

Oleocanthal exerts antitumor effects on human liver and colon cancer cells through ROS generation

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Abstract. The beneficial health properties of the Mediterranean diet are well recognized. The principle source of fat in Mediterranean diet is extra-virgin olive oil (EVOO). Oleocanthal (OC) is a naturally occurring minor phenolic compound isolated from EVOO, which has shown a potent anti-inflammatory activity, by means of its ability to inhibit the cyclooxygenase (COX) enzymes COX-1 and COX-2. A large body of evidence indicates that phenols exhibit anticancer activities. The aim of the present study was to evaluate the potential anticancer effects of OC in hepatocellular carcinoma (HCC) and colorectal carcinoma (CRC) models. A panel of human HCC (HepG2, Huh7, Hep3B and PLC/PRF/5) and CRC (HT29, SW480) cell lines was used. Cells were treated with OC, and cell viability and apoptosis were evaluated. Compared with classical commercially available COX inhibitors (ibuprofen, indomethacin, nimesulide), OC was more effective in inducing cell growth inhibition in HCC and CRC cells. Moreover, OC inhibited colony formation and induced apoptosis, as confirmed by PARP cleavage, activation of caspases 3/7 and chromatin condensation. OC treatment in a dose dependent-manner induced expression of γ H2AX, a marker of DNA damage, increased intracellular ROS production and caused mitochondrial depolarization. Moreover, the effects of OC were

suppressed by the ROS scavenger N-acetyl-L-cysteine. Finally, OC was not toxic in primary normal human hepatocytes. In conclusion, OC treatment was found to exert a potent anticancer activity against HCC and CRC cells. Taken together, our findings provide preclinical support of the chemotherapeutic potential of EVOO against cancer.

Introduction

In recent years, the health-promoting and disease prevention properties of the Mediterranean diet (MD) have been highlighted (1). The Mediterranean populations, which follow this pattern of eating for cultural and natural reasons, present a reduced incidence of chronic inflammation-derived diseases. The view that the MD is associated with a reduced incidence of inflammatory diseases is supported by a large number of studies (2). Extra-virgin olive oil (EVOO) is a common component of MD, which for some time has been examined in studies that have highlighted its health benefits (3-6). In EVOO a number of phenolic compounds have been identified, including tyrosol (7), hydroxytyrosol (8), oleuropein (9), and many others, as well as Oleocanthal (OC) [(-)-deacetoxyligstroside aglycone]. In 2005, Beauchamp *et al* identified in OC the pungent component of EVOO that induces a strong prickling sensation in the throat, similar to that caused by the non-steroidal anti-inflammatory drug (NSAID) ibuprofen (10). The authors showed that OC exhibits anti-inflammatory activity, as it is an inhibitor of cyclooxygenases (COXs), COX1 and COX2, two enzymes involved in the synthesis of prostaglandins and thromboxanes from arachidonic acid (10).

Recently, various studies have shown that OC exhibits anticancer activities by inhibiting cell proliferation, migration, and invasion in different human cancer cell types (11-16). In the breast cancer model OC suppresses cell proliferation, invasiveness and tumor growth by inhibiting the HGF-induced phosphorylation of c-Met and suppressing the Brk/paxillin/Rac1 signaling pathway, via inhibition of Brk phosphorylation (11). *In vivo* studies in mice have shown that

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OC treatment suppresses tumor cell growth (11). Moreover, further studies have demonstrated that OC inhibits the growth of several breast cancer cell lines by inhibiting the enzymatic activity of mTOR, a serine/threonine kinase which is involved in cell survival and proliferation in cancer cells (17).

Hepatocellular carcinoma (HCC), is an inflammation-related cancer that arises in the context of hepatic damage and inflammation. HCC is the fifth most common cancer worldwide, characterized by an increasing incidence and a poor prognosis (18-22). It is largely asymptomatic until it is in the advanced state, when the treatments available are often unsuccessful, the standard treatments being surgical resection and liver transplantation. Other treatments, such as chemoembolization and ultrasound ablation techniques also rarely lead to a complete recovery (22). Standard cancer drugs such as doxorubicin, cisplatin, and 5-fluorouracil have a very limited efficacy (22). Moreover, the latest new targeted therapy approved for the treatment of patients with advanced HCC, e.g. sorafenib, has a poor efficacy (23).

In the inflammation process an important role is attributed to the COX enzymes, although the role of the cyclooxygenases in hepatocellular carcinogenesis is still unclear. Some studies have shown an increased expression of COX-2 in patients with different types of liver disease, suggesting its possible role in hepatocarcinogenesis, especially in the early stages (24-26). Results from our laboratory have confirmed the antitumor and pro-apoptotic effects of COX inhibitors used alone or in combination with other targeted specific drugs (27-32). However, our results and those of other authors suggest that often the anticancer activities of COX inhibitors might be due to COX-independent effects (32).

Colorectal cancer (CRC) is one of most common cancers worldwide, with a number of different etiologies. However, the largest proportion of CRC cases has been linked to environmental causes, such as chronic intestinal inflammation (33). Elevated COX-2 expression has been found in most CRC cancer tissues and it is associated with poor prognosis (34-37). Large epidemiological studies have demonstrated that NSAIDs reduce the risk of CRC cancer in humans and, recently, also an antitumoral activity of NSAIDs has been described in CRC (38-40).

Although OC has already been shown to inhibit growth and metastasis as well as tumorigenicity in different tumor cell types, the underlying molecular mechanism of action in HCC and CRC is not yet fully understood.

In this study, we investigated the anticancer effects of OC in HCC and CRC cell lines. Interestingly, OC caused a loss of cell viability and induced apoptosis in both liver and colon cancer cells, without affecting the cell viability of healthy primary hepatocytes, through ROS generation and independently of COX-2 expression.

Materials and methods

Cell lines, cell culture and reagents. The human hepatocarcinoma cell lines HepG2, Hep3B, Huh7, PLC/PRF/5 and the colon carcinoma cell lines SW480 and HT29 used in this study were maintained in RPMI medium (Sigma-Aldrich, St. Louis, MO, USA), containing 10% (v/v) Fetal Bovine Serum (FBS) (Gibco, Life Technologies, Monza, Italy).

The HCC cell lines have different characteristics of differentiation, biological behavior, and genetic defects (31). HepG2 and Hep3B cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The PLC/PRF/5 cells used in this study were a gift from Professor O. Bussolati (University of Parma, Parma, Italy). The other cell lines used were gifts from various sources: the Huh-7 cells from Professor M. Levrero (Department of Internal Medicine, Sapienza University, Rome, Italy); SW480 cells from Dr J.L. Iovanna (Inserm, Marseille, France); and HT29 cells from Professor S. Travali (University of Catania, Catania, Italy). All cell lines were authenticated by short tandem repeat (STR) profiling (BMR Genomics, Padua, Italy), and used within 6 months of receipt.

OC was synthesized as previously described (41). Nimesulide, SC560 and ibuprofen were purchased from Cayman Chemical (Ann Arbor, MI, USA). All the reagents were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich)

Cell viability assays. Cells (5×10^3 /well) were distributed into each well of 96-well microtiter plates and then incubated overnight. At time 0, the medium was replaced with fresh complete medium plus 1% (v/v) FBS, and different doses of OC, nimesulide, SC560 and ibuprofen were added. Cells were cultured for 24, 48 and 72 h. At the end of treatment, MTS assays were performed using the CellTiter Aqueous OneSolution kit (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Cell viability was expressed as a percentage of the absorbance measured in the control cells. Values were expressed as means \pm SD of three separate experiments, each performed in triplicate. In some experiments, cells were treated with OC plus the antioxidant N-acetyl-L-cysteine (NAC) (Sigma-Aldrich).

Colony formation assays. The effects of different inhibitor concentrations on cell growth were also assessed using a clonogenic assay. For this analysis, $1.0-1.5 \times 10^3$ cells were plated in 6-well plates in growth medium, and after overnight attachment cells were exposed either to OC or vehicle for 48 h in fresh complete medium with 1% (v/v) FBS. The cells were then washed and allowed to grow for 14 days in drug-free complete medium with 10% (v/v) FBS, after which the cell colonies were fixed with 70% ethanol at 4°C for 20 min. and stained with crystal violet (0.1% in H₂O) for 5 min. The plates were rinsed with water, air-dried, photographed and evaluated for colony formation. Colonies containing more than 50 cells were counted.

Data are expressed as a percentage of colonies in untreated cells and are the means \pm SD of three separate experiments, each of which was performed in duplicate.

