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Oxidized derivative of docosahexaenoic acid preferentially inhibit cell proliferation in triple negative over luminal breast cancer cells

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Abstract

Omega-3 polyunsaturated fatty acids (PUFAs) exert an anticancer effect by affecting multiple cellular mechanisms leading to inhibition of proliferation and induction of apoptosis. It is well known that breast cancer comprises distinct molecular subtypes which differ in their responsiveness to therapeutic and preventive agents. We tested the hypothesis that n-3FA may preferentially affect triple-negative breast cancer cells for which no targeted intervention is presently available. The in vitro antiproliferative effects of n-3 PUFA docosahexaenoic acid (DHA) and its metabolite, 4-OH-DHA as well as its putative metabolite 4-OXO-DHA, were tested in five triple-negative human basal breast cell lines at different stages of transformation (MCF-10F, trMCF, bsMCF, MDA-MB-231, and BT-549) and three luminal breast cancer cell lines (MCF-7, T-47D, and SK-BR-3). Cell proliferation was measured with the tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay. DHA and its oxidized derivatives significantly inhibited cell proliferation (20–90% reduction) of both basal and luminal breast cancer cell lines. The inhibitory effect was more pronounced on triple-negative basal breast cancer cell lines as compared to luminal breast cancer cell lines after 4-OXO-DHA treatment. Our data provide novel information regarding the preferential antitumor effect of oxidized derivatives of DHA on basal type breast cancer.

Keywords

Triple-negative breast cancer; Fish oil; DHA

Introduction

The role of diet as a preventative measure for breast cancer remains controversial (Signori *et al.* 2011). Current literature suggests that the risk of developing breast cancer may decrease or increase with the intake of omega-3 (*n*-3) and omega-6 (*n*-6) fatty acids, respectively (Carroll and Braden 1984). *n*-6 polyunsaturated fatty acids (PUFAs) have been suggested to promote cancer while *n*-3 PUFAs have been implicated in cancer suppression (Chapkin *et al.* 2007; Larsson *et al.* 2004). It has been reported that *n*-3 PUFAs alter the cell membrane phospholipid composition and as a result, *n*-3 PUFAs may exert an anticancer effect by affecting multiple cellular mechanisms (Signori *et al.* 2011). Among these, the expression and function of multiple receptors, proteins, and lipid-derived signaling molecules may be affected. *n*-3 PUFAs may mediate mammary cancer prevention by affecting eicosanoid metabolism, oxidative stress, cell membrane structure, cell proliferation, and apoptosis (Signori *et al.* 2011). Altering these processes will eventually lead to the inhibition of cell

proliferation and increased cell death (Signori *et al.* 2011). Currently, the molecular mechanisms of these alterations are not well understood (Berquin *et al.* 2008).

Dietary long-chain *n*-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are found primarily in cold-water fish (Berquin *et al.* 2008). The amount of EPA and DHA varies from species of fish and geographical location. Fish such as mackerel, tuna, and salmon are from deep, cold water and tend to have the highest concentration of EPA and DHA (Larsson *et al.* 2004). DHA has been shown to promote an anticancer effect on multiple breast cancer cell lines in vitro (Liu *et al.* 2007; Schley *et al.* 2007; Sun *et al.* 2008). DHA has also been reported to induce apoptosis via multiple pathways (Berquin *et al.* 2008; Sun *et al.* 2008; Blanckaert *et al.* 2010; Kang *et al.* 2010; Ravacci *et al.* 2013). In vivo studies using animal models also found that *n*-3 PUFAs reduced tumor growth, slowed histopathological progression, and increased survival (Berquin *et al.* 2007). Indeed, epidemiologic studies show an inverse association between percent calories from fish and incidence of breast cancer, suggesting a protecting role (Kaizer *et al.* 1989). The protective effects of fish oil have been shown also in other cancer types, such as colorectal cancer (Anti *et al.* 1992). Nevertheless, analytic epidemiologic studies having a case-control or cohort design have not yielded clear conclusions concerning the protective effect of fish consumption or n-3 PUFAs intake against cancer. Some studies published in the literature have failed to show an inverse association between the intake of n-3 PUFAs or fish and cancer risk (Vatten *et al.* 1990; Chajes *et al.* 1999; Holmes *et al.* 1999). Previous studies conducted in vitro and in vivo models of mammary carcinogenesis clearly demonstrated that DHA (Fig. 1*a*) is a superior chemopreventive agent to EPA (Noguchi *et al.* 1997; Yuri *et al.* 2003; Kang et al. 2010; Rahman *et al.* 2013). Promising literature indicated that DHA and its metabolite 4-OH-DHA (Fig. 1*b*) may play a major role in the antitumor properties of omega-3 (Itoh *et al.* 2006; Sapieha *et al.* 2011).

