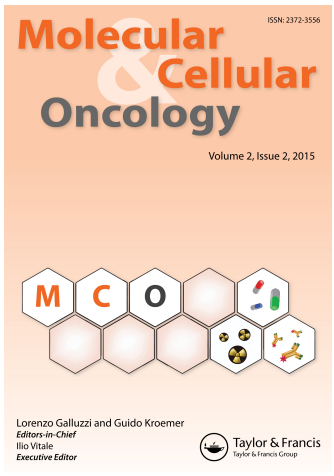

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(-)-Oleocanthal rapidly and selectively induces cancer cell death via lysosomal membrane permeabilization (LMP)

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

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Key words: Oleocanthal, lysosomal membrane permeabilization, apoptosis, necrosis, extra virgin olive oil

Abbreviations used: ASM, acid sphingomylenase; BMP, bis(monoacylglycero)phosphate; EVOO, extravirgin olive oil; LMP, Lysosomal membrane permeabilization;

OC, -(-)oleocanthal; PARP, poly-ADP-ribose polymerase

Abstract

(-)-Oleocanthal (OC), a phenolic compound 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. Amazingly, OC induced cell death in all cancer cells examined ï as rapidly as 30 minutes after treatment in the absence of serum. OC treatment of non-transformed cells suppressed 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 necessary for lysosomal membrane stability. The data presented here indicates that cancer cells having fragile lysosomal membranes ï as compared to non-cancerous cells ï are susceptible to lysosomotropic agent-induced cell death. Therefore, targeting lysosomal membrane stabiltiy represents a novel approach to induce 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 of several types of human cancers.[1] Polyphenolic secoiridoids of EVOO have been shown to decrease cell viability in HER2-overexpressing breast cancer cells by selectively inducing apoptotic cell death.[2] (-)-Oleocanthal (OC), a di-aldehydic form of ligostride aglycone isolated from EVOO, possesses a wide range of biological effects ð a potent anti-oxidant; 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, associated with the debilitating effects of Alzheimerôs disease; an inhibitor of cell proliferation, migration, and invasion of human breast and prostate cancer through c-Met inhibition; an inhibitor of AMPK in colon cancer cells; and an inhibitor of macrophage inflammatory protein-1Ŭin multiple myeloma.[3-8]

To investigate the anti-cancer effects of OC, we examined its impact on the viability and survival of cancerous and non-cancerous cells. Interestingly, OC rapidly (within 30 minutes) causes loss of cell viability in cancer cells in a dose dependent manner. Under serum withdrawal, OC promotes primary necrotic cell death in cancer cells, which is 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 induces a reversible cell cycle arrest in non-cancerous cells that retain their viability. Our findings indicate that OC-mediated cancer cell death is promoted by the destabilization of the lysosomal

membrane leading to the induction of lysosomal membrane permeabilization (LMP). The OC-induced LMP is mediated by the inhibition of acid sphingomyelinase (ASM) activity, which can be derepressed by up-regulation of Hsp70 or dual treatment with anionic lipids. These data provide evidence that the source of EVOOs anti-cancer activity is due, in part, to the ability of OC to rupture lysosomal membranes in cancer cells leading to cell death via necrosis and/or apoptosis. Importantly, non-cancerous cells remain viable due to the integrity of their lysosomal membranes.

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 cell proliferation, migration and invasion in breast and prostate cancer cells via inhibition of c-Met phosphorylation.[5] OC has also been shown to inhibit cell proliferation in multiple myeloma cells via induction of apoptosis and inhibiting 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 μM OC rapidly induced a loss of cell adhesion within 30 minutes post treatment as well as 100% non-viable cells in all cancer cell lines after 24 hour treatment (**Fig. 1A**). Interestingly, OC increased the levels of phosphorylated p44/42 (P-p44/42), but did not significantly increase the levels of cleaved poly-ADP-ribose polymerase (PARP) ï an indicator of apoptotic death ï 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. Also, OC treatment resulted in a complete loss of mitochondrial activity at low micro-molar 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 along with the absence of PARP and caspase-3 cleavage suggests that OC induces primary necrotic cell death in the absence of serum for all cancer cells.