Caspase activity assays. Cells (5×10^3 /well) were treated with 25 μ M OC, and after 24 h the activity of caspases 3 and 7 were measured by the Caspase-Glo[®] 3/7 (Promega Corp.) Assay according to the manufacturer's instructions. Results were expressed as arbitrary units (AU). Values were the mean \pm SD of three separate experiments, each performed in duplicate.

Western blot analysis. Cell/well (35×10^4) were plated in 6-well plates. After 24 h of treatment whole cellular lysates from cells

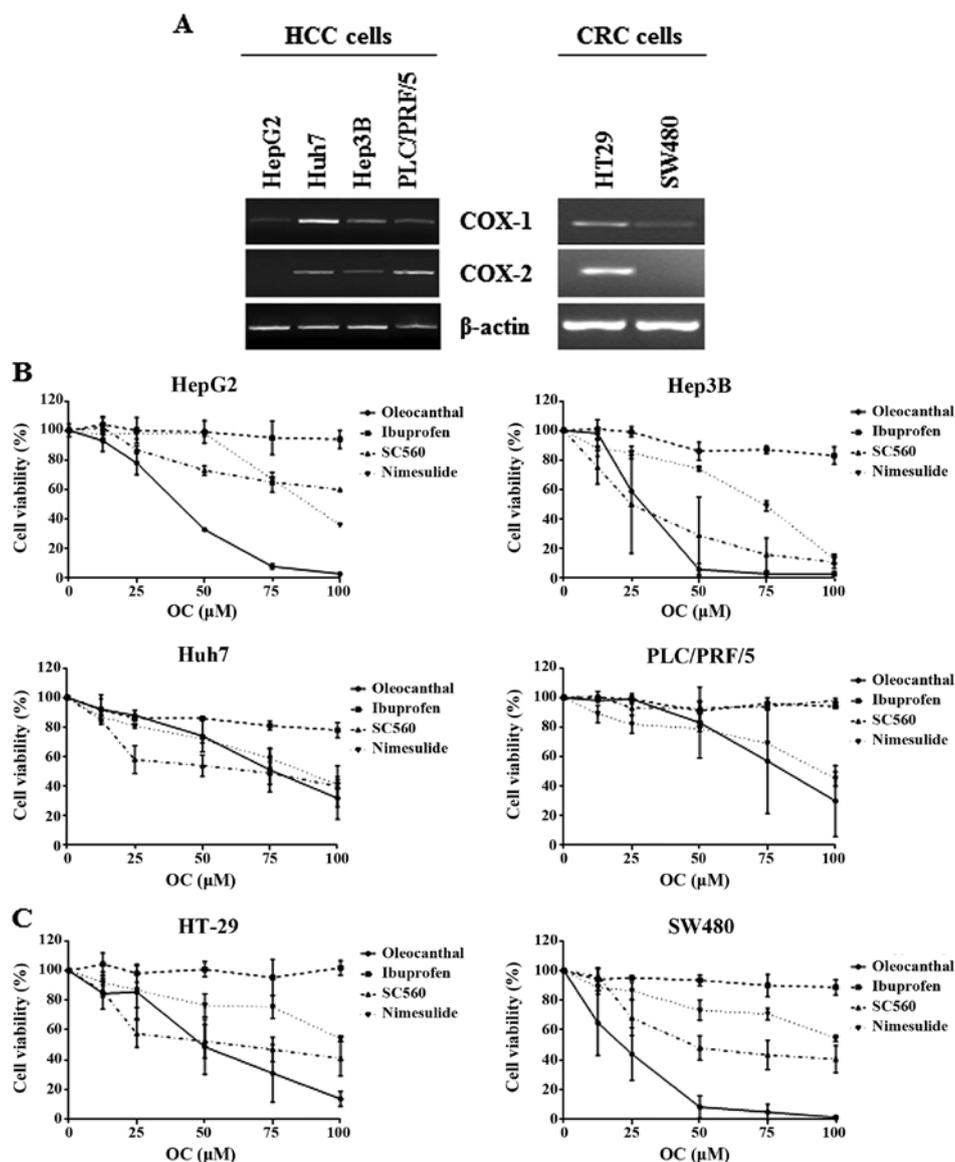


Figure 1. Oleocanthal (OC) treatment inhibits cell viability in HCC and CRC cells. (A) COX-1 and COX-2 mRNA were evaluated by RT-PCR in a panel of HCC and CRC cell lines. (B) HCC and (C) CRC cells were treated with OC, ibuprofen (COX-2 inhibitor), SC560 (COX-1 inhibitor) and nimesulide (dual COX-1 and COX-2 inhibitor) at the indicated concentrations and cell viability was evaluated using MTS assays after 72 h. Data are expressed as a percentage of control cells and are the means \pm SD of three separate experiments, each of which was performed in triplicate.

were obtained using RIPA buffer (Cell Signaling Technologies Inc., Beverly, MA, USA) and western blots were performed using the methodology for the Odyssey[®] infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA), as previously described (42). Membranes were scanned and analyzed with an Odyssey infrared imaging system (LI-COR Biosciences) using Odyssey 3.0 imaging software. Antibody signals were analyzed as integrated intensities of regions defined around the bands of interest in either channel, with primary antibodies raised against β -actin (Sigma-Aldrich), phospho-p38, p38, PARP and γ H2AX (Cell Signaling Technologies Inc.).

Flow cytometry analysis. After 24 h of OC treatment, 0.5×10^6 cells were collected and stained with FITC-conjugated Annexin V antibody, and propidium iodide (Apoptosis detection kit; Dojindo, Munich, Germany). The number of viable, apoptotic and necrotic cells were determined using the

FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Results are presented as percentage. Values represent the mean \pm SD of two separate experiments.

Measurement of reactive oxygen species (ROS). The intracellular accumulation of ROS was determined using the fluorescent probe 2',7'-difluorodihydrofluorescein diacetate (H₂DCFDA) and MitoSOX[™] Red mitochondrial superoxide indicator (Invitrogen Corp., Camarillo, CA, USA). Cells (2×10^4) were treated with 25-50 μ M OC for 24 h and then incubated with the probe in the dark at 37°C in 5% CO₂ incubator according to the manufacturer's instructions. Cells were observed with fluorescence microscopy (Axioskop; Zeiss, Oberkochen, Germany) and photographed.

TUNEL assays. Cells were cultured in 8-well chamber slides overnight. After treatment for 24 h with 25-50 μ M OC, cells

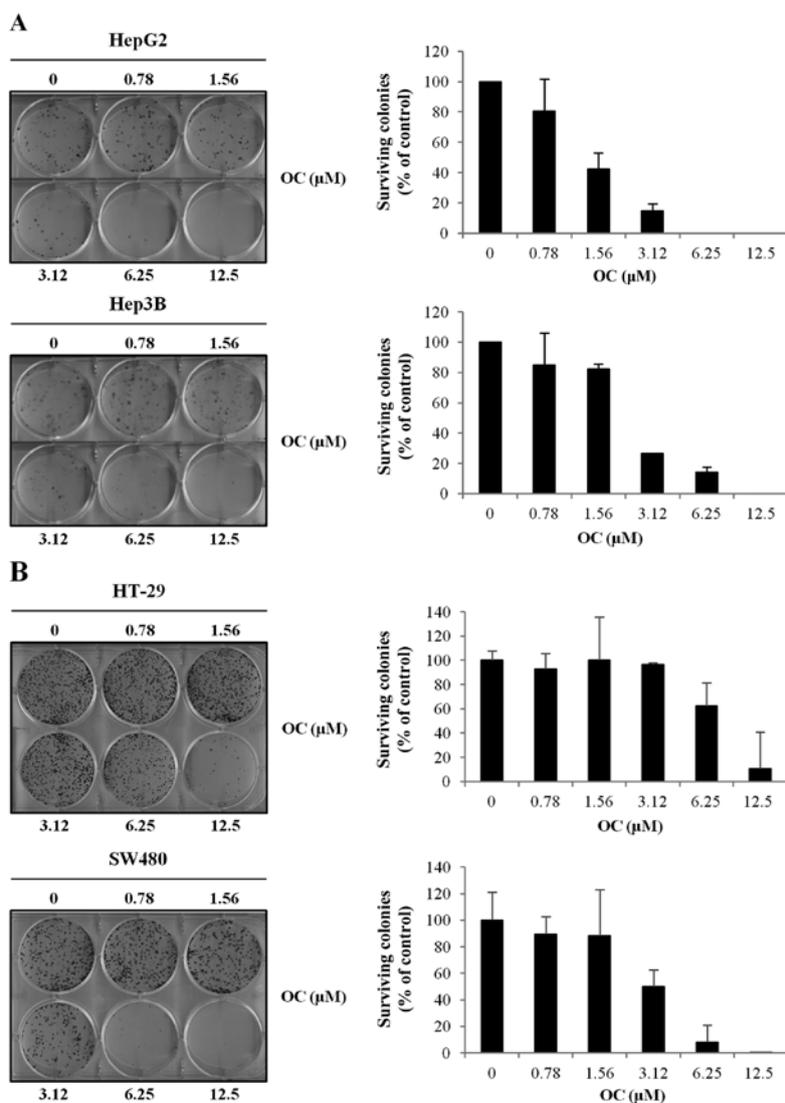


Figure 2. Oleocanthal (OC) treatment inhibits colony formation capacity of HCC and CRC cells. (A) HCC and (B) CRC cells were plated overnight and exposed to OC for 48 h, followed by growth in fresh culture media for 14 days, as described in Materials and methods. Surviving colonies were stained (left panel) and counted (right panel). Data are expressed as a percentage of colonies in untreated cells and are the means of three separate experiments, each of which was performed in duplicate.

were washed twice with PBS and fixed in 4% paraformaldehyde solution for 25 min at room temperature. Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay using the DeadEnd™ Colorimetric TUNEL System kit from Promega Corp., following the manufacturer's instructions. Cells were visualized with an Axioskop microscope (Zeiss).

