# Anticancer effects of n-3 EPA and DHA and their endocannabinoid derivatives on breast cancer cell growth and invasion.

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## Abstract

The anticancer effects of the omega-3 long chain polyunsaturated fatty acids (LCPUFA), EPA and DHA may be due, at least in part, to conversion to their respective endocannabinoid derivatives, eicosapentaenoyl-ethanolamine (EPEA) and docosahexaenoyl-ethanolamine (DHEA). Here, the effects of EPEA and DHEA and their parent compounds, EPA and DHA, on breast cancer (BC) cell function was examined. EPEA and DHEA exhibited greater anti-cancer effects than EPA and DHA in two BC cells (MCF-7 and MDA-MB-231) whilst displaying no effect in non-malignant breast cells (MCF-10a). Both BC lines expressed $CB_{1/2}$ receptors that were responsible, at least partly, for the observed anti-proliferative effects of the omega-3 endocannabinoids as determined by receptor antagonism studies. Additionally, major signalling mechanisms elicited by these CB ligands included altered phosphorylation of p38-MAPK, JNK, and ERK proteins. Both LCPUFAs and their endocannabinoids attenuated the expression of signal proteins in BC cells, albeit to different extents depending on cell type and lipid effectors. These signal proteins are implicated in apoptosis and attenuation of BC cell migration and invasiveness. Furthermore, only DHA reduced *in vitro* MDA-MB-231 migration whereas both LCPUFAs and their endocannabinoids significantly inhibited invasiveness. This finding was consistent with reduced integrin β3 expression observed with all treatments and reduced MMP-1 and VEGF with DHA treatment. Attenuation of cell viability, migration and invasion of malignant cells indicates a potential adjunct nutritional therapeutic use of these LCPUFAs and/or their endocannabinoids in treatment of breast cancer.



**Keywords:**

*Omega (N)-3 Fatty Acids; N-acylethanolamides (NAEs); Endocannabinoids; Breast Cancer; Cannabinoid Receptors (CBRs); MAP kinase signalling; cell proliferation.*

48 **Introduction**

49 We previously showed that n-3-long-chain polyunsaturated fatty acids (n-3 LCPUFA) can
50 enhance apoptosis in breast cancer cells (BC) and that docosahexaenoic acid (DHA; 22:6n-
51 3) can improve the efficacy of chemotherapy drugs commonly used in breast and prostate
52 cancer treatment [1,2]. Suggestions that n-6 LCPUFA, that predominate in Western diets,
53 can elicit pro-cancer effects, as observed in both cell studies and in animal models of cancer
54 [3], instead of the anticancer effects attributed to n-3 LCPUFA, are still controversial. For
55 example, the ethanolamide of n-6 arachidonic acid (C20:4 n-6), arachidonoylethanolamide
56 (anandamide), is an endocannabinoid that activates cannabinoid $CB_1$ and $CB_2$ receptors,
57 both when administered exogenously and when synthesised and released endogenously [4].
58 Despite deriving from an n-6 fatty acid *in situ*, which is a putative pro-cancer fatty acid,
59 anandamide displays many anticancer and anti-proliferative properties in various types of
60 cancer cells [3,5].

61 The endocannabinoid system in general appears to play an inhibitory role in the initiation
62 and progression of certain types of cancer, for example prostate, colorectal and breast
63 cancer. $CB_1$ and $CB_2$ receptors are often over-expressed in these types of cancer
64 tissues/cells. Synthetic and/or endogenous cannabinoid receptor agonists can inhibit both
65 proliferation and invasion of cancer cells and this effect can be prevented by selective $CB_1$
66 and/or selective $CB_2$ receptor antagonist [5,6]. Inhibiting the degradation of these
67 endocannabinoids, by inhibiting fatty acid amide hydrolase (FAAH) *in vitro*, resulted in
68 their increased availability for binding to CB receptors. FAAH inhibition attenuated
69 colorectal cancer cell proliferation and prostate cancer cell invasion *in vitro* and the
70 development of pre-cancerous colonic lesions *in vivo* in animals [7]. Inhibition of cancer
71 cell migration and invasion of tissues/organs would attenuate cancer metastasis, the major
72 cause of mortality in cancer patients, and allow for longer treatment periods for the primary

73 tumour. Our previous and current findings suggest that n-3 LCPUFA and their
74 endocannabinoid derivatives could be important as an adjunct or primary treatment
75 modalities in the amelioration of cancer metastasis.

76 Dietary n-3 and n-6 LCPUFAs can be converted to ethanolamide (NAEs) and 2-
77 monoacylglyceride (2-AG) derivatives *in situ* [8,9]. Our group first showed that levels of
78 ethanolamide derivatives of n-3 LCPUFA, DHEA and EPEA, increased in breast and
79 prostate cancer cells treated with the respective precursor LCPUFAs, EPA and DHA [9-11].
80 We were also first to demonstrate that these n-3 ethanolamides are true endocannabinoids
81 that bind the cannabinoid receptors $CB_1$ and $CB_2$ and elicit anticancer/anti-proliferative/
82 pro-apoptotic effects in prostate cancer cells, regardless of the hormone receptor status
83 (positive or negative) of the cells. This included demonstrating their anti-proliferative
84 effects on the cell cycle and induction of apoptosis by flow cytometry [10].

85 We previously hypothesised that the anti-proliferative, anticancer effects of the EPA and
86 DHA n-3 LCPUFA *in vitro* and *in vivo* may be due, at least in part, to conversion to their
87 respective CB receptor binding endocannabinoid derivatives [10]. CB receptor activation by
88 specific ligands results in activation of the MAP kinase pathway through regulation of
89 p38MAPK, JNK, and ERK phosphorylation. This was observed for cannabinoids, synthetic
90 cannabinoids and the n-6 endocannabinoid, anandamide. The MAP kinases are
91 serine/threonine kinases involved in control of cell differentiation, growth and proliferation,
92 cell death and responses to cellular stress [12]. Activation of the MAPK pathway has been
93 observed after treatment of prostate, liver, malignant pancreatic and leukemia cells with Δ9-
94 THC (Δ9 tetrahydrocannabinol), cannabidiol and WIN 55,212-2 (a synthetic CB agonist)
95 [13-16]. Activation appears to be both CB receptor dependent and independent, as shown by
96 studies with CB receptor antagonists [15]. This work demonstrated that activation of
97 JNK/p38MAPK in liver cancer cells by WIN 55,212-2 was associated with up-regulation of

98 pro-apoptotic factors [15]. Conversely, it was demonstrated that in Δ9-THC-treated
99 leukemia cells, inhibition, but not activation of the $ERK_{1/2}$ signalling pathway was
100 independent of p38MAPK, or JNK [14]. In rat glioma C6 cells, WIN 55,212-2 treatment
101 reduced tumour growth and induced apoptosis, with a corresponding down-regulation of
102 $ERK_{1/2}$ signalling [17]. It is not known if these pathways are also regulated by n-3
103 ethanolamides in BC cells.
104 To clarify this question, we determined the role of n-3 LCPUFA and their endocannabinoids
105 in eliciting anti-proliferative, anti-migratory and anti-invasive effects in non-malignant
106 breast cells and in hormone-sensitive and insensitive BC cells. We also investigated their
107 effects on major underlying MAPK signalling regulation and integrin expression levels that
108 might help explain their potentially anticancer effects on BC cells *in vitro*.
109