In the presence of serum, however, OC treatment increased the level of cleaved PARP and cleaved caspase-3, which correlated with increased numbers of non-viable cells in all cancer cells examined (**Fig. 1B**). OC did not have a significant effect on the level of P-p44/42 in the presence of serum, where baseline levels of P-p44/42 tended to be high (**Fig. 1B**) and only partially inhibiting mitochondrial activity (data not shown), which correlates with the loss of cell viability observed in **Figs 1B**. 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 **Fig. 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 three non-cancerous cells examined as determined by cell number after 72 hour treatment. Rapamycin was used as a positive control due to 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 hours of OC treatment in BJ cells (**Fig. 1D,** upper and lower left panel).

Phosphorylation of Rb is a critical step for 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 as necessary for the reduced binding affinity of E2F to Rb.[16] We therefore examined OCôs effect on the levels of phospho-Rb at Ser608 in the non-cancerous cells. As shown in **Fig. 1D** (right panel), non-cancerous BJ and 3Y1 (embryonic rat fibroblast) cells treated with OC resulted in decreased levels of phospho-Rb at Ser608 after 24 hours, which continued for 72 hours in the presence of serum. IMR90 cells did not show a significant decrease in Rb phosphorylation at Ser 608 ï likely 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 adverse effects of OC treatment.

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

The morphological and biochemical changes associated with apoptotic cell death

occur between 6-12 hours post traumatic event leading to apoptotic body formation between 24-48 hours.[18] The rapidity with which OC induces cell rounding and loss of substrate adherence in the absence of serum (observed within 30 minutes post treatment) suggests a non-apoptotic form of cell death. As was seen in **Fig. 1A**, 20 μM OC induced elevated ERK1/2 phosphorylation ï which has been associated with necrotic cell death [9] ï 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 dosage of OC (10 μM) was used to reduce the rate of cell rounding in order to determine the specific method of cell death. As shown in **Fig. 2A**, OC treatment induced a significant number of necrotic cells post 24 hour treatment. The high number of necrotic cells observed explains the large loss of cell viability in the absence of cleaved caspase-3 and limited increase in cleaved PARP observed in **Fig. 1A**, which further verifies cell death via a primary necrotic pathway. While PARP cleavage was not observed in the absence of serum, due to activation of the primary necrotic cell death pathway, increased cleaved PARP and caspase-3 levels 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, **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 which is reversed by anionic lipids.

When caused by severe insult, either extracellularly or intracellularly, primary necrosis can be identified by the rapid permeabilization of the plasma membrane.[19] Lysosomes have recently been implicated in cell death due to the release of lysosomal hydrolytic enzymes into the cytosol leading to apoptosis (via mitochondrial outer membrane permeabilization (MOMP) and caspase activation) or necrosis (via cytosolic acidification) cell death.[21] To determine whether LMP is mediating OC-induced cancer cell death, we examined the integrity of the lysosomal membrane post OC treatment using acridine orange. Acridine orange is a lysosomotropic metachromatic fluorochrome that emits a red fluorescence when in high concentration within intact lysosomes.[22] OC significantly reduced the red fluorescence in both the absence and presence of serum indicating that OC-induced cell death is mediated by the induction of LMP in all cancer cell lines and conditions (**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 amphiphillic drug, induces LMP due to the 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 10 μM OC treatment for 4 hours. In the presence of serum, 10 μM OC induced a maximum of 10% inhibition of ASM activity, (**Fig. 3B**, right panel). The level by which ASM activity is inhibited correlates with the specific cell

death pathway that is activated upon OC treatment and the degree of LMP. Massive or complete LMP rapidly liberated 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 essential as a co-factor for lysosomal sphingomyelin metabolism.[27] 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 would inhibit OC-induced LMP. Cerulenin, a fatty acid synthase inhibitor, has been shown to upregulate Hsp70 expression due to Hsp70ôs ability 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 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 the absence of serum in PC3 cells. The inhibition of OC-induced LMP also correlated with an increase in cell viability as measured by percent attached cells, **Fig. 3D** (right panel). Dual treatment of lipids and OC did not result in cell death as observed by the

lack of floating cells after 24 up to 72 hours OC treatment ï decreased cell number is due to a decrease in cell proliferation. These data indicate that maintaining or enhancing ASM activity ï via addition of anionic lipids ï increases the stability of the lysosomal membrane thus preventing LMP-induced cell death in cancer cells. Collectively, data in **Figs. 1-3** indicates that, in cancer cells, OC inhibits ASM activity which leads to complete or partial LMP, 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 the downregulation of ASM activity leading to LMP, while reversibly arresting non-cancerous cells. In non-cancerous cells, OC induced G1 cell cycle arrest via inhibition of Rb phosphorylation at Ser608 preventing cell cycle progression into S phase.[33, 34] Importantly, non-cancer cells arrested by OC resumed proliferation after 72 hours of OC treatment.

