

# 1 Oleocanthal and oleocanthal-rich olive oils induce 2 lysosomal membrane permeabilization in cancer cells

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24

## 25 Abstract

26

27 Oleocanthal is a phenolic compound found in varying concentrations in extra virgin olive  
28 oil. Oleocanthal has been shown to be active physiologically, benefiting several diseased  
29 states by conferring anti-inflammatory and neuroprotective benefits. Recently, we and  
30 other groups have demonstrated its specific and selective toxicity toward cancer cells;  
31 however, the mechanism leading to cancer cell death is still disputed. The current study  
32 demonstrates that oleocanthal, as well as naturally oleocanthal-rich extra virgin olive  
33 oils, induced damage to cancer cells' lysosomes leading to cellular toxicity *in vitro* and *in*  
34 *vivo*. Lysosomal membrane permeabilization following oleocanthal treatment in various  
35 cell lines was assayed via three complementary methods. Additionally, we found  
36 oleocanthal treatment reduced tumor burden and extended lifespan of mice engineered  
37 to develop pancreatic neuroendocrine tumors. Finally, following-up on numerous  
38 correlative studies demonstrating consumption of olive oil reduces cancer incidence and  
39 morbidity, we observed that extra virgin olive oils naturally rich in oleocanthal sharply  
40 reduced cancer cell viability and induced lysosomal membrane permeabilization while  
41 oleocanthal-poor oils did not. Our results are especially encouraging since tumor cells  
42 often have larger and more numerous lysosomes, making them especially vulnerable to  
43 lysosomotropic agents such as oleocanthal.

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46

## 47 **Introduction**

48           Olive oil has been consumed by humans for millennia and is frequently  
49 associated with health-related properties. The Mediterranean diet and olive oil  
50 consumption in particular are correlated with lower cancer incidence and mortality (1-3).  
51 A meta analyses of nineteen observational studies performed between 1990 and 2011  
52 that included approximately 35,000 individuals found that olive oil intake is inversely  
53 related to cancer prevalence (4). More recently, a randomized trial found that women  
54 who adhered to a Mediterranean diet supplemented with extra virgin olive oil (EVOO)  
55 had 62% less invasive breast cancer incidence than a control group that was advised to  
56 restrict dietary fats (5). These studies, however, did not distinguish between the  
57 protective effects of EVOO's triglycerides and its phenolic components. Furthermore, to  
58 the best of our knowledge, no controlled study tested the effect of high phenolic EVOO  
59 on cancer.

60           (-)-Oleocanthal, also known as deacetoxy-ligstroside aglycon, was first identified  
61 as a minor phenolic compound in the fruit of the olive tree by Motedoro et al. in 1993  
62 (6). A few years later, it was reported to be the primary agent that conveys strong  
63 stinging sensation at the back of the throat when ingesting certain EVOOs (7). In 2005,  
64 Beauchamp and colleagues published the first paper to refer to this compound as  
65 oleocanthal [Oleo - for oil, Canth – Greek for stinging or literally prickly (named for the  
66 throat irritation caused by oleocanthal), and Al – for the two aldehyde groups that are  
67 believed to be responsible for oleocanthal's reactivity] (8). Beauchamp and colleagues  
68 also identified oleocanthal as a potent inhibitor of cyclooxygenase enzymes, conferring

69 anti-inflammatory activity that was more potent than ibuprofen (8). Since this pioneering  
70 oleocanthal paper was published, several groups looked at its medicinal neuroprotective  
71 properties. Pitt et al. observed that low doses of oleocanthal altered Alzheimer's-  
72 associated amyloid- $\beta$  oligomers (9), and Li et al reported that oleocanthal inhibited tau  
73 fibrillization (10). In 2018, Batarseh et al. reported that high-oleocanthal EVOO reduced  
74 amyloid- $\beta$  load and related toxicity in a mouse model of Alzheimer's disease. (11)

75         Since oleocanthal inhibits cyclooxygenase enzymes that mediate inflammation,  
76 which is associated with cancer initiation and progression, there was increasing interest  
77 in studying the anti-cancer properties of Oleocanthal. The first reports of oleocanthal  
78 attenuating tumorigenicity were in 2011. Elnagar et al. (12) demonstrated oleocanthal's  
79 ability to prevent cell migration in a metastatic model for the breast carcinoma cell line  
80 MDA-MB-231. Khanal et al.(13) showed that oleocanthal inhibits phorbol ester-induced  
81 cell transformation in murine JB6 Cl41 cells. Additionally, the authors showed that  
82 oleocanthal inhibits proliferation and the ability to form colonies formation in soft agar of  
83 HT-29 colon cancer cells. Other reports illustrated the anti-proliferative activity of  
84 oleocanthal using various cancer cell lines – including breast carcinoma (14), multiple  
85 myeloma (15), hepatocellular carcinoma (16), melanoma (17), and prostate cancer (12).  
86 The proposed mechanisms for oleocanthal activity differ among the different research  
87 groups. El Sayed and colleagues published several papers illustrating that oleocanthal  
88 acts a c-Met inhibitor (12, 14, 18). c-Met is a receptor tyrosine kinase that is activated  
89 by hepatocyte growth factor. c-Met acts upstream of both the PI3K and the MAPK  
90 pathways. The El Sayed group demonstrated oleocanthal inhibitory effects on c-Met  
91 downstream targets when cells were stimulated with hepatocyte growth factor. In their

92 studies, the mode of death was apoptotic as evident by the induction of cleaved  
93 caspase-3 and cleaved PARP. Pei et al. reported that oleocanthal inhibits growth and  
94 metastasis of hepatocellular carcinoma by blocking activation of the transcription factor  
95 STAT3 (16). Interestingly, one of the canonical ways in which STAT3 is activated is via  
96 receptor tyrosine kinases, including c-Met. Yet the upstream initiating event that  
97 promotes cancer cell death remains uncertain.

98         Whereas we previously reported that oleocanthal induces cancer cell death via  
99 lysosomal membrane permeabilization (LMP) (19), other mechanisms for cancer cell  
100 death – notably apoptosis – in response to oleocanthal have been reported (16, 20, 21).  
101 Different forms of stress can induce LMP, which causes release of intra-lysosomal  
102 enzymes to the cytoplasm – resulting in lysosome-dependent cell death (22). This newly  
103 appreciated cell-death mechanism is gaining interest, since transformed cells are often  
104 characterized by a large increase to their lysosomal compartment and are strongly  
105 dependent on lysosomal function (23). Lysosomes contain over 50 different hydrolases,  
106 and many of these are up-regulated and utilized by cancer cells, often in secreted  
107 forms, for purposes of invasion, angiogenesis, and progression (24, 25). The increased  
108 reliance on lysosomal processes might also represent an Achilles' heel for cancer. As  
109 Christian DeDuve noted – the high concentration of degradative enzymes in lysosomes  
110 make them in essence “suicide bags” (26). Lysosomes in transformed cells are more  
111 susceptible to rupture, causing release of hydrolases such as cathepsin (generic name  
112 for lysosomal proteases) into the cytosol (27). Depending upon the degree of LMP, both  
113 apoptotic and non-apoptotic death can be observed (22, 28). Low levels of LMP injures

114 cells and triggers apoptotic death mechanisms, whereas high levels of LMP kills cells  
115 rapidly and directly as a form of necrosis.