110 **Materials and Methods**

111 *Cell lines*

112 Human BC cell line MCF-7 was purchased from the European Collection of Cell Cultures
113 (Health Protection Agency, Salisbury, UK) and MDA-MB-231 and MCF-10a cell lines
114 were obtained from American Type Culture Collection and LGC Standards (Middlesex,
115 UK), respectively. Cells were purchased fresh from these facilities specifically for this study
116 in order to ensure their fidelity and kept at passage numbers less than 30. MCF-7 and MDA
117 cell lines were cultured in RPMI 1640 medium (Lonza, Basel, Switzerland) containing 10%
118 (v:v) foetal bovine serum and 1% (v:v) Penicillin-Streptomycin (10,000 units/ml penicillin,
119 10 mg/ml streptomycin, Sigma-Aldrich, Dorset, UK). MCF-10a cells were cultured in 50:50
120 DMEM/Hams F12 with 5% horse serum (both Thermofisher Scientific, Loughborough,
121 UK), 20ng/ml human Epidermal Growth Factor (hEGF) 100ng/ml cholera toxin and

122 500ng/ml hydrocortisone (all from Sigma Aldrich). Cells were grown under standard
123 conditions of 5% $CO_2$ at 37ºC in a humidified incubator.

124 *Biochemical reagents*

125 Docosahexaenoic acid (DHA; 22:6 n-3, >99% purity), and eicosapentaenoic acid (EPA;
126 20:5 n-3 >98% purity) were purchased from Sigma-Aldrich (Dorset, UK). Eicosapentaenoyl
127 ethanolamide (EPEA, ≥98% purity) and docosahexaenoyl ethanolamide (DHEA, ≥98%
128 purity) were obtained from Cayman Chemicals (distributed by Cambridge Bioscience Ltd,
129 Cambridge, UK). Fatty acids and ethanolamides were dissolved in 100% ethanol, and stored
130 at 100mM stock solutions, -80°C under nitrogen and diluted to appropriate concentrations in
131 media when required.

132 *Receptor antagonists*

133 Selective antagonists of $CB_1$ (AM281) and $CB_2$ (AM630) were used to block activity of CB
134 receptors. AM281 and AM630 were obtained from Insight Biotechnology UK and stored at
135 50mM and 3mM and in DMSO and ethanol, respectively. Each antagonist was used at a
136 final concentration of 1µM, as described previously [10]. Cells were treated with each
137 antagonist only at this concentration which showed that there was no effect on cell viability
138 in either of the breast cancer cell lines under these conditions (data not shown).

139 *Cell viability*

140 A standard MTT dye reduction assay was used to assess the cytotoxicity of the respective
141 compounds in the BC cells. Briefly, cells were plated in 96 well plates at seeding densities
142 of $5x10^4$ cells/ml. Cells were treated the following day with appropriate agents for 24 and 48
143 hours. MTT solution (5mg/ml in PBS) was then added and incubated for 4 hours. The
144 media/MTT were removed and replaced with 200µl DMSO to dissolve the MTT formazan
145 crystals. Plates were immediately read at 570nM in a multiwell plate reader (DynaTech
146 MR5000, Dynex Laboratories, Worthing, UK). Each experiment contained 6 replicates and

147 was repeated at least three times. Cytotoxicity was expressed as mean percentage increase
148 relative to unexposed control ± SD. Control values were set at 0% cytotoxicity. Cytotoxicity
149 data (where appropriate) were fitted to a sigmoidal curve and a logistic regression model
150 was used to calculate $IC_{50}$, which is the concentration of fatty acid/cannabinoid causing 50%
151 inhibition compared to untreated controls. Mean $IC_{50}$ is the concentration of agent which
152 reduces cell growth by 50% under the experimental conditions and is the average of at least
153 three independent reproducible statistically significant measurements. The $IC_{50}$ values were
154 reported at ± 95% confidence intervals (± 95% CI). This analysis was performed using
155 GraphPad Prism (San Diego, USA)

156 *Protein extraction*

157 Cells were homogenised in lysis buffer (20mM Tris, 0.25M sucrose, 10mM EGTA, 2mM
158 EDTA, 1mM sodium orthovanadate, 25mM sodium β-glycerophosphate, 50mM sodium
159 fluoride, 0.1% (v:v) Protease Inhibitor Cocktail (Sigma, Dorset, UK), pH 7.5) and sonicated
160 for 10 seconds at frequency of 10kHz. Concentrations for each sample were determined by
161 the Biorad DC Protein Assay (Biorad, Hertfordshire, UK), according to manufacturer's
162 instructions.

163 *Migration and invasion determinations*

164 The less aggressive MCF-7 cells were regarded as too non-invasive, therefore only MDA-
165 MB-231 cell migration and invasion was determined using a commercially available
166 migration and invasion assay (CytoSelect$^{TM}$, Cell Biolabs Inc, USA). Cell migration was
167 determined using medium containing serum (chemo-attractant) in the bottom of the chamber
168 while the upper chamber contained re-suspended cells in serum-free medium. Cells were
169 treated with n-3 LCPUFAs or their ethanolamides for 24h prior to the assessment of
170 migration through the polycarbonate membrane (pore size 8uM) inserted into the wells
171 between upper and lower chamber. Cells that were able to cross the polycarbonate membrane

172 towards chemo-attractants were stained and quantified using a plate reader at 560nm.
173 Concentrations were used such that physiological effects could be observed (based on
174 previous experiments), but limited cell death was induced (maximum of 10-15% cell death).
175 This would ensure effects seen were from anti-invasion/migration causes, rather than simply
176 reduced cell numbers. Invasiveness of the MDA-MB-231 cells was determined in a similar
177 manner to migration except that the polycarbonate membrane was coated with a synthetic
178 basement membrane matrix which the cells had to degrade in order to move (invade) into the
179 lower chamber.

180 *Western blot analysis*

181 Cells were treated for 24h with the $IC_{50}$ concentration of each compound. A total of 25-30µg
182 of protein was electrophoresed through precast 4-20% polyacrylamide gels (Invitrogen,
183 Paisley, UK) for 1-2h and the separated proteins transferred to nitrocellulose membranes
184 (Biorad, Hertfordshire, UK), then blocked with 5% (w:v) skimmed milk in tris-buffered
185 saline (TBS) with 0.1% (v:v) Tween 20 (TBST solution) at room temperature and incubated
186 individually , with either 1:100 dilution of anti-FAAH antibody, 1:100 $CB_1$, 1:200 $CB_2$, 1:200
187 ERK, 1:200 p-ERK, 1:200 JNK, 1:200 p-JNK, (Insight Biotechnology, UK), 1:200 p38 and
188 pp38 (Abcam, UK), 1:100 Integrin β3 and VEGF (Santa Cruz, USA), 1:100 MMP-1 (Abcam,
189 UK) at 4°C overnight. β-actin (1:20,000) was used as an internal loading control to normalize
190 between lanes during densitometry (Abcam, UK). Appropriate secondary antibodies (Insight
191 Biotechnology, UK) anti-mouse, rabbit (1:5000), or goat (1:10,000) were incubated at room
192 temperature for 1h. Proteins were visualized using ECL+plus™ chemiluminescent detection
193 kit (Amersham Pharmacia, Buckinghamshire, UK), according to manufacturer's instructions
194 and a Fluor S phosphorimager (Biorad, Hertfordshire, UK). Experiments were repeated with
195 proteins isolated from at least three independent extractions.

