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(-)-Oleocanthal rapidly and selectively induces cancer cell death via lysosomal membrane permeabilization

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Abbreviations: ASM, acid sphingomyelinase; BMP, bis(monoacylglycero)phosphate, EVOO, extra virgin olive oil; LMP, lysosomal membrane permeabilization; OC, (-)-Oleocanthal; PARP, poly(ADP-ribose) polymerase.

(-)-Oleocanthal (OC), a phenolic compound present in extra-virgin olive oil (EVOO), has been implicated in the health benefits associated with diets rich in EVOO. We investigated the effect of OC on human cancer cell lines in culture and found that OC induced cell death in all cancer cells examined as rapidly as 30 minutes after treatment in the absence of serum. OC treatment of non-transformed cells suppressed their proliferation but did not cause cell death. OC induced both primary necrotic and apoptotic cell death via induction of lysosomal membrane permeabilization (LMP). We provide evidence that OC promotes LMP by inhibiting acid sphingomyelinase (ASM) activity, which destabilizes the interaction between proteins required for lysosomal membrane stability. The data presented here indicate that cancer cells, which tend to have fragile lysosomal membranes compared to non-cancerous cells, are susceptible to cell death induced by lysosomotropic agents. Therefore, targeting lysosomal membrane stability represents a novel approach for the induction of cancer-specific cell death.

Introduction

Extra-virgin olive oil (EVOO), a central component of the Mediterranean diet, contains an abundance of phenolic antioxidants that are potent inhibitors of reactive oxygen species and is associated with a reduced risk for several types of human cancer.¹ Polyphenolic secoiridoids of EVOO have been shown to decrease viability of HER2-overexpressing breast cancer cells by selectively inducing apoptotic cell death.² (-)-Oleocanthal (OC), a di-aldehydic form of ligostride aglycone that has been isolated from EVOO, possesses a wide range of biological effects. Previous studies have reported its activity as a potent antioxidant; a non-steroidal anti-inflammatory agent that inhibits COX-1 and COX-2; a neuroprotectant that alters the structure and function of the neurotoxins β -amyloid and Tau, which are associated with the debilitating effects of Alzheimer disease; an inhibitor of proliferation, migration, and invasion of human breast and prostate cancer cells through c-Met inhibition; an inhibitor of AMPK in colon cancer cells; and an inhibitor of macrophage inflammatory protein-1 α in multiple myeloma.³⁻⁸

To investigate the anticancer effects of OC, we examined its effect on the viability and survival of cancerous and non-cancerous cells. Interestingly, OC rapidly (within 30 minutes) induced loss of viability in cancer cells in a dose-dependent manner. Under serum withdrawal, OC promoted primary necrotic cell death in cancer cells, which correlated with elevated levels of phosphorylated ERK1/2 in the absence of cleaved caspase-3 expression. In the presence of serum, a combination of apoptosis and secondary necrosis was observed. Importantly, OC induced a reversible cell cycle arrest in non-cancerous cells but did not affect their viability. Our findings indicate that OC-mediated cancer cell death is promoted by destabilization of the lysosomal membrane, leading to the induction of lysosomal membrane permeabilization (LMP). OC-induced LMP is mediated by the inhibition of acid sphingomyelinase (ASM) activity, which can be derepressed by upregulation of Hsp70 or dual treatment with anionic lipids. These data provide evidence that the anticancer benefits of EVOO result, in part, from the ability of OC to rupture lysosomal membranes in cancer cells leading to cell death via