116 In this report, we demonstrate oleocanthal's ability to induce severe LMP in a  
117 variety of cancer cells lines, leading to rapid necrotic cell death *in vitro* and shrinkage of  
118 tumors and extension of lifespan in an *in vivo* mouse model for pancreatic  
119 neuroendocrine tumors (PanNET). Strikingly, we were also able to replicate the  
120 beneficial effects of purified oleocanthal by treating cells with EVOOs that naturally  
121 contain high levels of oleocanthal.

122

## 123 **Materials and methods**

124

### 125 **Reagents**

126 Oleocanthal extracted from EVOO was obtained from Dr. Alexios-Leandros  
127 Skaltsounis at the University of Athens, Department of Pharmacology. The structure  
128 and purity (97%) of the oleocanthal was determined by HPLC and H1 NMR analysis.  
129 The Governor premium EVOO limited edition (Corfu, Greece) and Atsas EVOO  
130 (Cyprus) were a gift from the producers. California Olive Ranch™ EVOO (California,  
131 USA), Colavita mild olive oil (Italy), Colavita EVOO (Italy), and Mazola corn oil (USA)  
132 were purchased at a New York City grocery store. All treatments used EVOO from  
133 newly opened bottles that were kept in the dark at room temperature within one month  
134 of opening. Oleocanthal concentration was determined by H1 NMR analysis by a third  
135 party (Numega Labs, San Diego, California). All other reagents, unless noted otherwise,  
136 were purchased from Fisher Scientific.

## 137 **Cells and cell culture conditions**

138 PC3, MDA-MB-231, MCF7, HEK-293T, MCF10A, and BJ-hTert cells used in this  
139 study were obtained from the American Type Tissue Culture Collection. Mouse PanNET  
140 N134 cells were generated by the Du laboratory(29). PC3 cells were maintained in F-  
141 12K medium, MCF10A cells were maintained in MEGM Mammary Epithelial Cell  
142 Growth Medium Bullet Kit (Lonza) supplemented with 100 ng/ml cholera toxin. other  
143 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented  
144 with 10%, or 15% (N134) fetal bovine serum (Hyclone). No further authentication was  
145 performed.

146

## 147 **Antibodies**

148 Mouse anti human galectin-3 antibody (BD Biosciences, 556904), goat anti-human  
149 Cathepsin B antibody (R&D systems AF953), goat anti human cathepsin-D antibody  
150 (Santa Cruz sc-6486), goat anti mouse Cathepsin L antibody (R&D systems AF1515),  
151 mouse-anti human LAMP2 antibody (abcam 25631), rat anti-mouse Lamp2 antibody  
152 (Hybridoma bank 1B4D), rabbit anti-GAPDH antibody (Cell signaling 2118S), rabbit anti-  
153 HSP70 antibody (Proteintech 10995).

154

## 155 **Cell viability**

156 (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (XTT)  
157 reduction assay was used to measure cells viability. In brief,  $5 \times 10^4$  cells/500  $\mu$ l/well  
158 were seeded into 24-well plates in triplicates. After 24 hours, cells were given treatment

159 medium containing 20  $\mu$ M oleocanthal, or vehicle only and incubated at 37°C with 5%  
160 CO<sub>2</sub>. After a 24 h incubation period, cells were treated with 150  $\mu$ l XTT (Invitrogen™  
161 Molecular Probes™ XTT cat. no. x6493) for 2 h. Then, plates were read at 480 nm  
162 wavelength by a spectrophotometer (Molecular devices, SpectraMax i3). After  
163 subtracting blank well absorbance, the absorbance of vehicle treated cells was set to  
164 100%, and the relative absorbance of oleocanthal treated cells was reported as %  
165 viable cells.

166

## 167 **Lentiviral-based overexpression of HSP70**

168 PC3 cells were transduced with either HSP70-1 (Santa Cruz biotechnology sc-  
169 418088-LAC) or control (Santa Cruz biotechnology sc-437282) lentiviral CRISPR  
170 activation particles per manufacturer protocol. Stable cell lines of HSP70  
171 overexpressing and mock transduced control cells were generated via antibiotic  
172 selection. Viability assay was performed as described above.

173

## 174 **$\beta$ -hexosaminidase latency assay**

175 To determine possible direct effects of oleocanthal on lysosome stability, we  
176 examined  $\beta$ -hexosaminidase release from lysosomes. Briefly, fractions enriched in  
177 lysosomes were incubated with oleocanthal. After incubation lysosomes were separated  
178 from the incubating media by filtration through a 96-well plate with 0.22  $\mu$ m filter using a  
179 vacuum manifold.  $\beta$ -hexosaminidase activity in the media was measured using a  
180 colorimetric assay as described previously (30). Broken lysosomes were calculated as  
181 the percentage of total lysosomal hexosaminidase activity detected in the flow-through.



## 182 **NMR**

183 Oleocanthal content in oil was assessed via H-1 NMR as previously described  
184 (31). Briefly, oil samples ( $240 \pm 20$  mg) and Syringaldehyde internal standard were  
185 dissolved in 0.6 ml of CDC13. H1 NMR experiments (NS=512) were recorded on Bruker  
186 AV500. Proton signals of aldehydes from oleocanthal (9.18 ppm) and Syringaldehyde  
187 (9.77 ppm) were integrated.

188

## 189 **Apoptosis / Necrosis assay**

190 Mode of death was detected by flow cytometric analysis of annexin V-FITC and  
191 propidium iodide staining (Vibrant apoptosis assay, Molecular Probes V-13242) per  
192 manufacturer's protocol.

193

## 194 **Immunohistochemistry**

195 The Aits, Jaattela, and Nylandsted protocol for detection of damaged lysosomes  
196 by Galectin-3 translocation was performed as previously described (32, 33). Slides were  
197 visualized on confocal microscope (Nikon Instruments A1 Confocal Laser Microscope  
198 Series equipped with NIS-Elements acquisition Software).

199

## 200 **LysoTracker assay**

201  $2.5 \times 10^5$  cells per well were grown in a 6 well plates. The next day, the media was  
202 changed and cells were incubated with treatment media containing 20  $\mu$ M oleocanthal,  
203 2 mM LLOMe, or DMSO for the indicated amounts of time. In the last 15 minutes of the

204 treatment, 50 nM LysoTracker green (Invitrogen™ Molecular Probes™ LysoTracker™  
205 green DND-26 L7526) was added to the media. Cells were harvested with trypsin  
206 EDTA, and re-suspended to  $1 \times 10^6$  cells/ml. Green fluorescent intensity was  
207 immediately analyzed by flow-cytometry (Orflo MoxiGo II).