Measurement of mitochondrial membrane potential. For $\Delta\Psi_m$ measurement, cells (2×10^4 cells/well) in chamber slides were treated with 25–50 μM OC for 24 h. JC-1 staining solution (5 $\mu\text{g}/\text{ml}$, Thermo Fisher Scientific Inc., Waltham, MA, USA) was added to both treated and untreated cells at 37°C for 15 min, according to the manufacturer's instructions. After washing twice with PBS, mitochondrial membrane potential was monitored by determining the relative amounts of dual emission with a multiple fluorescence reader (Victor; Perkin Elmer, Waltham, MA, USA) and cells were visualized with a fluorescence microscope.

Purification of normal human hepatocytes. All human tissues were collected with informed consent following ethical and institutional guidelines. Liver tissue dissociation and subsequent hepatocyte isolation procedures were performed as previously described (43). Cell viability was assessed by Trypan Blue (Sigma-Aldrich) exclusion method, and plating efficiency assessed as previously described (43).

After 24–48–72 h of exposure to OC, hepatocyte status (ATP content) was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corp.). Fresh medium with drugs was changed daily.

Double-stranded DNA (dsDNA) quantification was performed with a Quant-iT™ PicoGreen® dsDNA ultrasensitive fluorescent nucleic acid staining kit (Molecular Probes; Invitrogen Corp.), as previously described (44). Briefly, after CellTiter-Glo measurement, each well was incubated with 80 μl Quant-iT PicoGreen in Tris-Ethylenediaminetetraacetic acid (EDTA) buffer, and the fluorescence intensity was read on a fluorescent spectrometer (Synergy HT; BioTek Instruments,

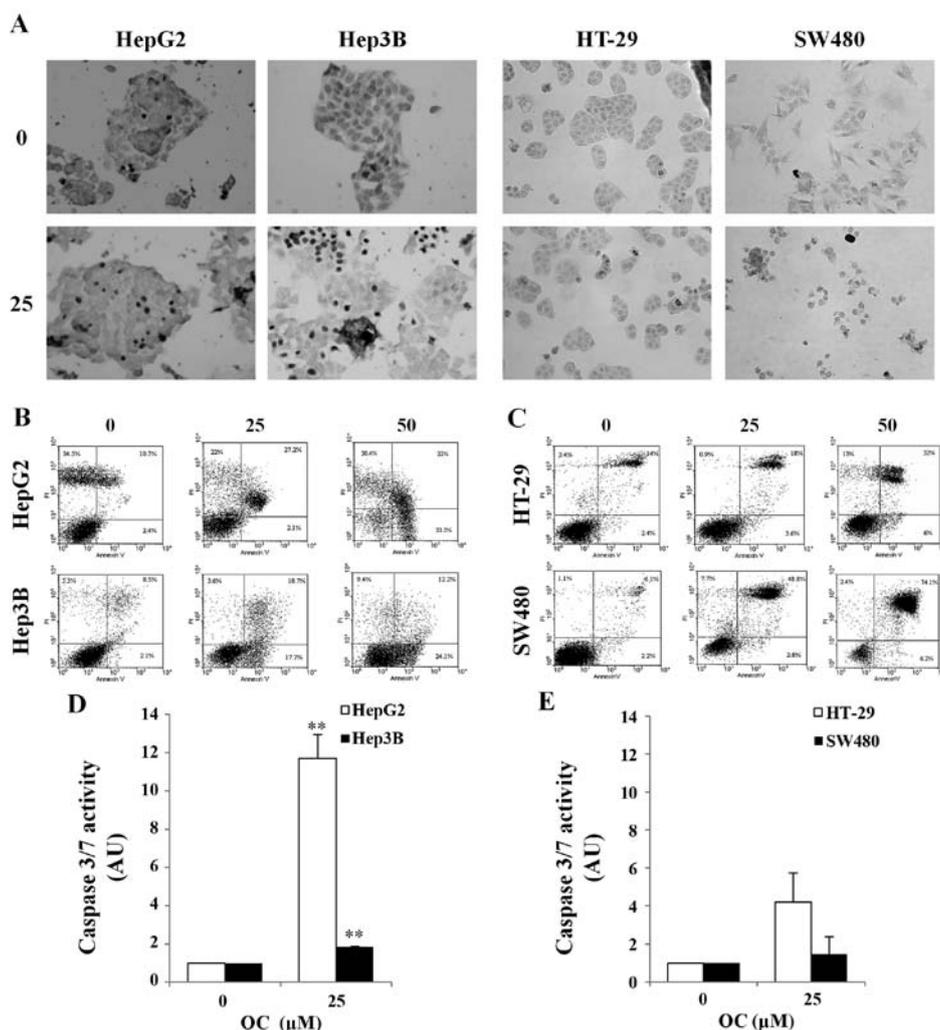


Figure 3. Oleocanthal (OC) treatment induces apoptosis in HCC and CRC cells. (A) Detection of apoptosis by TUNEL assay as described in Materials and methods. Photomicrographs of HepG2, Hep3B, HT29 and SW480 cells treated for 24 h with 25 μM OC. (B and C) Representative dot plot of flow cytometry analysis. Apoptotic cell death was determined by Annexin V/propidium iodide double labeling in HCC and CRC cells. (D) HCC and (E) CRC cells were treated with OC for 24 h, and levels of caspase activity were measured by Caspase-Glo® 3/7 assays. Data are expressed as arbitrary units (AU) normalized to control value and are the means ± SD of three separate experiments, each of which was performed in duplicate. **p<0.01 versus untreated cells.

Inc., Winooski, VT, USA) at an excitation wavelength of 488/15 nm and an emission wavelength of 528/20 nm. dsDNA concentration was quantified by interpolating the A528 values for the unknowns from a standard curve of λ DNA using the equation (dsDNA (mg/ml) = 0.1057 × A528 - 61.322; R2=0.9941). The luminescence produced was normalized to the amount of DNA in each well, and normalized to control cells (0.1% DMSO). Three separate experiments using different batches of primary isolated human hepatocytes were used, each experiment performed in triplicate.

Statistical analysis. Statistical analysis was performed using Student's two-tailed t-test. Statistical significance was assessed at p<0.05.

Results

Oleocanthal inhibits cell viability and colony formation capacity of HCC and CRC cells. To investigate the potential anticancer effects of OC in hepatocellular carcinoma (HCC), we exposed 3 human HCC cell lines, characterized by different

properties such as: differentiation status, biological behavior and genetic defects (27). In particular, as regards COX-1 and COX-2 mRNA expression, all cell lines expressed COX-1 mRNA, whereas each type of the cell lines showed different expression levels of COX-2 mRNA (Fig. 1A); PLC/PRF/5 cells expressed the highest COX-2 mRNA levels while in HepG2 cells COX-2 mRNA was undetectable (Fig. 1A).

To determine whether the effects of OC were cell-type specific we also used a different cancer model, i.e. human colorectal carcinoma (CRC). For this purpose, we used the HT29 and SW480 cells, which are known to be COX-2-positive and COX-2-negative, respectively (45) (Fig. 1A).

Since OC displays anti-inflammatory properties similar to the nonsteroidal anti-inflammatory drug (NSAID) ibuprofen (10), a dual inhibitor of cyclooxygenase (COX) enzymes COX-1 and COX-2, we compared the effects on cell viability and cell survival of OC with that of ibuprofen, as well as, the COX-2 inhibitor nimesulide and the COX-1 inhibitor SC560.

