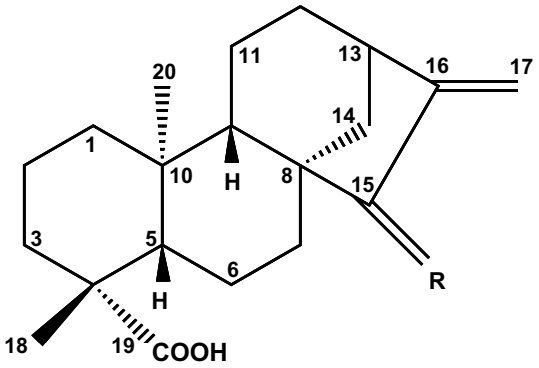
Although many of this type of compounds have been reported to have the capacity to induce apoptosis in different cell lines, their molecular targets differed significantly (Morales et al., 2005; Castrillo et al., 2001; Lee et al., 2002), and showed dissimilar levels of drug sensitivity (Kaur et al., 2005).

Prostate cancer is recognized as one of the five move frequent forms of neoplasia affecting the male population worldwide (Jemal et al., 2005). With adenocarcinoma progression towards androgen-independence, like the PC3 cell line, tumors become resistant to a variety of conventional treatments (Tilley et al., 1990), demanding new therapeutic alternatives including activation of apoptosis or programmed cell death (Honda et al., 2002; Nguyen and Wells, 2003). The identification of potential targets that are activated during apoptosis onset is made possible by virtue of the selectivity of natural compounds and their derivatives to these targets (Morales et al., 2005; Castrillo et al., 2001; Lee et al., 2002). Considering the physiological function that *ent*-kauranes may have as precursors of plant cell proliferation regulators (Vieira et al., 2005), it is interesting that they may affect animal cell proliferation as well.

In the present work, we provide evidence of the proapoptotic effect of EOKA (**1c**) on the human prostate carcinoma epithelial cell line PC-3, which induces a differential expression of proteins associated with the execution and regulation of apoptosis.

2. Results and discussion

We tested EOKA (**1c**) for its cytotoxicity against human prostate carcinoma epithelial cell line PC-3. As can be seen in Fig. 2a, PC-3 cells exposed to the kaurenoic acid showed a lower viable cell number in a dose-dependent manner, reaching an IC_{50} value of 3.7 μg/ml. This concentration value is 4 times lower than that corresponding to a primary cell culture of fibroblasts (14.8 μg/ml) (data not shown), cells that we used to evaluate the differential effect of the



1a R = β H,α-$OCOCH_3$
1b R = β H,α-OH
1c R = O

Fig. 1. Chemical structures of the *ent*-15α-acetoxy-kaur-16-en-19-oic acid (**1a**), *ent*-15α-hydroxy-kaur-16-en-19-oic acid (grandiflorolic acid, **1b**), and *ent*-15-oxo-kaur-16-en-19-oic acid (**1c**).