In contrast to the cell cycle arrest observed in non-cancerous cells, cancer cells rapidly underwent either primary necrotic cell death correlating with increased ERK1/2 phosphorylation in the absence of cleaved caspase-3 and PARP under serum withdrawal conditions 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 transformations, which lead to increased size and greater cathepsin activity than 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 enzymes released 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.

While fragility of the lysosomal membrane increases the susceptibility of a cancer cell to LMP, cancer cells have overcome this vulnerability by overexpressing Hsp70, a regulator of multi-protein 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, cancer cells that overexpress Hsp70 promote cell survival by increasing lysosomal integrity in order to prevent LMP.[35, 37] This study states that increasing Hsp70 expression using cerulenin, a fatty acid synthesis inhibitor, suppresses OC-induced LMP in PC3 cells due to increased lysosomal membrane stability.[28]

While cationic lipid 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 also provided evidence that anionic free fatty acids are also capable of stabilizing the lysosomal membrane and prevent 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 ï 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. Whether LMP was involved in cell death or PARP cleavage observed was not clear in this study. However, this study demonstrates that OC has similar effects as this present study in an animal model showing minimal effects of OC on normal cells, which reinforces the potential for 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 enlarged lysosomes in cancer cells. This study suggests that the chemopreventive activity of EVOO is due to the ability of its bioactive phenolic components, especially OC, to induce cell death upon entering the lysosome and inhibiting ASM activity, which induces LMP. Therefore, the ability of OC to induce LMP in cancer but not normal cells represents a novel therapeutic strategy for treating a large number of cancers where lysosomes are larger and apparently 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 H^1NMR analysis. Acridine orange was obtained from Life Technologies. The Promokine Apoptotic/Necrotic/Healthy cell detection kit and Cerulenin was obtained from Fisher Scientific. The Sphingomyelinase Fluorometric Assay kit was obtained from Cayman Chemicals. Cleaved Caspase-3, cleaved PARP, Phospho-p44/42 (Thr202/Tyr204), Total p44/42, β-actin and Phospho-Rb (Ser 608) primary antibodies were obtained from Cell Signaling. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Hsp70 antibodies were obtained from Santa Cruz Biotechnology.

Lipid mix supplementation

Fatty acid mixture was obtained from Invitrogen (11905) and was supplied to cells as 1:200 dilution complexed with 5% fatty acid free bovine serum albumin (BSA; Sigma Aldrich) in 2:1 ratio for the final concentration of lipids in the media of 0.375 mg/L. Composition of fatty acid mixture: Arachidonic acid (20:4 ; 2 mg/L), Cholesterol (220 mg/L), DL-α-Tocophenol 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 (90000 mg/L), Stearic acid (18:0 ; 10 mg/L), Tween 80 (2200 mg/L).

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

Cell viability, analyzed by the level of mitochondrial activity, was determined via the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay according

to the vendor's instructions (Sigma Aldrich). Cell proliferation and percent non-viable cells were determined by calculating percent cells attached compared to control as previously described.[39] ASM activity was determined using the Sphingomyelinase Fluorometric Assay kit obtained from Cayman Chemicals. Cell death was evaluated via two separate methods: examination of cleavage of the caspase-3 substrate PARP as well as detection of apoptotic, necrotic and healthy cells by the Promokine Apoptotic/Necrotic/Healthy Cells Detection Kit following the manufacture guidelines. Briefly, 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 are sensitive to 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 were performed on a cell suspension. Cell death assay: following incubation with FITC-Annexin V, Ethidium Homodimer III and Hoechst 33342, cells were washed twice with 1x Binding Buffer then resuspended in 30 μL 1x Binding Buffer; lysosomal integrity assay: post OC treatment, cells were washed twice with PBS then resuspended in 30 μL PBS. 5 μL of the cell suspension was placed on a microscope slide and covered with a coverslip. Images were collected with an Olympus BX61 fluorescence microscope using 10x objective connected to a Hamamatsu ORCA-ER CCD camera, 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 subjected to SDS-PAGE separating gels. Electrophoresed proteins were then 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

The authors disclose no potential conflicts of interest.