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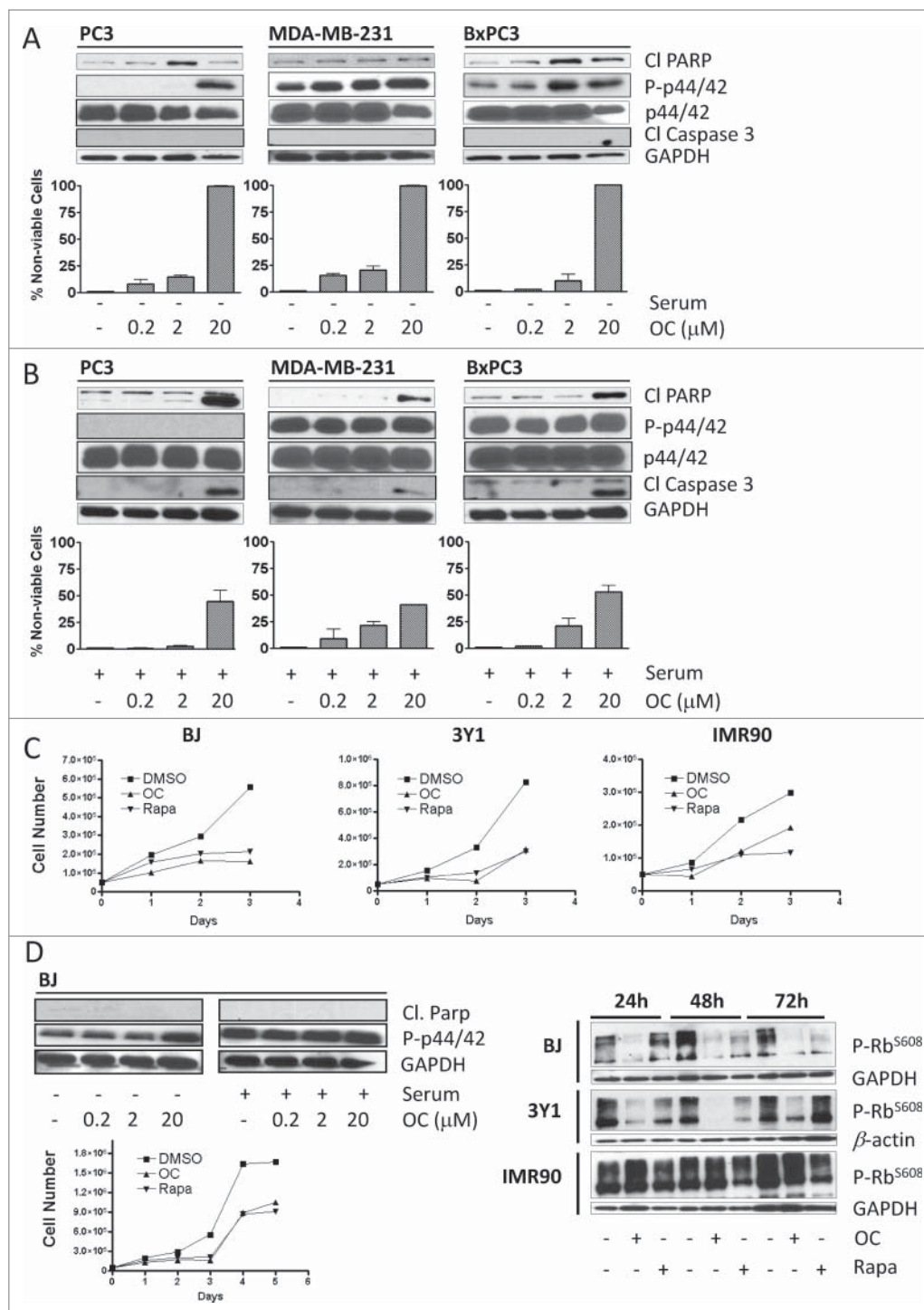


Figure 1. OC induces loss of cell viability in cancer cells but reversible cell cycle arrest in non-cancerous cells. (**A**, **B**) PC3, MDA-MB-231, and BxPC3 cells were plated at a density of 3×10^5 cells/35-mm plate. After 24 h the medium was replaced with fresh medium containing 0% serum (**A**) or 10% serum (**B**) and the indicated concentration of OC. After incubation for 4 h, levels of cleaved PARP, phospho-p44/42 (Thr202/Tyr204), total p44/42, cleaved caspase 3, and GAPDH were determined. Cell viability was determined after 24 h of treatment. (**C**) BJ, 3Y1, and IMR90 cells were plated at a density of 5×10^4 cells/35-mm plate. After 24 h the cells were provided with fresh media containing 10% serum and the indicated concentration of OC or rapamycin (Rapa). After incubation for 24, 48, or 72 h the attached cells were counted to determine cell proliferation. (**D**) (Upper left panel) BJ cells were plated at a density of 2×10^5 cells/35-mm plate. After 24 h the cells were provided with fresh media containing 10% serum and the indicated concentration of OC or Rapa. After a further 24, 48, 72, 96, or 120 h, the cells were counted to determine cell proliferation. (Right panel) BJ, 3Y1, and IMR90 cells were plated as above. After 24 h the cells were provided with fresh media containing 10% serum and the indicated concentration of OC or Rapa. After a further 24, 48, or 72 h, the cells were subjected to western blot analysis for phosphorylated Rb (Ser608) and GAPDH or β -actin. Error bars for all graphs represent the standard deviation from two independent experiments. All data shown are representative of at least two independent experiments.

necrosis and/or apoptosis. Importantly, due to high lysosomal membrane integrity, non-cancerous cells remain viable.