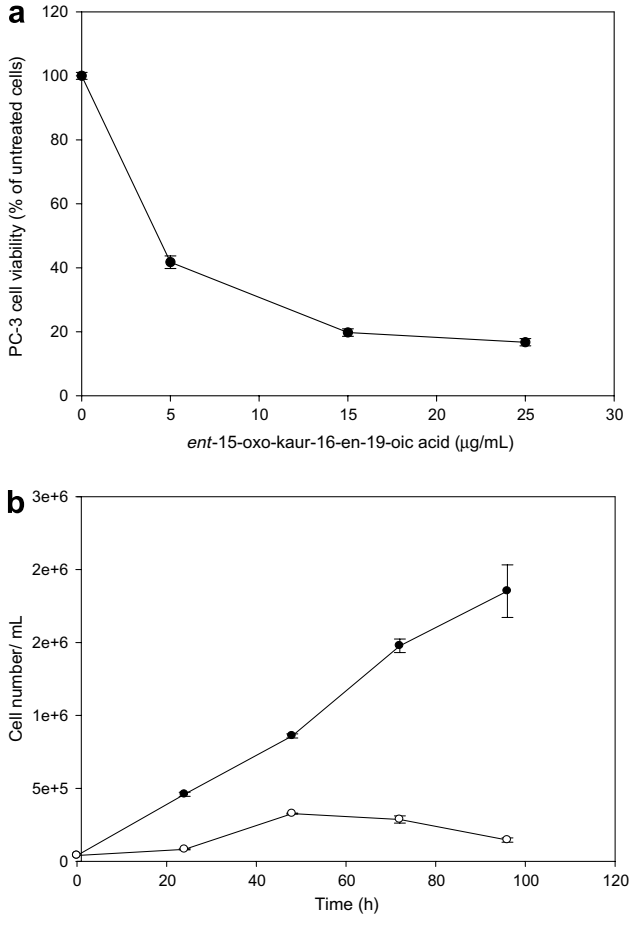


Fig. 2. Cytotoxic activity of *ent*-15-oxo-kaur-16-en-19-oic acid (**1c**) on human prostate PC-3 cell line. (a) Dose response curve for cells treated with EOKA (**1c**). The estimated IC_{50} value for this compound was 3.75 μg/mL. (b) Inhibition of cell growth after treatment with EOKA (**1c**) at IC_{50} concentration (open circles). Close circles, cell growth curve without EOKA (**1c**) treatment. ANOVA: $F(5, N = 5) = 996.6$, $p = 0.00001$.

isolated natural compound between tumoral and normal cells.

Fig. 2b shows how EOKA (**1c**) exerts an antiproliferative effect on PC-3 cells when compared with untreated cells. Such an event is more evident after 48 h of drug exposure.

The preferential cytotoxicity of EOKA (**1c**) on prostate cancerous cells over normal fibroblasts indicates a higher sensitivity of the tumoral cells to this compound. This finding is in agreement with recently reported activities for other kauranoids such as *ent*-16β-17α-dihidroxykaurene (Morales et al., 2005), *ent*-15-oxo-1,7,14,20-tetrahydroxykaur-16-ene or Kamebakaurin (Lee et al., 2002), and *ent*-16-kaur-16-en-19-oic acid (Castrillo et al., 2001), derivatives from kaura-9(11),16-dien-19-oic acid (Alonso, 2006), among many others, where they are sensitizing the tumoral cells to undergo apoptosis. Rundle et al. (2006) recently reported that EOKA (**1c**) induces mitotic arrest on human epithelial cell lines at early drug exposure times. This group did not find evidence of apoptosis induction; however, other compounds such as mebendazole (methyl 5-benzoyl-2-benzimidazole-carbamate) (Sasaki et al., 2002), or arsenic trioxide (Ling et al., 2002), besides inducing mitotic arrest, also provoked apoptosis in human tumor cell lines.

To determine the possible involvement of EOKA (**1c**) in apoptosis induction, we evaluated the generation of the typical DNA ladder pattern of internucleosomal fragmentation when cells undergo apoptosis. In this regard, DNA samples from PC-3 treated cells were run by conventional agarose gel (2.5%) electrophoresis to observe the nucleosomal fragmentation caused by the activation of apoptosis-dependent nucleases. Fig. 3 shows that cells treated with EOKA (**1c**) for at least 144 h manifested a DNA fragmentation pattern which is not observed in control cells. This electrophoretical pattern, as a ladder of 180-bp fragments, is typical of nuclesomal DNA fragmentation produced by apoptosis.

After this finding, we continued to determine whether caspase-3, the key effector caspace of apoptosis, was also being activated. When caspace-3 activity was specifically determined in cells treated with EOKA (**1c**), this compound was able to significantly increase (1.8 times) the specific activity of this protease compared to the untreated cells (see Fig. 4a).

Although basal levels of procaspase-3 have been reported for PC-3 cell line by Winter et al. (2001), in our study the activated forms of the cleaved polypeptide were only evident after exposing the cells to the kaurenoic acid. As can be seen in Fig. 4b, the active forms of the caspase-3 revealed by Western blot and represented by cleaved-polypeptide fragments of 17 kDa and 11 kDa are particularly evident after the 72-h treatment, which coincides with the activation time course of the caspace-3 enzymatic activity. After this time, caspase-3 activity seems to decline, probably due to a generalized proteolysis caused by the caspace cascade itself.

