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 β 3 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.

Introduction

We previously showed that n-3-long-chain polyunsaturated fatty acids (n-3 LCPUFA) can enhance apoptosis in breast cancer cells (BC) and that docosahexaenoic acid (DHA; 22:6n-3) can improve the efficacy of chemotherapy drugs commonly used in breast and prostate cancer treatment [1,2]. Suggestions that n-6 LCPUFA, that predominate in Western diets, can elicit pro-cancer effects, as observed in both cell studies and in animal models of cancer [3], instead of the anticancer effects attributed to n-3 LCPUFA, are still controversial. For example, the ethanolamide of n-6 arachidonic acid (C20:4 n-6), arachidonylethanolamide (anandamide), is an endocannabinoid that activates cannabinoid CB₁ and CB₂ receptors, both when administered exogenously and when synthesised and released endogenously [4]. Despite deriving from an n-6 fatty acid *in situ*, which is a putative pro-cancer fatty acid, anandamide displays many anticancer and anti-proliferative properties in various types of cancer cells [3,5].

The endocannabinoid system in general appears to play an inhibitory role in the initiation and progression of certain types of cancer, for example prostate, colorectal and breast cancer. CB₁ and CB₂ receptors are often over-expressed in these types of cancer tissues/cells. Synthetic and/or endogenous cannabinoid receptor agonists can inhibit both proliferation and invasion of cancer cells and this effect can be prevented by selective CB₁ and/or selective CB₂ receptor antagonist [5,6]. Inhibiting the degradation of these endocannabinoids, by inhibiting fatty acid amide hydrolase (FAAH) *in vitro*, resulted in their increased availability for binding to CB receptors. FAAH inhibition attenuated colorectal cancer cell proliferation and prostate cancer cell invasion *in vitro* and the development of pre-cancerous colonic lesions *in vivo* in animals [7]. Inhibition of cancer cell migration and invasion of tissues/organs would attenuate cancer metastasis, the major 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 CB₁ and CB₂ 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

pro-apoptotic factors [15]. Conversely, it was demonstrated that in Δ^9 -THC-treated leukemia cells, inhibition, but not activation of the ERK_{1/2} signalling pathway was independent of p38MAPK, or JNK [14]. In rat glioma C6 cells, WIN 55,212-2 treatment reduced tumour growth and induced apoptosis, with a corresponding down-regulation of ERK_{1/2} signalling [17]. It is not known if these pathways are also regulated by n-3 ethanolamides in BC cells.

To clarify this question, we determined the role of n-3 LCPUFA and their endocannabinoids in eliciting anti-proliferative, anti-migratory and anti-invasive effects in non-malignant breast cells and in hormone-sensitive and insensitive BC cells. We also investigated their effects on major underlying MAPK signalling regulation and integrin expression levels that might help explain their potentially anticancer effects on BC cells *in vitro*.

Materials and Methods

Cell lines

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

500ng/ml hydrocortisone (all from Sigma Aldrich). Cells were grown under standard conditions of 5% CO₂ at 37°C in a humidified incubator.

Biochemical reagents

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

Receptor antagonists

Selective antagonists of CB₁ (AM281) and CB₂ (AM630) were used to block activity of CB receptors. AM281 and AM630 were obtained from Insight Biotechnology UK and stored at 50mM and 3mM and in DMSO and ethanol, respectively. Each antagonist was used at a final concentration of 1μM, as described previously [10]. Cells were treated with each antagonist only at this concentration which showed that there was no effect on cell viability in either of the breast cancer cell lines under these conditions (data not shown).