Results

OC induces loss of cell viability in cancer cells but reversible cell cycle arrest in non-cancerous cells

OC has previously been shown to inhibit proliferation, migration, and invasion of breast and prostate cancer cells via

inhibition of c-Met phosphorylation.⁵ OC has also been reported to inhibit cell proliferation in multiple myeloma cells via induction of apoptosis and inhibition of macrophage inflammatory protein 1- α expression.⁷ To further explore the mechanism by which OC induces cell death in cancer cells, we investigated the effect of OC on cell viability in PC3 (prostate), MDA-MB-231 (breast), and BxPC3 (pancreatic) cancer cells. Under serum withdrawal, 20 μ M OC rapidly induced a loss of

cell adhesion within 30 min post treatment and resulted in 100% non-viability in all cancer cell lines after 24 h of treatment (Fig. 1A). Interestingly, OC increased the levels of phosphorylated p44/42 (also known as ERK1/ERK2), but did not significantly increase the levels of cleaved poly-ADP-ribose polymerase (PARP), an indicator of apoptotic death, in the absence of serum. It was previously shown that ERK activation is a critical mediator of mitochondrial dysfunction and necrotic cell death of renal epithelial cells following treatment with oxidizing agents.⁹ Importantly, OC did not induce expression of cleaved caspase-3 in the absence of serum. Caspase-3, an effector caspase necessary for the morphological and biochemical features associated with apoptosis, is cleaved during both intrinsic and extrinsic apoptotic cell death pathways.^{10, 11} The absence of cleaved caspase-3 expression upon OC treatment in the absence of serum indicates that the cancer cells have bypassed the apoptotic machinery leading to cell death. In addition, OC treatment resulted in a complete loss of mitochondrial activity at low micromolar concentrations in the absence of serum, as measured by the MTT assay (data not shown). Taken together, the rapid loss of viability caused by OC, together with the absence of PARP and caspase-3 cleavage, suggests that OC induces primary necrotic cell death in the absence of serum in a wide range of cancer cells.

In the presence of serum, however, OC treatment increased the levels of cleaved PARP and cleaved caspase-3, which correlated with increased numbers of non-viable cells in all cancer cell lines examined (Fig. 1B). OC did not have a significant effect on the level of phosphorylated p44/42 in the presence of serum, when baseline levels of phosphorylated p44/42 tended to be high (Fig. 1B), and only partially inhibited mitochondrial activity (data not shown). Thus, in the presence of serum, OC induced cell death via activation of apoptotic mechanisms. Overall, these data indicate that OC rapidly induces robust cancer cell death via different mechanisms depending on whether serum is absent or present.

Targeted cancer therapies that are cytotoxic to tumors and non-toxic to non-cancerous tissues are in high demand but in short supply. Given that the data in Figs. 1A and 1B demonstrate that OC significantly induces cell death in cancer cells, we examined the effect of OC on non-cancerous BJ human fibroblasts, 3Y1 rat fibroblasts, and IMR90 human lung fibroblasts. As shown in Fig. 1C, OC inhibited cell proliferation in all 3 non-cancerous cell lines examined as determined by cell number after 72 h of treatment. Rapamycin was used as a positive control because of its ability to induce G1 cell cycle arrest.¹² Importantly, OC did not induce PARP cleavage in the presence or absence of serum and cell proliferation was restored after 72 h of OC treatment in BJ cells (Fig. 1D, upper and lower left panel).

Phosphorylation of Rb is a critical step mediating progression through G1 to S phase of the cell cycle.¹³ Underphosphorylation of Rb inhibits E2F activity through sequestration, thus inhibiting cell cycle progression.^{14, 15} Rb phosphorylation at Ser608 is reported to be necessary for reduced binding affinity of E2F to Rb.¹⁶ We therefore examined the effect of OC on the levels of phospho-Rb at Ser608 in non-cancerous cells. As shown in Fig. 1D (right panel), treatment of non-cancerous BJ and 3Y1 cells with OC resulted in decreased levels of phospho-Rb at

Ser608 after 24 h, which continued for 72 h in the presence of serum. IMR90 cells did not show a significant decrease in Rb phosphorylation at Ser 608, probably reflecting cell cycle arrest outside of G1.¹⁷ These data suggest that OC treatment does not induce cell death in non-cancerous cells but rather reversibly induces cell cycle arrest via suppression of Rb phosphorylation, which serves to protect healthy cells against the adverse effects of OC.

OC differentially induces cell death in cancer cells in the absence and presence of serum