208

## 209 **Cell fractionation and western blot analysis**

210 Cytosolic and light membrane fractions containing lysosomes were obtained  
211 using a cell fractionation kit (Abcam ab109719) and procedure was carried according to  
212 manufacturer's protocol. Where indicated, highly purified lysosome enriched fractions  
213 were isolated through centrifugation in discontinuous gradients of metrizamide and  
214 Percoll as previously described. Cytosolic and light membrane fractions were obtained  
215 and protein concentration was estimated. Twenty micrograms of proteins were loaded  
216 into wells of freshly prepared polyacrylamide gel. Proteins were electrophoresed and  
217 transferred to a nitrocellulose membrane. The membranes were blocked in 5% milk in  
218 PBST and incubated overnight with indicated antibodies. The membranes were washed  
219 and incubated with the appropriate secondary antibodies for one hour at RT, washed  
220 again and visualized using KwikQuant™ Imager (Kindle Biosciences).

221

## 222 **Oleocanthal administration to animals**

223 5 mg of oleocanthal was dissolved in DMSO to prepare a stock solution of 50  
224 µg/µl. The stock solution was aliquoted to avoid multiple freeze-thaw cycles, and stored  
225 at -20°C. RIP-Tag mice were intraperitoneally injected with DMSO or oleocanthal (5  
226 mg/kg) daily starting at 9 weeks of age. Mice were weighted weekly starting from 9

227 weeks of age to calculate how much working solution (2.5 µg/µl) to make in normal  
228 0.9% saline and the same dose was used for that week. Kaplan-Meier survival curve  
229 was generated using GraphPad Prism. The pancreases of treated mice at 14 weeks of  
230 age were dissected, and macroscopic tumors (> 0.5 mm<sup>3</sup>) were counted and measured.  
231 Tumor volume (v) was calculated using the formula for a spheroid:  $v = 0.52 \times (\text{width})^2 \times$   
232 (length). All the tumor volumes from each mouse were summed up as the tumor  
233 burden. All procedures involving mice were approved by the Institutional Animal Care  
234 and Use Committee. There was no noticeable influence of sex on the results of this  
235 study (p value > 0.05). This study was carried out in strict accordance with the  
236 recommendations in the Guide for the Care and Use of Laboratory Animals of the  
237 National Institutes of Health. All mice were housed in accordance with institutional  
238 guidelines.

239

## 240 **Oil treatments**

241 Olive oil (or Corn oil) containing treatment media was freshly prepared before  
242 each experiment by mixing oil in serum free media in a 1:25 ratio (1 mL of oil in 24 mL  
243 Media). The mixture was vigorously vortexed on highest setting for one minute on a  
244 tabletop vortex (Scientific industries Vortex Genie-2) to allow the more hydrophilic  
245 components of the oil to be extracted into the aqueous medium. The treatment media  
246 was then allowed to rest for 5 minutes and the oil settled on the top of the tube. The  
247 resulting EVOO enriched treatment media was then collected from underneath the oil  
248 layer and was used to treat the cells.

## 250 **Results**

251

### 252 **Oleocanthal induces rapid necrotic cell death in a variety of** 253 **cancer cells**

254 As we and other groups have previously reported, oleocanthal is toxic to many  
255 cancer cells and causes rapid and extreme loss of cell viability without killing healthy  
256 cells (14, 16, 19). We treated a panel of cancer cells and normal human cells with 20  
257  $\mu$ M oleocanthal, and as expected, saw a sharp loss in viability within 24 hours among  
258 the cancer cells (MDA-MB-231 human breast cancer cells, PC3 human prostate cancer  
259 cell lines, and N134 murine PanNET cancer cells) while the non-cancerous cells  
260 (MCF10A human breast epithelial cells, HEK293T human kidney cells and BJ-hTERT  
261 human fibroblast cells) were less affected by oleocanthal treatment (Fig 1A). Phenotypic  
262 changes were observed as rapidly as one hour post treatment when cells start to round  
263 up and detach from the cell culture dishes. Moreover, loss in cell viability was induced in  
264 PC3 cells by a brief 60 min treatment of oleocanthal followed by removal of the  
265 treatment media (Fig 1B) – indicating that the cell death induced by oleocanthal is rapid.  
266 We previously reported that oleocanthal-induced cell death is due to a necrotic  
267 mechanism, whereas other groups have reported the mechanism of death to be  
268 apoptotic (15, 16).

269 To further establish the mechanism of cell death, we performed a well-  
270 established apoptosis assay using double staining for annexin-V-FITC (AV) and  
271 propidium iodide (PI) and compared the cell death caused by oleocanthal to the known  
272 apoptosis inducer staurosporine in MDA-MB-231 breast cancer cells (Fig 1C) and in

273 PC3 prostate cancer cells (Fig 1D). Whereas staurosporine treated cells single-stained  
274 for AV, a hallmark of apoptosis, the oleocanthal treated cells double stained for both AV  
275 and PI – clearly distinguishing the cell death induced by oleocanthal from the apoptotic  
276 death induced by staurosporine. In the literature, double staining by PI and AV is  
277 interpreted as necrosis – although there are occasional apoptotic phenotypes observed  
278 (34). The distinction depends on whether there is an earlier time point where cells are  
279 still not permeable to PI but already stain for AV. In our hands, regardless of how short  
280 of a treatment we performed, including a 15 min treatment, we never observed  
281 oleocanthal treated cells to be single stained for AV, indicating that they do not undergo  
282 classic apoptosis. We always observed double staining for both AV and PI upon  
283 oleocanthal treatment, which led us to conclude that the mode of death was  
284 predominantly necrosis.

285

286 **Fig 1. Oleocanthal induces rapid necrotic cell death in a variety of cancer cells.**

287 **(A)** The indicated cell lines were treated with 20  $\mu$ M oleocanthal (OC) for 24 hours and  
288 viability was measured via the reduction of XTT. \*\*P < 0.01 (One-way ANOVA). **(B)**  
289 PC3 cells were treated with 20 $\mu$ M oleocanthal or DMSO control for either 24 hours  
290 without media change, or 1 hour followed by a media change into full growth medium.  
291 Viability was measured 24 hours post treatment via the reduction of XTT. C and D)  
292 MDA-MB-231 cells **(C)** and PC3 cells **(D)** were treated with vehicle only (DMSO), or 20  
293  $\mu$ M oleocanthal for the indicated time points, and double-stained with Annexin-V FITC  
294 and PI. Fluorescence was measured on a flow cytometer (MoxiGo II). Treatment with  
295 1 $\mu$ M Staurosporine (St) for 4 hours is presented as a positive control for apoptotic cells.

296 Representative scatter plots from 3 independent experiments are shown, as well as bar  
297 graph quantifications: the lower right quadrant (apoptosis) is shown in green, and upper  
298 quadrant (necrosis) is shown in red. Bar graphs represent the mean  $\pm$  SEM (n=3).