Acknowledgments

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Figure Legends

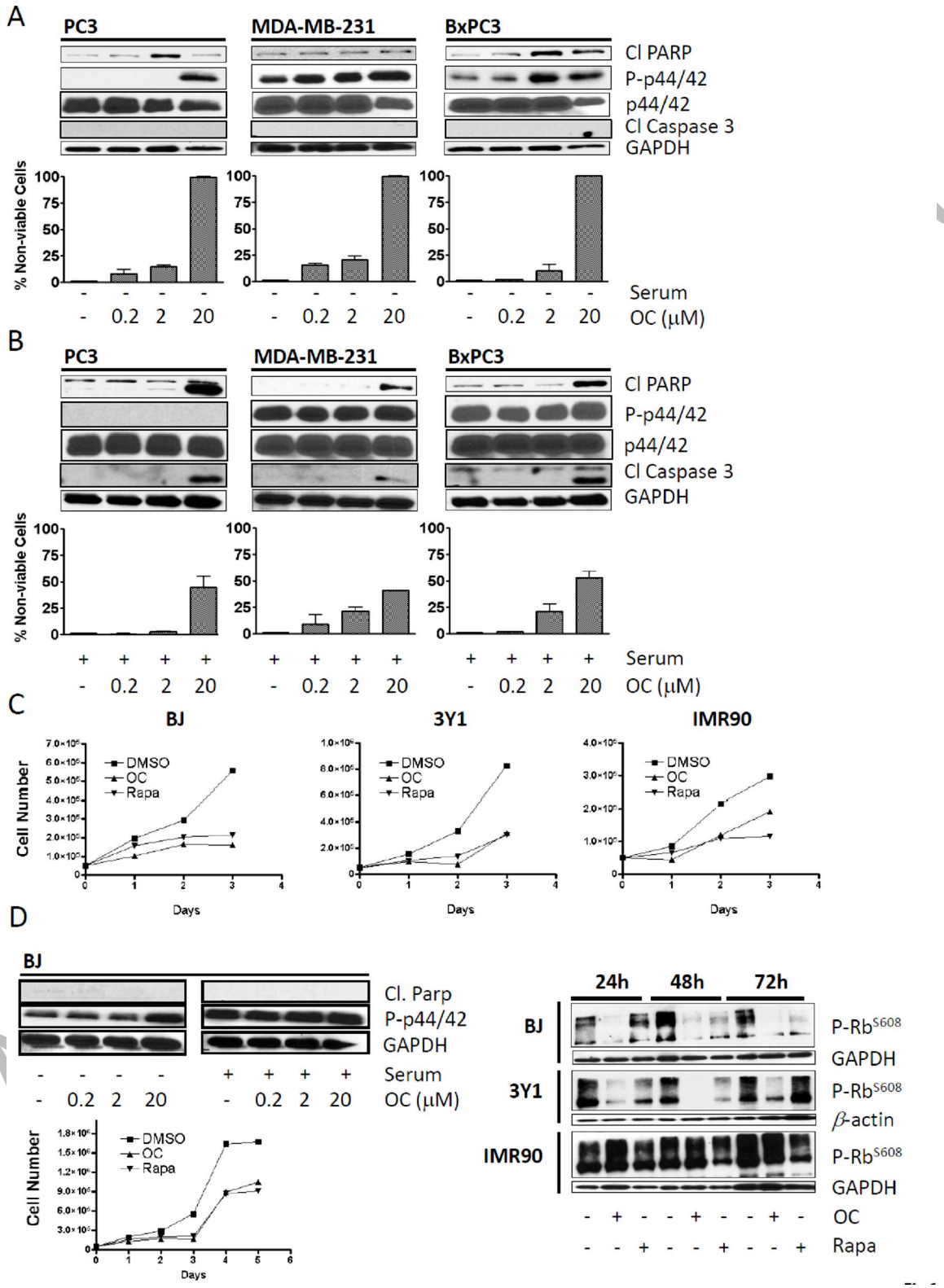

Figure 1. OC induces loss of cell viability in cancer cells but reversible cell cycle arrest in non-cancerous cells. (**A**) PC3, MDA-MB-231 and BxPC3 cells were plated at a density of 3 x 10^5 cells/35 mm plate. 24 hr later the cells were provided with fresh media containing 0% serum, fresh media containing 0% serum and the indicated concentration of OC. 4 hours later, levels of cleaved PARP, phosphor-p44/42 (Thr202/Tyr204), total p44/42, cleaved caspase 3 and GAPDH were determined. 24 hours later cell viability was determined. (**B**) PC3, MDA-MB-231 and BxPC3 cells were plated as above. 24 hr later the cells were provided with fresh media containing 10% serum, fresh media containing 10% serum and the indicated concentration of OC. 4 hours later, levels of cleaved PARP, phosphor-p44/42 (Thr202/Tyr204), total p44/42, cleaved caspase 3 and GAPDH were determined and cell viability was determined 24 hours later. (**C**) BJ, 3Y1 and IMR90 cells were plated at a density of 5 x 10^4 cells/35mm plate. 24 hr later the cells were provided with fresh media containing 10% serum, fresh media containing 10% serum and the indicated concentration of OC or rapamycin (Rapa). 24, 48 or 72 hours later, attached cells were counted to determine cell proliferation. (**D**) BJ cells were plated at a density of 2 x 10^5 cells/35 mm plate. 24 hr later the cells were provided with fresh media containing 10% serum, fresh media containing 10% serum and the indicated concentration of OC. 4 hours later, levels of cleaved PARP, phosphor-p44/42 (Thr202/Tyr204) and GAPDH was determined (upper left panel). BJ cells were plated at a density of 5 x 10^4 cells/35mm plate. 24 hr later the cells were provided with fresh media containing 10% serum, fresh media containing 10% serum and the indicated concentration of OC or Rapa. 24, 48, 72, 96 or 120 hours later, the cells were counted to determine cell proliferation (lower left panel). BJ, 3Y1

and IMR90 cells were plated as above. 24 hr later the cells were provided with fresh media containing 10% serum, fresh media containing 10% serum and the indicated concentration of OC or Rapa. 24, 48 or 72 hours later, the cells were subjected to Western blot analysis for phosphorylated Rb (Ser608) and GAPDH or β-actin (right panel). Error bars for all graphs represent the standard deviation from two independent experiments. The Western blots and all experiments are representative of at least two independent experiments.

