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Characterization of anticancer properties of 2,6-diisopropylphenol–docosahexaenoate and analogues in breast cancer cells

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ABSTRACT

The present study describes the characterization and evaluation of novel anticancer conjugates, 2,6-diisopropylphenol–docosahexaenoate (PP–DHA), and its analogues including 2,4-diisopropylphenol–docosahexaenoate (DIPP–DHA), 2-isopropylphenol–docosahexaenoate (IPP–DHA), 2-cyclohexanephenol-docosahexaenoate (CHP–DHA) and phenol–docosahexaenoate (P–DHA) on breast cancer cell lines. Representative breast cancer cell lines, based on estrogen α receptor (ER) and oncogene Her-2 expression, were used and include MDA-MB-231 (ER-negative, Her-2-negative), MCF-7 (ER-positive, Her-2-negative) AU565 (ER-negative, Her-2-positive) and MDA-MB-361 (ER-positive, Her-2-positive). The PP–DHA conjugate significantly inhibited cell growth and induced cell loss in the breast cancer cell lines similarly; however, this conjugate was not effective against normal mammary epithelial cells. The effect of various conjugates were in PP–DHA > IPP–DHA > DIPP–DHA > CHP–DHA >> P–DHA order. PP–DHA and IPP–DHA conjugates were stable in human and mouse serum. Furthermore, the non-hydrolyzable amide-linked conjugate analogues affected breast cancer cells in a manner similar to that of the ester-linked conjugates. This suggests that ester-linked PP–DHA and IPP–DHA conjugates were stable during treatment to breast cancer cells due to structural hindrance. PP–DHA did not affect PPARα or PPARγ activities but its anticancer effects appear to be mediated in part though the inhibition of histone deacetylase (HDAC) activity. Further experiments are needed to confirm their molecular target and to test the effectiveness of these compounds in an in vivo model for their anticancer properties. In conclusion, these results suggest that the novel PP–DHA and IPP–DHA conjugates and their amide derivatives may be useful for the treatment of breast cancer.

1. Introduction

One of the basic concerns for successful and effective use of anticancer therapeutics, which act on intracellular targets, is the propensity for uptake by tumors and cellular retention. While targeting through receptor-mediated endocytosis has shown utility, many drugs cross the plasma membrane through either existing transport systems, pinocytosis and/or diffusion to elicit the cellular actions. A common approach to enhancing the cellular uptake and membrane transport is to link drugs to lipophilic carriers.[1–3] Docosahexaenoic acid (22:6, DHA), a long chain dietary polyunsaturated fatty acid has recently been the subject of numerous studies used as a fatty acid of choice for conjugating drugs. This compound also possesses moderate anticancer activities against a variety of cancers and shows synergy in pre-clinical models when used in combination with certain drugs.[4–9]

Several investigations have pursued the synthesis and testing of novel compounds by conjugating naturally occurring lipids with known cancer chemotherapeutics. For example, chlorambucil-fatty acid conjugates (including DHA) were synthesized and tested on human lymphoma cell lines.[3] The conjugates selectively inhibited the growth of neoplastic lymphocytes with minimal effects on quiescent lymphocytes.[3] More extensive studies have been conducted with a paclitaxel-DHA ester-conjugate (Taxoprexin®), which has been shown to behave as a prodrug in vivo.[10–12] Pre-clinical testing of this agent demonstrated antitumor activity in a mouse lung cancer model. In addition, enhanced pharmacokinetic and tissue distribution properties were observed that appear to lead to improved efficacy with respect to paclitaxel alone. Early stage clinical results have warranted continued Phase II trials as a single agent. Other exploratory studies have been done with DHA conjugates to enhance the bio-availability and activity of the anticancer drug methotrexate (MTX).[2] More recently, conjugates of camptothecin with unsaturated fatty acids were developed as anticancer agents[13] and Ojima and co-workers have conjugated taxoids with

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DHA to induce antitumor effects in drug resistant human colon tumor xenografts in DLD-1 mice.[14] A final example shows a DHA-doxorubicin conjugate with improved anticancer efficacy over free doxorubicin in an experimental animal model of L1210 leukemia and B16 melanoma.[15]

