Cytotoxic activity of oleocanthal isolated from virgin olive oil on human melanoma cells

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Abstract

Oleocanthal is one of the phenolic compounds of extra virgin olive oil with important anti-inflammatory properties. Although a potential anticancer activity has been reported, only limited evidence has been provided in cutaneous malignant melanoma. The present study is aimed at investigating the selective in vitro anti-proliferative activity of oleocanthal against human malignant melanoma cells. Since oleocanthal is not commercial available, it was obtained as pure standard by direct extraction and purification from extra virgin olive oil. Cell viability experiments carried out by WST-1 assay demonstrated that oleocanthal had a remarkable and selective activity for human melanoma cells *versus* normal dermal fibroblasts with IC_{50}s in the low micromolar range of concentrations. Such an effect was paralleled by a significant inhibition of ERK1/2 and AKT phosphorylation and down-regulation of Bcl-2 expression. These findings may suggest that extra virgin olive oil phenolic extract enriched in oleocanthal deserves further investigation in skin cancer.

***Keywords**:* Olive oil, oleocanthal, melanoma cells, A375, dermal fibroblasts

INTRODUCTION

Malignant cutaneous melanoma is the most aggressive and chemo-resistant form of skin cancers. Novel systemic treatment options are now available [1] and intratumoral injection of cytokines represents a promising approach for patients with unresectable disease or continuous recurrence despite surgery [2]. In contrast to systemic treatment, topical application of drugs could result in high concentration of active species at the site of disease with the consequent improvement in therapeutic outcome.

Oleocanthal is an olive oil phenolic component responsible for the peculiar pungent and irritant sensation of olive oil. It is well proven that oleocanthal is able to inhibit cyclooxygenase enzymes, thus exerting an important anti-inflammatory activity [3], and very recent studies have demonstrated its potential for prevention of different types of cancer [4].

The present study is aimed at investigating the selective in vitro anti-proliferative activity of oleocanthal against human malignant melanoma cells compared to human dermal fibroblasts. Since oleocanthal is not commercial available, it was obtained as pure standard by direct extraction and purification from virgin olive oil.

MATERIALS AND METHODS

Extraction, Purification and Characterization of Oleocanthal

Extraction and purification of oleocanthal was performed using a slightly modified procedure reported in literature (17). Briefly about 190 g of olive oil was mixed together with *n*-hexan (760 mL) and acetonitrile (950 mL). The mixture was homogenized using a vortex mixer and centrifuged at 4000 rpm for 5 min, at 25 °C. The acetonitrile phase was collected and evaporates under reduced pressure, to afford a crude residue, which was purified by column chromatography using Si gel 60 Merck (40-63 μm) as stationery phase and mobile phase as shown in Table 1.

Fractions 185-204 (70.0 mg) containing oleocanthal were further purified by preparative TLC using petroleum ether/ethyl acetate 3:7 as mobile phase. The corresponding zones were extracted from the stationary phase using ethyl acetate under sonication for 15 min. After, the solvent was filtered and evaporated under reduce pressure. This procedure was performed twice until maximum purity of oleocanthal (10.0 mg) was achieved. Identification and purity of the extracted compound were based on ^{1}H NMR, LC-MS and HPLC analyses. The ^{1}H NMR spectra were recorded in $CDCl_3$ on a Bruker AVANCE III™ 400 spectrometer (operating at 400 MHz) using solvent as internal standard. LC-MS runs were performed on AB-Sciex (Concord, Ontario, Canada) API 4000 triple quadrupole mass spectrometer, equipped with Turbo V electrospray ionization source (ESI), coupled to an Agilent Technologies (Santa Clara, CA, USA) 1290 UHPLC system, consisting of a high pressure pump, auto-sampler and column oven. For HPLC analysis, a Varian Prostar HPLC system equipped with a PDA Detector set to 278 nm was used. Analyses were performed on a reverse phase C18 column (Phenomenex 250 x 4.6 mm, 5 µm particle size, Gemini). Mobile phase consisted of 0.1 % v/v trifluoroacetic acid in acetonitrile (A) and 0.1 % v/v aqueous trifluoroacetic acid (B). The gradient elution program at a flow rate of 0.7 mL/min was as follow: 0-3 min 20% of A; 3-30 min from 20% to 95% of A; 30-35 min 95% of A. The analyses revealed for oleocanthal a purity >95%.

Analytical Reagents

Solvents used for the purification procedure, gradient solvents used for HPLC analyses and NMR solvents were purchased from Sigma-Aldrich. Evaporation was carried out under vacuum using a rotating evaporator. Silica gel flash chromatography was performed using silica gel 60 Å (0.040-0.063 mm; MERK). Thin layer chromatography (TLC) analysis was carried on Merck aluminium silica gel (60 F254) TLC plates that were visualized under a UV

lamp (λ = 254 nm) or by spraying with a 10 % solution of phosphomolybdic acid in absolute ethanol. Preparative thin layer chromatography (Prep TLC) purification was performed using either 2 mm (20 x 10) and 1 mm (10 x 10) glass-backed sheets pre-coated with silica gel 60 F254 purchased from VWR.