299

## 300 **Oleocanthal induces lysosomal membrane permeabilization** 301 **and cathepsin release to the cytosol**

302 In the last few years there has been a growing appreciation for the importance of  
303 lysosome-dependent cell death (35), and with this appreciation new techniques and  
304 assays have been introduced to assess LMP. The galectin translocation assay, first  
305 described by Aits et al. (32), is emerging as a gold standard to identify and quantify  
306 LMP. Galectins are  $\beta$ -galactoside binding proteins that normally localize to the cytosol  
307 and feature a diffuse cytosolic staining when observed in a confocal microscope. Upon  
308 damage to the lysosomal membrane, galectins translocate to damaged lysosomes and  
309 get trapped because of their affinity to luminal lysosomal  $\beta$ -galactoside sugars (32, 33).  
310 We performed the galectin translocation assay on MCF7 human breast cancer cells, as  
311 indicated by Aits et al., because of the high levels of galectin-3 in these cells (32, 33).  
312 We used the well-described LMP inducer, L-leucyl-L-leucine methyl ester (LLOMe) as a  
313 positive control. Within 2 hours of treatment with oleocanthal, we observed robust  
314 lysosomal staining for galectin-3, similar to LLOMe treatment (the positive control) and  
315 unlike treatment with vehicle only (DMSO) (Fig 2A). The translocation of galectin-3 from  
316 diffuse cytosolic staining to strong punctate perinuclear staining is indicative of  
317 damaged lysosomal membranes (33).

318 We further looked at the integrity of the lysosomal compartment by performing a  
319 LysoTracker retention assay. LysoTracker is a fluorescent acidotropic probe for labeling  
320 and tracking acidic organelles in live cells. In healthy cells, staining with LysoTracker  
321 results in a strong fluorescence signal. Loss of fluorescence is associated with either  
322 damage or de-acidification of lysosomes (36). Known LMP inducers such as LLOMe  
323 lead to decreased LysoTracker fluorescence signal within a short time post treatment  
324 (37). We, therefore, treated PC3 prostate cancer cells with oleocanthal, LLOMe, or  
325 vehicle only, and stained with LysoTracker green. oleocanthal induced a sharp  
326 reduction in fluorescence intensity (Fig 2B). Although LLOMe treated cells showed a  
327 more pronounced reduction in fluorescence intensity, oleocanthal's effect was highly  
328 significant and further implicates LMP as the immediate cause of death in cancer cells  
329 induced by oleocanthal.

330 To further test whether the observed damage to lysosomes was a result of loss  
331 of acidity or actual permeability of the membrane and to assess the functional  
332 consequences of damage to lysosomes, we looked at the distribution of lysosomal  
333 enzymes in the cell. Prior to oleocanthal treatment, lysosomal hydrolases such as  
334 cathepsin B and cathepsin D were entirely excluded from the cellular cytosol (Fig 2C,  
335 2<sup>nd</sup> lane). Upon oleocanthal treatment, however, we observed a substantial release of  
336 these proteases to the cytosol (Fig 2C, 5<sup>th</sup> lane), indicating that oleocanthal treatment  
337 causes cathepsins to be released from the lysosomes to the cytosol.

338 Interestingly, incubating purified lysosomes isolated from PC3 cells *in vitro* with  
339 increasing concentrations of oleocanthal (as we don't know the final cytosolic  
340 concentration of oleocanthal inside cells) had no appreciable difference in lysosomal

341 stability as compared to vehicle (Fig 2D). This indicates that oleocanthal does not act  
342 directly as a membrane disrupting agent on lysosomes, but rather induces lysosomal  
343 permeability only in a cellular context – likely through oleocanthal metabolites.

344 The heat shock protein HSP70 is known to stabilize lysosomal membranes (28)  
345 and in various models of LMP, HSP70 provides protection from subsequent cell death  
346 (38). We, therefore, overexpressed HSP70 in PC3 prostate cancer cells and examined  
347 the effect on oleocanthal-induced loss of cell viability. Indeed, oleocanthal-induced loss  
348 of cell viability was partially rescued by HSP70 overexpression (Fig 2E), further  
349 supporting a role for LMP as the cause of oleocanthal-induced cell viability.

350 Collectively the data provided in Fig 2 strongly suggest that oleocanthal triggers  
351 rapid damage to lysosomes, which causes them to become permeable and leaky,  
352 allowing cytosolic proteins into the lysosome (galectin-3) and lysosomal proteins  
353 (cathepsins) out into the cytosol. The rapid assault on lysosomes, on which cancer cells  
354 are highly metabolically dependent supports the idea that the cellular toxicity caused by  
355 oleocanthal is due to LMP. All other observed effects of oleocanthal on apoptotic and  
356 necrotic forms of cell death in cancer cells are likely down-stream of the LMP and  
357 dependent on the corresponding degree of LMP.

358

359 **Fig 2. Oleocanthal induces LMP and cathepsin leakage. (A)** MCF-7 cells were  
360 treated with DMSO, 30 $\mu$ M oleocanthal for 2 hours, or 2 mM LLOMe and stained for  
361 Galectin-3. Nuclei were labeled with Hoechst 33,342. Scale bars 20  $\mu$ M. Green Galectin  
362 punctea indicate compromised lysosomes. **(B)** PC3 cells were treated with 20  $\mu$ M  
363 oleocanthal for one hour, or 2mM LLOMe for 15 minutes, then loaded with LysoTracker



364 green. Fluorescence intensity was measured via flow cytometry. Histogram shows a  
365 representative shift in LysoTracker fluorescence associated with perturbation to the  
366 lysosomal compartment. Bar graph shows mean fluorescence intensity of three  
367 replicate experiments. **(C)** PC3 cells were treated with 20  $\mu$ M oleocanthal, and two  
368 hours later their cytosolic fractions (Cyto), and light membrane fractions containing  
369 lysosomes (Lyso) were separated. Level of cathepsin B (CTSB) and cathepsin D  
370 (CTSD) in the various fractions or whole cell lysates is shown. LAMP2 is a lysosomal  
371 marker and GAPDH is a cytosolic marker. **(D)** Lysosomes isolated from overnight  
372 serum-deprived PC3 cells were incubated for 20 min with the indicated concentrations  
373 of oleocanthal or vehicle (DMSO). At the end of the incubation, lysosomes were filtered  
374 through a vacuum manifold and b-hexosaminidase activity was measured in the flow  
375 through and in the total lysosomal fraction. Broken lysosomes were calculated as the  
376 percentage of total lysosomal hexosaminidase activity detected in the flow-through and  
377 plotted in logarithmic scale. **(E)** PC3 cells were infected with HSP70-1 Lentiviral  
378 Activation Particles, or control (scrambled) particles, and treated with 20  $\mu$ M  
379 oleocanthal. Viability was assayed using reduction of XTT. \*P < 0.05, \*\*P < 0.01 (Two-  
380 tailed unpaired t-test). Bar graphs represent the mean  $\pm$  SEM (n=3).