The focus of the present work was to determine whether DHA and 4-OH-DHA exert a differential effect in at least two subtypes of breast cancer, the luminal and the basal type. Furthermore, because of its superior PPARγ agonistic activity to that of 4-OH-DHA, we included the putative metabolite 4-OXO-DHA (Fig. 1*c*) for comparison (Itoh *et al.* 2008). Our laboratory has developed a model of human breast epithelial cell transformation using the normal-like basal cell-type MCF-10F and 17-β-estradiol as the carcinogen (Russo et al. 2003, 2006a, b). This model represents the progression of basal breast cancer from normal cell (MCF-10F), transformed cell (trMCF), and invasive-metastatic (bsMCF) (Soule *et al.* 1990; Russo *et al.* 2006a, b; Huang *et al.* 2007). The uniqueness of this model is that all cells have the same genetic lineage and stable phenotypes. In addition to these cell lines, we included two basal (MDA-MB-231 and BT-549) and three luminal cell lines (MCF-7, T-47D, and SK-BR-3) well characterized in literature. Our results show that oxidized derivative of DHA, 4-OXO-DHA, preferentially inhibited the growth of basal-like breast cancer for which currently there is no targeted therapy available.

Materials and Methods

Cell cultures

The antiproliferative effect of DHA and its oxidized derivatives were tested on eight human breast-derived cell lines. Among the eight cell lines, one normal-like breast epithelial cell line (MCF-10F), one transformed breast epithelial cell line (trMCF), three basal breast cancer cell lines bsMCF, MDA-MD-231, and BT-549), and three luminal breast cancer cell lines (MCF7, T-47D, and SK-BR- 3) were chosen. Basal cell lines were classified as ER−, PR−, and HER2−. MCF7 and T-47D were classified as luminal A (ER+, PR+, and HER2−) while SK-BR-3 is classified as luminal B (ER+, PR+, HER2+). MCF10F, trMCF, and bsMCF cells were maintained in DMEM/F12 media (1:1 Gibco/BRL, Gaithersburg, MD) supplemented with 5% horse serum (Gibco), 100 ng/ml cholera toxin (ICN Biomedicals, Cleveland, OH), 10 μg/ml insulin (Sigma, St. Louis, MO), 0.5 μg/ml hydrocortisone (Sigma), 20 ng/ml epidermal growth factor (Gibco), 1.05 mM $CaCl_2$, and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml; amphotericin, 0.25 μg/ml; Sigma). MCF7 cells were maintained in DMEM media supplemented with 10% fetal bovine serum (FBS). MDA-MB-231, BT-549, and T-47D breast cancer cells were grown in RPMI-1640 media supplemented with 10% FBS. SK-BR-3 was grown in McCoy's 5a media supplemented with 10% FBS. All cell lines were maintained in a 37°C, 5% CO_2 incubator. All cell lines and media were obtained from the Tissue Culture Facility at Fox Chase Cancer Center.

Preparation of DHA and oxidized metabolites

Stock concentrations of 2.5 mM of DHA and oxidized derivatives were bovine serum albumin (BSA)-conjugated. A solution of 5 mg DHA/0.05 M Na_2CO_3 was prepared and flushed with nitrogen gas. This solution was then vortexed extensively and left at room temperature for 1 h. The vial was vortexed intermittently to insure that all DHA was dissolved completely. A 2.5-mM fatty acid (FA) BSA complex was prepared at FA/BSA 3:1 mole ratio using 15% BSA dissolved in normal cell culture medium. Solutions were prepared in a conical tube and were flushed with nitrogen gas before being placed on a shaker for 30 min. The FA-BSA stock solution was filter-sterilized under a sterile cell culture hood, aliquoted, and stored at −20°C.

Treatment

DHA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). DHA derivatives, 4-OH-DHA and 4-OXO-DHA, were synthesized and provided by Dr. Karam El-Bayoumy laboratory using a previously established synthetic approach (Itoh *et al.* 2006). DHA and its metabolites were BSA-conjugated in culture medium as previously described. Stock solutions (2.5mM) were prepared and stored at −20°C. Based on published literature, five concentrations, 0, 10, 25, 50, and 100 μM, of DHA and its derivatives were tested for a total of 4 days. Medium was changed daily over the course of treatment time.