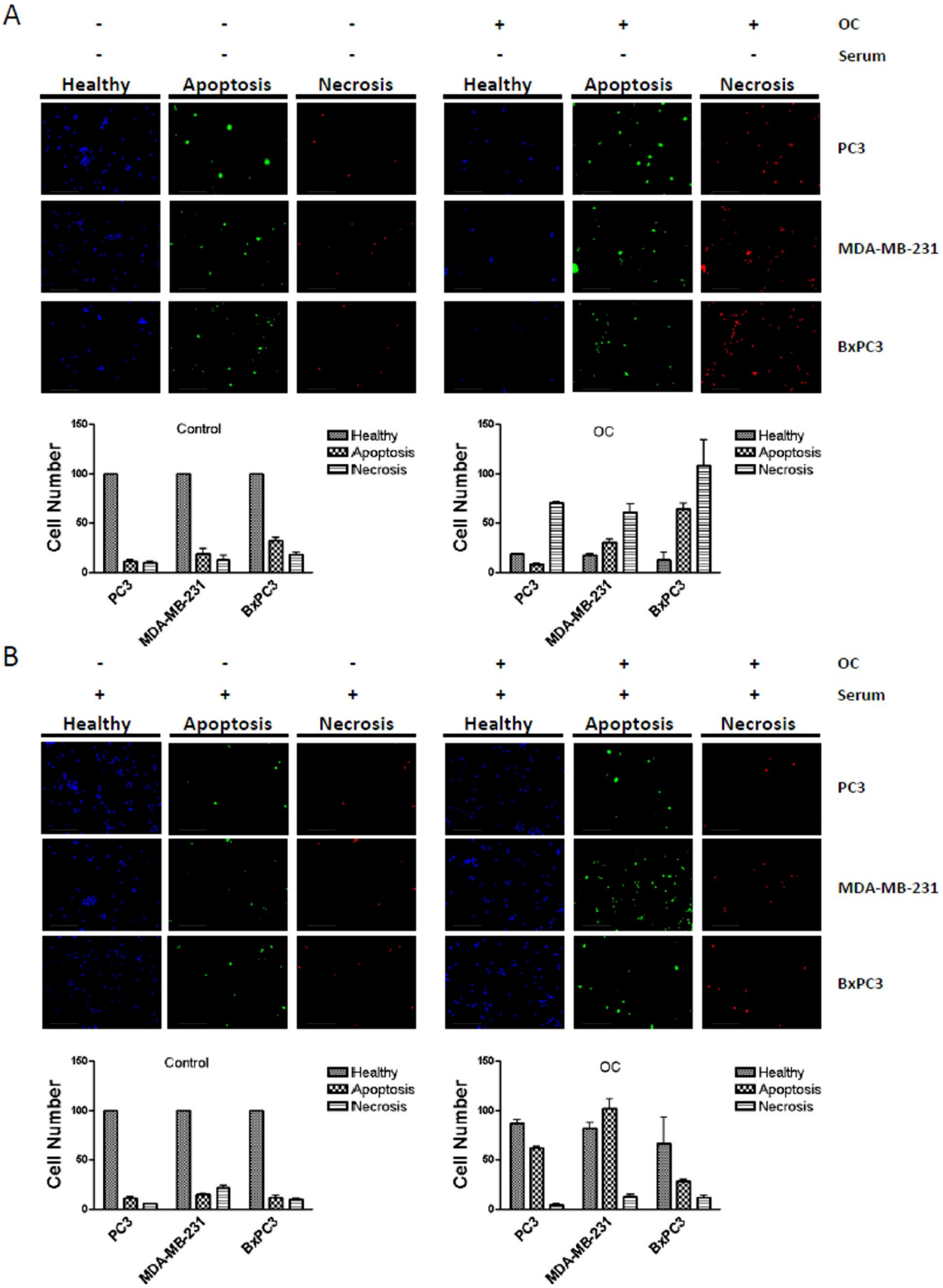

Figure 2. OC differentially induces cell death in the absence and presence of serum in cancer cells. (**A**) PC3, MDA-MB-231 and BxPC3 cells were plated at a density of 2 x 10^5 cells/60 mm plate. 24 hr later the cells were provided with fresh media containing 0% serum, fresh media containing 0% serum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