Cell viability assays were performed in HCC and CRC cells using different concentrations of OC and other anti-inflammatory compounds (Fig. 1B and C). Of note, after

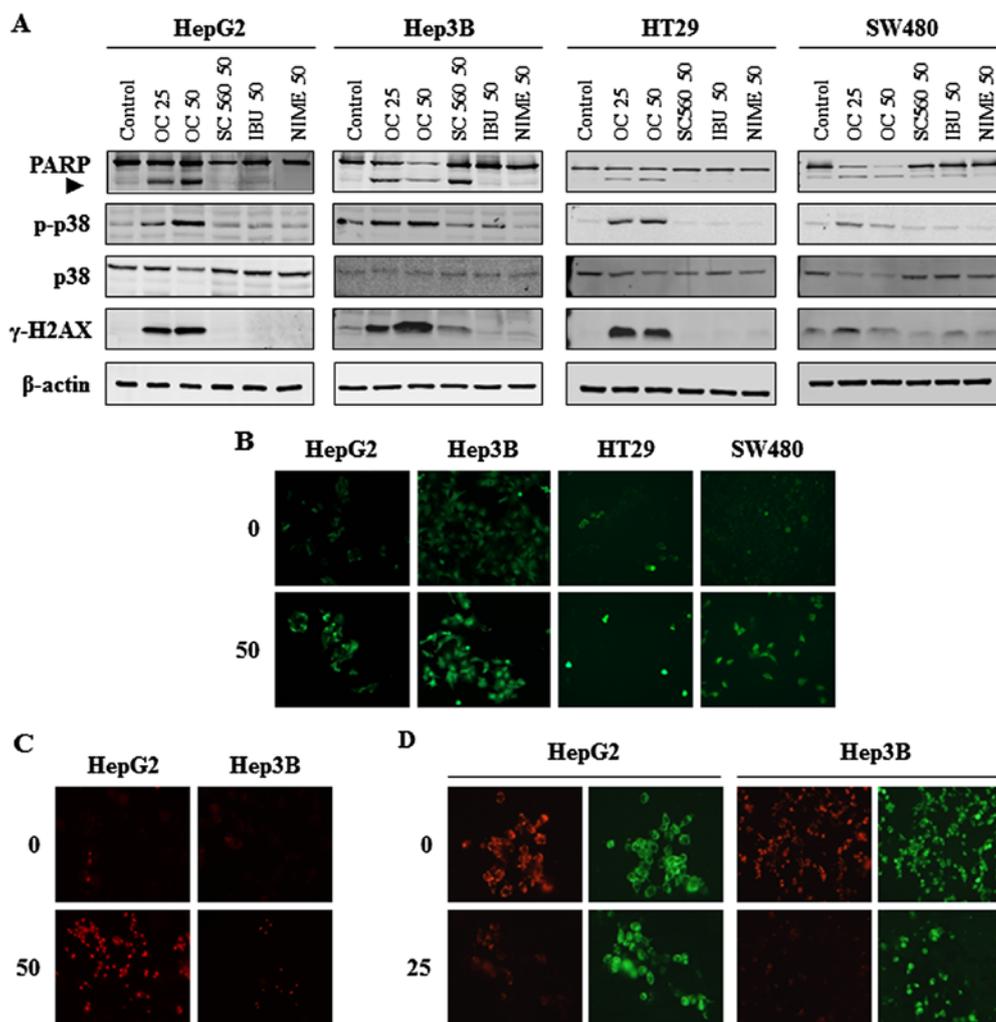


Figure 4. Oleocanthal (OC) treatment induces apoptosis, DNA damage, ROS generation in HCC and CRC cells, and alters mitochondrial membrane potential in HCC cells. (A) Western blot analysis of PARP, phospho-p38 and γ -H2AX expression in HCC and CRC cells after treatment with OC and NSAIDs at the indicated concentrations (μ M) for 24 h. The data shown represent three independent experiments with comparable outcomes. The arrowhead indicates the 85 kDa form of PARP. (B) Cells were untreated or treated with OC at the indicated concentrations (μ M) for 24 h, and intracellular ROS levels were evaluated by H_2 DCFDA staining. The data represent three different experiments with comparable outcomes. (C) Cells were treated with OC at the indicated concentrations (μ M) for 24 h and the presence of mitochondrial superoxide anions was evaluated using a MitoSOX-red fluorescent probe. The data represent two different experiments with comparable outcomes. (D) HCC cells were treated with 25 μ M OC for 24 h and mitochondrial membrane depolarization was determined by using a MitoProbe JC-1 assay. The JC-1 fluorescence probe exhibits a potential-dependent accumulation in functional mitochondrial membrane where it exhibits a red fluorescence. A decrease in the red/green fluorescence intensity ratio indicates mitochondrial depolarization. Data are representative of two different experiments with comparable outcomes.

treatment for 72 h, OC was more effective than ibuprofen in inhibiting proliferation in all the cell lines analyzed. In Hep3B cells, treatment with OC showed the strongest inhibition of cell viability with an IC_{50} value of 26.6 μ M, followed by HepG2 cells (IC_{50} =41.9 μ M), whereas in Huh7 and PLC/PRF/5 cells, OC displayed an effect comparable to that of nimesulide and SC560 (Fig. 1B).

In addition, as shown in Fig. 1C, OC inhibited cell viability in a dose-dependent manner in both CRC cell lines. OC was more effective than the NSAIDs nimesulide and ibuprofen, and also SC560, as already observed in HCC cells (Fig. 1B). Time course analyses of cell viability assays did not show any time-dependent effects of OC (data not shown).

Several lines of evidence have pointed out that the effect of NSAIDs does not always depend on their effect on the inhibition of COX enzyme activities, i.e. the mechanism of action of NSAIDs is also COX-2-independent (32,45-47). Therefore,

we continued all our studies using the HCC cell line HepG2 cells and the CRC SW480 cells, as models for COX-2 negative cells, and Hep3B cells and HT-29, respectively, as models for COX-2 positive cells.

We then investigated the effect of OC on colony formation in HCC and CRC cells. In both cancer types, OC displayed a strong dose-dependent inhibition of colony-forming capacity that was independent of COX-2 expression (Fig. 2A and B).

Oleocanthal induces apoptosis in HCC and CRC cells.

To further explore the mechanism of loss of cell viability observed after OC treatment, we analyzed the activation of apoptotic response in HCC and CRC cell lines. The results in Fig. 3A show that inhibition of cell viability in HCC and CRC cells after OC treatment is related to the induction of apoptosis as confirmed by the increased number of apoptotic cells (brown/dark nuclei). We also quantified apoptosis after 24 h of