Cell viability

A standard MTT dye reduction assay was used to assess the cytotoxicity of the respective compounds in the BC cells. Briefly, cells were plated in 96 well plates at seeding densities of 5x10⁴ cells/ml. Cells were treated the following day with appropriate agents for 24 and 48 hours. MTT solution (5mg/ml in PBS) was then added and incubated for 4 hours. The media/MTT were removed and replaced with 200μl DMSO to dissolve the MTT formazan crystals. Plates were immediately read at 570nm in a multiwell plate reader (DynaTech MR5000, Dynex Laboratories, Worthing, UK). Each experiment contained 6 replicates and

was repeated at least three times. Cytotoxicity was expressed as mean percentage increase relative to unexposed control $\pm$ SD. Control values were set at 0% cytotoxicity. Cytotoxicity data (where appropriate) were fitted to a sigmoidal curve and a logistic regression model was used to calculate IC₅₀, which is the concentration of fatty acid/cannabinoid causing 50% inhibition compared to untreated controls. Mean IC₅₀ is the concentration of agent which reduces cell growth by 50% under the experimental conditions and is the average of at least three independent reproducible statistically significant measurements. The IC₅₀ values were reported at $\pm$ 95% confidence intervals ($\pm$ 95% CI). This analysis was performed using GraphPad Prism (San Diego, USA)

Protein extraction

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

Migration and invasion determinations

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

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

Western blot analysis

Cells were treated for 24h with the IC₅₀ concentration of each compound. A total of 25-30µg of protein was electrophoresed through precast 4-20% polyacrylamide gels (Invitrogen, Paisley, UK) for 1-2h and the separated proteins transferred to nitrocellulose membranes (Biorad, Hertfordshire, UK), then blocked with 5% (w:v) skimmed milk in tris-buffered saline (TBS) with 0.1% (v:v) Tween 20 (TBST solution) at room temperature and incubated individually, with either 1:100 dilution of anti-FAAH antibody, 1:100 CB₁, 1:200 CB₂, 1:200 ERK, 1:200 p-ERK, 1:200 JNK, 1:200 p-JNK, (Insight Biotechnology, UK), 1:200 p38 and pp38 (Abcam, UK), 1:100 Integrin β3 and VEGF (Santa Cruz, USA), 1:100 MMP-1 (Abcam, UK) at 4°C overnight. β-actin (1:20,000) was used as an internal loading control to normalize between lanes during densitometry (Abcam, UK). Appropriate secondary antibodies (Insight Biotechnology, UK) anti-mouse, rabbit (1:5000), or goat (1:10,000) were incubated at room temperature for 1h. Proteins were visualized using ECL+plusTM chemiluminescent detection kit (Amersham Pharmacia, Buckinghamshire, UK), according to manufacturer's instructions and a Fluor S phosphorimager (Biorad, Hertfordshire, UK). Experiments were repeated with proteins isolated from at least three independent extractions.

Data analysis

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

Results

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

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

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

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

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

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

Breast cancer cells differentially express CB₁ and CB₂ receptors and FAAH

Previously reported studies suggested MCF-7 and MDA-MB-231 cells either do or do not express CB₁ and CB₂ [18]. These discrepancies are possibly due to differences in the cell lines used, natural mutations of high passage number cells, or the use of different antibodies from different companies. We observed that both cell lines clearly expressed CB₁ with different proportions of glycosylated and non-glycosylated forms present between cell lines; MCF-7 cells, but not MDA-MB-321 cells, showed higher levels of the glycosylated form of CB₁ (Figure 3A and B). CB₂ was also expressed in both cell lines, with MCF-7 cells expressing this receptor at a significantly higher level (~3-fold) than MDA-MB-321 cells (Figure 3C, p=0.034). Glycosylated forms of CB₂ were not detected in either cell line. This contrasted with our previous observations in prostate cancer cells where both hormone-positive and negative cell lines demonstrated glycosylated forms of CB₂, but not of CB₁ [10]. FAAH protein was highly expressed in MCF-7 cells but was not detectable, at least by Western blotting, in MDA-MB-321 cells (Figure 3D).