Poly(ADP-ribose) polymerase (PARP) is commonly used as a native substrate of caspace-3 to follow the apoptosis onset. As depicted in Fig. 5, after the immunodetection of PARP by Western blotting, we observed that PC-3 cells treated with EOKA (**1c**) produced the specific cleavage of PARP, generating a fragment of 85 kDa,

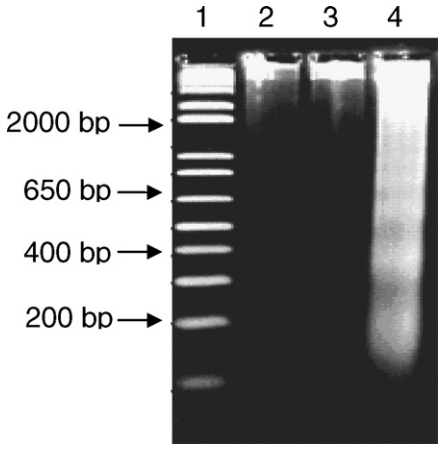


Fig. 3. Induction of DNA fragmentation by EOKA (**1c**) on PC-3 cells. DNA fragmentation was assessed on cells treated or untreated with this compound at IC_{50}; DNA bands were resolved by agarose gel (2.5%) electrophoresis. (1) Molecular weight standards, (2) untreated cells (144-h treatment with vehicle only), (3) 120-h treatment, (4) 144-h treatment.

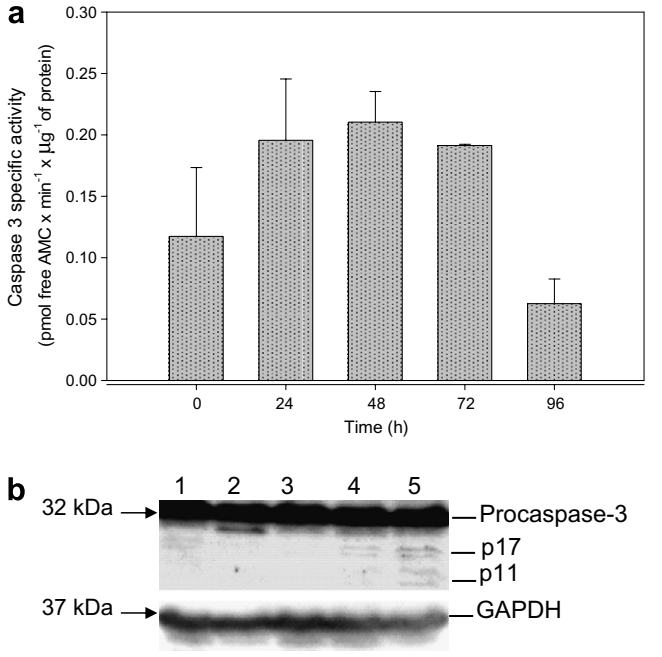


Fig. 4. Caspase 3 activation. (a) Activity levels of caspase 3 detected in PC-3 cells assayed with EOKA. ANOVA: H (4,$N = 15$) = 9.3. $p = 0.0540$. (b) Western blot of caspase-3 and procaspase-3 on PC3 cells treated with *ent*-15-oxo-kaur-16-en-19-oic acid (**1c**). (1) Untreated cells (96-h treatment with vehicle only), (2) 24-h treatment, (3) 48-h treatment, (4) 72-h treatment, (5) 96-h treatment. The immunoblot of GAPDH is shown as protein loading reference for each treatment. AMC = 7-amino-4-methyl coumarin.