381

## 382 **Oleocanthal extends the life span of mice bearing PanNET**

### 383 **tumors**

384 To assess the benefit of oleocanthal treatment in a genetically engineered mouse  
385 model of PanNET, *RIP-Tag* mice (39), we performed a mouse survival trial. The *RIP-*  
386 *Tag* mice inevitably develop tumors that progress through well-defined stages that

387 closely mimic those found in human pancreatic neuroendocrine tumorigenesis (i.e.,  
388 hyperplasia, angiogenesis, adenoma, and invasive carcinoma) (39). We treated mice  
389 with 5 mg/kg oleocanthal or DMSO vehicle daily through intraperitoneal injection starting  
390 at 9 weeks of age. The median survival of vehicle-treated mice was 14 weeks of age  
391 (Fig 3A). In contrast, the oleocanthal-treated animals had a significant extension of life  
392 surviving a median period of 18 weeks – or an additional 4 weeks. To determine the  
393 effect of oleocanthal on tumor sizes, we treated another cohort of mice with 5 mg/kg  
394 oleocanthal or vehicle DMSO daily through intraperitoneal injection starting at 9 weeks  
395 of age and euthanized them at 14 weeks of age (5 week treatment). Although the effect  
396 on tumor burden did not reach statistical significance, there was a trend toward smaller  
397 tumor burden with oleocanthal treatment (Oleocanthal: 14.7 mm<sup>3</sup> vs. DMSO: 24.8 mm<sup>3</sup>)  
398 (Fig 3B).

399 To determine whether the tumors were smaller due to LMP induced cell death,  
400 we checked for cytosolic cathepsin release in the murine cells. We treated an  
401 established cell line derived from a *Rip-Tag* neuroendocrine tumor (N134) *in vitro* and  
402 found cathepsin L (which is a highly expressed cathepsin in N134 cells) present in the  
403 cytosol upon oleocanthal treatment (Fig 3C). The data presented in Fig 3 provide  
404 evidence that oleocanthal suppresses tumorigenesis in a mouse model for PanNET  
405 pancreatic neuroendocrine tumors.

406

407 **Fig 3. Oleocanthal increases life span of mice with PanNET tumors. (A)** Kaplan-  
408 Meier survival curve for RIP-Tag mice receiving DMSO or oleocanthal. The mice were  
409 treated with DMSO (n= 11) or oleocanthal (5 mg/kg, n=15), 7 days a week. Mice were

410 treated starting from 9 weeks of age. Both the Gehan-Breslow-Wilcoxon method and  
411 the Log-rank (Mantel-Cox) method were used to calculate statistical significance \*P <  
412 0.05. **(B)** Tumor burden from mice treated with DMSO or oleocanthal (n =7 for each  
413 group) starting from 9 weeks of age and ending at 14 weeks of age. ns P > 0.05. **(C)** A  
414 cell-line derived from a murine PanNET tumor, was established (N134). Cells were  
415 treated with DMSO or oleocanthal and analyzed for cytosolic cathepsin L (CTSL) via  
416 Western blot as in Fig 2C.

417

## 418 **Oleocanthal-rich EVOOs are toxic to cancer cells via LMP**

419 The use of EVOO in the Mediterranean diet has been associated with cancer  
420 protective effects (4). However, the concentration of oleocanthal in EVOOs varies  
421 greatly (31). We, therefore, examined the effect of EVOOs with varying oleocanthal  
422 concentrations on cancer cell viability. We hypothesized that EVOOs with high levels of  
423 oleocanthal will show greater toxicity towards cancer cells than EVOOs with lower levels  
424 of oleocanthal. The levels of oleocanthal present in several EVOOs, a non-virgin olive  
425 oil, and corn oil were determined by <sup>1</sup>H-NMR as described in Materials and Methods  
426 (Fig 4A). Two EVOOs (Colavita EVOO, and Olive Ranch) had average content of  
427 oleocanthal. Two EVOOs (The Governor and Atsas) had levels of oleocanthal that was  
428 5 or 6-fold higher than the other EVOOs. The non-virgin olive oil (Colavita mild) and the  
429 corn oil (Mazola) had no detectable oleocanthal and were used as negative controls.  
430 We then prepared cellular treatment media that consisted of cell culture media and  
431 EVOO in a ratio of 25:1. We used this specific ratio because it would make the  
432 maximum oleocanthal level in the treatment media in the 20 μM range for the most

433 potent EVOO. To ensure that the oleocanthal was transferred to the media, we  
434 vortexed the mixture vigorously, in essence extracting the more polar components (the  
435 phenolic content of the oil) into the media. We then treated PC3 prostate cancer cells  
436 (Fig 4B) and MDA-MB-231 breast cancer cells (Fig 4D) with this enriched media.  
437 Strikingly, the ability of the EVOO enriched media to kill the cells was directly and  
438 linearly correlated to the EVOO's oleocanthal content. The oils with the highest  
439 oleocanthal content reduced cell viability for both PC3 and MDA-MB-231 cells to a  
440 similar degree to that observed in response to purified oleocanthal. The oils with the  
441 next two highest oleocanthal concentrations reduced viability in a manner  
442 corresponding with oleocanthal concentration; and the oils with no measurable amounts  
443 of oleocanthal did not affect cell viability relative to the no-oil negative control treatment.  
444 We also analyzed the ability of EVOO to induce LMP as determined by cathepsin  
445 release. As shown in Figs 4C and 4E, the EVOOs with the highest concentration of  
446 oleocanthal induced cathepsin release caused leakage of both cathepsin D and B into  
447 the cytosol of PC3 cells (Fig 4C) and MDA-MB-231 cells (Fig 4E). In contrast, the other  
448 oils caused minimal cytosolic cathepsin release - indicating that the oleocanthal content  
449 in EVOOs is a major determinant for EVOO's cancer-protective properties. These data  
450 demonstrate that oleocanthal is able to exert this beneficial effect when delivered via  
451 whole EVOO and not only in a purified phenolic form.

452

453 **Fig 4. Oleocanthal-rich olive oils are toxic to cancer cells via LMP. (A)** Relative  
454 oleocanthal concentration in various oils was measured by H1 NMR as described in  
455 Materials and Methods. **(B and C)** PC3 cells **(B)** and MDA-MB-231 cells **(C)** were

456 treated with 20  $\mu$ M OC, or the specified oils for 24 hours. Viability was measured via the  
457 reduction of XTT. **(D and E)** Cytosolic lysates were collected as in Fig 2C and subjected  
458 to Western blot analysis of cathepsin B (CTSB) and cathepsin D (CTSD) in the cytosol.  
459 Bar graphs represent the mean  $\pm$  SEM (n=3)

460

## 461 **Discussion**

462 Although several groups have demonstrated oleocanthal's ability to inhibit key proteins  
463 that promote cell growth and survival (12, 14-17, 40), a unifying mechanism for the  
464 specific and irreversible cellular death-inducing properties of oleocanthal has not been  
465 established. In this report, we observed that a transient exposure of cancer cells to  
466 oleocanthal for one hour resulted in the loss of cell viability after 24 hours. Although a  
467 classic apoptotic mechanism has been proposed (16, 20, 21), in our hands the rapid cell  
468 death caused by oleocanthal was necrotic. Specifically, viable cells were not observed  
469 to display phosphatidylserine on the outer membrane leaflet as evidenced by staining  
470 with AV, a well-established phase in the apoptotic cascade. Furthermore, using three  
471 different and complementary methods, we demonstrated that oleocanthal-treated cells  
472 undergo LMP. The latest, most robust method to assess LMP is the galectin  
473 translocation assay (33). We observed that oleocanthal treated MCF7 breast cancer  
474 cells showed robust galectin-3 translocation to lysosomes, similar to that observed with  
475 the established LMP inducer LLOMe. In a biochemical assay that checks the leakage of  
476 lysosomal enzymes into the cytosol, we observed a pronounced leakage of both  
477 cathepsin D and cathepsin B to the cytosol in PC3 prostate and MDA-MB-231 breast  
478 cancer cells. The translocation of cathepsins of two different sizes suggests that the