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

AM281 and AM630 are CB₁ and CB₂ selective antagonists, respectively. In MCF-7 cells blockage of CB₁ had little effect on EPA or DHEA, but significantly decreased the effectiveness of DHA (p<0.001) and EPEA (p<0.05) on cell viability (Figure 4A). Blocking the CB₂ receptor significantly decreased the efficacy of EPA and EPEA (both p<0.01), had no effect on DHA, but significantly increased the efficacy of DHEA (p<0.01) on cell

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

In MDA cells, blocking CB₁ significantly decreased the efficacy of EPEA ($p<0.05$), DHA ($p<0.01$) and DHEA ($p<0.001$, completely inhibited), but EPA was without effect on cell viability (Figure 4B). Blocking CB₂ significantly decreased the effectiveness of EPA and EPEA ($p<0.05$, $p<0.001$ respectively), but increased the efficacy of DHA and DHEA ($p<0.01$, $p<0.05$ respectively). When both receptors were blocked with antagonists, the effectiveness of EPA and EPEA was significantly decreased ($p<0.001$ and $p<0.01$ respectively), but the efficacy of DHA and DHEA was again significantly increased (both $p<0.01$). These effects were similar to those observed with CB₂ inhibition alone.

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

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

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

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

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

Reduction in expression of migration- and invasion-related proteins

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

their ethanolamides, showed a significant reduction in integrin $\beta 3$ expression elicited by all treatments ($p < 0.01$) (Figure 6C). Interestingly, only DHA elicited a reduction in MMP-1 ($p < 0.05$) and VEGF ($p < 0.01$) protein expression in the cells.

Discussion

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

the BC cells (Figure 3) contrasted with our previous observations in prostate cancer cells where glycosylated CB₂, but not glycosylated CB₁, was detected in both hormone sensitive and hormone insensitive prostate cell lines [10]. The physiological significance of these differences in glycosylated CB_{1/2} receptor distribution and the role of the glycosylated forms in BC of the receptors *per se* is not clear at present and warrants further study.

The IC₅₀ concentrations of endocannabinoids required to inhibit BC cell proliferation in this study appear somewhat higher than levels of other endocannabinoids which have been measured *in vivo*, in serum [21]. One study by Rovito et al [19] in BC cells suggested that the potency of DHAE and EPEA can be increased by around an order of magnitude with increasing incubation time (from 24h to 96h), suggesting that length of treatment may be important factor. However, in the present study, whilst it was observed that the potency of EPEA was increased at 48h, we did not observe any change with DHEA. The physiological concentration of endocannabinoids at the site of formation is likely extremely difficult to determine precisely. Furthermore, since DHEA and EPEA are synthesised from DHA and EPA from the diet, the *in vivo* levels of the omega3-ethanolamides (DHAE and EPEA) will likely depend on the levels of omega3 PUFA in an individuals' diet. This area concerning the conversion of PUFAs to endocannabinoids and how these relate to circulatory levels of these compounds certainly requires further investigation.

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

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

Activation of CB₁ and CB₂ receptors by endocannabinoids in BC cells in this study displayed both similarities and differences from that previously found in prostate cancer cells [10]. If a derivative is functioning as a ligand of CB₁ or CB₂, then inhibiting the receptors with potent antagonists should decrease its potency in binding to the receptor. This has been observed for the effects of anandamide (AEA) on cell death in other cell lines [23-25]. However, Kuc *et al.*, [26] observed that CB₁, CB₂ and TRPV-1 receptor blockade in JWF2 keratinocytes, which over-express COX-2, did not inhibit AEA-mediated cell death, suggesting these receptors were not involved in the apoptotic signalling in the JWF2 cells. The latter study however, only looked at one cell line, and an n-6 ethanolamide, whereas our previous study in prostate cancer cells with n-3 endocannabinoids showed that blocking CB₁ or CB₂ with antagonists did indeed attenuate their anti-proliferative effects [10]. This study suggested the effect was complex and possibly dependent on cell type, relative levels of CB receptors, type of endocannabinoid ligand used and the integrity of the important molecular signalling pathways present. In further contrast to the work of Kuc *et al.*, blocking CB₁ receptor in the present study significantly decreased the efficacy of DHA and EPEA in MCF-7 cells and also of EPEA, DHA and DHEA in MDA-MB-231 cells, suggesting that these compounds do exert at least some of their antiproliferative, anticancer effects through the CB₁ receptor. Similarly, inhibition of CB₂ significantly affected the action of EPA and EPEA in both cell lines, again demonstrating that EPA and EPEA can exert some of their effects through CB₂ in these cells. From these findings it appeared that EPA/EPEA are better ligands of the CB₂ receptor and DHA/DHEA of the CB₁ receptor.