The morphological and biochemical changes associated with apoptotic cell death occur between 6 and 12 h after a traumatic event that leads to apoptotic body formation between 24 and 48 h.¹⁸ The rapidity with which OC induces cell rounding and loss of substrate adherence in the absence of serum (observed within 30 min post treatment) suggests a non-apoptotic form of cell death. As shown in Fig. 1A, 20 μ M OC induced elevated ERK1/2 phosphorylation, which has been associated with necrotic cell death,⁹ in the absence of cleaved PARP and cleaved caspase-3 expression. We therefore investigated the method by which OC induces cell death in the absence of serum using a cell death-specific assay kit that distinguishes apoptosis from necrosis. A lower concentration of OC (10 μ M) was used for this assay to reduce the rate of cell rounding in order to determine the specific method of cell death. As shown in Fig. 2A, OC induced a significant number of necrotic cells after 24 h of treatment. The large number of necrotic cells observed explains the substantial decrease in cell viability in the absence of cleaved caspase-3 and limited increase in cleaved PARP (shown in Fig. 1A). Although PARP cleavage was not observed in the absence of serum due to activation of the primary necrotic cell death pathway, increased levels of cleaved PARP and caspase-3 were observed upon OC treatment in the presence of serum (Fig. 1B), which resulted in more apoptotic than necrotic cells (Fig. 2B). Signaling pathways that regulate cell death participate in both apoptosis and necrosis; thus, it is possible to have apoptotic and secondary necrotic cell death within the same cell population.^{19,20} Collectively, data in Figs. 1 and 2 suggest that OC induces cell death in cancer cells via both apoptotic and necrotic mechanisms depending on the presence or absence of serum.

OC downregulates ASM activity to induce LMP and cell death in cancer cells that is reversed by anionic lipids

Primary necrosis caused by a severe insult, either extracellular or intracellular, can be identified by rapid permeabilization of the plasma membrane.¹⁹ Lysosomes have recently been implicated in cell death through the release of lysosomal hydrolytic enzymes into the cytosol, which leads to apoptosis (via mitochondrial outer membrane permeabilization [MOMP] and caspase activation) or necrosis (via cytosolic acidification).²¹ To determine whether LMP mediates OC-induced cancer cell death, we examined the integrity of the lysosomal membrane after OC treatment using acridine orange, a lysosomotropic metachromatic fluorochrome that emits a red fluorescence when in present at a high concentration within intact lysosomes.²² OC significantly reduced the red fluorescence in both the absence and presence of