MTT assay

Cells were seeded in 100 μL culture medium into costar 96-well flat bottom tissue culture plates at an optimal density per cell line (1000–4000 cells/well) to have a 70–80% confluent

culture by 5 days. Cells were treated in adherent conditions on the following day. Medium containing the tested treatment doses was changed daily.

Cell proliferation was measured with the tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay (Mosmann 1983). Cell proliferation was assessed every 24 h of treatment for a total of 96 h using Vybrant MTT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). Media was removed and replaced with 100 μL phenol-red-free media containing 12 mM MTT. The plate was incubated in a 37°C, 5% CO_2 incubator for 4 h, followed by 4 h of incubation after the addition of HCl-SDS to solubilize the formazan product. Optical density was read at 570 nm using Epoch Microplate Spectrophotometer (Biotek, Winnoski, VT). Data was analyzed on Sigma Plot v12. A one-way analysis of variance (ANOVA) was performed using the Holm-Sidak method to calculate a relevant p value where p values less than 0.05 were considered to be statistically significant.

Statistics

For each of the treatments under consideration, 100 μM DHA, 4-OH-DHA, and 4-OXO-DHA, Kruskal-Wallis tests indicated a significant effect of cell line on growth relative to untreated control after treatment. Tukey honestly significant difference (HSD) tests with α=0.05 on the ranks of cell growth relative to untreated control after treatment was used to identify cell lines responsible for the significance of Kruskal-Wallis tests. Results of this post hoc analysis are shown in Tables 1 and 2; cell lines labeled with the same letter are not significantly different. For each treatment, the Tukey HSD test controls the overall type I error rate at 0.05; no adjustment for multiple comparisons was made between treatments for means of comparing individual treatment effect on cell lines.

Results

DHA, 4-OH-DHA, and 4-OXO-DHA inhibit in vitro cell proliferation of human breast cancer cells

Treatment of DHA, 4-OH-DHA, and 4-OXO-DHA resulted in an inhibitory effect on the proliferation of MCF-10F, trMCF, bsMCF, MDA-MB-231, BT-549, T-47D, and SK-BR-3 (Fig. 2). Proliferation was significantly increased after DHA and 4-OXO-DHA treatment on MCF-7. DHA and its oxidized metabolites produced an inhibitory effect that was dose dependent after 96 h of treatment (Fig. 3). Cell proliferation was significantly inhibited within 48 h of 100 μM treatment, but the most prominent effect was observed after 96 h of 100 μM treatment (Fig. 4).

The effect of 100 μM 4-OXO-DHA on the proliferation of all cultured cell lines differed according to the cell type. The three luminal cell lines, MCF-7, T-47D, and SK-BR-3, were significantly different from the four basal cell lines tested, trMCF, bsMCF, BT-549, and MDA-MB-231 as described in Table 1. DHA and 4-OH-DHA treatment did not show a clear preferential effect. Treatment of 4-OXO-DHA was more effective than DHA and 4-OH-DHA in MDA-MB-231, BT-549, and SK-BR-3 cell lines (Table 2). 4-OXO-DHA was more

potent than DHA in trMCF and bsMCF. 4-OH-DHA treatment was more potent than DHA in trMCF and bsMCF.

Discussion

Triple-negative breast cancer remains a difficult and aggressive cancer to treat. The possibility of preventing triple-negative breast cancer by dietary n-3 PUFAs supplements such as DHA or its metabolites may be a potential approach in cancer prevention or treatment. A new concept is emerging which is based on the combination of chemotherapy and nutrition intervention. Preclinical evidence of the preventive benefits of n-3 PUFAs in breast cancer continues to fuel interest in the potential role of dietary fat content in reducing breast cancer risk. There are several clinical trials that seek to gain knowledge of the role of dietary fatty acids in the prevention of breast cancer. Yee et al. tested different concentrations of n-3 PUFAs in a 6-month randomized open-label study with 48 women with increased breast cancer risk (Yee *et al.* 2010). The authors concluded that daily doses up to 7.56 g DHA+EPA were well tolerated with excellent compliance. Body mass index and baseline fatty acid concentrations modulated the dose-response effects of n-3 PUFAs supplements on serum EPA and DHA and breast adipose tissue DHA. In a recent report by our team, we demonstrated the expected rise in serum n-3 PUFAs in women supplemented with Lovaza (4 g daily) for 12 mo (Signori *et al.* 2012); these results further support the excellent compliance and the oral supplementation of n-3 PUFAs as a feasible strategy in future breast cancer chemoprevention trials. n-3 PUFA consumption has also been studied in colorectal cancer (CRC). Two large epidemiological observational studies have demonstrated significant inverse relationships between n-3 PUFA intake/levels and risk of colorectal neoplasia. The studies showed a significant dose-dependent reduction in CRC risk for total n-3 PUFA intake (Kim *et al.* 2010). Recent evidence supports a broader role of n-3 fatty acid supplements in cancer patients. Early administration of this nutritional supplement helps to preserve muscle tissue and prevent cachexia as well as tolerate better the chemotherapy treatment (Murphy *et al.* 2012). The dose of fish-oil/omega-3 PUFAs needed to achieve maximal target tissue effects for breast cancer prevention remains undefined. Our data show that DHA and its metabolites have a preferential antiproliferative effect on basal breast cancer compared to luminal breast cancer.