479 lysosomal membrane undergoes severe and unrepairable permeabilization. Agents that  
480 are known to cause LMP with only minimal cathepsin release, such as LLMOe (37)  
481 enable cells to survive the initial LMP and repair their lysosomal membrane. Other  
482 agents that cause the release of cathepsin D (a small hydrolase) but not the release of  
483 cathepsin B (a larger hydrolase) are often associated with apoptosis (22). We,  
484 therefore, conclude that the degree of lysosomal damage in the case of oleocanthal is  
485 massive and leads to rapid necrosis in the affected cancer cells with less and survivable  
486 damage to normal cells.

487         It was previously suggested that many cancer cells are more vulnerable to  
488 attacks on their lysosomes because they have larger and more fragile lysosomes (41)  
489 and are more reliant on lysosomal processes metabolically (27). Furthermore, many  
490 cancer cells upregulate lysosomal biogenesis and lysosomal enzyme turnover (27).  
491 Therefore, once lysosomal enzymes and acids are released into the cytosol en mass,  
492 rapid cell toxicity ensues (42). The effect of oleocanthal was observed in both cell  
493 culture and a live mouse model for the development of PanNETs (39) where lifespan  
494 was extended by 4 weeks (29%). It has been reported that 2.6 adult mice days are  
495 equivalent to one human year (43). Based on this life-span conversion, oleocanthal  
496 might extend life 10.4 years for PanNET pancreatic neuroendocrine cancer patients.  
497 Importantly, the cancer cells from the PanNETs when put in culture released cathepsin  
498 upon oleocanthal treatment and died rapidly.

499         In addition to looking into the effects of purified oleocanthal, we were very  
500 interested to see if oleocanthal in a more natural form can cause a similar outcome.  
501 Since different olive oils are known to have varied oleocanthal concentrations as a

502 function of their origin, harvest time, and processing methods (7), we examined several  
503 olive oils with varied concentrations of oleocanthal from very low to very high. For our *in*  
504 *vitro* experiments, we used two EVOOs with average low oleocanthal content and two  
505 with very high oleocanthal content (about 5 times the average), and for our negative  
506 control we used two oils that contained no measurable oleocanthal. Upon treatment of  
507 cultured cancer cells with oil enriched cell culture media we observed that the  
508 concentration of oleocanthal in the oil was directly related to the toxicity of the oils  
509 towards cancer cells. The oils with the high oleocanthal content completely killed the  
510 cancer cells in a manner similar to purified oleocanthal. The oils with the average  
511 oleocanthal content, also reduced viability but to a lesser extent. The non EVOOs with  
512 no oleocanthal had no effect on cell viability. Furthermore, by looking at cytosolic  
513 cathepsin release, the EVOOs mechanism of promoting cancer cell death also involved  
514 LMP, similar to the effects of purified oleocanthal.

515 Many studies have linked consumption of EVOO with reduced incidence of  
516 cancer (4), most significantly a randomized trial in which elevated EVOO in the diet led  
517 to a 62% reduction in the incidence of breast cancer in Spain over a 5 year period (5).  
518 Data provided here link the cytotoxic effects of EVOOs to their level of oleocanthal. The  
519 cytotoxic effects were due to the ability of oleocanthal to induce LMP and necrotic cell  
520 death preferentially in cancer cells. Whereas pure oleocanthal can also have negative  
521 effects on non-cancerous cells, EVOO is considered safe and healthy and, therefore,  
522 could be both preventative as well as a potential treatment – as indicated by the  
523 Spanish study (5). Since the apparent target for oleocanthal-induced necrosis is the  
524 lysosome, the reason for the elevated sensitivity of cancer cells to oleocanthal could be

525 due to the increased size and fragility of the lysosomal compartment of cancer cells  
526 (27). If the enlarged fragile lysosomal compartment (23, 41) is the reason for increased  
527 sensitivity to oleocanthal, it is likely that EVOOs with high oleocanthal could be  
528 preventative for many cancers – in addition to reduced breast cancer in Spain (5).  
529 Whether purified oleocanthal could be used therapeutically remains to be evaluated.  
530 How can one determine whether there are high levels of oleocanthal in an EVOO?  
531 EVOOs with high oleocanthal levels produce a unique stinging sensation in the back of  
532 the throat and not elsewhere in the mouth, as well as eliciting a brief coughing that has  
533 been used to determine the presence of oleocanthal in EVOO (8). Tasting EVOO for  
534 this signature stinging sensation and cough elicitation could allow people to identify  
535 EVOOs with high oleocanthal content without sophisticated equipment. In light of the  
536 results presented in this report, and since EVOOs have been safely used in the diet for  
537 millennia and are associated with good health, the authors believe that consuming more  
538 EVOO with high oleocanthal content is a prudent dietary approach to cancer prevention  
539 with the caveat that dietary oils convey calories and consequently other caloric sources  
540 will have to yield to avoid obesity.

541

542

## 543 **Acknowledgments**

544 We thank Dr. Ana Maria Cuervo for valuable guidance, and to Daniela Mikhaylov,  
545 Lucy Pascarosa, and Ismat Zerín for experimental assistance.