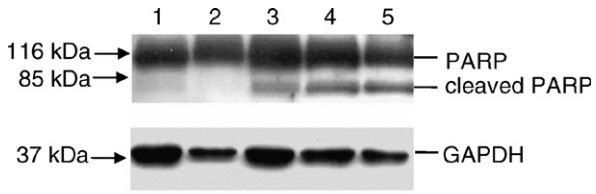


Fig. 5. Western blot of PARP from PC-3 cells treated with *ent*-15-oxo-kaur-16-en-19-oic acid (**1c**). (1) Untreated cells (96-h treatment with vehicle only), (2) 24-h treatment. (3) 48-h treatment, (4) 72-h treatment, (5) 96-h treatment. The immunoblot of GAPDH is shown as protein loading reference for each treatment.

containing the C-terminal end of the protein recognized by the antibody. Control cells only showed the native uncleaved PARP (116 kDa). The polypeptide bands corresponding to fragments of cleaved PARP became evident after the 48-h treatment, also coinciding with the activation time course of the caspace-3 enzymatic activity.

The antiapoptotic protein Bcl-2 has been proposed as one of the key players in apoptosis regulation, particularly in the intrinsic or mitochondrial pathway, where its higher protein levels compared to those of proapoptotic proteins prevent the cell to undergo apoptosis (Cory and Adams, 2002). As shown in Fig. 6a, cells exposed to the kaurenoic acid showed lower protein levels of Bcl-2 when compared with untreated cells. This protein levels diminished with the time course of the treatment. Tumor progression has been associated with an over-expression of Bcl-2, and this is generally linked to cells that are resistant to chemotherapy (Johnstone et al., 2002). Similarly, in our case, EOKA (**1c**) produced a clear reduction of Bcl-2 protein levels when compared to the untreated PC-3 cells. However, the levels of the proapoptotic protein Bak, a Bcl-2 antagonist, remained unchanged upon treatment with EOKA (**1c**) (see Fig. 6b). This imbalance between the antiapoptotic protein Bcl-2 and the prosurvival protein Bak could explain the commitment of the tumoral cell to undergo apoptosis under the effect provoked by EOKA (**1c**). *ent*-Kaurene diterpenoids isolated from *Jungermannia truncata* (Nagashima et al., 2003) have the same enone moiety as EOKA (**1c**) combined with hydroxyl groups at C-3, C-7, C-11, C-14, or C-20. Since each one of these compounds can similarly induce apoptosis, it could be inferred that the enone moiety might be responsible for the biological activity. Moreover, although EOKA (**1c**) has been proven to cause mitotic arrest by inhibiting RanBP2 function at early drug exposure times (Rundle et al., 2006), the specific mechanisms of action of this compound to unchain apoptosis still remain to be elucidated.

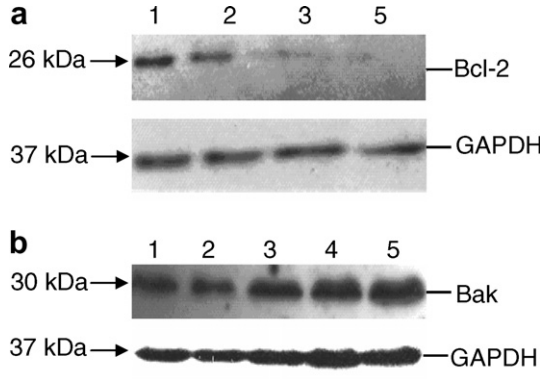


Fig. 6. Western blot of Bcl-2 and Bak proteins on PC-3 cells treated with *ent*-15-oxo-kaur-16-en-19-oic acid (**1c**). (1) Untreated cells (96-h treatment with vehicle only), (2) 24-h treatment, (3) 48-h treatment, (4) 72-h treatment, (5) 96-h treatment. The immunoblot of GAPDH is shown as protein loading reference for each treatment.

3. Concluding remarks

In this work we reported the differential cytotoxic and proapoptotic effect of EOKA (**1c**), an *ent*-kaurenoic acid obtained from a natural kaurene diterpene isolated from an endemic plant of the Venezuelan Andes. Our study provides evidence for the role of EOKA (**1c**) as a potential antitumoral agent by its ability to activate apoptosis in PC-3 cells. We found that EOKA (**1c**) promotes activation of caspace-3 and caspace specific cleavage of PARP, and also induces nucleosomal DNA fragmentation as well as reduction of the protein levels of Bcl-2.