Cell cultures

A375 (CRL-1619^{TM}, American Type Culture Collection, Rockville, MD, USA) is a human melanoma cell line homozygous for V600E-B-RAF with high tumorigenic and metastatic potential. 501Mel is a stage IV, lymph node-derived heterozygous V600E cell line kindly gifted by Barbara Stecca (Istituto Toscano Tumori, Florence, Italy). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI supplemented with l-glutamine (2 mM), 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma-Aldrich, Milan, Italy). Adult human dermal fibroblasts (HDFa; Ref. C-013-5C, Gibco, Life Technologies, CA, USA) were cultured in an optimized medium containing 5% FBS and 1% penicillin/streptomycin, as recommended by the manufacturer.

Cell viability assay

Cell viability was measured using a method based on the cleavage of the 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium)-1,3-benzene disulfonate (WST-1) to formazan by mitochondrial dehydrogenase activity following manufacturer's instructions (Cell proliferation reagent WST-1; Roche, Mannheim, Germany). Briefly, cells (3×10^4/well) were seeded in 96-well plate in 10% FBS medium; after 24 h, the complete medium was replaced by 1% FBS medium containing oleocanthal or vehicle. Oleocanthal was dissolved in DMSO (final concentration never exceed 0.1%) and tested in a concentration range of 0.01–50 μM for 72 h.

At the end of treatment, WST-1 was added and absorbance measured at 450 nm using Infinite® M200 NanoQuant instrument (Tecan, Salzburg, Austria). Optical density values from vehicle-treated cells were considered as 100% cell viability.

RT-PCR and quantitative real-time PCR analyses

Total RNA from cells was extracted using the RNeasy Mini kit, following the manufacturer's instructions, and reverse-transcribed using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, California, USA). Real-time PCR was performed using SsoFast Eva Green Supermix (Ref. 172–5201; Bio-Rad, California, USA). Samples were amplified using the following thermal profile: 95°C for 30 s, 40 cycles of denaturation at 95°C 15 s, followed by annealing for 30 s and 72°C for 30 s, with a final step at 65°C for 5 s. Primer sequences are as follows: 5′-AACTGGAACGGTGAAGGTGAC-3′ (F) and 5′-GACTTCCTGTAACAACGCATCTC-3′ (R) for β-Actin (used as housekeeping gene); 5′-TCCATGTCTTTGGACAACCA-3′ (F) and 5′-CTCCACCAGTGTTCCCATCT-3′ (R) for Bcl-2, 5′-TCTGACGGCAACTTCAACTG-3′ (F) and 5′-TTGAGGAGTCTCACCCAACC-3′ (R) for Bax.

Western blot analysis

Cell lysates were collected after treatment with oleocanthal or vehicle at 10 μM for 30 min. Samples containing the same amount of protein (40 mg) were separated on a 10% SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane (Sigma–Aldrich, Milan, Italy), blocked with 5% non-fat milk in TBE and probed with specific antibodies. Incubation was performed at 4°C overnight with anti-ERK 1/2 (Ref. sc-514302, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p-ERK (Ref. sc-7383), anti-p-Akt1/2/3 (ser 473) (Ref. Sc-7985-R, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and

anti-β-actin (Ref. #MAB1501, Merck Millipore, Darmstadt, Germany) antibodies. Membranes were then washed with blocking solution and probed with specific secondary antibodies, as reported previously [5]. Quantification of proteins was performed using ImageJ densitometry software and signal intensities were normalized to those for β-actin.

Statistical analysis

All experiments were performed in triplicate and results analyzed by GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Data were shown as mean values ± standard error of the mean (SEM) obtained from at least three separate experiments. The IC_{50} values (concentrations that induce 50% cell growth inhibition) were determined using non-linear regression curve fit. The level of statistical significance was $p<0.05$.

RESULTS

Cell growth inhibition

Our findings demonstrated for the first time that oleocanthal induces cell growth inhibition in A375 and 501Mel cells in a concentration-dependent manner with IC_{50} mean values of 13.6±1.5 and 20±1.5 μM, respectively (Fig. 1). Of note, oleocanthal treatment did not exert significant changes in the human dermal fibroblasts viability (Fig. 1), suggesting selective activity for cancer cells versus normal cells.

Down regulation of the anti-apoptotic Bcl-2

To investigate the role of apoptosis in the mechanism of action of oleocanthal, we quantitatively assessed the effects on two important apoptotic genes by Real Time PCR. At a concentration that approximate the IC_{50} mean values obtained in cell growth experiments

(i.e., 10 μM), oleocanthal specifically down-regulated gene expression of the anti-apoptotic Bcl-2 protein both in A375 and 501Mel cells without affecting Bax expression (Fig. 2A).

Inhibition of oncogenic pathways

To find the potential molecular mechanism of action involved in cell growth inhibition, we tested the effects of oleocanthal on ERK1/2 and AKT by western blot. Activation of oncogenic targets was higher in A375 than 501Mel control cells (Fig. 2B). Such a difference was most probably due to the status of V600E-B-RAF mutation (homozygous for A375 and heterozygous for 501Mel) and/or related to other genotype/phenotype characteristics of the two cell lines tested. Oleocanthal at 10 μM for 30 min caused a remarkable reduction in ERK phosphorylation in both cell lines and such an effect was paralleled by inhibition of AKT phosphorylation (Fig. 2B).