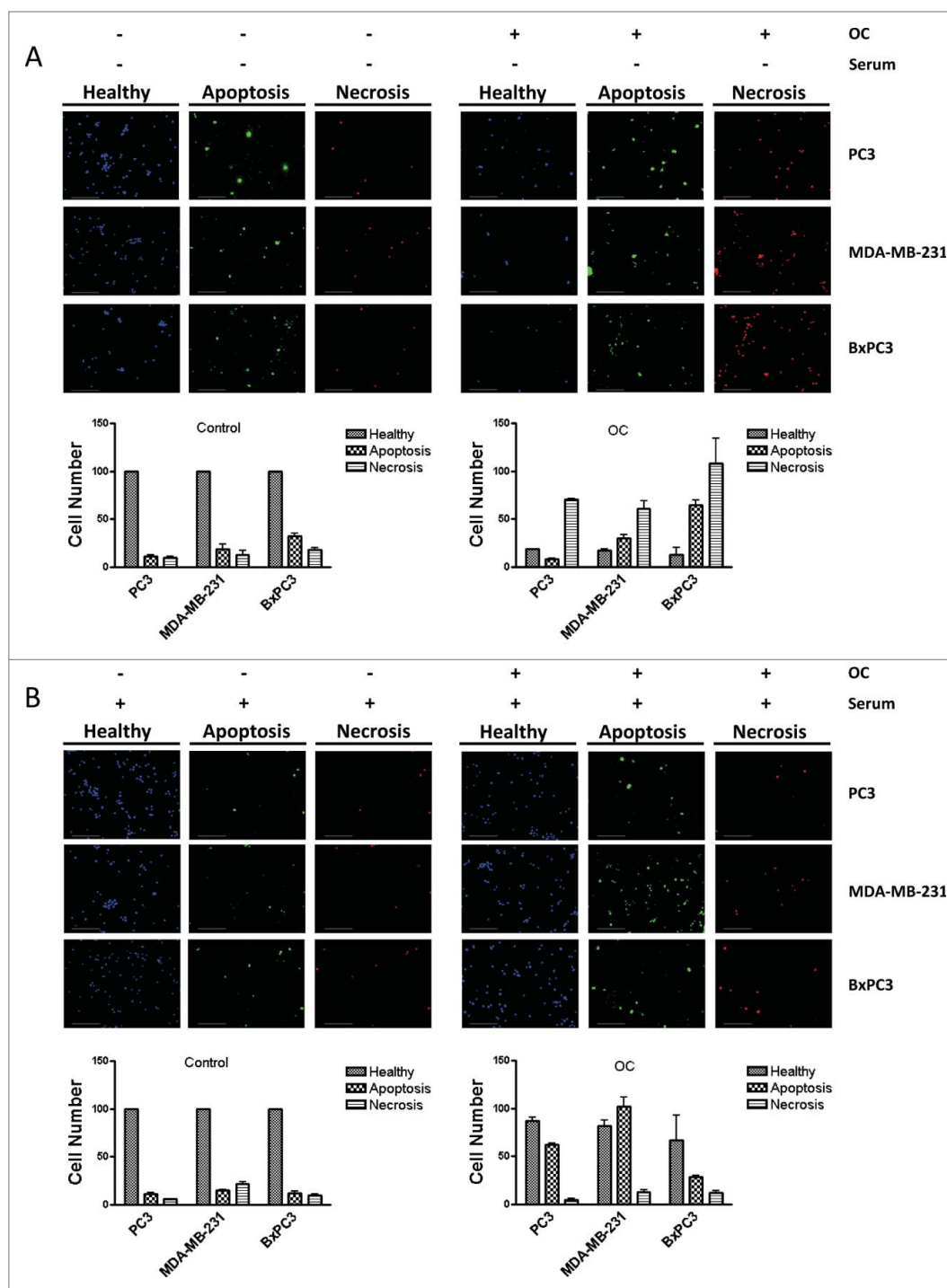


Figure 2. OC differentially induces cell death in cancer cells in the absence and presence of serum. (A) PC3, MDA-MB-231, and BxPC3 cells were plated at a density of 2×10^5 cells/60-mm plate. After 24 h the cells were provided with fresh media containing 0% serum and 10 μ M OC as indicated. After a further 24 h the cells were subjected to the Apoptosis/Necrosis/Healthy cell assay and analyzed by fluorescence microscopy using FITC, Texas Red, and DAPI filter settings. (B) PC3, MDA-MB-231, and BxPC3 cells were plated as above. After 24 h the cells were provided with fresh media containing 10% serum and 10 μ M OC as indicated. After a further 24 h, the cells were analyzed using the Apoptosis/Necrosis/Healthy cell assay kit as above. Cell numbers were determined using the ImageJ cell counter. Data shown are representative of at least two independent experiments. Scale bars = 200 μ m.

(Fig. 3A). However, OC did not reduce the red fluorescence in the absence or presence of serum in BJ cells, indicating that OC does not induce LMP in these non-cancerous cells (Fig. 3A lowest panel).

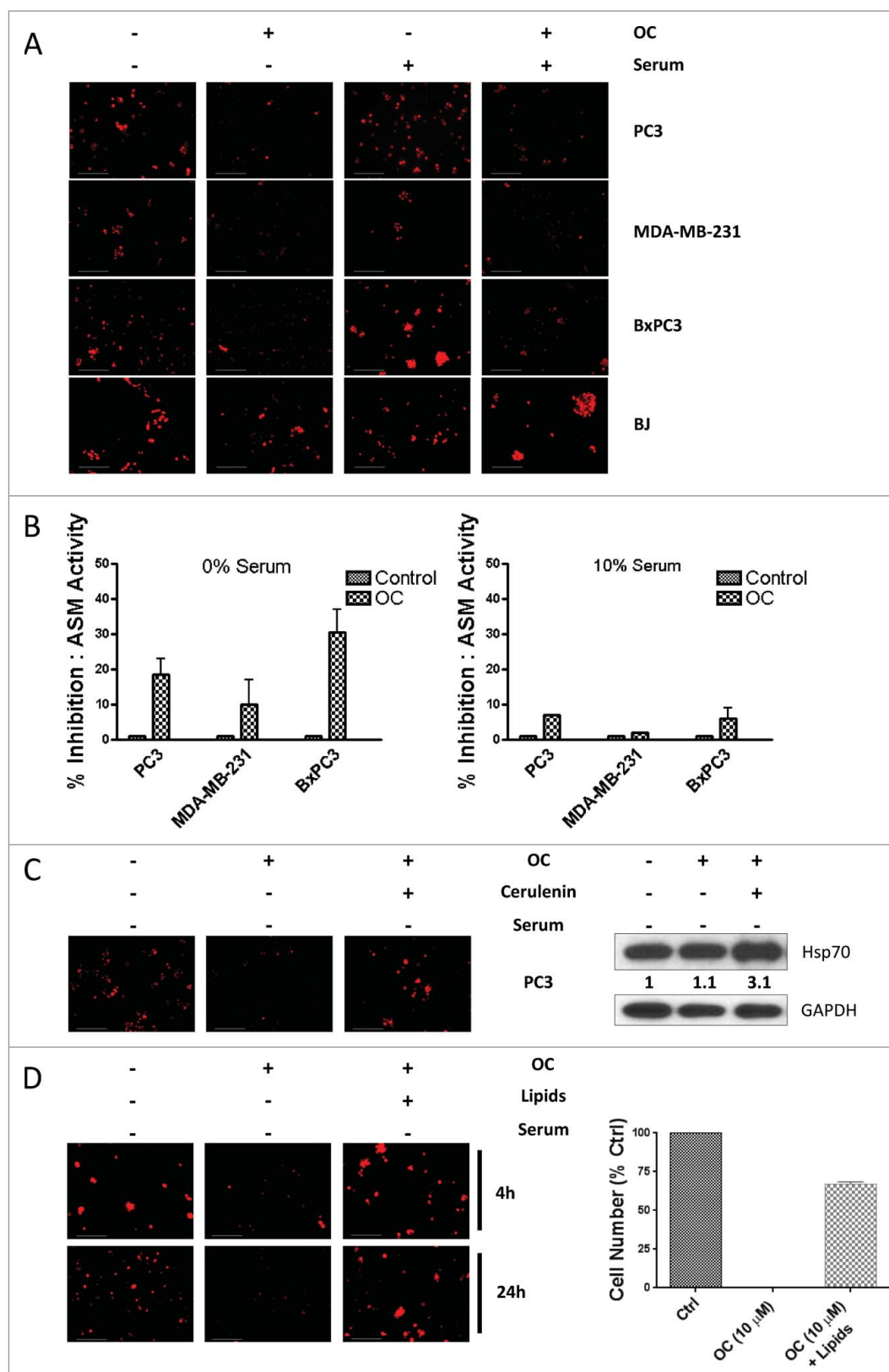
Lysosomal membrane integrity is regulated by the activity of ASM, a lysosomal lipase responsible for the hydrolysis of sphingomyelin (SM) to ceramide.²³ Petersen et al. have shown that siramesine, a cationic amphiphilic drug, induces LMP as a result of inhibition of ASM activity.²⁴ We therefore examined the effect of OC on the activity of ASM. As shown in Fig. 3B (left graph), under serum withdrawal conditions ASM activity was inhibited up to 40% following treatment with 10 μ M OC for 4 h. In the presence of serum, 10 μ M OC induced a maximum 10% inhibition of ASM activity (Fig. 3B, right panel). The degree by which ASM activity is inhibited correlates with the specific cell death pathway that is activated upon OC treatment and the extent of LMP; massive or complete LMP rapidly liberates proteases from the lysosomes and has been shown to induce necrotic cell death, whereas partial or selective LMP liberates proteases from the lysosome in a manner that activates programmed apoptotic cell death.^{25, 26} These findings reveal a correlation between OC-induced necrotic or apoptotic cell death and the level of ASM activity.