196 *Data analysis*

197 Unpaired Students t-test was used to compare means between two treatments. Levene's test
198 was used to assess equality of variance. One-way ANOVA with post-hoc analysis
199 (Bonferonni correction or Dunnett's test where appropriate) was also used. A value of p
200 $\leq$0.05 was taken as being significant. SPSS Statistics, version 20 (IBM) was used for
201 analysis.

202

203 **Results**

204 *N-3 fatty acids and their ethanolamides do not affect cell viability of non-cancer breast cells*
205 *(MCF-10a)*

206 It is important to determine that any anti-proliferative/anticancer effects of n-3 LCPUFA
207 and their ethanolamides are without significant effect in non-cancer, breast cell lines in
208 order for these compounds to be considered in possible anti-tumour therapies. Results
209 clearly demonstrated that none of the lipid treatments had any significant effect on the
210 growth of MCF-10a cells, either after 24 or 48h treatment, even at concentrations in excess
211 of those believed to be physiologically achievable (Figure 1).

212 *Anti-proliferative, anti-growth effects in breast cancer cell lines*

213 EPEA was significantly more effective than EPA in inhibiting cell growth in both MDA-
214 MB-231 ($p \leq 0.01$) and MCF-7 ($p \leq 0.01$) cells after 24h incubation. Similarly, DHEA was
215 more effective than DHA in both cell types ($p \leq 0.001$) after 24h treatment (Figure 2, A-B).
216 Overall, DHA was more effective than EPA in both cell types whilst the anti-proliferative
217 efficacy of EPEA was greater than that of DHEA, which differed from that observed with
218 the respective parent fatty acids (i.e. DHA had a lower $IC_{50}$ than EPA).

219 After 48h treatment, EPEA was again more effective than EPA in reducing cell viability in
220 both MDA-MB-231 ($p \leq 0.01$) and MCF7 ($p \leq 0.001$) cells. However, there was no significant
221 difference between DHA and DHEA in either cell type after the longer treatment period

222 (Figure 2, C-D) irrespective of differences in detectable FAAH protein levels between the
223 cell types (see Figure 3). In addition, the effect of DHEA on cell viability compared to DHA
224 was no longer evident after 48h compared with 24h treatment suggesting that degradation of
225 DHEA, but not surprisingly of EPEA, had occurred. Subsequently, all further experiments
226 were performed after 24h treatment.

227 *Breast cancer cells differentially express $CB_1$ and $CB_2$ receptors and FAAH*

228 Previously reported studies suggested MCF-7 and MDA-MB-231 cells either do or do not
229 express $CB_1$ and $CB_2$ [18]. These discrepancies are possibly due to differences in the cell
230 lines used, natural mutations of high passage number cells, or the use of different antibodies
231 from different companies. We observed that both cell lines clearly expressed $CB_1$ with
232 different proportions of glycosylated and non-glycosylated forms present between cell lines;
233 MCF-7 cells, but not MDA-MB-321 cells, showed higher levels of the glycosylated form of
234 $CB_1$ (Figure 3A and B). $CB_2$ was also expressed in both cell lines, with MCF-7 cells
235 expressing this receptor at a significantly higher level (~3-fold) than MDA-MB-321 cells
236 (Figure 3C, p=0.034). Glycosylated forms of $CB_2$ were not detected in either cell line. This
237 contrasted with our previous observations in prostate cancer cells where both hormone-
238 positive and negative cell lines demonstrated glycosylated forms of $CB_2$, but not of $CB_1$ [10].
239 FAAH protein was highly expressed in MCF-7 cells but was not detectable, at least by
240 Western blotting, in MDA-MB-321 cells (Figure 3D).

241 *Differential involvement of $CB_{1/2}$ receptors in the effect of endocannabinoid on cell viability*

242 AM281 and AM630 are $CB_1$ and $CB_2$ selective antagonists, respectively. In MCF-7 cells
243 blockage of $CB_1$ had little effect on EPA or DHEA, but significantly decreased the
244 effectiveness of DHA (p<0.001) and EPEA (p<0.05) on cell viability (Figure 4A). Blocking
245 the $CB_2$ receptor significantly decreased the efficacy of EPA and EPEA (both p<0.01), had
246 no effect on DHA, but significantly increased the efficacy of DHEA (p<0.01) on cell

247 viability. Antagonism of both receptors at the same time significantly decreased the action
248 of EPA ($p<0.05$), EPEA ($p<0.001$), DHA ($p<0.01$) and DHEA ($p<0.001$); under these
249 conditions, DHEA was ineffective even at 400uM (far beyond physiologically achievable
250 levels).

251 In MDA cells, blocking $CB_1$ significantly decreased the efficacy of EPEA ($p<0.05$), DHA
252 ($p<0.01$) and DHEA (($p<0.001$), completely inhibited), but EPA was without effect on cell
253 viability (Figure 4B). Blocking $CB_2$ significantly decreased the effectiveness of EPA and
254 EPEA ($p<0.05$, $p<0.001$ respectively), but increased the efficacy of DHA and DHEA
255 ($p<0.01$, $p<0.05$ respectively). When both receptors were blocked with antagonists, the
256 effectiveness of EPA and EPEA was significantly decreased ($p<0.001$ and $p<0.01$
257 respectively), but the efficacy of DHA and DHEA was again significantly increased (both
258 $p<0.01$). These effects were similar to those observed with $CB_2$ inhibition alone.

259 *Effect of n-3 LCPUFA/n-3 ethanolamides in modulating MAPK signalling pathways in*
260 *breast cancer cells*

261 In MCF-7 BC cells (Figure 5A), p38 MAPK protein levels were reduced by all treatments
262 i.e. EPA, EPEA, DHA and DHEA ($p<0.01$, $p<0.01$, $P<0.01$ and $p<0.001$ respectively).
263 Phosphorylated p38 MAPK was also reduced by all the treatments, although this was only
264 significant for EPEA, DHA and DHEA (all $p<0.01$). ERK expression was generally
265 unaffected by n-3 LCPUFA and their ethanolamides, apart from the decrease elicited by
266 DHEA treatment ($p<0.05$). Phosphorylated ERK was significantly decreased by both DHA
267 and DHEA ($p<0.05$, $p<0.01$ respectively). There was no significant effect on JNK
268 expression with any of the treatments, except for a small reduction with EPEA treatment
269 ($p<0.05$). However, there were significant reductions in the expression of phosphorylated
270 pJNK with EPEA, DHA and DHEA treatments ($P<0.05$, $p<0.05$ and $p<0.01$ respectively)
271 but this reduction was non-significant for EPA treatment.

272 Similar general trends to the above were seen in MDA-MB-231 cells, with the exception of
273 p38 MAPK expression, which was slightly increased by EPA treatment ($p=0.055$) and was
274 not affected by EPEA treatment (Figure 5B). DHA and DHEA treatment, however,
275 significantly reduced p38 MAPK expression (both $p<0.01$). Phosphorylated p38 MAPK
276 was significantly reduced by EPA and DHEA ($p<0.01$ respectively) but not by EPEA or
277 DHA. ERK expression was reduced significantly only by DHA ($p<0.05$) and non-
278 significantly by EPA and EPEA but was not changed by DHEA. Phosphorylated ERK was
279 reduced by all treatments, but significantly by EPA, EPEA and DHEA ($p<0.05$, $p<0.01$ and
280 $p<0.001$ respectively), with the reduction by DHA approaching significance ($p=0.051$). JNK
281 expression was not significantly changed by any treatment, whereas DHA and DHEA
282 significantly reduced phosphorylated JNK ($p<0.05$).