546



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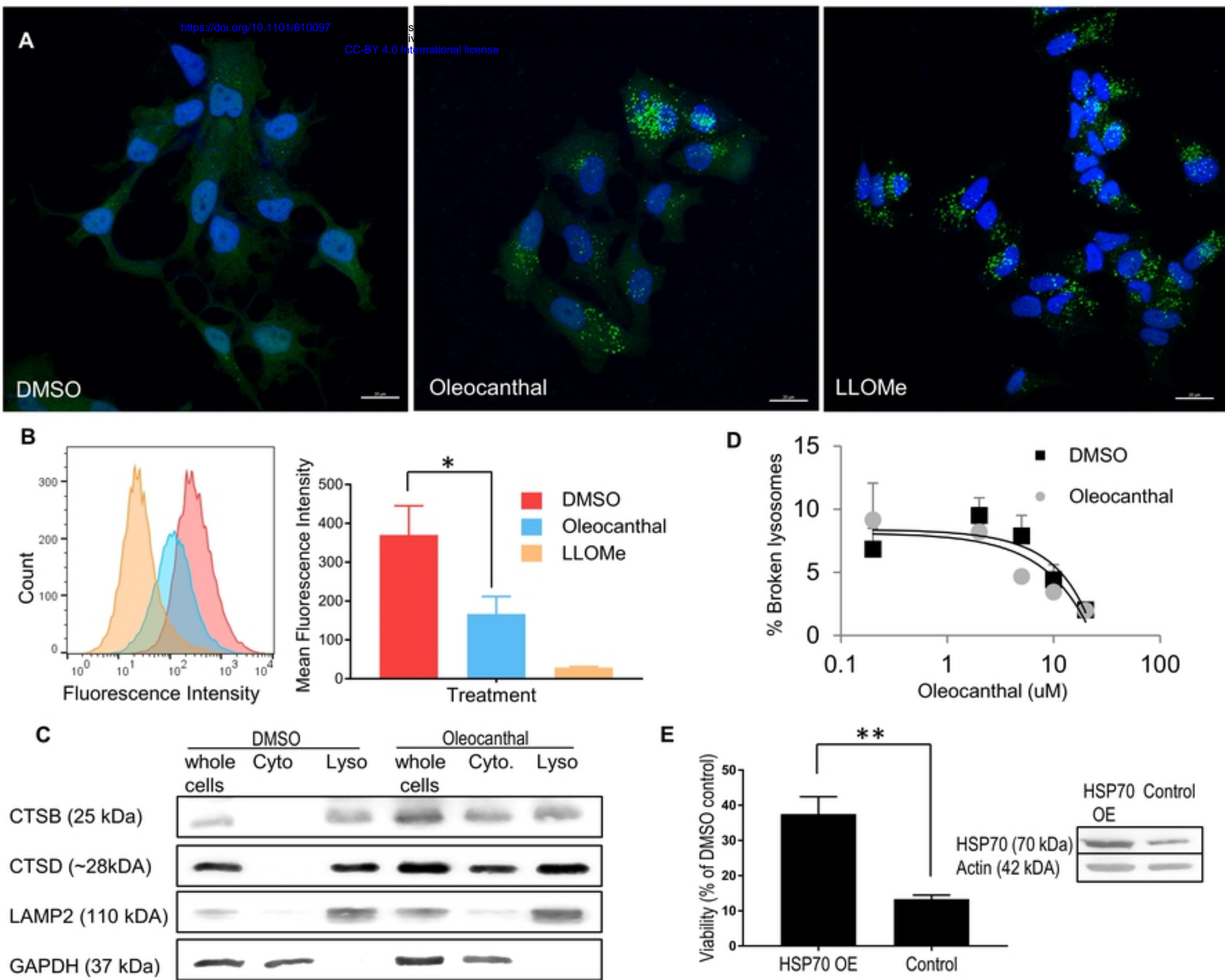