This intriguing differential effect of two major n-3 LCPUFA and their NAE derivatives warrants further investigation. Similar differential effects of the two n-3 NAEs were found in our previous study with prostate cell lines. Interestingly, it was found that blocking the CB₂ receptor potentiated the activity of DHEA, lowering the LC₅₀ in both MCF-7 and MDA-MB-231 cells. This effect was also evident in MDA-MB-231 cells with the parent fatty acid, DHA, but not with EPA. These findings may not be unexpected since it has been reported that anandamide-induced anti-proliferative effects in some cancer cell lines can be potentiated by CB₁- or CB₂-selective antagonists [27,28]. In this case, as has been proposed for anandamide, blockade of cannabinoid receptors in the presence of DHEA may increase the ability of DHEA to inhibit cancer cell proliferation through one or more cannabinoid receptor-independent mechanisms (reviewed in [29]). Indeed, the extent to which DHEA targets non-CB₁ and non-CB₂ receptors, particularly transient receptor potential V1 cation channels (TRPV1) needs to be established. These channels can be activated by both anandamide and omega-3 PUFAs and Maccarrone *et al.* (26) demonstrated that cannabinoid receptor activation can prevent apparent TRPV1-mediated apoptosis induced by anandamide [30]. Consequently, it is conceivable that by blocking the CB cannabinoid receptors there is reduced cannabinoid receptor-mediated protection of MCF-7 and MDA cells, thereby increasing the ability of DHEA to induce apoptosis through receptors such as TRPV1, which has been shown to be expressed in these cells [31]. Interestingly, whereas DHA is a potent TRPV1 agonist, EPA inhibits the activation of this cation channel by various agonists [32] and this may, at least in part, explain the lowering of the LC₅₀ observed with DHA/DHEA but not with EPA/EPEA in the BC cells. However, why this effect occurs specifically with the CB₂ receptor antagonist and not with the CB₁ receptor antagonist requires further investigation.

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

Our observations of a reduction in ERK and pERK by some, but not all, n-3 LCPUFA and n-3 endocannabinoids particularly in MDA-MB-231 cells, correlated with findings of Ellert-Miklaszewska *et al.*, who demonstrated inactivation (reduced phosphorylation) of ERK after treatment of glioma cells with the synthetic cannabinoid agonist WIN 55,212-2, and linked this inactivation to enhanced apoptosis through activation of the pro-apoptotic Bad protein [39]. In our study, DHEA in particular, significantly reduced phosphorylated ERK, even though unphosphorylated ERK did not change. Other studies have also shown inactivation of ERK in response to cannabinoids and CB₂ receptor agonists [40]. Findings of

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

Our findings of a general reduction in active pJNK, but not JNK, particularly with DHA and DHEA treatment in both cell types, can be regarded as inhibitory for cell proliferation/apoptosis [42,43]. However, JNK activation can also act as a “double edged sword” having both pro- and anti-apoptotic effects, perhaps depending on duration of activation [42,43]. CB₁ activation was coupled to JNK activation which supports our suggestion that DHEA acts mainly through CB₁. Our findings contrast with observations that anandamide-induced cell death required p38 and JNK activation in PC12 medulla cells [37,43]. Instead, we observed anti-proliferative effects when JNK phosphorylation was decreased, suggesting that pJNK was supporting a proliferative role; it could be that activation/inactivation of the other MAPKs, as described above, could be the more dominant factors.