283 *Attenuation of migration and invasion of MDA-MB-231 cells in vitro*

284 The changes elicited by the n-3 LCPUFA and their ethanolamides on signal protein
285 expression in the MDA-MB-231 cells, particularly reduced p38 expression, suggested that
286 the migration and invasion potential of these cells may be modulated. MCF-7 cells are non-
287 invasive and consequently were not studied. Employing a commercial migration assay kit
288 we demonstrated that only DHA was capable of severely attenuating the migration of these
289 cells ($p<0.001$) as other treatments were without effect (Figure 6B). By contrast, all the
290 treatments markedly reduced the invasiveness of these cells by around 60% ($p<0.01$ and
291 $p<0.001$) (Figure 6A).

292 *Reduction in expression of migration- and invasion-related proteins*

293 Increased expression of integrins, matrix metalloproteinases (MMPs) and vascular
294 endothelial growth factor (VEGF) proteins are implicated in augmentation of migration and
295 invasion of malignant cells into surrounding tissues. Western blot analyses of the levels of
296 these proteins expressed in MDA-MD-231 cells after treatment with the n-3 LCPUFA or

297 their ethanolamides, showed a significant reduction in integrin β3 expression elicited by all
298 treatments ($p<0.01$) (Figure 6C). Interestingly, only DHA elicited a reduction in MMP-1
299 ($p<0.05$) and VEGF ($p<0.01$) protein expression in the cells.

300

301 **Discussion**

302 In the current study we have demonstrated that EPEA and DHEA had greater anti-
303 proliferative effects than their precursor fatty acids in two breast cancer cell lines *in vitro*
304 (MCF-7 and MDA-MB-231). Importantly, these endocannabinoids did not affect growth of
305 non-malignant MCF-10a cells, even at high, non-physiological concentrations. Rovito et al,
306 have also reported that EPEA and DHEA reduced the viability of MCF-7 BC cells [19], an
307 effect that mirrored our earlier findings with prostate cancer cells [10] and supported the
308 current findings with MCF-7 cells. However, Rovito *et al.,* did not investigate
309 endocannabinoid effects on the viability of the aggressively invasive MBA-MD-231 cell
310 line, as shown here, nor did they determine the important effects of these endocannabinoids
311 or their parent fatty acids on migration and invasiveness of MDA-MB-231 cells. Our
312 observation and that of Rovito et al, [19] that n-3 endocannabinoids did not influence cell
313 growth of non-malignant MCF10a cells suggested a specificity towards cancer cells alone;
314 an important consideration if these treatments *in vitro* are to be translated to *in vivo* clinical
315 nutritional therapies. More recently, Gaston *et al.* [20], have also described the effects of
316 adding EPA, and AA to MCF-7 BC cells and T98G human glioblastoma cells. Their results
317 agreed with our previous study showing that EPA supplementation reduced cell growth of
318 MCF-7 cells, however, they did not investigate effects of EPEA or DHA/DHEA. They also
319 showed that AA supplementation did not significantly alter AEA levels in the MCF-7 cells,
320 agreeing with our previous studies [11] but they did not assess effects on invasion or other
321 signalling pathways. The expression of glycosylated and non-glycosylated CB receptors in

322 the BC cells (Figure 3) contrasted with our previous observations in prostate cancer cells
323 where glycosylated $CB_2$, but not glycosylated $CB_1$, was detected in both hormone sensitive
324 and hormone insensitive prostate cell lines [10]. The physiological significance of these
325 differences in glycosylated $CB_{1/2}$ receptor distribution and the role of the glycosylated forms
326 in BC of the receptors *per se* is not clear at present and warrants further study.

327 The $IC_{50}$ concentrations of endocannabinoids required to inhibit BC cell proliferation in this
328 study appear somewhat higher than levels of other endocannabinoids which have been
329 measured *in vivo*, in serum [21]. One study by Rovito et al [19] in BC cells suggested that
330 the potency of DHAE and EPEA can be increased by around an order of magnitude with
331 increasing incubation time (from 24h to 96h), suggesting that length of treatment may be
332 important factor. However, in the present study, whilst it was observed that the potency of
333 EPEA was increased at 48h, we did not observe any change with DHEA. The physiological
334 concentration of endocannabinoids at the site of formation is likely extremely difficult to
335 determine precisely. Furthermore, since DHEA and EPEA are synthesised from DHA and
336 EPA from the diet, the *in vivo* levels of the omega3-ethanolamides (DHAE and EPEA) will
337 likely depend on the levels of omega3 PUFA in an individuals' diet. This area concerning
338 the conversion of PUFAs to endocannabinoids and how these relate to circulatory levels of
339 these compounds certainly requires further investigation.

340 FAAH is the major enzyme for catabolizing, and therefore regulating, the concentration of
341 endocannabinoids *in situ*. Our detection of FAAH in MCF-7 (hormone sensitive), but not in
342 MDA-MB-231 (hormone insensitive) cells, agrees with our findings in prostate cells with
343 similar hormone sensitivity status [10]. This seems to be different in colon cancer cells as a
344 study by Wasilewski *et al.* [22] showed that inhibiting FAAH with a selective inhibitor (PF-
345 3845) decreased the viability, migration and invasiveness of the Colo-205 cell line. In
346 contrast, the similar efficacies of endocannabinoids in inhibiting proliferation of both BC

347 cell types observed in our study, irrespective of their high or low FAAH expression,
348 suggested the enzyme was perhaps unimportant in this process or that sufficient
349 endocannabinoid was always present in our *in vitro* system irrespective of FAAH activity.
350 Clearly further work in this area is required.

351 Activation of $CB_1$ and $CB_2$ receptors by endocannabinoids in BC cells in this study
352 displayed both similarities and differences from that previously found in prostate cancer
353 cells [10]. If a derivative is functioning as a ligand of $CB_1$ or $CB_2$, then inhibiting the
354 receptors with potent antagonists should decrease its potency in binding to the receptor.
355 This has been observed for the effects of anandamide (AEA) on cell death in other cell lines
356 [23-25]. However, Kuc *et al.,* [26] observed that $CB_1$, $CB_2$ and TRPV-1 receptor blockade
357 in JWF2 keratinocytes, which over-express COX-2, did not inhibit AEA-mediated cell
358 death, suggesting these receptors were not involved in the apoptotic signalling in the JWF2
359 cells. The latter study however, only looked at one cell line, and an n-6 ethanolamide,
360 whereas our previous study in prostate cancer cells with n-3 endocannabinoids showed that
361 blocking $CB_1$ or $CB_2$ with antagonists did indeed attenuate their anti-proliferative effects
362 [10]. This study suggested the effect was complex and possibly dependent on cell type,
363 relative levels of CB receptors, type of endocannabinoid ligand used and the integrity of the
364 important molecular signalling pathways present. In further contrast to the work of Kuc et
365 al., blocking $CB_1$ receptor in the present study significantly decreased the efficacy of DHA
366 and EPEA in MCF-7 cells and also of EPEA, DHA and DHEA in MDA-MB-231 cells,
367 suggesting that these compounds do exert at least some of their antiproliferative, anticancer
368 effects through the $CB_1$ receptor. Similarly, inhibition of $CB_2$ significantly affected the
369 action of EPA and EPEA in both cell lines, again demonstrating that EPA and EPEA can
370 exert some of their effects through $CB_2$ in these cells. From these findings it appeared that
371 EPA/EPEA are better ligands of the $CB_2$ receptor and DHA/DHEA of the $CB_1$ receptor.