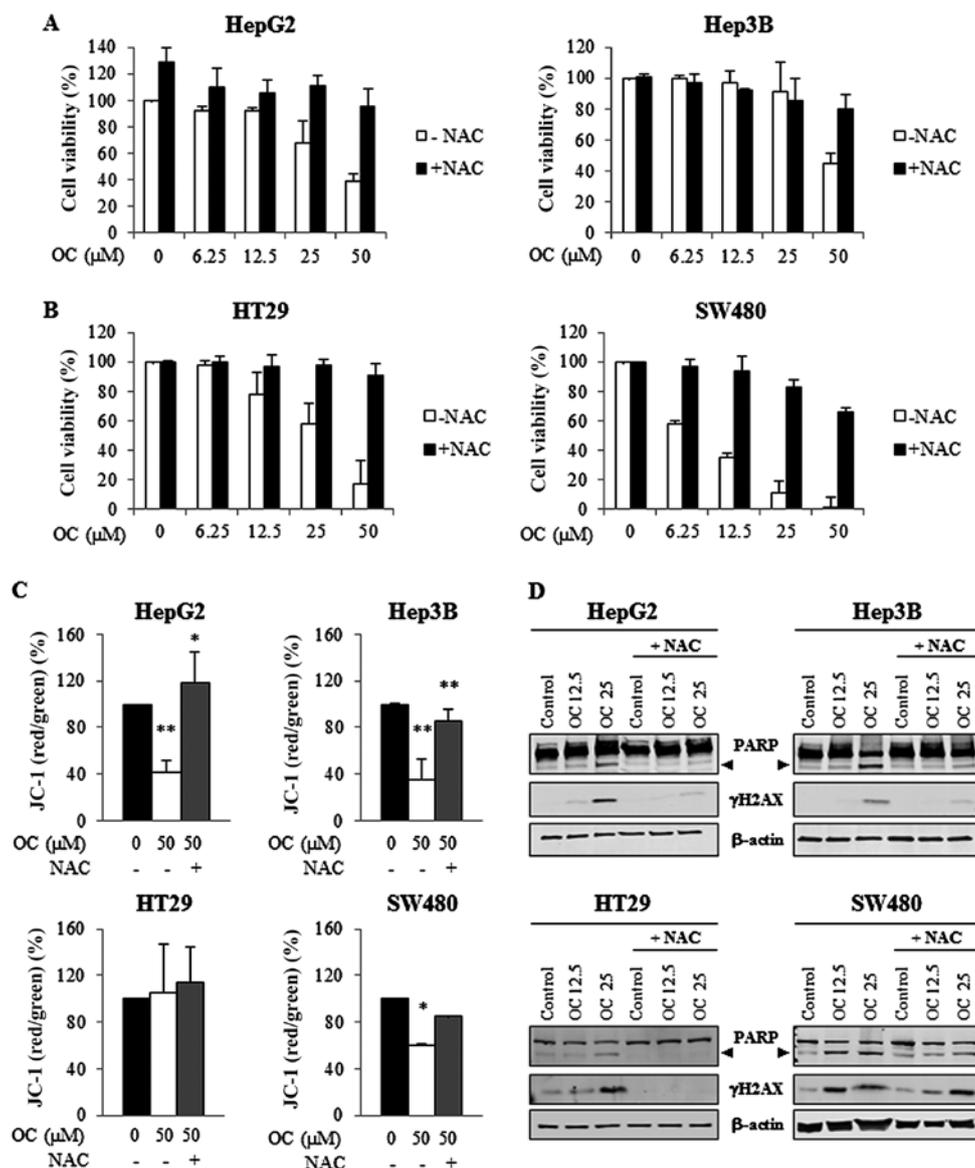


Figure 5. The antioxidant NAC reverses oleocanthal (OC) effects in HCC and CRC cells. (A) HCC and (B) CRC cells were pre-treated (2 h) with NAC (1 mM) before and during OC treatment for 24 h, and cell viability was assessed by MTS. The data are expressed as a percentage of control cells and are the means \pm SD of three separate experiments, each of which was performed in triplicate. (C) Mitochondrial membrane depolarization was determined using a MitoProbe JC-1 assay. Cells were treated with OC with or without 1 mM NAC for 24 h and the fluorescence values measured were reported in a histogram as a percentage of control values. A decrease in the red/green fluorescence intensity ratio indicates mitochondrial depolarization. The data are the means \pm SD of two separate experiments, each of which was performed in duplicate. * p <0.05 and ** p <0.01 versus untreated cells. (D) Western blot analysis of PARP and γ -H2AX in HCC and CRC cells treated with the indicated concentrations of OC (μ M) for 24 h with or without NAC (1 mM). Data shown are representative of independent experiments with comparable outcomes. The arrowhead indicates the 85 kDa form of PARP.

treatment with OC by flow cytometry analysis after staining cells with Annexin V/PI (Fig. 3B and C). The percentage of apoptotic cells increased after OC treatment from 10% in untreated Hep3B cells to 30% in Hep3B cells treated with 50 μ M OC, and from 10 to 56% in HepG2 cells (Fig. 3B).

In CRC cells, the percentage of apoptotic cells increased from 14% to 29% in HT29 and from 17 to 65% in SW480 after treatment with 50 μ M OC (Fig. 3C). All experiments were performed in 1% FCS, a growth condition that could explain the presence of some dead cells among the untreated cells (control).

Apoptosis induction was also investigated by analyzing caspase activation. The caspase assay displayed an activation of caspases 3/7 after 24 h of treatment with 25 μ M OC

(Fig. 3D and E) in all cell lines. In addition, western blot analyses of HCC and CRC cell lysates showed cleavage of Poly (ADP-ribose) polymerase (PARP) after treatment with OC in HepG2, HT29 and SW480 cells, and also after treatment with OC and SC560 in Hep3B cells. In contrast, a dose of either 50 μ M ibuprofen or nimesulide did not induce the cleavage of PARP in any of the cell lines (Fig. 4A). Western blot analyses after OC treatment also indicated an increase in phosphorylated levels of the stress kinase p38, known to be involved in death signaling (Fig. 4A). Taken together these data confirm that OC induced apoptosis in HCC and CRC cells.

Oleocanthal increases reactive oxygen species (ROS) generation in HCC and CRC cells. A number of studies have

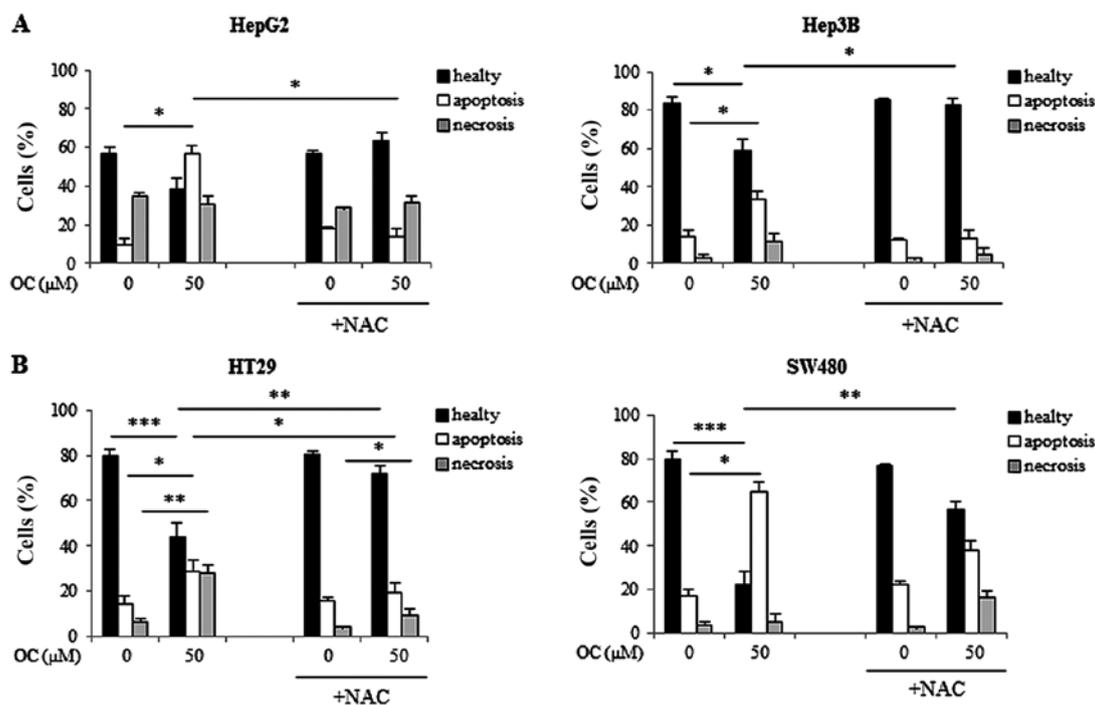


Figure 6. The antioxidant NAC rescues cell viability after oleocanthal (OC) treatment. (A) HCC and (B) CRC cells were pre-treated (2 h) with NAC (1 mM) before and during OC treatment for 24 h. Flow cytometry analysis was performed using Annexin V/propidium iodide double staining as indicated in Materials and methods. Percentage values of healthy, apoptotic and necrotic cells are reported in histograms. Data are the means \pm SD of three separate experiments performed in duplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus untreated cells.

shown that phenolic compounds may have pro-oxidant activities via the production of reactive oxygen species (ROS), and that ROS generation in cells is an effective apoptotic inducer (48-50).

To better understand the mechanism of apoptosis induced by OC, we evaluated ROS production, using the cell-permeable fluorescent probe H_2DCFDA . OC treatment induced intracellular ROS production in both HCC and CRC cells (Fig. 4B). Moreover, to identify the type of ROS produced on OC treatment, we evaluated the presence of superoxide anions with the MitoSOX-red fluorescent probe. As shown in Fig. 4C, the production of superoxide anions increased after treatment with OC in HCC cell lines.

Since high levels of ROS are known to produce DNA damage and impair mitochondrial integrity, we next evaluated the effect of OC treatment on phospho-H2AX (γ -H2AX) expression levels and on mitochondrial membrane potential. Western blot analysis showed a strong increase in γ -H2AX histone levels on OC treatment in all cell lines (Fig. 4A). These results indicate a strong induction of ROS production which caused DNA damage after treatment with OC.

$\Delta\psi_m$ were assessed by staining OC-treated HCC cells with the membrane permeable dye JC-1, a widely-used probe for determining changes of $\Delta\psi_m$ (Fig. 4D). JC-1 specifically shows potential-dependent accumulation in depolarized mitochondria, displaying a red to green fluorescence shift. The intensity of JC-1 red fluorescence was lower in the OC-treated cells than in the control, indicating a depolarization of the mitochondrial membrane (Fig. 4D).