ASM activity is regulated by its ability to bind to bis (monoacylglycero)phosphate (BMP), an anionic lipid that is an essential co-factor for lysosomal sphingomyelin metabolism.²⁷

serum, indicating that OC-induced cell death is mediated by the induction of LMP in all cancer cell lines and conditions

(monoacylglycero)phosphate (BMP), an anionic lipid that is an essential co-factor for lysosomal sphingomyelin metabolism.²⁷

Figure 3. OC downregulates ASM activity to induce LMP and cell death in cancer cells that is reversed by anionic lipids. **(A)** PC3, MDA-MB-231, BxPC3, and BJ cells were plated at a density of 3×10^5 cells/60-mm plate. After 24 h the cells were provided with fresh media containing 0 or 10% serum and 10 μ M OC as indicated. After a further 4 h the cells were treated with 5 μ M acridine orange for 15 min and then subjected to fluorescence microscopy using a Texas Red filter setting. **(B)** PC3, MDA-MB-231, and BxPC3 cells were plated at a density of 5×10^6 cells/10-cm plate. After 24 h the cells were provided with fresh media containing 0 or 10% serum and 10 μ M OC as indicated. Acid sphingomyelinase activity was measured after a further 4 h. **(C)** PC3 cells were plated at a density of 2×10^5 cells/60-mm plate. After 24 h the cells were provided with fresh media containing 10% serum and 30 μ M cerulenin as indicated. After a further 24 h, the cells were provided with fresh media containing 0% serum and 10 μ M OC as indicated and incubated for 4 h. The cells were treated with 5 μ M acridine orange for 15 min and subjected to fluorescence microscopy using a Texas Red filter setting or western blot analysis of Hsp70 and GAPDH. **(D)** PC3 cells were plated at a density of 2×10^5 cells/60-mm plate. After 24 h the cells were provided with fresh media containing 0% serum, 10 μ M OC, and lipids as indicated. After 4 or 24 h the cells were treated with 5 μ M acridine orange for 15 min and subjected to fluorescence microscopy using a Texas Red filter setting. Cell viability was determined after 24 h. ImageJ software was used to quantify the western blots. Experiments shown are representative of at least two independent experiments. Scale bars = 200 μ m.