372 This intriguing differential effect of two major n-3 LCPUFA and their NAE derivatives
373 warrants further investigation. Similar differential effects of the two n-3 NAEs were found
374 in our previous study with prostate cell lines. Interestingly, it was found that blocking the
375 $CB_2$ receptor potentiated the activity of DHEA, lowering the $LC_{50}$ in both MCF-7 and
376 MDA-MB-231 cells. This effect was also evident in MDA-MB-231 cells with the parent
377 fatty acid, DHA, but not with EPA. These findings may not be unexpected since it has been
378 reported that anandamide-induced anti-proliferative effects in some cancer cell lines can be
379 potentiated by $CB_1$- or $CB_2$-selective antagonists [27,28]. In this case, as has been proposed
380 for anandamide, blockade of cannabinoid receptors in the presence of DHEA may increase
381 the ability of DHEA to inhibit cancer cell proliferation through one or more cannabinoid
382 receptor-independent mechanisms (reviewed in [29]). Indeed, the extent to which DHEA
383 targets non-$CB_1$ and non-$CB_2$ receptors, particularly transient receptor potential V1 cation
384 channels (TRPV1) needs to be established. These channels can be activated by both
385 anandamide and omega-3 PUFAs and Maccarrone *et al.* (26) demonstrated that cannabinoid
386 receptor activation can prevent apparent TRPV1-mediated apoptosis induced by
387 anandamide [30]. Consequently, it is conceivable that by blocking the CB cannabinoid
388 receptors there is reduced cannabinoid receptor-mediated protection of MCF-7 and MDA
389 cells, thereby increasing the ability of DHEA to induce apoptosis through receptors such as
390 TRPV1, which has been shown to be expressed in these cells [31]. Interestingly, whereas
391 DHA is a potent TRPV1 agonist, EPA inhibits the activation of this cation channel by
392 various agonists [32] and this may, at least in part, explain the lowering of the $LC_{50}$
393 observed with DHA/DHEA but not with EPA/EPEA in the BC cells. However, why this
394 effect occurs specifically with the $CB_2$ receptor antagonist and not with the $CB_1$ receptor
395 antagonist requires further investigation.

396 Many studies investigating the role of cannabinoids and n-6 endocannabinoids in inhibiting
397 cancer cell proliferation suggested involvement of the MAP kinase family of signal
398 transducers [14, 15, 33-36)]. Our findings generally demonstrated that both n-3 LCPUFA
399 and their respective n-3 endocannabinoids can also elicit changes in MAPK signalling by
400 decreasing expression of p38 MAPK and levels of activated (phosphorylated) p38 MAPK
401 (pp38) in BC cells. Generally, both non-phosphorylated and phosphorylated p38 were
402 reduced, though phosphorylated p38 more so. In the case of EPA treatment, phosphorylated
403 (activated) pp38 was significantly reduced, even though non-phosphorylated p38 levels
404 were increased. Activation of p38 MAPK by cannabinoids has been reported previously
405 [16,17,34-37] and it is usually thought this activation leads to increased apoptosis, but here
406 we show down-regulation of both total p38 MAPK and activated pp38 by n-3 LCPUFA and
407 their endocannabinoids in both BC cells, a finding that correlated with decreased cell
408 viability of these cells. It is known that p38 MAPK can play a dual role in regulating cell
409 death, either mediating cell survival or cell death through different mechanisms, including
410 apoptosis. Specific functions of p38 MAPKs in cell growth inhibition and apoptosis appear
411 to depend on the stimuli, cell type, and/or the particular enzyme isoforms present (reviewed
412 in [38]). In the current study we did not determine the latter.

413 Our observations of a reduction in ERK and pERK by some, but not all, n-3 LCPUFA and
414 n-3 endocannabinoids particularly in MDA-MB-231 cells, correlated with findings of Ellert-
415 Miklaszewska *et al.*, who demonstrated inactivation (reduced phosphorylation) of ERK
416 after treatment of glioma cells with the synthetic cannabinoid agonist WIN 55,212-2, and
417 linked this inactivation to enhanced apoptosis through activation of the pro-apoptotic Bad
418 protein [39]. In our study, DHEA in particular, significantly reduced phosphorylated ERK,
419 even though unphosphorylated ERK did not change. Other studies have also shown
420 inactivation of ERK in response to cannabinoids and $CB_2$ receptor agonists [40]. Findings of

421 reduced activation of ERK agree with the pro-apoptotic effects of inhibiting the ERK
422 pathway, as stated in the studies above, rather than the pro-apoptotic effects of activating the
423 pathway as suggested by some authors (see above). In the review by Cagnol and Chambard,
424 2009, it was suggested that different effects can arise from activation or inactivation of ERK
425 depending on ERK location, duration of activation, and the presence or absence of reactive
426 oxygen species [41].

427 Our findings of a general reduction in active pJNK, but not JNK, particularly with DHA and
428 DHEA treatment in both cell types, can be regarded as inhibitory for cell
429 proliferation/apoptosis [42,43]. However, JNK activation can also act as a "double edged
430 sword" having both pro- and anti-apoptotic effects, perhaps depending on duration of
431 activation [42,43]. $CB_1$ activation was coupled to JNK activation which supports our
432 suggestion that DHEA acts mainly through $CB_1$. Our findings contrast with observations
433 that anandamide-induced cell death required p38 and JNK activation in PC12 medulla cells
434 [37,43]. Instead, we observed anti-proliferative effects when JNK phosphorylation was
435 decreased, suggesting that pJNK was supporting a proliferative role; it could be that
436 activation/inactivation of the other MAPKs, as described above, could be the more dominant
437 factors.

438 Metastasis of tumours, where tumour cells migrate from the primary tumour and invade
439 surrounding tissues, is a major causal factor in the high mortality rates reported for various
440 cancers. Preventing the dispersal of malignant tumour cells throughout the body would
441 confer a huge clinical benefit in combating cancer *per se.* During the metastatic process
442 circulating cancer cells adhere to the extracellular matrix (ECM) of different tissues and
443 organs, facilitated by various adhesion molecules (e.g. integrins) expressed at the surface of
444 most cells and in particular on vascular endothelial cells. The ECM at the site of attachment
445 is then degraded by various zinc matrix metalloproteinases (MMPs) thereby allowing the