Figure 2



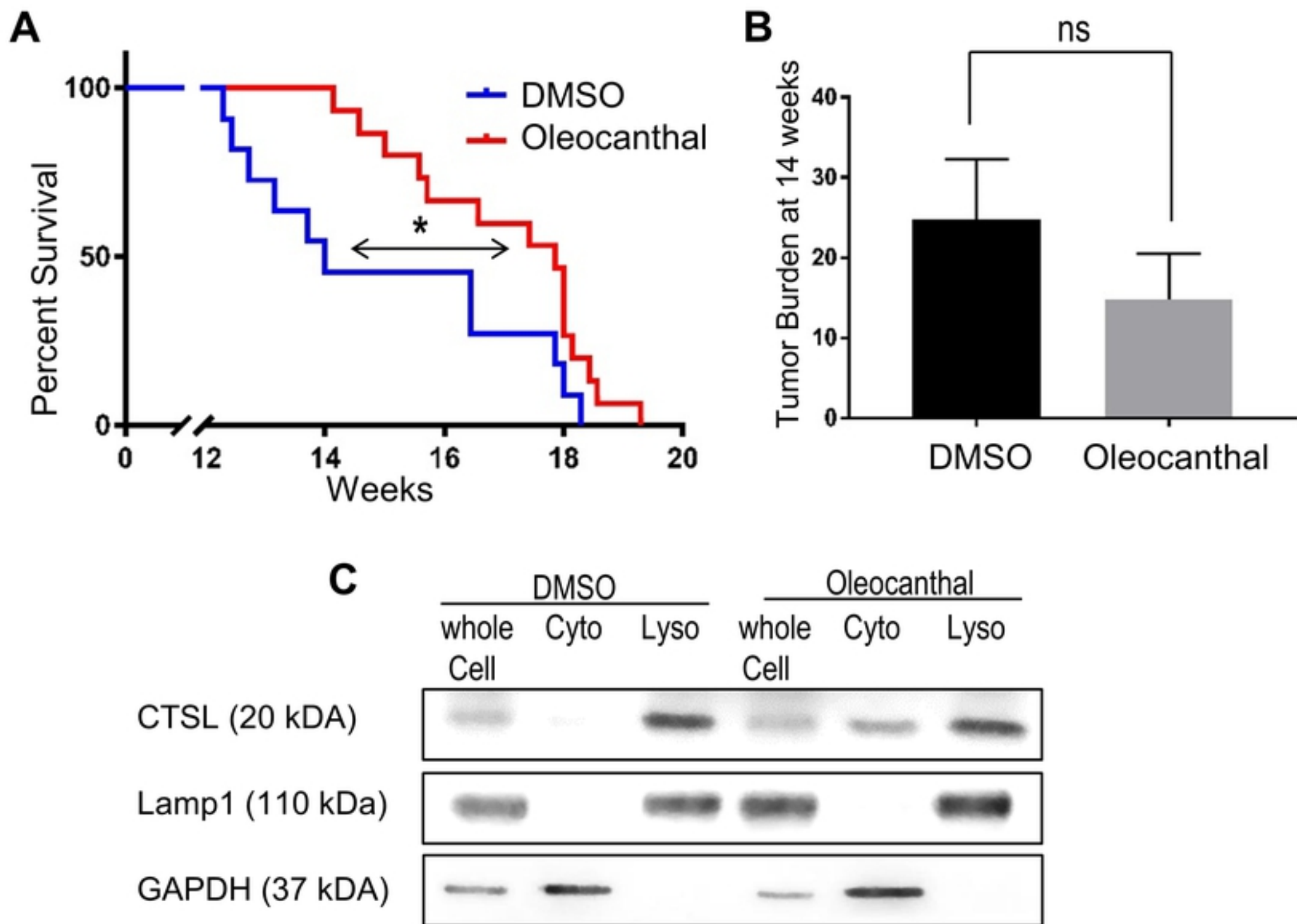


Figure 3



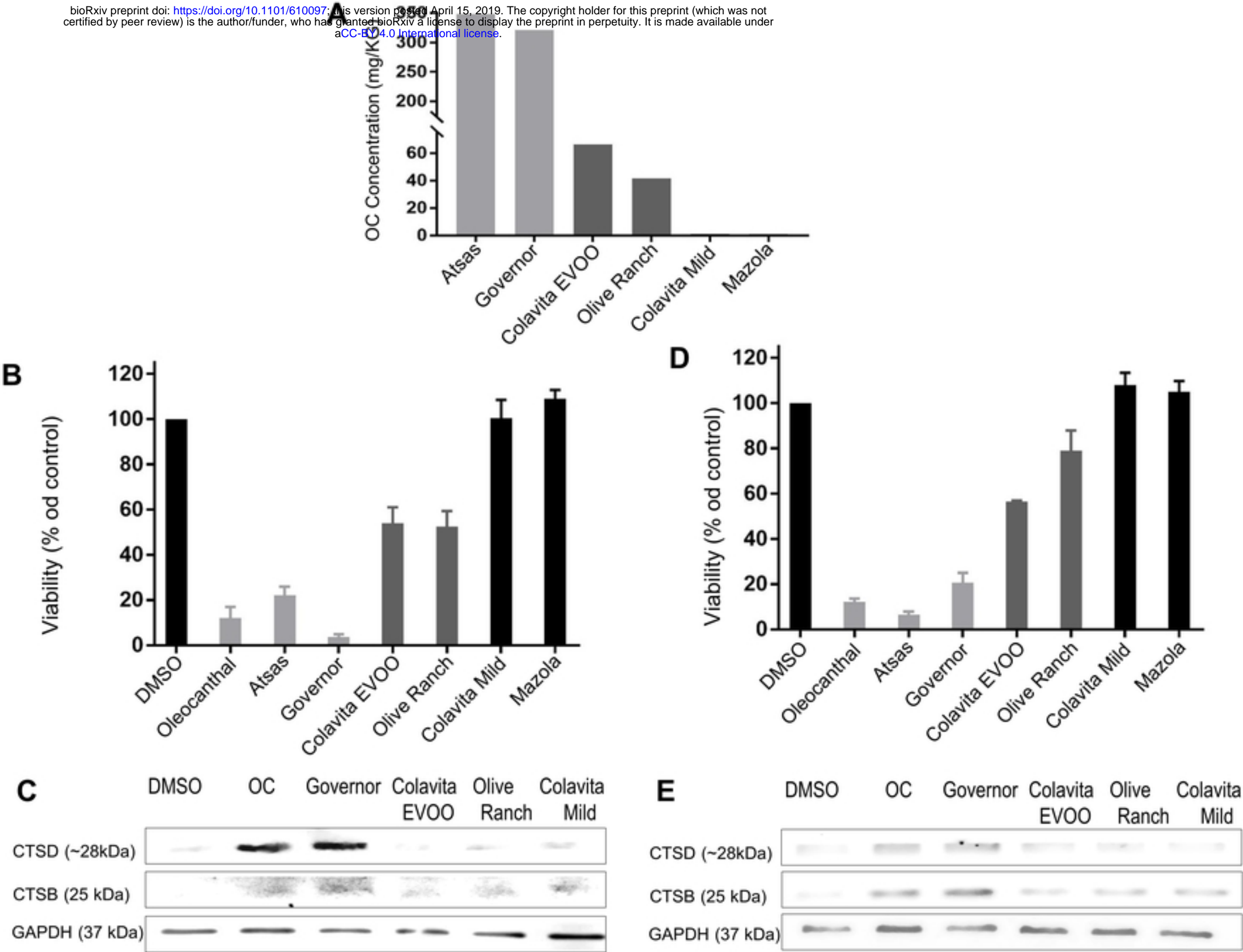


Figure 4

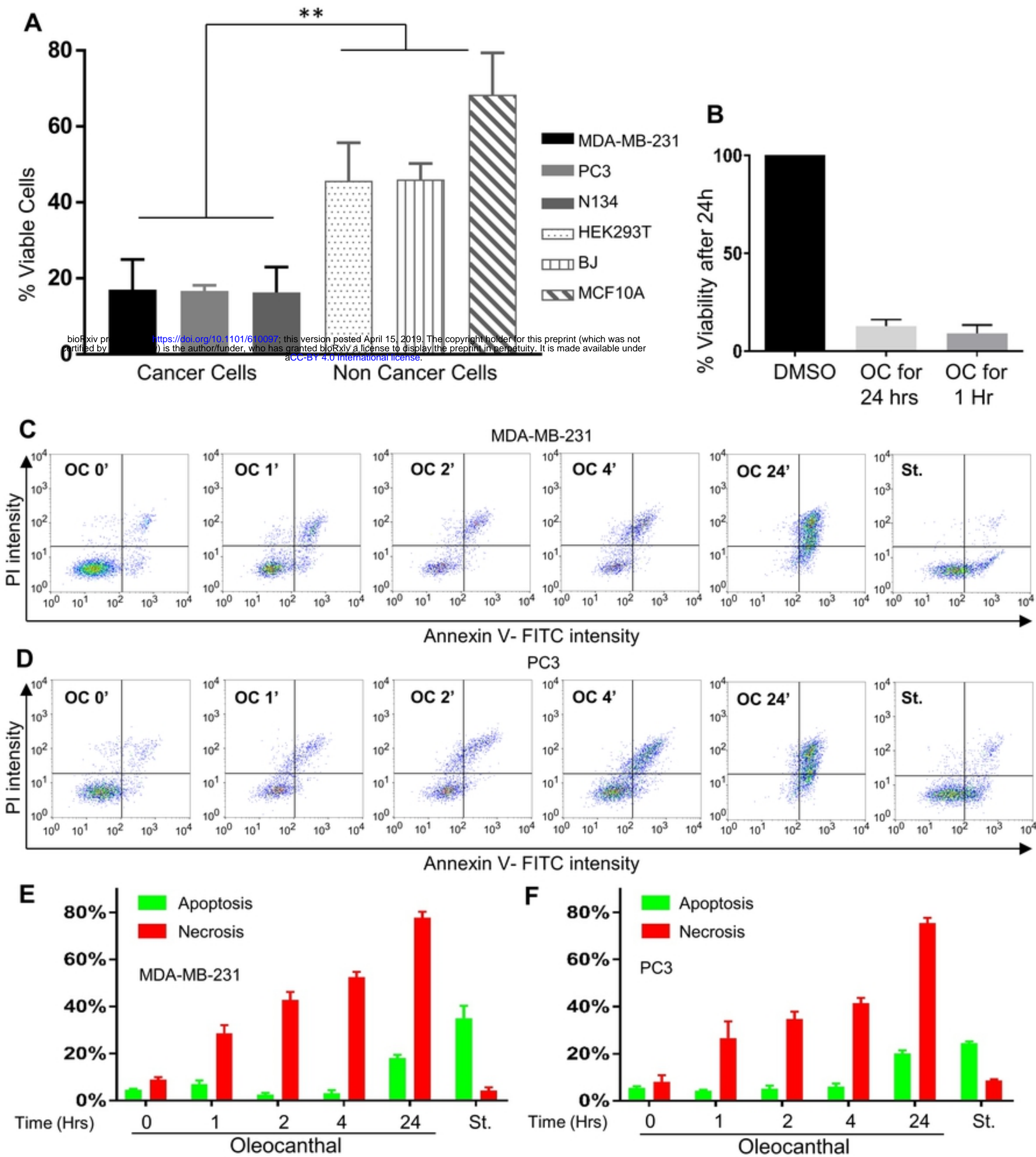


Figure 1