Metastasis of tumours, where tumour cells migrate from the primary tumour and invade surrounding tissues, is a major causal factor in the high mortality rates reported for various cancers. Preventing the dispersal of malignant tumour cells throughout the body would confer a huge clinical benefit in combating cancer *per se*. During the metastatic process circulating cancer cells adhere to the extracellular matrix (ECM) of different tissues and organs, facilitated by various adhesion molecules (e.g. integrins) expressed at the surface of most cells and in particular on vascular endothelial cells. The ECM at the site of attachment 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 CB₁ 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

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479

480 **Declaration of interests**

481 The authors confirm that there are no known conflicts of interest associated with this
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483 influenced its outcome.

Figure Legends

Figure 1: Cell viability of MCF-10a cells

Viability of cells treated with LCPUFA and ethanolamides for (A) 24 hours or (B) 48 hours, expressed as a percentage of untreated control cells. Error bars represent standard error of means, $n=3$.

Figure 2: IC₅₀ values of n-3 fatty acids and ethanolamides

IC₅₀ values (μM) determined by MTT assay $\pm$ standard error of means in (A) MCF-7 and (B) MDA-MB-231 cells treated with fatty acids (EPA and DHA) or ethanolamides (EPEA and DHEA) for 24h, and (C), (D) for 48 hrs. Student's unpaired t-test was used for comparisons between treated and control cells and the level of statistical significance expressed as $*p\leq 0.05$, $**p\leq 0.01$, $***p\leq 0.001$, ($n\geq 3$).

Figure 3: Protein expression of cannabinoid receptors CB₁ and CB₂ and FAAH

(A) Representative blots for CB₁, CB₂ and FAAH. (B-D) Graphical representations of (B) CB₁ (C) CB₂ and (D) FAAH in MCF-7 and MDA-MB-231 breast cancer cells. Graphical representations are expressed as percentage of expression compared to β -actin (marked as 42 kDa). Error bars represent standard error of means.

Figure 4: Effects on cell viability of inhibiting CB receptors

IC₅₀ values (μM) as determined by MTT assay $\pm$ SEM in (A) MCF-7 cells and (B) MDA-MB-231 cells treated with fatty acids or their ethanolamides with and without CB receptor antagonists for 24h $*p\leq 0.05$, $**p\leq 0.01$, $***p\leq 0.001$, ($n\geq 3$), '=' is where IC₅₀ could not be determined.

Figure 5: MAP kinase pathway expression

Protein expression (as a ratio of protein to actin) expressed as a percentage of untreated control in (A) MCF-7 and (B) MDA-MB-231 breast cancer cells. Representative blots including β -actin loading control. Bars indicate blots linked to β -actin expression from the same blot. Student's unpaired t-test was used for comparisons between treated and control cells and the level of statistical significance expressed as $*p\leq 0.05$, $**p\leq 0.01$,

Figure 6: Effects of n-3 fatty acids and ethanolamides on invasion/migration

Effect of 24h treatment on MDA-MB-231 cells (expressed as a percentage of untreated cells) on (A) Invasion and (B) Migration. (C) Protein expression of integrin $\beta 3$, MMP-1 and VEGF (as a ratio of protein to β -actin) expressed as percentage of untreated MDA-MB-231 cells. One-way ANOVA and Dunnett's post hoc tests were used for comparisons between treated and control cells and the level of statistical significance was expressed as $*p\leq 0.05$, $**p\leq 0.01$, $***p\leq 0.001$, ($n\geq 3$)

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Figure 1

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Figure 2

Figure 3

Figure 4

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Figure 5