446 malignant cells to enter tissues/organs and initiate new tumours [44]. Activation of the
447 MAPK pathway is also involved positively in the metastatic process and our findings that n-
448 3 LCPUFA and their endocannabinoids attenuate this pathway suggested that these lipids
449 could reduce the migration and invasion of MDA-MB-231 BC cells. We clearly
450 demonstrated that both n-3 LCPUFA and their respective endocannabinoids significantly
451 reduced the invasive potential of these BC cells *in vitro*, whereas only DHA reduced their
452 migratory potential. These effects were also reflected in the significantly reduced expression
453 of an integrin adhesion molecule protein in these cells. DHA was also the only lipid to
454 attenuate both MMP-1 and VEGF in these cells. This contrasts with the findings of McCabe
455 *et al.*, who showed that DHA did not attenuate MMP-2 expression in Caki-1 cells, although
456 it reduced their invasiveness, similar to our findings [45]. Mohammadpour *et al.,* [46]
457 recently demonstrated that the selective $CB_1$ receptor agonist (arachidonyl-2´-
458 chloroethylamide) exhibited anti-invasive potential in MDA-MB-231 cells and stem cells
459 derived from them, whereas AM251 (selective CB1 antagonist) showed the opposite effects.
460 These authors did not determine effects of n-3 endocannabinoids on invasiveness of their
461 cells.
462 In summary, we have shown that both n-3 LCPUFAs and their ethanolamides can attenuate
463 cell viability of hormone-sensitive and hormone-insensitive BC cells and invasiveness of
464 insensitive MDA-MB-231 cells. Cell migration was only attenuated by DHA. These
465 changes in function were mediated, at least in part, by $CB_{1/2}$ receptors and were reflected in
466 the attenuation of p38, ERK and JNK MAPK pathways. These findings supported our
467 hypothesis that the effects of LCPUFA on BC cells are mediated, at least in part, by their
468 endocannabinoids. However, further studies are needed to determine whether similar
469 findings occur *in vivo* and to understand the exact role that omega-3 endocannabinoids play

470 in the known health benefits of omega-3 LCPUFAs including their pro-cardiovascular and
471 anticancer effects.

472

473 **Acknowledgements**

474 We would like to acknowledge NHS Grampian Endowment funds and TENOVUS Scotland
475 for funding. JL was funded by a scholarship from Fraserburgh Moonlight Prowl and AAS
476 was funded by the Scottish Government's Rural and Environment Science and Analytical
477 Services Division (RESAS). We also thank Dr Raphael Mechoulam, University of
478 Jerusalem, for the gift of the endocannabinoids.

479

480 **Declaration of interests**

481 The authors confirm that there are no known conflicts of interest associated with this
482 publication and there has been no significant financial support for this work that could have
483 influenced its outcome.

484 **Figure Legends**

485

486 **Figure 1: Cell viability of MCF-10a cells**
487 Viability of cells treated with LCPUFA and ethanolamides for (A) 24 hours or (B) 48 hours,
488 expressed as a percentage of untreated control cells. Error bars represent standard error of
489 means, n=3.
490
491 **Figure 2: $IC_{50}$ values of n-3 fatty acids and ethanolamides**
492 $IC_{50}$ values (μM) determined by MTT assay ± standard error of means in (A) MCF-7 and
493 (B) MDA-MB-231 cells treated with fatty acids (EPA and DHA) or ethanolamides (EPEA
494 and DHEA) for 24h, and (C), (D) for 48 hrs. Student's unpaired t-test was used for
495 comparisons between treated and control cells and the level of statistical significance
496 expressed as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ($n \geq 3$).
497
498 **Figure 3: Protein expression of cannabinoid receptors $CB_1$ and $CB_2$ and FAAH**
499 (A) Representative blots for $CB_1$, $CB_2$ and FAAH. (B-D) Graphical representations of (B)
500 $CB_1$ (C) $CB_2$ and (D) FAAH in MCF-7 and MDA-MB-231 breast cancer cells. Graphical
501 representations are expressed as percentage of expression compared to β-actin (marked as
502 42 kDa). Error bars represent standard error of means.
503
504 **Figure 4: Effects on cell viability of inhibiting CB receptors**
505 $IC_{50}$ values (μM) as determined by MTT assay ± SEM in (A) MCF-7 cells and (B) MDA-
506 MB-231 cells treated with fatty acids or their ethanolamides with and without CB receptor
507 antagonists for 24h *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ($n \geq 3$), '=' is where $IC_{50}$ could not be
508 determined.
509
510 **Figure 5: MAP kinase pathway expression**
511 Protein expression (as a ratio of protein to actin) expressed as a percentage of untreated
512 control in (A) MCF-7 and (B) MDA-MB-231 breast cancer cells. Representative blots
513 including β-actin loading control. Bars indicate blots linked to β-actin expression from the
514 same blot. Student's unpaired t-test was used for comparisons between treated and control
515 cells and the level of statistical significance expressed as *$p \leq 0.05$, **$p \leq 0.01$,
516
517 **Figure 6: Effects of n-3 fatty acids and ethanolamides on invasion/migration**
518 Effect of 24h treatment on MDA-MB-231 cells (expressed as a percentage of untreated
519 cells) on (A) Invasion and (B) Migration. (C) Protein expression of integrin β3, MMP-1 and
520 VEGF (as a ratio of protein to β-actin) expressed as percentage of untreated MDA-MB-231
521 cells. One-way ANOVA and Dunnett's post hoc tests were used for comparisons between
522 treated and control cells and the level of statistical significance was expressed as *$p \leq 0.05$,
523 **$p \leq 0.01$, ***$p \leq 0.001$, ($n \geq 3$)
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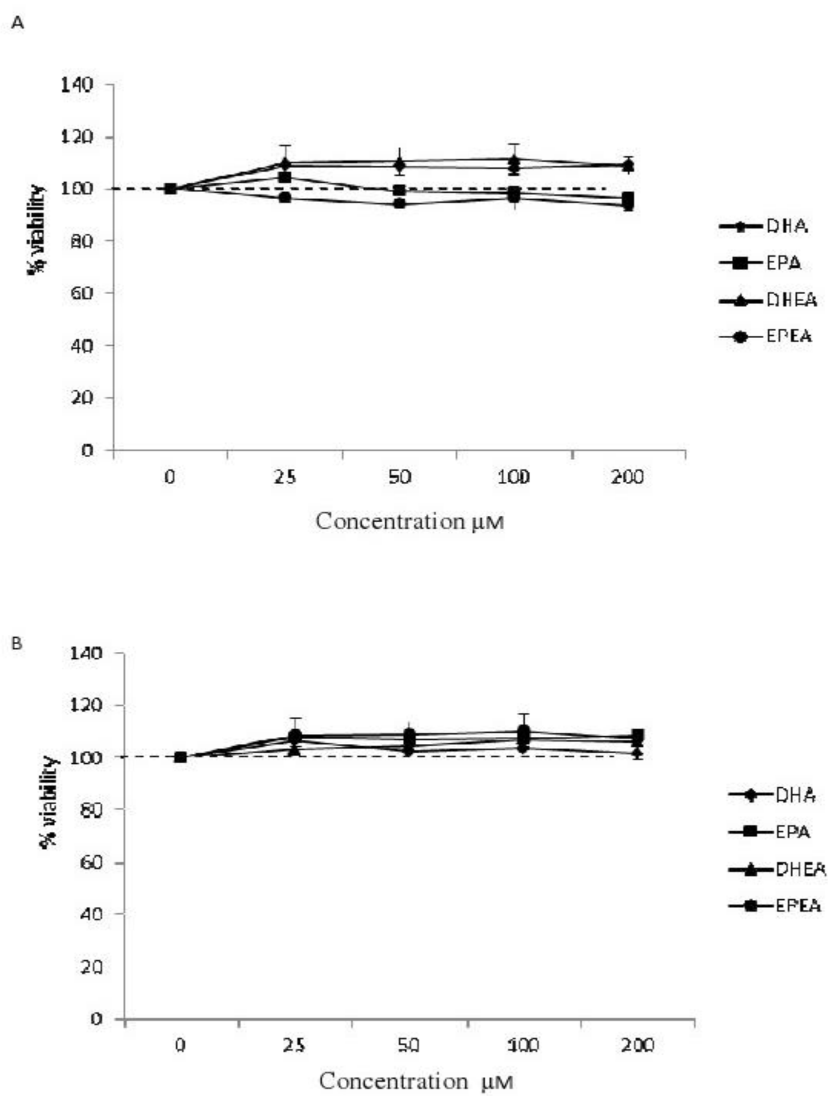