Treatment with N-acetyl-L-cysteine (NAC) reverses the cytotoxic effects of oleocanthal in HCC and CRC cells. In

the previous experiments, we observed an induction of ROS generation due to treatment with OC in HCC and CRC cells. To determine whether OC induces cytotoxic effects via ROS generation, we tested the effects of the ROS scavenger N-acetyl-L-cysteine (NAC) on cell viability, on apoptosis activation and on DNA damage. For these purposes, cells were pre-treated with NAC (1 mM) for 2 h and subsequently treated with different concentrations of OC for an additional 24 h in the presence of NAC.

The cell morphology of HCC and CRC cell lines after 24 h of OC treatment was examined by light microscopy with or without the presence of NAC (not shown). In samples treated with both OC and NAC there was a reduction in the number of floating cells, cells remained spread as non-treated cells, suggesting a recovery of cell vitality, otherwise in samples treated with OC alone cells were shrunken, detached, and fragmented into membrane-bound apoptotic bodies. The recovery in cell viability was confirmed by MTS assays. In all cell lines, NAC significantly decreased the OC-induced inhibition of cell viability (Fig. 5A and B). In addition, treatment with NAC abrogated OC-induced mitochondrial membrane depolarization and the ratio of red/green JC-1 fluorescence was restored to normal levels (Fig. 5C).

Furthermore, as shown by western blot analysis (Fig. 5D), treatment with NAC also prevented OC-induced PARP cleavage and γ -H2AX activation.

Finally, apoptosis inhibition by NAC treatment was also confirmed by flow cytometry analysis. Flow cytometry analysis of cells treated with 50 μM OC in the presence or absence of NAC confirmed that NAC prevented OC-induced apoptosis, leading to the recovery of viable cells (Fig. 6A and B). In Hep3B cells, the percentage of apoptotic cells declined from

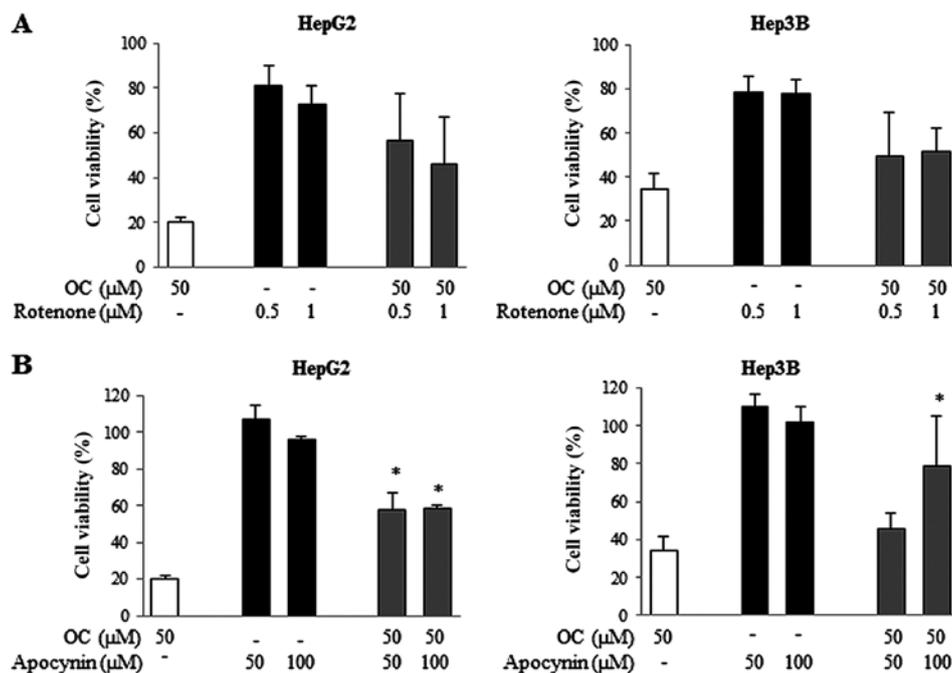


Figure 7. NADPH oxidase enzyme is the main source of ROS in HCC cells. (A) Cells were co-treated with oleocanthal (OC) and MRC complex I inhibitor rotenone at the indicated concentrations for 24 h and cell viability was evaluated using MTS assays. Data are expressed as a percentage of control cells and are the means \pm SD of three separate experiments, each of which was performed in triplicate. (B) Cells were co-treated with OC and NADPH oxidase inhibitor apocynin at the indicated concentrations for 24 h and cell viability was evaluated using MTS assays. Data are expressed as a percentage of control cells and are the means \pm SD of three separate experiments, each of which was performed in triplicate. * $p < 0.05$ versus cells treated with OC.

33 \pm 8% after treatment with OC to 13 \pm 1% after treatment with OC plus NAC. In HepG2 cells the percentage of apoptotic cells decreased from 56 \pm 6% after treatment with OC to 13 \pm 9% after treatment with OC plus NAC (Fig. 6A). In SW480 cells, the percentage of apoptotic cells diminished from 65 \pm 18% after treatment with OC to 38 \pm 2% after treatment with OC plus NAC. In HT29 cells, the percentage of apoptotic cells declined from 29 \pm 4% after treatment with OC to 19 \pm 4% after treatment with OC plus NAC (Fig. 6B).

Taken together, these results indicated that the oxidative stress induced by OC treatment reduced the cell viability of HCC and CRC cells, and that apoptosis activation, mitochondrial and DNA damage are downstream of the oxidative stress.

NADPH oxidase enzyme is the main source of ROS in HCC cells treated with OC. We then investigated the source(s) of ROS in cells treated with OC. The major recognized sources of ROS in cells are the mitochondrial respiratory chain (MRC) complexes (51) and the NADPH oxidase (NOX) enzyme (52,53). We analyzed the effects of specific inhibitors of each component of MRC and of NADPH oxidase in cells treated with OC. The MRC complex I inhibitor rotenone, complex II inhibitor thenoyltrifluoroacetone (TTFA), complex III inhibitor antimycin, complex IV inhibitor sodium azide and complex V inhibitor oligomycin were used. HCC cells were treated with OC alone or in combination with each single MRC inhibitor at different concentrations for 24 h, after which cell viability assays were performed. All of them failed to revert cell growth inhibition induced by OC (data not shown), with the exception of rotenone, which reversed the effect of OC, however, the inhibition did not reach statistical significance (Fig. 7A).

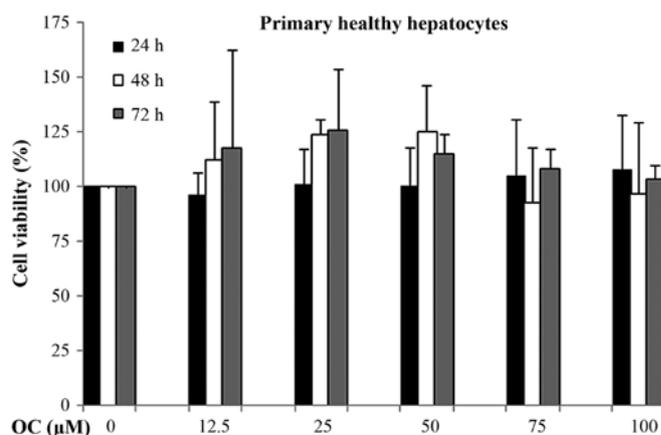


Figure 8. Oleocanthal (OC) is not cytotoxic for primary human hepatocytes. Primary human hepatocytes were treated with OC at the indicated concentrations and cell viability was evaluated using the CellTiter-Glo[®] assay after 24, 48 and 72 h. Data are expressed as a percentage of control cells and are the means \pm SD of three separate experiments, each of which was performed in triplicate.

Next, the effects of apocynin, an inhibitor of NADPH oxidase, were examined. The results shown in Fig. 7B demonstrate that in HCC cell lines apocynin significantly prevented OC-induced cell growth inhibition. In Hep3B cells cultured in the presence of apocynin, cell viability increased from 36% to 78.8% in samples treated with OC, whereas in HepG2 cells it increased from 20 to 58%.

These results suggest that both MRC complex I and NADPH oxidase are the main sources of ROS in HCC cells treated with OC.

OC is not cytotoxic in primary normal human hepatocytes. Finally, we tested the effects of OC on primary normal human hepatocytes, measuring the levels of cytotoxicity by cell viability assays. Surprisingly, prolonged treatment with OC (72 h) did not result in reduced hepatocyte viability (Fig. 8). The percentage of hepatocyte viability was unaffected even at higher OC doses (100 μ M). These results suggest that OC, a natural compound, is cytotoxic for cancer cells but does not have any effect on the cell viability of normal healthy hepatocytes.