Hsp70 has been shown to bind with high affinity and specificity to BMP, thereby enhancing the stability of the BMP–ASM complex, which in turn inhibits LMP and promotes cell survival.²³

Therefore, we examined whether Hsp70 inhibits OC-induced LMP. Cerulenin, a fatty acid synthase inhibitor, has been shown to upregulate Hsp70 expression through the ability of Hsp70 to

interact directly with fatty acids.²⁸ Under serum withdrawal conditions, cerulenin-mediated overexpression of Hsp70 partially inhibited OC-induced LMP in PC3 cancer cells (Fig. 3C). Cationic lipids or cationic amphiphilic drugs have been shown to destabilize the lysosomal membrane by inhibiting the hydrolysis of SM via downregulation of ASM activity.^{24,29} However, anionic amphipathic lipids or free fatty acids have been shown to enhance ASM activity.^{30–32} We therefore examined the effect of an anionic lipid mixture on OC-induced LMP. As shown in Fig. 3D (left panel), treatment with anionic lipids inhibited OC-induced LMP in PC3 cells in the absence of serum. The inhibition of OC-induced LMP also correlated with an increase in cell viability as measured by the percentage of attached cells Fig. 3D (right panel). Dual treatment with lipids and OC did not result in cell death, as observed by the lack of floating cells after 24 to 72 h of OC treatment, although there was a decrease in cell proliferation. These data indicate that maintaining or enhancing ASM activity through the addition of anionic lipids increases the stability of the lysosomal membrane, thus preventing LMP-induced cell death in cancer cells. Collectively, the data in Figs. 1–3 indicate that OC inhibits ASM activity in cancer cells, leading to complete or partial LMP and resulting in necrotic or apoptotic cell death in the absence and presence of serum, respectively.

Discussion

The data presented here demonstrate that OC selectively induces cell death in cancer cells via downregulation of ASM activity leading to LMP, while reversibly arresting non-cancerous cells. In non-cancerous cells, OC induces G1 cell cycle arrest via inhibition of Rb phosphorylation at Ser608, preventing cell cycle progression into S phase.^{33,34} Importantly, non-cancerous cells that are arrested by OC resume proliferation after 72 h of OC treatment.

In contrast to the cell cycle arrest observed in non-cancerous cells, cancer cells rapidly underwent either primary necrotic cell death under serum withdrawal conditions, correlating with increased ERK1/2 phosphorylation in the absence of cleaved caspase-3 and PARP, or caspase-3-dependent apoptotic cell death in the presence of serum via induction of LMP. During the development of cancer cells, lysosomes undergo morphological transformation that leads to increased size and greater cathepsin activity compared with those in normal cells.³⁵ The increased lysosomal size renders cancer cells vulnerable to treatment with anticancer agents.³⁶ LMP causes a release of cathepsins (lysosomal proteases) into the cytosol leading to degradation of cellular proteins.²¹ Accordingly, the amount and rate of lysosomal enzyme release into the cytosol mediates the activation of necrotic or apoptotic signaling pathways.²⁶ We have shown here that the degree of OC-mediated suppression of ASM activity correlates with activation of specific cell death pathways.

Although fragility of the lysosomal membrane increases the susceptibility of cancer cells to LMP, cells can overcome this vulnerability by overexpressing Hsp70, a regulator of multiprotein

complex assembly and protein transport across cellular membranes.³⁷ Hsp70 localizes on the lysosomal membrane and protects against LMP by enhancing ASM activity and stabilizing the interaction between ASM and BMP.^{23,35} Therefore, overexpression of Hsp70 in cancer cells favors cell survival by increasing lysosomal integrity and preventing LMP.^{35,37} Increasing Hsp70 expression with cerulenin, a fatty acid synthesis inhibitor, suppresses OC-induced LMP in PC3 cells due to increased lysosomal membrane stability.²⁸

Whereas cationic lipids destabilize the lysosomal membrane by regulating the interaction between ASM and the lysosomal membrane, anionic lipids such as BMP and phosphatidylinositol (PI) have been shown to activate ASM activity as measured by enhanced hydrolysis of SM.^{29,31,32} We have provided evidence that anionic free fatty acids are also capable of stabilizing the lysosomal membrane and preventing LMP. Our findings suggest that OC-mediated cancer cell death is due to its ability to act as a lysosomotropic agent and facilitate complete LMP in the absence of serum and partial LMP in the presence of serum, which is related to the rate of ASM activity inhibition.

Importantly, it was recently reported that OC prevents tumor growth in an orthotopic model of breast cancer in athymic nude mice.³⁸ This study presented data implicating suppression of c-Met as a critical consequence of OC treatment although it was not clear whether LMP was involved in cell death or PARP cleavage was observed. Nonetheless, the study demonstrated that OC has similar effects in an animal model to those described in the current study with minimal effects on normal cells, thus reinforcing the potential of OC as a therapeutic agent in breast and other cancers.