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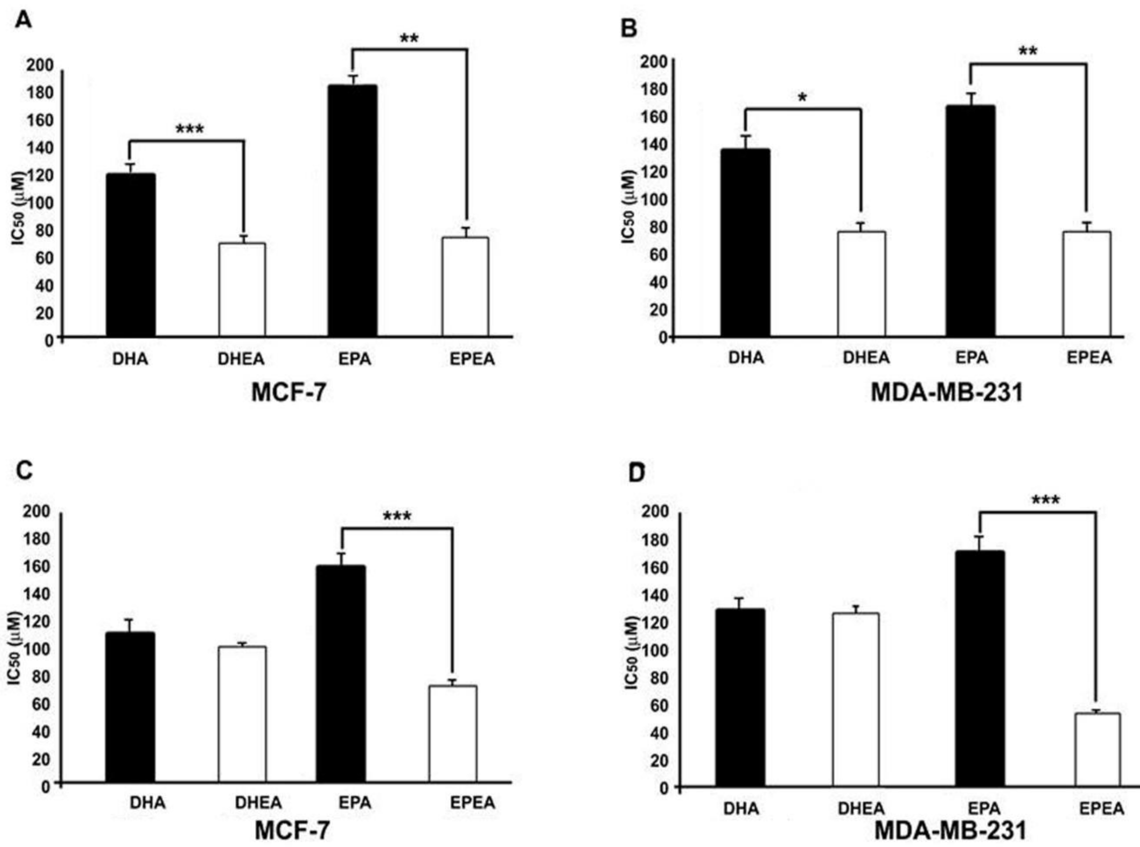


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698 **Figure 3**

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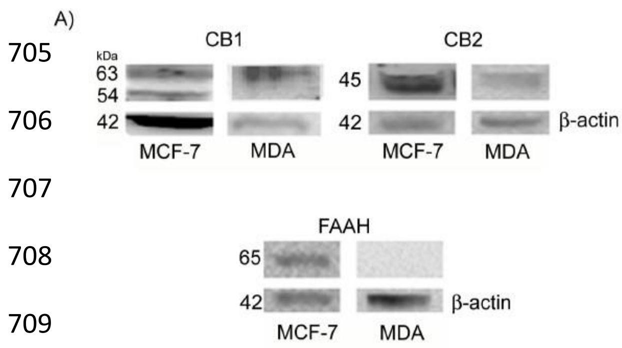


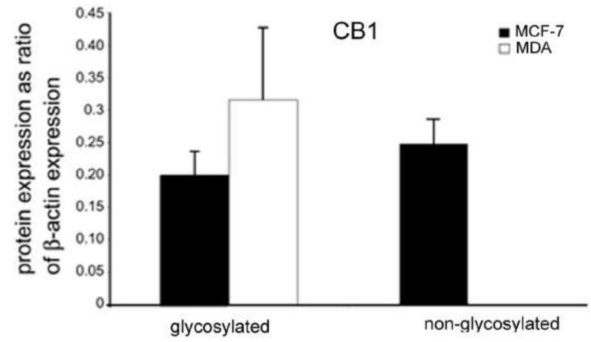


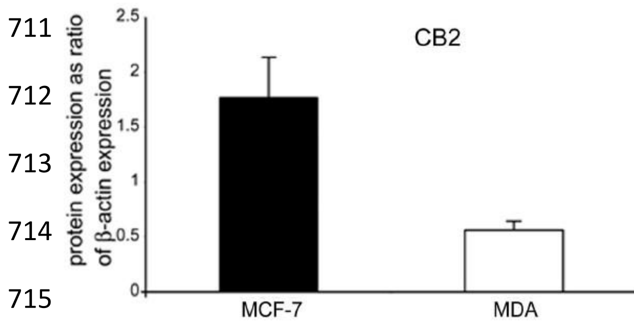


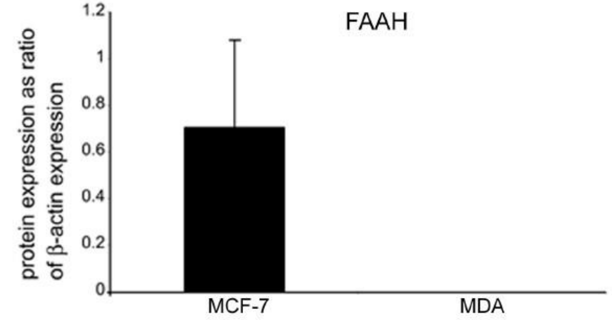


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**Figure 4**

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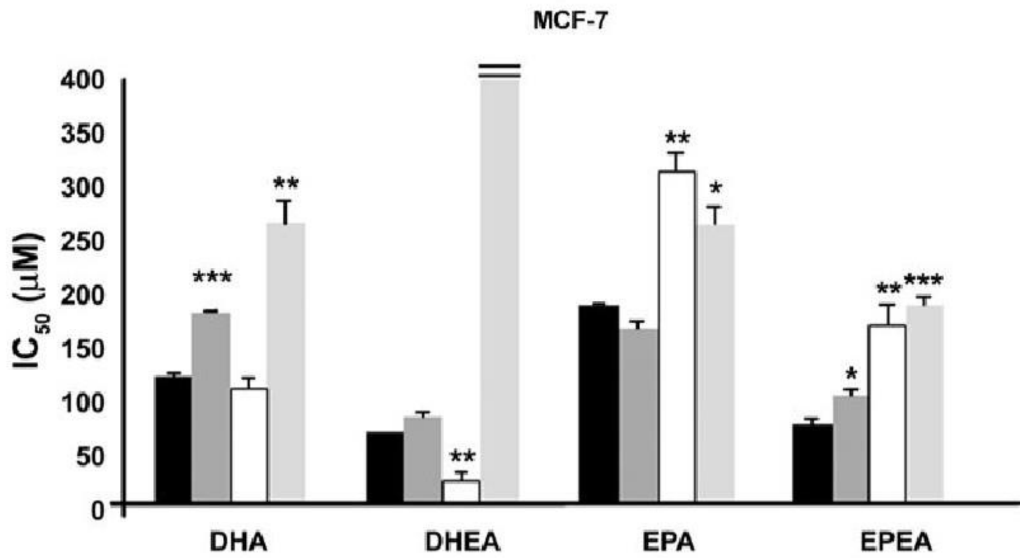