Discussion

Oleocanthal (OC) was first described as anti-inflammatory ibuprofen-like compound (10). We and other research groups have demonstrated that ibuprofen and other NSAID molecules can act as anticancer drugs (27,28,45-47). In the current study, we compared the effects on cell viability of OC in comparison to other NSAIDs, such as the COX-1 inhibitor SC560, the dual COX-1/COX-2 inhibitor ibuprofen and the COX-2 inhibitor nimesulide. Moreover, to better investigate the role of COX-2 we used HCC and CRC cell lines which expressed different levels of COX-2, i.e. Hep3B and HT29 as COX-2 positive cells, and HepG2 and SW480 as COX-2 negative cells. We demonstrated that OC exerted antitumor activities in HCC and CRC cells. Our results demonstrated that the OC inhibitory effect on cell viability was more effective than the effects of NSAIDs in all the cell lines tested, and, moreover, its effects were COX-2-independent. Interestingly at the same dose, OC had no effect on normal human hepatocytes, suggesting that it inhibits cancer cell viability while sparing normal cells.

OC inhibited the capacity of HCC and CRC cells to form colonies and induced apoptosis as demonstrated by the induction of PARP cleavage as well as the activation of caspases 3/7. Moreover, OC treatment induced intracellular ROS production in HCC and CRC cells. In particular, in HCC cells we observed a specific induction in superoxide anions.

It is well known that high levels of ROS cause damage in all cellular compartments. For example, they can impair mitochondrial integrity. The γ -H2AX histone is a well-known and established marker of DNA damage, recruited in DNA double strand break (DSBs) foci to allow the assembling of repair machinery. Mitochondrial membrane potential ($\Delta\psi$ m) represents an important parameter of mitochondrial function and integrity. After OC treatment, the stability of mitochondrial membrane potential was significantly impaired in all cell lines.

ROS also causes DNA damage, and we observed a strong induction of γ -H2AX levels, a marker of DNA damage, in all cell lines treated with OC. Consequently, the ROS scavenger NAC rescued cell viability, reduced the number of apoptotic cells and prevented PARP cleavage after OC treatment in all cell lines. Furthermore, the increase in γ -H2AX expression, observed after treatment with OC, was reversed by treatment with NAC, and mitochondrial function was also restored after co-treatment with NAC.

We identified NADPH oxidase as a main source of ROS induced by OC treatment, as demonstrated by the improvement in cell viability of cells co-treated with OC and the NADPH oxidase inhibitor apocynin. However, our results suggest that also the MRC complex I might be a source of

ROS, particularly in the form of superoxide anions. In fact, cells co-treated with OC and the MRC complex I inhibitor rotenone showed a tendency to recover cell viability in HepG2 and in Hep3B cells.

Polyphenols were first indicated as antioxidant components of the diet (54) and initial evidence supported a role of polyphenols in the prevention of cardiovascular and neurodegenerative disorders, cancers and other diseases due to their antioxidant activities. Despite this, the results of the present study demonstrate that oleocanthal, a phenolic compound of EVOO, exerts a pro-oxidant action on HCC and CRC cells. In agreement with this finding, numerous studies in recent years have suggested that polyphenols might also exhibit pro-oxidant effects by generating ROS (49,50,55). Therefore, ROS could be said to have a Janus face nature, as it might contribute on the one hand to carcinogenesis, but, on the other, might induce cancer cell growth arrest, apoptosis, or necrosis (56). The precise mechanism(s) of this behavior is, however, as yet unclear. It has been speculated that malignant cells, living under an increased level of oxidative stress, could be more vulnerable to further ROS increases (57). Studies indicate that normal cells show a lower steady-state level of ROS than cancer cells and a constant level of reducing equivalents (58). The different redox status of normal and cancer cells would allow the development of new promising therapeutic strategies based on drugs that might alter redox equilibrium.

In our present study, OC proved to be an excellent anti-cancer drug for its ability to kill cancer cells without affecting normal cells. Overall, our results highlight the potential of OC in the treatment of HCC and CRC, and provide a basis for future investigation into its use in humans.

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References

- Colomer R and Menéndez JA: Mediterranean diet, olive oil and cancer. *Clin Transl Oncol* 8: 15-21, 2006.
- Tripoli E, Giammanco M, Tabacchi G, Di Majo D, Giammanco S and La Guardia M: The phenolic compounds of olive oil: Structure, biological activity and beneficial effects on human health. *Nutr Res Rev* 18: 98-112, 2005.
- Cicerale S, Lucas LJ and Keast RS: Antimicrobial, antioxidant and anti-inflammatory phenolic activities in extra virgin olive oil. *Curr Opin Biotechnol* 23: 129-135, 2012.
- Cicerale S, Conlan XA, Sinclair AJ and Keast RS: Chemistry and health of olive oil phenolics. *Crit Rev Food Sci Nutr* 49: 218-236, 2009.
- Cicerale S, Lucas L and Keast R: Biological activities of phenolic compounds present in virgin olive oil. *Int J Mol Sci* 11: 458-479, 2010.
- Caramia G, Gori A, Valli E and Cerretani L: Virgin olive oil in preventive medicine: From legend to epigenetics. *Eur J Lipid Sci Technol* 114: 375-388, 2012.
- Rafehi H, Ververis K and Karagiannis TC: Mechanisms of action of phenolic compounds in olive. *J Diet (Suppl)* 9: 96-109, 2012.