Our data show that DHA-oxidized derivative, 4-OXO-DHA, had a differential effect on the two main subtypes of breast cancer cell lines tested. Our results indicated that not only 4-OXO-DHA had an inhibitory effect on breast cancer, but also it produced a preferential inhibitory effect on basal cell lines over luminal cell lines. Under the experimental conditions used in this study, there was a stimulatory effect of DHA and metabolites on MCF7 cell line. However, the other luminal A cell line, T-47D, showed an inhibitory effect; thus, we cannot assume that DHA may enhance the growth of luminal A breast cancer. Recent studies demonstrated that DHA intake has been shown to promote an anticancer effect on multiple breast cancer cell lines in vitro (Liu *et al.* 2007; Schley *et al.* 2007; Sun *et al.* 2008). Specifically, Blankaert et al. reported that intake of DHA decreased cell proliferation, increased apoptosis, and decreased the invasive potential of the MDA-MB-231 cell line, a highly malignant triple-negative breast cancer cell line (Blanckaert et al. 2010). Multiple pathways have been proposed to explain how DHA has also been reported to

induce apoptosis via multiple pathways (Berquin *et al.* 2008; Sun *et al.* 2008; Blanckaert et al. 2010; Kang *et al.* 2010; Ravacci *et al.* 2013). The mechanism of DHA remains unclear and controversial (Gago-Dominguez *et al.* 2003; Stripp *et al.* 2003; Schley *et al.* 2007; Zhang *et al.* 2012). Furthermore, no mechanism has been identified to explain why triple-negative breast cancer cells are more sensitive to DHA, 4-OH-DHA, and 4-OXO-DHA treatment. This finding warrants further investigation.

Using the MCF-10F model, we indicated that more transformed cells were more sensitive to DHA and metabolite treatment. For example, both trMCF and bsMCF, which represent a transformed and metastatic cell line, were more sensitive to DHA, 4-OH-DHA, and 4-OXO-DHA treatment compared to their parent normal-like MCF-10F cell line. This observation is consistent with a previous report where they showed that a transformed cell, MCF-7, responded better to DHA ethanolamine derivatives than the normal-like breast epithelial cell line MCF-10A (Rovito *et al.* 2013). MCF- 10A and MCF-10F were both derived from the same patient, and both express phenotypes of a normal-like breast epithelial cell line (Soule *et al.* 1990). The pronounced inhibitory effect we observed on the trMCF cell line compared to MCF-10F and bsMCF suggests that DHA and its oxidized derivatives may be beneficial in treating cancer in early stages or possibly breast cancer prevention.

This study supports the hypothesis that treatment of DHA and its metabolites inhibits breast cancer cell proliferation. DHA produced an antiproliferative effect on multiple cell lines in our model; however, the effect was more pronounced when cells received 4-OXO-DHA treatment. Additionally, 4-OXO-DHA treatment preferentially affected triple-negative breast cancer cells. The larger inhibition of cell proliferation produced by this metabolite suggests that metabolites of DHA may be more beneficial in cancer treatment than DHA. The largest inhibition of cell proliferation was produced by 4-OXO-DHA (100 μM). Since DHA is metabolized into 4-OH-DHA and likely to 4-OXO-DHA, these metabolites may be the potential compounds that are in fact inhibiting cell proliferation. Thus, understanding the metabolism of DHA and how the metabolites react with the cell is essential in understanding the anticancer effect produced by DHA.