Our study provides a mechanism by which OC selectively and rapidly induces cell death in cancer cells without being cytotoxic to non-cancerous cells. Compounds that induce lysosomal membrane destabilization, such as OC, represent a viable method to exploit the vulnerability of the enlarged lysosomes in cancer cells. Our data suggest that the chemopreventive activity of EVOO is due to the ability of its bioactive phenolic components, especially OC, to induce cell death by entering the lysosome and inhibiting ASM activity, which induces LMP. Therefore, the ability of OC to induce LMP in cancer cells, but not normal cells, represents a novel therapeutic strategy for treating a large number of cancer types in which lysosomes are enlarged and more sensitive to lysosomotropic agents.

Materials and methods

Cells and cell culture conditions

The BxPC3, PC3, MDA-MB-231, BJ, 3Y1, and IMR90 cells used in this study were obtained from the American Type Tissue Culture Collection and were maintained in Roswell Park Memorial Institute Medium (RPMI) and Dulbecco's Modified Eagle Medium (DMEM), respectively, supplemented with 10% or 15% (IMR90) fetal bovine serum (Hyclone). No authentication was performed by the authors.

Materials

Rapamycin was obtained from LC Labs. (-)-OC, extracted from EVOO, was obtained from Dr. Alexios-Leandros Skaltsounis at the University of Athens Department of Pharmacology. The structure and purity (97%) of (-)-oleocanthal was determined by HPLC and ¹H-NMR analysis. Acridine orange was obtained from Life Technologies. The Promokine Apoptotic/Necrotic/Healthy cell detection kit and Cerulenin were obtained from Fisher Scientific. The Sphingomyelinase Fluorometric Assay kit was obtained from Cayman Chemicals. Primary antibodies specific for cleaved caspase-3, cleaved PARP, phospho-p44/42 (Thr202/Tyr204), total p44/42, β -actin, and phospho-Rb (Ser 608) were obtained from Cell Signaling Technology. Antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Hsp70 were obtained from Santa Cruz Biotechnology.

Lipid mix supplementation

Fatty acid mixture was obtained from Invitrogen (11905) and was supplied to cells as a 1:200 dilution complexed with 5% fatty acid free bovine serum albumin (BSA; Sigma Aldrich) in a 2:1 ratio for a final lipid concentration in the media of 0.375 mg/L. The composition of the fatty acid mixture was arachidonic acid (20:4; 2 mg/L), cholesterol (220 mg/L), DL- α -Tocopherol acetate (70 mg/L), linoleic acid (18:2; 10 mg/L), linolenic acid (18:3; 10 mg/L), myristic acid (14:0; 10 mg/L), oleic acid (18:1; 10 mg/L), palmitic acid (16:0; 10 mg/L), puronic F-68 (90,000 mg/L), stearic acid (18:0; 10 mg/L), Tween 80 (2,200 mg/L).

Cell viability and proliferation, acid sphingomyelinase activity, apoptosis/necrosis cell assay

Cell viability was determined by the level of mitochondrial activity using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay according to the vendor's instructions (Sigma Aldrich). Cell proliferation and percentage of non-viable cells were determined by calculating the percentage of attached cells compared to control as previously described.³⁹ ASM activity was determined using the Sphingomyelinase Fluorometric Assay kit from Cayman Chemicals. Cell death was evaluated by 2 separate methods: examination of cleavage of the caspase-3 substrate PARP and detection of apoptotic, necrotic, and healthy cells by the Promokine Apoptotic/Necrotic/Healthy Cells Detection Kit following the manufacturer's guidelines.

Briefly, by using a combination of Annexin V, ethidium homodimer III, and Hoechst 33342, apoptotic, necrotic, and healthy cells can be respectively quantified within the same cell population. Healthy cells are only stained by membrane permeable Hoechst 33342, with blue fluorescence. Apoptotic cells exhibit Annexin V green staining. Ethidium homodimer III, which is impermeant to live or apoptotic cells, stains necrotic cells intensely with red fluorescence. Cells entering late apoptosis (also known as secondary necrosis) are stained both green and blue.

Immunofluorescence

Immunostaining for the cell death assay or lysosomal integrity was performed in a cell suspension. For the cell death assay, cells were incubated with FITC-Annexin V, ethidium Homodimer III, and Hoechst 33342, washed twice with a 1 \times Binding Buffer, and then resuspended in 30 μ L 1 \times Binding Buffer. For the lysosomal integrity assay, cells were washed twice with PBS and then resuspended in 30 μ L PBS. A 5- μ L sample of the cell suspension was placed on a microscope slide and covered with a coverslip. Images were collected with an Olympus BX61 fluorescence microscope with a 10 \times objective, connected to a Hamamatsu ORCA-ER CCD camera and controlled by the SlideBook 5.1 image capture software.

Western blot analysis

Proteins were extracted from cultured cells in modified RIPA buffer (Upstate Biotechnology). Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to western blot analysis as described previously.⁴⁰ Western blots were quantified using ImageJ software.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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