Although chemotherapeutic agents have been used to specifically kill tumor cells by inducing apoptosis, many of these tumors acquired drug resistance to the apoptotic stimuli during tumorigenesis. Therefore, an effective strategy for overcoming the resistance to anticancer agents is an important field of study still to be exploited. Experiments are being conducted to establish the sensitizing ability of EOKA (**1c**) when combined with traditional drugs for cancer treatment.

4. Experimental part

4.1. Plant material

Resinous exudates (100 g) caused by wounds of moth caterpillars on *E. Schultzii* Wedd were collected from plants in Mucubají, Merida State, at 3500 m above sea level in March 2004. A voucher specimen of the plant is kept at the MERF Herbarium (No. 1038).

4.2. Instrumentation

Melting points were measured on a Fisher Johns hot stage and are uncorrected. Optical rotations were taken on a Jasko electropolarimeter Model DIP 370. IR spectra were measured on a Perkin Elmer 1720X apparatus as KBr discs. ^{1}H and ^{13}C NMR measurements were performed on a Bruker Advance DRX-400. UV spectra were

measured on a Shimadzu UV-1700 spectrometer. EIMS were obtained on a Hewlett–Packard model 5973 spectrometer at 70 eV. Flash chromatography was performed on silica gel Merck 60 (230–400 mesh), TLC was carried out on silica gel Merck 60 F254.

4.2.1. ent-15-Oxo-kaur-16-en-19-oic acid (EOKA) (**1c**)

Resin (100 g) from *E. schultzii* was extracted twice at room temperature with *n*-hexane (0.5 l). The solvent was vacuum distilled to yield 58 g of extract. This extract was dissolved Et_2O (1 l) and extracted with 0.5 N aqueous NaOH (2×0.5 l). The aqueous phase was neutralized by addition of diluted HCl (1 l) to pH 3.0. Upon cooling, the acid fraction was recovered by extracting shaking twice with Et_2O. The combined ether extract were dried (Na_2SO_4) and distilled to yield 28 g of a yellow mixture. The acid fraction (20 g) was submitted to flash cc on silica gel (500 g). Elution started with *n*-hexane:diethylether (3:1, v/v) and 200 ml fractions were taken and inspected by TLC. Fractions 45–87 yielded 2.7 g of pure *ent*-15α-acetoxy-kaur-16-en-19-oic acid (Fig. 1a), mp 172–174 °C (recrystallized from EtOH), IR (KBr) ν_{max} cm^{-1}: 1725, 1690, 1650, 690; and ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 5.27 (1H, s, H-17a), 5.10 (2H, s, H-17b and H-15), 2.81 (1H, br s, H-13), 2.08 (3H, s, $COCH_3$), 1.25 (3H, s, H-18), 0.95 (3H, s, H-20). Melting point, IR and ^{1}H NMR signals agree with those reported by Brieskorn and Pöhlmann (1968). *ent*-15α-Acetoxy-kaur-16-en-19-oic acid (**1a**) (0.35 g) was dissolved in 25 ml of 0.5 N KOH in MeOH and left overnight at room temperature. The solvent was distilled; the residue was treated with diluted HCl (25 ml); and then extracted with Et_2O (2×50 ml). Distillation of the Et_2o extract yielded *ent*-15α-hydroxy-kaur-16-en-19-oic acid (308 mg) (Fig. 1b) which was crystallized from MeOH, mp 224–228 °C, IR (KBr) ν_{max} cm^{-1}: 3350, 1695, 896. The IR spectrum was identical to that reported by Piozzi et al. (1968) for grandiflorolic acid (**1b**).