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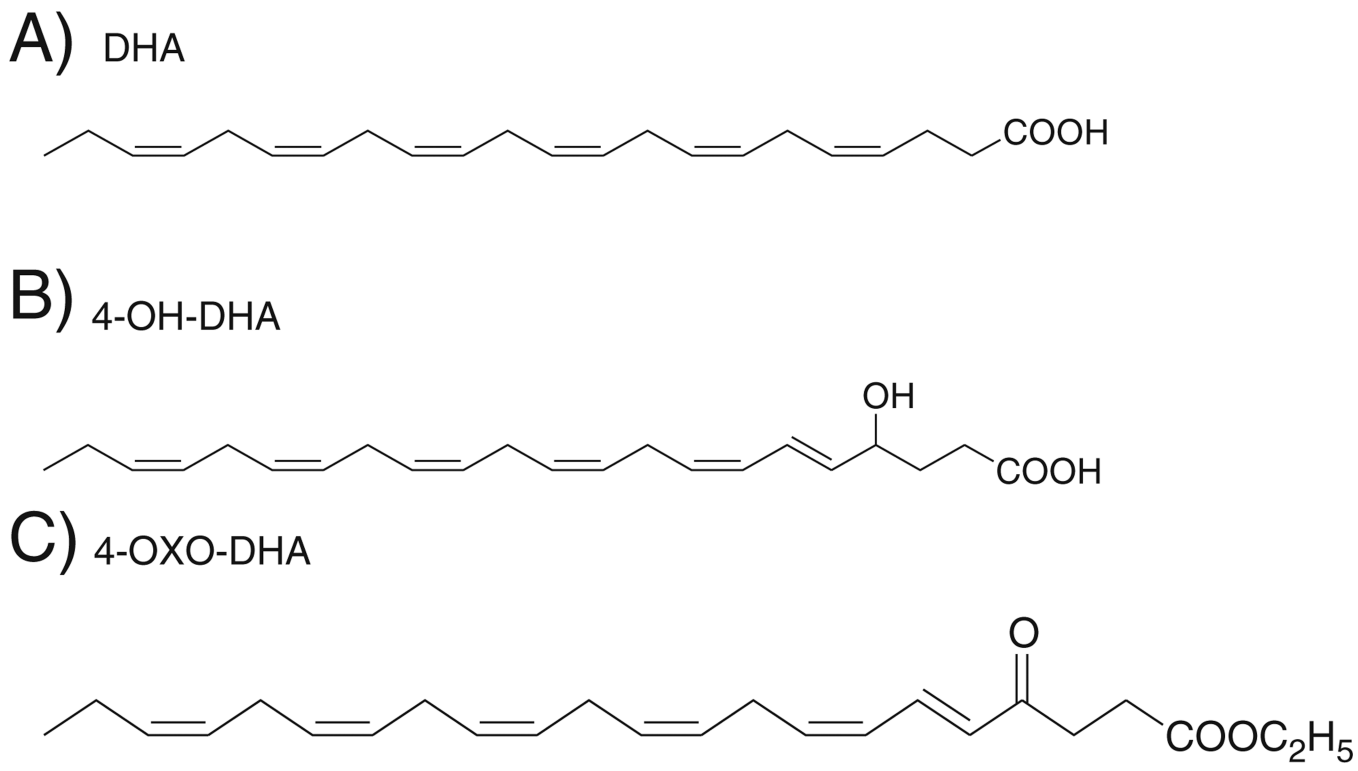

Figure 1.
Chemical structures of *a* DHA, *b* 4-OH-DHA, and *c* 4-OXO-DHA.