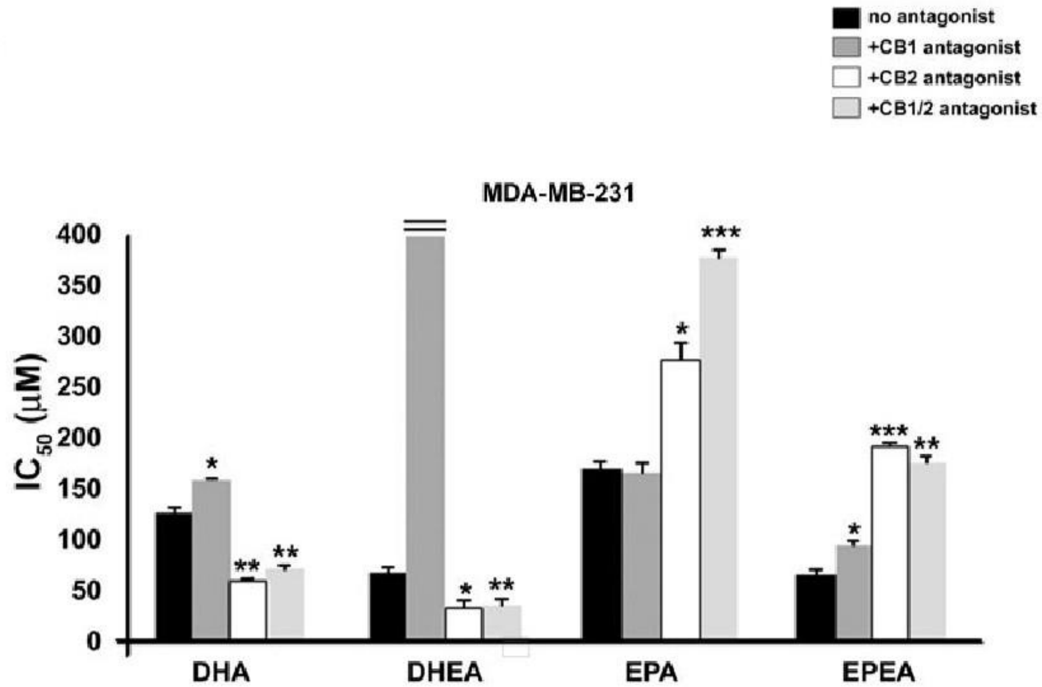

752 **Figure 5**

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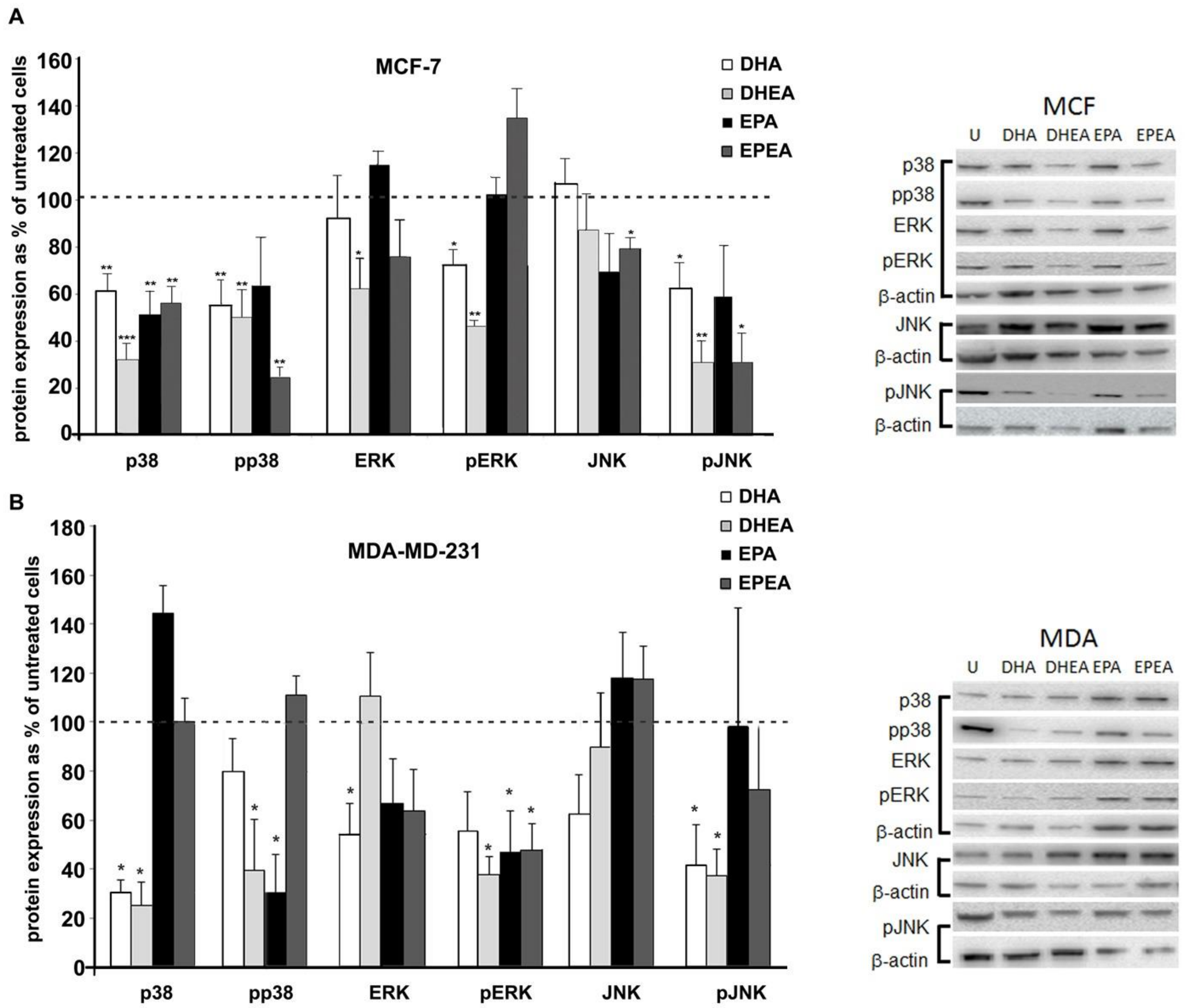


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764 **Figure 6**