While these studies demonstrate the utility of a polyunsaturated fatty acid ester for prodrugs to enhance efficacy of cancer chemotherapy, there is a lack of examples of chemicals that utilize an enhanced DHA effect on cancer cells. This is despite the epidemiological evidence for dietary DHA leading to reduced risks of certain cancers. In addition, the mechanism of cancer cell inhibition by DHA remains unclear. One strategy that recently employed the conjugation of DHA with 2,6-diisopropylphenol (propofol)-DHA and 2,6-diisopropylphenol–EPA conjugates resulted in enhanced anticancer activities against breast tumor cell lines.[16] The selection of propofol was based upon the fact that it is known to be a well tolerated and non-toxic anesthetic that has shown some elements of being an anti-metastatic agent in pre-clinical models.[17] Both conjugates exhibited anticancer effects that include the inhibition of cell migration and adhesion and the induction of apoptosis within MDA-MB-231 breast cancer cells. The present study focuses on establishing the chemical features responsible for the enhanced anticancer activity of DHA. In addition, the chemosensitivity of a panel of breast cancer cell lines to a series of conjugates as well biochemical studies have been investigated in an effort to understand the mode of action of the conjugates. Our studies suggest that 2,6-diisopropylphenol–DHA is the most effective compound in comparison to other analogues and that cleavage of the ester linkage is not required for the cellular activity. The conjugates are also stable in both human and mouse serum. The overall effects of these compounds are not attributed to a major re-distribution of the lipid components of the plasma membrane. The anticancer effects of the conjugates appear to be mediated in part through the inhibition of histone deacetylase (HDAC) activity.

2. Chemistry

2.1. Synthesis of 2,6-diisopropylphenol–docosahexanoate and its analogues

2,6-Diisopropylphenol–docosahexanoate and its analogues were synthesized with a one step reaction procedure as described previously[16] and outlined in Scheme 1.

The fatty acid anhydride was formed through a 1,3-dicyclohexylcarbodiimide (DCC) dehydration under an anhydrous condition, which is then reacted with the hydroxyl group of 2,6-diisopropylphenol or its analogues through a catalyzed esterification in the present of *N*,*N*-dimethylpyridin-4-amine (DMAP). These ester-conjugates were isolated and purified through normal phase silica gel chromatography with a yield of 74–94%.[16] The amides of 2,6-diisopropylphenol its analogues were prepared similarly by amidation in the presence of 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU) and diisopropyl ethyl amine (DIEA) in the presence of inert condition at room temperature. The amide conjugates were purified by flash chromatography on a silica gel column with a yield of 66–90%. The structure of the phenolic-fatty acids conjugates are depicted in Scheme 2.

2.2. Characterization of 2,6-diisopropylphenol–docosahexanoate and its analogues

The formation of the 2,6-diisopropylphenol–docosahexanoate and its analogues were confirmed with ^{1}H, ^{13}C NMR and HR MS.

2.2.1. Compound 1: 2,6-diisopropylphenyl-docosahexaenoate (PP–DHA)

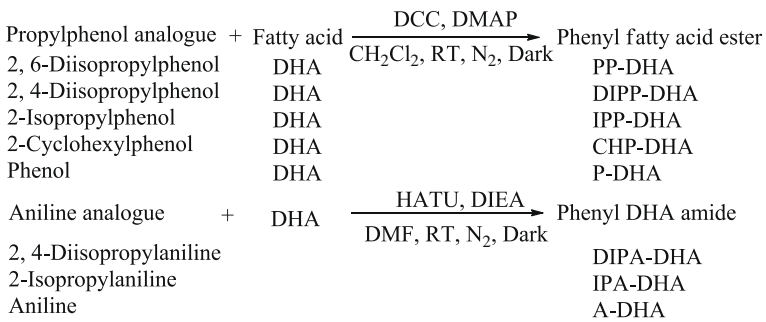
The PP–DHA conjugate was purified by chromatography on a preparative thin layer silica gel. The compound was eluted with 95:5 hexane/ethyl acetate as a colorless oil. The yield was 80.5%. ^{1}H NMR ($CDCl_3$) δ 1.02 (tri, 3H, J = 7.5 Hz), 1.24 (d, 12H, J = 6.9 Hz), 2.12 (m, 2H), 2.62 (m, 2H), 2.74 (m, 2H), 2.84–3.00 (m, 12H), 5.38–5.44 (m, 10H), 5.53 (tri, 2H, J = 4.7 Hz), 7.18–7.25 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 14.2, 20.5, 22.8, 25.5, 25.6, 27.5, 34.0, 123.8, 126.4, 127.0, 127.7, 127.8, 127.9, 128.0, 128.2, 128.3, 128.5, 129.7, 132.0, 140.2, 145.5, 171; Mass spectrum (ESI), m/z 489.3738 $(M+H)^+$ ($C_{34}H_{48}O_2 + H^+$ requires 489.3733).