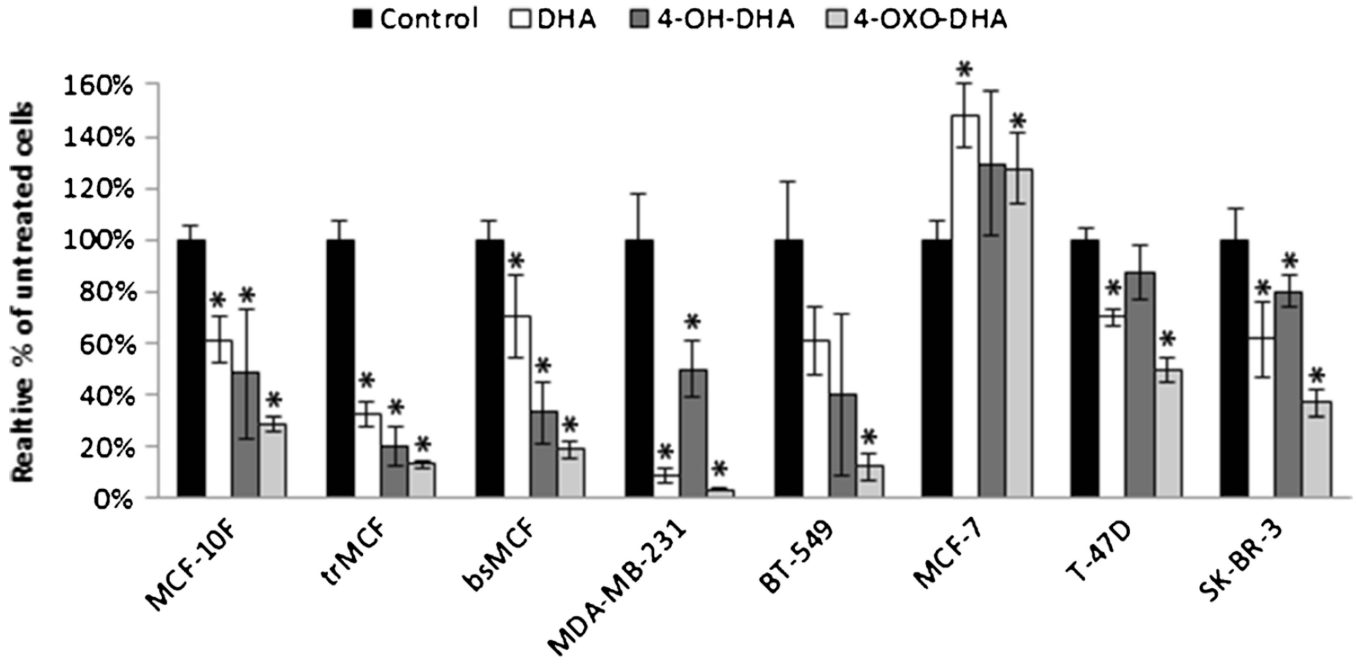

Figure 2.

The antiproliferative effect of DHA, 4-OH-DHA, and 4-OXO-DHA (100 μM) on a normal breast epithelial and breast cancer cell lines. Cell proliferation was measured by MTT assay 96 h after treatment as described in "Materials and Methods." All values were compared to a relative percentage of the untreated control. *Bars* represent the mean± standard deviation of triplicate experiments with experimental quadruplicate replicates. *$p \geq 0.05$ indicates a significant difference compared to 0 μM control.