DISCUSSION

In the current study, we clearly demonstrated that oleocanthal induces cytotoxicity against human melanoma cells with no effect on normal cells. We used two human melanoma cell lines with different stage of progression/invasiveness and status of BRAF mutations. Specifically, A375 cells are homozygous for V600E-B-RAF and have high tumorigenic and metastatic potential [6], while 501Mel, is a stage IV, lymph node-derived heterozygous V600E cell line[7]. Interesting to note, mutated B-RAF is associated with an aggressive disease phenotype[8] and our findings demonstrated that oleocanthal was able to induce cytotoxicity in both cell lines with similar IC_{50}s. In line with our results are those showing that oleocanthal inhibits the growth of human breast cancer cell lines MDA-MB-231, MCF-7 and BT-474, without affecting normal human MCF10A cell growth [9].

Noteworthy, selective antitumor effect by oleocanthal has been reported to be associated, at least partially, to its ability to induce lysosomal membrane permeabilization leading to apoptosis and/or necrosis. Indeed, cancer cells tend to have fragile lysosomal membranes compared to non-cancerous cells, making them sensitive to cell death induced by lysosomotropic agents [10].

Our findings showed a remarkable oleocanthal-mediated down-regulation of Bcl-2 gene expression both in the highly tumorigenic BRAF V600E mutant melanoma cell line, A375, and in the metastatic melanoma cell line, 501Mel. Such an effect was observed at a concentration of oleocanthal that approximate the IC_{50}s obtained in cell proliferation experiments. This is important from the pharmacological viewpoint because in resistant melanoma cells, defined by no loss of phosphorylated ERK after treatment with vemurafenib, inhibition of anti-apoptotic BCL-2 proteins can sensitize cells to both conventional and targeted therapies [11,12].

Down-regulation of ERK1/2 and AKT signal transduction pathways has been reported to play a role in the oleocanthal-induced cytotoxicity in multiple myeloma cells [13] and activation of the parallel PI3K/AKT pathway represents an important mechanism of resistance to B-RAF inhibitors [14]. We demonstrated by western blot analyses that oleocanthal significantly reduced ERK and AKT phosphorylation in human melanoma cells. Therefore, taking into account downstream targets of these pathways and their role in cell signaling and regulation, it is conceivable that inhibition of ERK and AKT pathways may lead to suppression of cell growth. The fact that in our study, oncogenic target inhibition occurred at 10 μM, i.e. a concentration that approximates the half-maximum concentration (IC_{50}) in cell growth experiments, may support this notion.

Other possible mechanisms of action reported in breast cancer models include blocking cell migration, invasion and G1/S cell cycle progression via inhibition of Hepatocyte growth

factor (HGF)-induced c-Met activation [9], a mechanism suggested as a therapeutic target also for melanoma metastasis [15]. Noteworthy, HGF has been implicated as an autocrine growth factor in A375 and 501 Mel cells[16] and, AKT and ERK1/2 signaling pathways are necessary for c-Met-mediated regulation of cell adhesion and invasion[17]. These evidence suggest that the potential effect on c-MET could account, at least partially, for the anticancer action of oleocanthal observed in our studyNoteworthy, additional findings supporting this notion are those showing that olive oil secoiridoids, including oleocanthal, are excellent scaffolds for the design of novel c-MET inhibitors [18].

It is well proven that oleocanthal inhibits cyclooxygenase (COX) enzymes exerting an important anti-inflammatory activity similar to that of ibuprofen [3]. We did not test the possible involvement of these enzymes in the mechanism of action of oleocanthal. However, we recently demonstrated that treatment of A375 cells with selective (i.e., rofecoxib) or nonselective (i.e., indomethacin) COX-2 inhibitors did not significantly affect A375 cell viability [19]. Although this specific point needs to be experimentally addressed, the abovementioned evidence together with the widely recognized COX-2–independent anticancer effects of selective COX-2 inhibitors [20], suggest a little or no role of COX enzymes in the antitumor activity of oleocanthal observed in our experimental setting.

In conclusion, the ability of oleocanthal to inhibit ERK1/2 and AKT phosphorylation and to down-regulate Bcl-2 expression represents a pharmacological profile that deserves further investigation in skin cancer.

FUNDING

This work was supported by Association against Melanoma Onlus (Italy).

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FIGURE LEGENDS

Figure 1. Cell viability after treatment with oleocanthal for 72 h. A375 and 501Mel: human melanoma cells; HDFa: human dermal fibroblasts, adult. *$p<0.05$ (n=3).

Figure 2. (A) Real-Time PCR analysis of Bax and Bcl-2 expression in A375 and 501Mel cells. (B) Western blot analysis of total and phosphorylated ERK 1/2 and phosphorylated AKT in A375 and 501Mel cells. **$p<0.01$; ***$p<0.001$; One-way ANOVA followed by Newman-Keuls multiple comparison test.