A solution containing 200 mg (0.63 mmol) of *ent*-15α-hydroxy-kaur-16-en-19-oic acid (**1b**) in pyridine (5 ml) was treated with CrO_3 (250 mg, 2.5 mmol) in pyridine (Sarett′s reagent) and left overnight at room temperature. The following day, in order to end the reaction, H_2O was added to the reaction mixture (25 ml) which was filtered and extracted with Et_2O (3×50 ml). The condensed ether extracted were dried over (anhyd Na_2SO_4) and evaporated to dryness. The crude product was submitted to flash chromatography, which was eluted with 1-hexane:ether (3:1, v/v) to yield *ent*-15-oxo-kaur-16-en-19-oic acid (190 mg) (Fig. 1c), mp 184–185 °C, $[\alpha]_D^{25}$-165 (*c* 0.15, $CHCl_3$), UV λ_{max} 234 nm (log ε 3.85) typical of an enone system on a five member ring, IR (KBr) ν_{max} cm^{-1}: 2940, 2866, 1723, 1690, 1645, 1466, 1256, 939, ^{1}H NMR (CD_3Cl, 400 MHz): 5.91(1H, s, H-17a), 5.22 (1H, s, H-17b), 3.02 (1H, br s, H-13), 2.37 (1H, d, $J = 11$; 9 Hz, H-14a), 2.12 (1H, m, H-3a), 1.84 (1H, m, H-1b), 1.80 (1H, m, H-6b), 1.78 (1H, m, H-7a), 1.65 1H, m, H-11b), 1.63 (1H, m, H-12b), 1.45(1H, m, H-2a), 1.44 (1H, m, H-6a), 1.40 (1H, m, H-2b), 1.38 (1H, m, H-14b), 1.32 (1H, m, H-7b), 1.22 (3H, s, H-18), 1.17 (1H, m, H-9), 1.0 (1H, m, H-3b), 0.99 (3H, s, H-20), 0.78 (1H, dt, $J = 4$, 14 Hz, H-1a), ^{13}C NMR ($CDCl_3$, ppm): 211.0 (C-15), 184.4 (C-19), 149.5 (C-16), 114.5 (C-17), 56.0 (C-9), 52.5 (C-8), 51.6 (C-5), 43.7 (C-4), 40.3 (C-10), 39.8 (C-1), 38.1 (C-13), 37.6 (C-3), 36.5 (C-14), 32.6 (C-7), 32.2 (C-12), 28.9 (C18), 20.0 (C-6), 18.8 (C-2), 18.4 (C-11), 15.5 (C-20) ; MS (*m*/*z*, %): 316 (M^+, $C_{20}H_{28}O_3$, 100), 301 (28), 283 (16), 255 (26), 207 (17), 148 (52). 121 (30).

4.3. Culture cell lines

Androgen-independent human prostate carcinoma epithelial cell line PC-3 used in this study was kindly provided by Dr. Marie France Poupon at Institute Curie, Paris-France, and was maintained in RPMI (Roswell Park Memorial Institute) medium supplemented with 10% fetal bovine serum, 1% of L-glutamin and 1% streptomycin (all obtained from Sigma–Aldrich, USA). Cells were grown in a humidified incubator with 5% CO_2 and 95% air at 37 °C.

4.4. Cytotoxicity assay

A 96-well microtiter plate (tissue culture grade) containing 0.2 ml of growth medium/per well (RPMI) was seeded with sufficient PC-3 cells to provide approximately 70% growth confluence after 24–48 h of culture. At this point, cells were exposed to EOKA for 72 h at concentrations ranging from 5 to 25 μg/ml, and then evaluated for cytotoxicity. In all cases, although EOKA (**1c**) was dissolved in dimethylsulfoxide (DMSO), the final concentration of this compound in the culture medium was lower than 1%, a concentration that has neither cytotoxic effect nor causes any interference with the colorimetric detection method.

Cytotoxicity assays were carry out by colorimetry following the reduction of 3-(4,5-dimethylthiazol-2-yI)-2,5-diphenyltetrazolium bromide (MTT assay) (Denizot and Lang, 1986). This colorimetric assay is based on the ability of live, but not dead, tumor cells to reduce dissolved MTT into an insoluble purple compound, formazan, by cleavage of the tetrazolium ring by dehydrogenase enzymes. Cells grown in microtiter plates were first incubated with MTT at 37 °C for 3 h, then they were washed with phosphate buffered saline (PBS), and finally the colorimetric detection was done by the addition of Formazan (dissolved in 1% dimethyl sulfoxide, DMSO). Absorbance was measured by a microplate reader (SepctraFluor®, Tecan) set at a wavelength of 570 nm. The IC_{50} value in the MTT assay was defined as the concentration of test compound resulting in a 50% reduction of absorbance compared to untreated cells. Further evaluations of the EOKA on the cell line were performed using the IC_{50}.