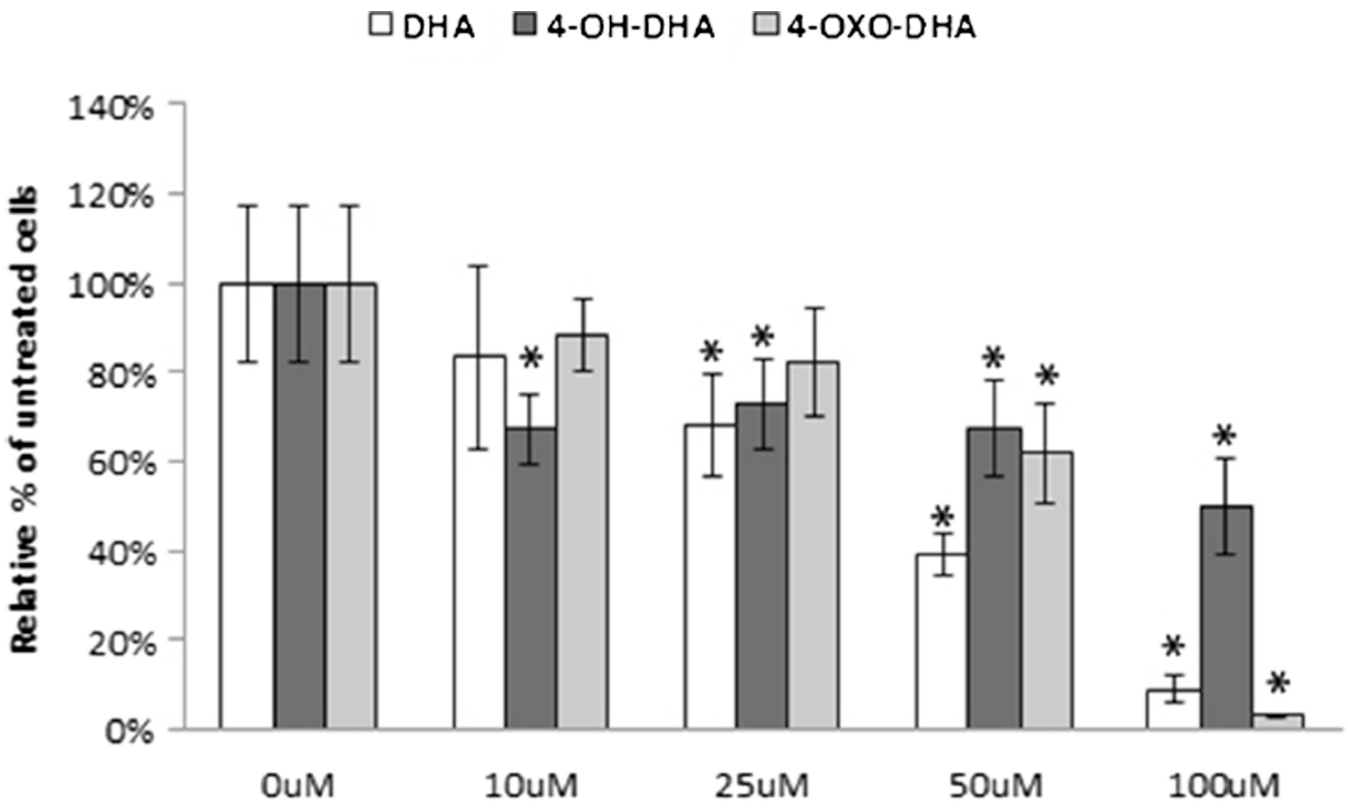

Figure 3.

The dose-dependent antiproliferative effect of DHA, 4-OH-DHA, and 4-OXO-DHA (100 μM) on MDA-MB-231 basal breast cancer cell line. Cell proliferation was measured by MTT assay 96 h after treatment as described in "Materials and Methods." All values were compared to a relative percentage of the untreated control. *Bars* represent the mean±standard deviation of triplicate experiments with experimental quadruplicate replicates. *$p \geq 0.05$ indicates a significant difference compared to 0 μM control.