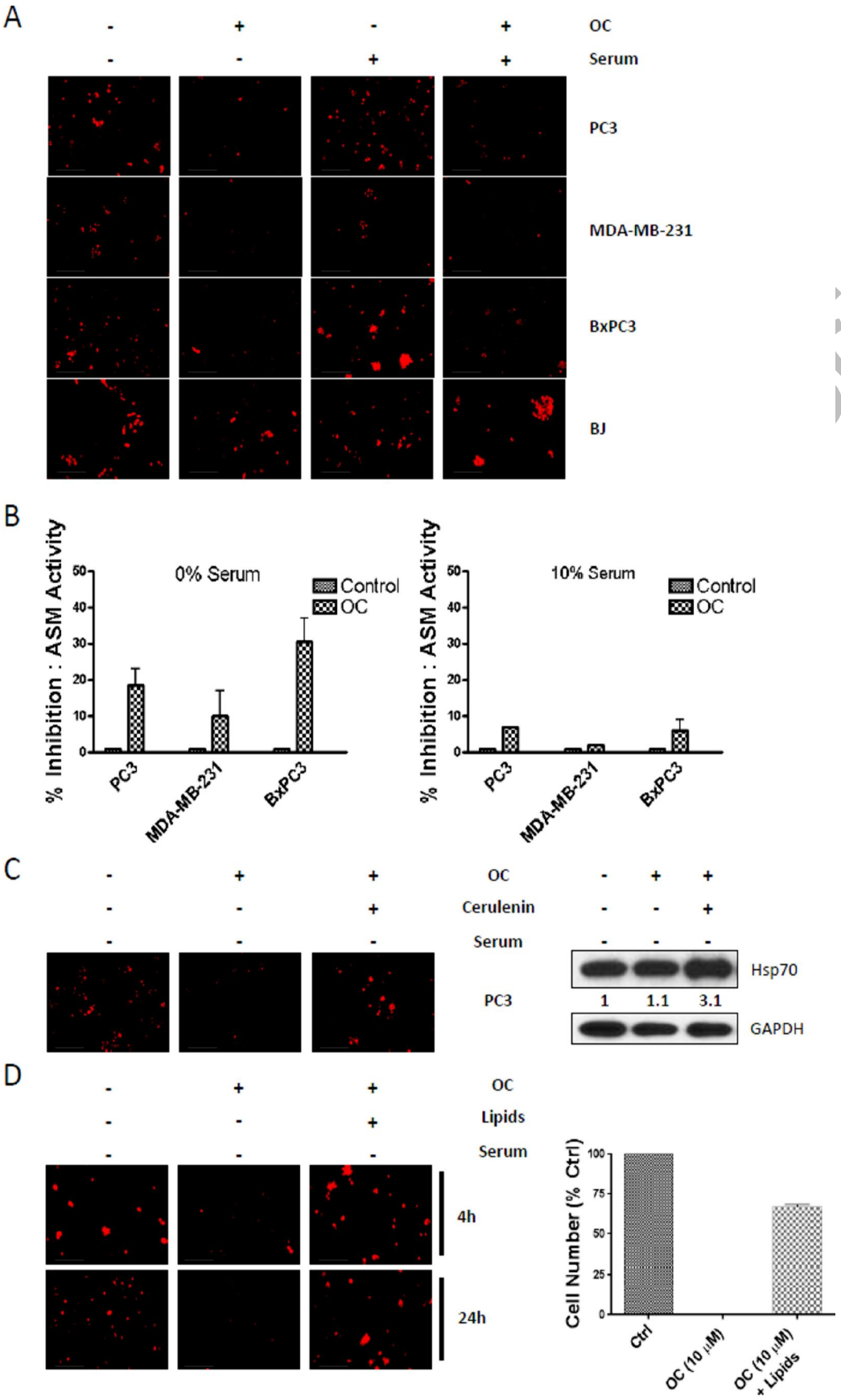

Figure 3. OC downregulates ASM activity to induce LMP and cell death in cancer cells which is reversed by anionic lipids. (**A**) PC3, MDA-MB-231, BxPC3 and BJ cells were plated at a density of 3 x 10^5 cells/60 mm plate. 24 hr later the cells were provided with fresh media containing 0 or 10% serum and 10 μM OC where indicated. 4 hours later, the cells were treated with 5μM acridine orange. 15 minutes later, the cells were collected and subjected to fluorescence microscopy using Texas Red filter setting. (**B**) PC3, MDA-MB-231 and BxPC3 cells were plated at a density of 5 x 10^6 cells/10 cm plate. 24 hr later the cells were provided with fresh media containing 0 or 10% serum and 10 μM OC where indicated. 4 hours later, the cells were collected and subjected to the Acid Sphingomyelinase Fluorometric Assay kit to determine the activity of acid sphingomyelinase. (**C**) PC3 cells were plated at a density of 2 x 10^5 cells/60 mm plate. 24 hr later, the cells were provided with fresh media containing 10% serum or fresh media containing 10% serum and 30 μM Cerulenin as indicated. 24 hours later, the cells were provided with fresh media containing 0% serum and 10 μM OC where indicated. 4 hours later, the cells were treated with 5 μM acridine orange. 15 minutes later, the cells were collected and subjected to fluorescence microscopy using Texas Red filter setting or western blot analysis for levels of Hsp70 and GAPDH. (**D**) PC3 cells were plated at a density of 2 x 10^5 cells/60 mm plate. 24 hours later, the cells were provided with fresh media containing 0% serum, 10 μM OC and 10 μM OC with lipids where indicated. 4 or 24 hours later, cells were treated with 5 μM acridine orange. 15 minutes later, the cells were collected and subjected to fluorescence microscopy using Texas Red filter setting ï 24 hours later cell viability was also determined. Imagej software was used to quantify the Western blots. Experiments shown are

representative of at least two independent experiments. Scale bars = 200 μm.