4.5. Effect of EOKA (**1c**) on cell growth

Cells (10^6) were plated in 35-mm tissue culture dishes at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) containing EOKA at 3.7 μg/ml (IC_{50}). The number of viable cells per dish was counted with a hemocytometer at 24-h intervals for a period of 96 h. Cells were collected from culture dishes after a trypsin-EDTA treatment for 10 min at 37 °C. All experiments were done in duplicate and each set was repeated at least twice.

4.6. Statistical analysis

Comparisons between the means of various treatment groups were analyzed by one-way ANOVA. The difference among means was considered significant when $p < 0.05$.

4.7. Nucleosomal DNA fragmentation assay

PC-3 cells were grown until 70% confluent and were then treated with EOKA (**1c**) or vehicle (1% DMSO) as negative control. Cells were collected by centrifugation ($1500g \times 20$ min), washed with cold phosphate-buffered saline and lysed in Lysis Buffer (50 mM Tris, pH 7.4, 5 mM EDTA and 1% SDS) for 20 min on ice. The supernatant was treated with 50 μg RNase A at 37 °C for 30 min and then with Proteinase K at 0.1 mg/ml for another hour. After phenol/$CHCl_3$ (1:1, v/v) extraction, DNA was precipitated with *i*-PrOH. The DNA pellet was rinsed with EtOH–H_2O (7:3, v/v) air dried, and dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.3). The DNA fragments (10 μg) were separated by conventional electrophoresis on a 2.5% agarose gel and visualized after SYBRGreen® staining (Sigma–Aldrich).

4.8. SDS-PAGE and western blot analysis

PC-3 cells (2×10^6) treated and untreated with *ent*-OKA (**1c**) were lysed by adding Lysis Buffer RIPA (150 mM NaCl; 50 mM Tris pH 8; 0,1% SDS; 0.5% sodium deoxicolate; 1% Nonidet P40), centrifuged to separate non-soluble material, and then equal amounts of soluble proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a Hybond-P membrane (Amersham Pharmacia) with the use of a Mini Trans-Blot Cell® (Bio-Rad). The membranes were blocked with TBST (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.001% Tween 20) containing 5% (w/v) nonfat milk, washed, and probed with the appropriate concentration of primary antibodies (1:400 dilution, Santa Cruz Biotechnology) for 2 h at 4 °C. After washing with TBST, the membrane was incubated with 1:2000 diluted peroxidase-conjugated goat anti-(mouse IgG) Ig or goat anti-(rabbit IgG) Ig (Santa Cruz Biotechnology) for 1 h at 25 °C. The antibody reactive bands were revealed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer's instructions.

4.9. Caspace-3 enzymatic activity determination

Cultured cells (10^6) were washed with PBS at 37 °C and resuspended in PBS at 4 °C. Cells were harvested by centrifugation at 4 °C ($450\times g$, 10 min) and tested with the fluorometric CasPACE Assay System (Promega). Briefly, the cell pellet was resuspended in hypotonic cell lysis buffer at a final concentration of 10^8 cells/ml and centrifuged ($16{,}000\times g$, 4 °C, 20 min). The supernatant was preincubated for 30 min at 30 °C with the caspase assay buffer. The samples were then incubated with the caspase-3 substrate Ac-DEVD-AMC for 1 h at 30 °C. Fluorescence intensity was measured with the aid of a microplate reader. The specificity of the assay was verified by adding the caspase-3 inhibitor Ac-DEVD-CHO to the incubation mixture. Caspase-3 specific activity was measured as the amount of the free fluorochrome 7-amino-4-methyl coumarin (AMC) released $\times \min^{-1} \times \mu g^{-1}$ of protein.

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