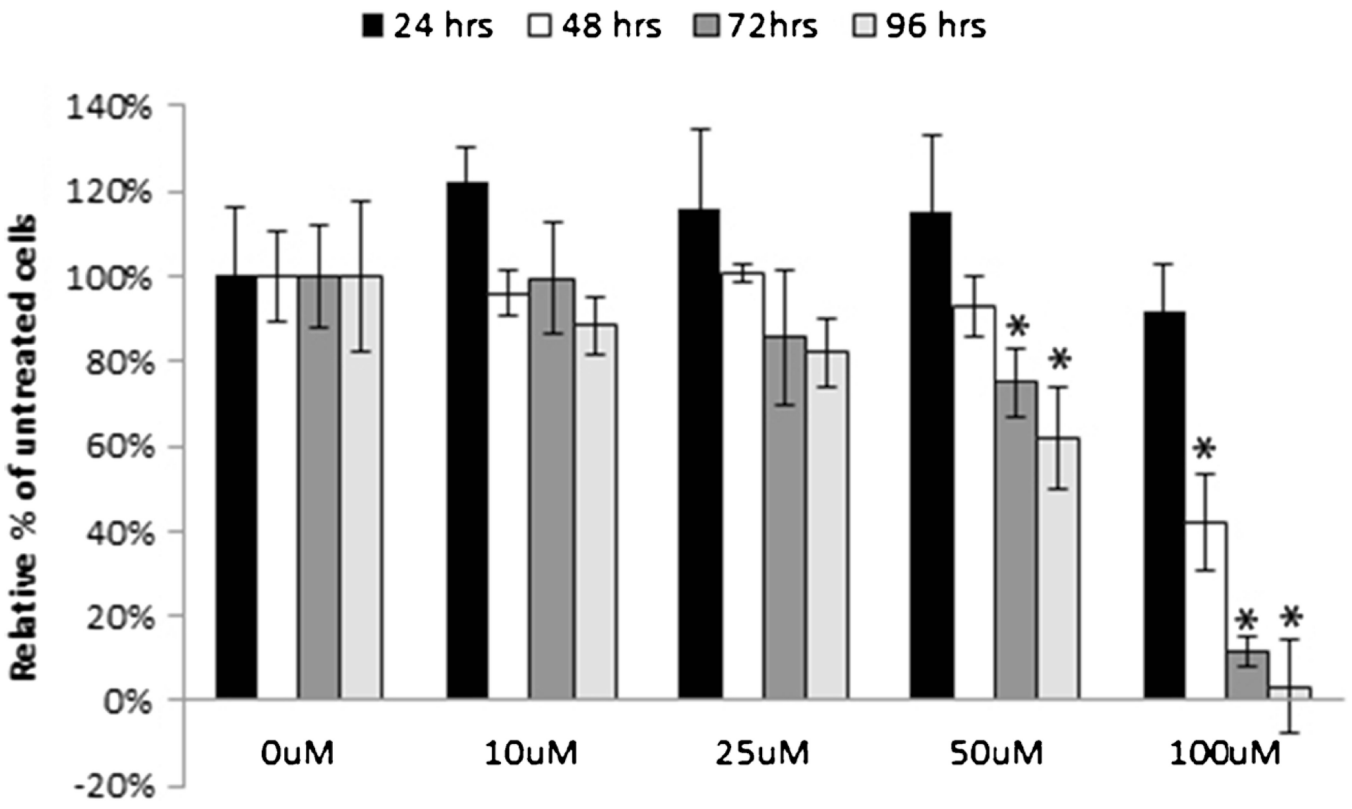

Figure 4.

The antiproliferative effect of 4-OXO-DHA on MDA-MB-231 basal breast cancer cell line. Cell proliferation was measured by MTT assay every 24 h after treatment for a total of 96 h as described in “Materials and Methods.” All values were compared to a relative percentage of the untreated control. *Bars* represent the mean±standard deviation of triplicate experiments with experimental quadruplicate replicates. *$p \geq 0.05$ indicates a significant difference compared to 0 μM control.

Table 1

Results of the post hoc analysis from MTT results of 96 h 100 μM DHA, 100 μM 4-OH-DHA, and 100 μM 4-OXO-DHA treatment

DHA			4-OH-DHA			4-OXO-DHA		
Labels	**Cell line**	**Rank means**	**Labels**	**Cell line**	**Rank means**	**Labels**	**Cell line**	**Rank means**
a	MCF-7	30.5	a	MCF-7	30	a	MCF-7	30.50
ab	bsMCF	21	ab	SK-BR-3	23.25	ab	T-47D	25.50
ab	T-47D	20	abc	T-47D	20.5	bc	SK-BR-3	23.50
ab	BT-549	19.5	abc	BT-549	17.25	cd	MCF-10 F	18.50
bc	MCF-10 F	16.75	bcd	MDA-MB-231	15.25	de	bsMCF	13.25
bc	SK-BR-3	15.25	bcd	MCF-10 F	13.5	e	BT-549	9.50
cd	trMCF	6.5	cd	bsMCF	8.75	e	trMCF	8.75
d	MDA-MB-231	2.5	d	trMCF	3.5	f	MDA-MB-231	2.50
($\chi^2(7)=24.32$; $p=0.0010$)			($\chi^2(7)=22.00$; $p=0.0025$)			($\chi^2(7)=29.36$; $p=0.0001$)		

Kruskal-Wallis test indicated a significant effect of cell line on growth relative to untreated control after treatment. Tukey HSD tests with $\alpha=0.05$ on the ranks of cell growth relative to untreated control after treatment was used to identify cell lines responsible for the significance of Kruskal-Wallis tests. This post hoc analysis yielded the following, where cell lines labeled with the same letter are not significantly different

Table 2

Results of post hoc analysis from MTT results of 96 h DHA, 4-OH-DHA, and 4-OXO-DHA treatment

Kruskal-Wallis test								
	MCF-10F	**trMCF**	**bsMCF**	**MDA-MB-231**	**BT-549**	**MCF-7**	**T-47D**	**SK-BR-3**
0 μM control	a	a	a	a	a	b	a	a
100 μM DHA	ab	b	b	c	a	a	b	b
100 μM 4-OH-DHA	b	c	c	b	a	ab	b	b
100 μM 4-OXO-DHA	b	c	c	d	b	ab	b	c

Kruskal-Wallis test indicated a significant effect of cell line on growth relative to untreated control after treatment. Tukey HSD tests with α=0.05 on the ranks of cell growth relative to untreated control after treatment was used to identify cell lines responsible for the significance of Kruskal-Wallis tests. This post hoc analysis yielded the following, where cell lines labeled with the same *letter* are not significantly different. Labels in each *column* are determined independently, and in each case a>b>c>d, showing the directionality of significant differences in each case