8. Visioli F and Bernardini E: Extra virgin olive oil's polyphenols: Biological activities. *Curr Pharm Des* 17: 786-804, 2011.
9. Omar SH: Oleuropein in olive and its pharmacological effects. *Sci Pharm* 78: 133-154, 2010.
10. Beauchamp GK, Keast RSJ, Morel D, Lin J, Pika J, Han Q, Lee C-H, Smith AB and Breslin PAS: Phytochemistry: Ibuprofen-like activity in extra-virgin olive oil. *Nature* 437: 45-46, 2005.
11. Akl MR, Ayoub NM, Mohyeldin MM, Busnena BA, Foudah AI, Liu Y-Y and Sayed KA: Olive phenolics as c-Met inhibitors: (-)-Oleocanthal attenuates cell proliferation, invasiveness, and tumor growth in breast cancer models. *PLoS One* 9: e97622, 2014.
12. Elnagar AY, Sylvester PW and El Sayed KA: (-)-Oleocanthal as a c-Met inhibitor for the control of metastatic breast and prostate cancers. *Planta Med* 77: 1013-1019, 2011.
13. Scotece M, Gómez R, Conde J, Lopez V, Gómez-Reino JJ, Lago F, Smith AB III and Gualillo O: Oleocanthal inhibits proliferation and MIP-1 α expression in human multiple myeloma cells. *Curr Med Chem* 20: 2467-2475, 2013.
14. Fogli S, Arena C, Carpi S, Polini B, Bertini S, Digiacoio M, Gado F, Saba A, Saccomanni G, Breschi MC, *et al*: Cytotoxic activity of oleocanthal isolated from virgin olive oil on human melanoma cells. *Nutr Cancer* 68: 873-877, 2016.
15. Pei T, Meng Q, Han J, Sun H, Li L, Song R, Sun B, Pan S, Liang D and Liu L: (-)-Oleocanthal inhibits growth and metastasis by blocking activation of STAT3 in human hepatocellular carcinoma. *Oncotarget* 7: 43475-43491, 2016.
16. Khanal P, Oh W-K, Yun HJ, Namgoong GM, Ahn S-G, Kwon S-M, Choi H-K and Choi HS: p-HPEA-EDA, a phenolic compound of virgin olive oil, activates AMP-activated protein kinase to inhibit carcinogenesis. *Carcinogenesis* 32: 545-553, 2011.
17. Khanfar MA, Bardaweel SK, Akl MR and El Sayed KA: Olive oil-derived oleocanthal as potent inhibitor of mammalian target of rapamycin: Biological evaluation and molecular modeling studies. *Phytother Res* 29: 1776-1782, 2015.
18. Dhanasekaran R, Limaye A and Cabrera R: Hepatocellular carcinoma: Current trends in worldwide epidemiology, risk factors, diagnosis, and therapeutics. *Hepat Med* 4: 19-37, 2012.
19. El-Serag HB: Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* 142: 1264-1273.e1, 2012.
20. Ferlay J, Shin H-R, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 127: 2893-2917, 2010.
21. Schütte K, Bornschein J and Malfertheiner P: Hepatocellular carcinoma - epidemiological trends and risk factors. *Dig Dis* 27: 80-92, 2009.
22. Thomas MB and Zhu AX: Hepatocellular carcinoma: The need for progress. *J Clin Oncol* 23: 2892-2899, 2005.
23. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc J-F, de Oliveira AC, Santoro A, Raoul J-L, Forner A, *et al*: SHARP Investigators Study Group: Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 359: 378-390, 2008.
24. Cervello M and Montalto G: Cyclooxygenases in hepatocellular carcinoma. *World J Gastroenterol* 12: 5113-5121, 2006.
25. Koga H, Sakisaka S, Ohishi M, Kawaguchi T, Taniguchi E, Sasatomi K, Harada M, Kusaba T, Tanaka M, Kimura R, *et al*: Expression of cyclooxygenase-2 in human hepatocellular carcinoma: Relevance to tumor dedifferentiation. *Hepatology* 29: 688-696, 1999.
26. Leng J, Han C, Demetris AJ, Michalopoulos GK and Wu T: Cyclooxygenase-2 promotes hepatocellular carcinoma cell growth through Akt activation: Evidence for Akt inhibition in celecoxib-induced apoptosis. *Hepatology* 38: 756-768, 2003.
27. Foderà D, D'Alessandro N, Cusimano A, Poma P, Notarbartolo M, Lampiasi N, Montalto G and Cervello M: Induction of apoptosis and inhibition of cell growth in human hepatocellular carcinoma cells by COX-2 inhibitors. *Ann NY Acad Sci* 1028: 440-449, 2004.
28. Lampiasi N, Foderà D, D'Alessandro N, Cusimano A, Azzolina A, Tripodo C, Florena AM, Minervini MI, Notarbartolo M, Montalto G, *et al*: The selective cyclooxygenase-1 inhibitor SC-560 suppresses cell proliferation and induces apoptosis in human hepatocellular carcinoma cells. *Int J Mol Med* 17: 245-252, 2006.
29. Cusimano A, Foderà D, D'Alessandro N, Lampiasi N, Azzolina A, Montalto G and Cervello M: Potentiation of the antitumor effects of both selective cyclooxygenase-1 and cyclooxygenase-2 inhibitors in human hepatic cancer cells by inhibition of the MEK/ERK pathway. *Cancer Biol Ther* 6: 1461-1468, 2007.
30. Cusimano A, Foderà D, Lampiasi N, Azzolina A, Notarbartolo M, Giannitrapani L, D'Alessandro N, Montalto G and Cervello M: Prostaglandin E2 receptors and COX enzymes in human hepatocellular carcinoma: Role in the regulation of cell growth. *Ann NY Acad Sci* 1155: 300-308, 2009.
31. Cusimano A, Azzolina A, Iovanna JL, Bachvarov D, McCubrey JA, D'Alessandro N, Montalto G and Cervello M: Novel combination of celecoxib and proteasome inhibitor MG132 provides synergistic antiproliferative and proapoptotic effects in human liver tumor cells. *Cell Cycle* 9: 1399-1410, 2010.
32. Cervello M, Bachvarov D, Cusimano A, Sardina F, Azzolina A, Lampiasi N, Giannitrapani L, McCubrey JA and Montalto G: COX-2-dependent and COX-2-independent mode of action of celecoxib in human liver cancer cells. *OMICS* 15: 383-392, 2011.
33. Terzić J, Grivennikov S, Karin E and Karin M: Inflammation and colon cancer. *Gastroenterology* 138: 2101-2114.e5, 2010.
34. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S and DuBois RN: Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 107: 1183-1188, 1994.
35. Gupta RA and Dubois RN: Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. *Nat Rev Cancer* 1: 11-21, 2001.
36. Marnett LJ and DuBois RN: COX-2: A target for colon cancer prevention. *Annu Rev Pharmacol Toxicol* 42: 55-80, 2002.
37. Ogino S, Kirkner GJ, Nosho K, Irahara N, Kure S, Shima K, Hazra A, Chan AT, Dehari R, Giovannucci EL, *et al*: Cyclooxygenase-2 expression is an independent predictor of poor prognosis in colon cancer. *Clin Cancer Res* 14: 8221-8227, 2008.
38. Piazzuelo E and Lanás A: NSAIDs and gastrointestinal cancer. *Prostaglandins Other Lipid Mediat* 120: 91-96, 2015.
39. Ghanghas P, Jain S, Rana C and Sanyal SN: Chemopreventive action of non-steroidal anti-inflammatory drugs on the inflammatory pathways in colon cancer. *Biomed Pharmacother* 78: 239-247, 2016.
40. Wang D and DuBois RN: The role of COX-2 in intestinal inflammation and colorectal cancer. *Oncogene* 29: 781-788, 2010.
41. Smith AB III, Han Q, Breslin PA and Beauchamp GK: Synthesis and assignment of absolute configuration of (-)-oleocanthal: A potent, naturally occurring non-steroidal anti-inflammatory and anti-oxidant agent derived from extra virgin olive oils. *Org Lett* 7: 5075-5078, 2005.
42. Cusimano A, Puleio R, D'Alessandro N, Loria GR, McCubrey JA, Montalto G and Cervello M: Cytotoxic activity of the novel small molecule AKT inhibitor SC66 in hepatocellular carcinoma cells. *Oncotarget* 6: 1707-1722, 2015.
43. Gramignoli R, Green ML, Tahan V, Dorko K, Skvorak KJ, Marongiu F, Zao W, Venkataramanan R, Ellis ECS, Geller D, *et al*: Development and application of purified tissue dissociation enzyme mixtures for human hepatocyte isolation. *Cell Transplant* 21: 1245-1260, 2012.
44. Gramignoli R, Tahan V, Dorko K, Venkataramanan R, Fox IJ, Ellis ECS, Vosough M and Strom SC: Rapid and sensitive assessment of human hepatocyte functions. *Cell Transplant* 23: 1545-1556, 2014.
45. Smith ML, Hawcroft G and Hull MA: The effect of non-steroidal anti-inflammatory drugs on human colorectal cancer cells: Evidence of different mechanisms of action. *Eur J Cancer* 36: 664-674, 2000.
46. Zhang Y-J, Bao Y-J, Dai Q, Yang W-Y, Cheng P, Zhu L-M, Wang B-J and Jiang F-H: mTOR signaling is involved in indomethacin and nimesulide suppression of colorectal cancer cell growth via a COX-2 independent pathway. *Ann Surg Oncol* 18: 580-588, 2011.
47. Janssen A, Maier TJ, Schiffmann S, Coste O, Seegel M, Geisslinger G and Grösch S: Evidence of COX-2 independent induction of apoptosis and cell cycle block in human colon carcinoma cells after S- or R-ibuprofen treatment. *Eur J Pharmacol* 540: 24-33, 2006.
48. Khan HY, Zubair H, Ullah MF, Ahmad A and Hadi SM: A prooxidant mechanism for the anticancer and chemopreventive properties of plant polyphenols. *Curr Drug Targets* 13: 1738-1749, 2012.
49. Lecci RM, Logrieco A and Leone A: Pro-oxidant action of polyphenols as action mechanism for their pro-apoptotic activity. *Anticancer Agents Med Chem* 14: 1363-1375, 2014.
50. León-González AJ, Auger C and Schini-Kerth VB: Pro-oxidant activity of polyphenols and its implication on cancer chemoprevention and chemotherapy. *Biochem Pharmacol* 98: 371-380, 2015.

51. Richter C, Ghafourifar P, Schweizer M and Laffranchi R: Nitric oxide and mitochondrial Ca^{2+} . *Biochem Soc Trans* 25: 914-918, 1997.
52. Trudel S, Pâquet MR and Grinstein S: Mechanism of vanadate-induced activation of tyrosine phosphorylation and of the respiratory burst in HL60 cells. Role of reduced oxygen metabolites. *Biochem J* 276: 611-619, 1991.
53. Bedard K and Krause K-H: The NOX family of ROS-generating NADPH oxidases: Physiology and pathophysiology. *Physiol Rev* 87: 245-313, 2007.
54. Scalbert A, Johnson IT and Saltmarsh M: Polyphenols: antioxidants and beyond. *Am J Clin Nutr* 81 (Suppl): 215S-217S, 2005.
55. Ferroni F, Maccaglia A, Pietraforte D, Turco L and Minetti M: Phenolic antioxidants and the protection of low density lipoprotein from peroxynitrite-mediated oxidations at physiologic CO_2 . *J Agric Food Chem* 52: 2866-2874, 2004.
56. Gorrini C, Harris IS and Mak TW: Modulation of oxidative stress as an anticancer strategy. *Nat Rev Drug Discov* 12: 931-947, 2013.
57. Trachootham D, Lu W, Ogasawara MA, Nilsa RD and Huang P: Redox regulation of cell survival. *Antioxid Redox Signal* 10: 1343-1374, 2008.
58. Ivanova D, Bakalova R, Lazarova D, Gadjeva V and Zhelev Z: The impact of reactive oxygen species on anticancer therapeutic strategies. *Adv Clin Exp Med* 22: 899-908, 2013.