2.2.2. Compound 2: 2,4-diisopropylphenyl-docosahexaenoate (DIPP–DHA)

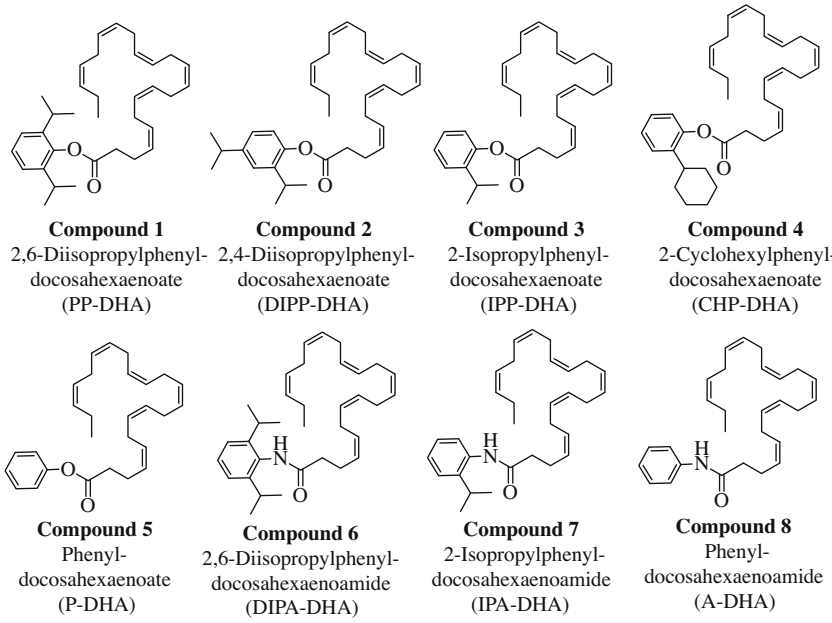
The DIPP–DHA conjugate was purified by chromatography on a preparative thin layer silica gel. The compound was eluted with 95:5 hexane/ethyl acetate as a colorless oil. The yield was 94%. ^{1}H NMR ($CDCl_3$) δ 1.00 (tri, 3H, J = 7.5 Hz), 1.23 (d, 6H, J = 6.9 Hz), 1.27 (d, 6H, J = 6.9 Hz), 2.11 (m, 2H), 2.57 (m, 2H), 2.67 (m, 2H), 2.82–2.94 (m, 10H), 3.02 (m, 2H), 5.36–5.45 (m, 10H), 5.50 (m, 2H,), 6.92 (d, 1H, J = 8.3 Hz), 7.24 (tetr, 2H, J = 8.3, J = 2.1 Hz), 7.16 (d, 1H, J = 2.1 Hz); ^{13}C NMR ($CDCl_3$) δ 14.2, 20.5, 22.8, 22.9, 25.5, 25.6, 27.4, 33.8, 34.2, 121.8, 124.3, 124.6, 127.0, 127.6, 127.8, 128.0, 128.2, 128.5, 128.0, 129.6, 131.9, 139.4, 145.9, 146.5, 171.8; Mass spectrum (ESI), m/z 489.3731 $(M+H)^+$ ($C_{34}H_{48}O_2 + H^+$ requires 489.3733).

2.2.3. Compound 3: 2-isopropylphenyl-docosahexaenoate (IPP–DHA)

The IPP–DHA conjugate was purified by chromatography on a preparative thin layer silica gel. The compound was eluted with 95:5 hexane/ethyl acetate as a colorless oil. The yield was 91%. ^{1}H NMR ($CDCl_3$) δ 1.02 (tri, 3H, J = 7.5 Hz), 1.25 (d, 6H, J = 7.0 Hz), 2.13 (m, 2H), 2.59 (m, 2H), 2.69 (m, 2H), 2.87–3.00 (m, 10H), 3.08 (m, 1H), 5.36–5.45 (m, 10H), 5.53 (m, 2H,), 7.04 (m, 1H), 7.24 (m, 2H), 7.34 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 14.3, 20.5, 22.8,



Scheme 1. Chemical reactions for synthesis of 2,6-diisopropylphenol–docosahexaenoate and its analogues.



Scheme 2. Structure of 2,6-diisopropylphenol (analogues)–fatty acid conjugates.

22.9, 25.5, 25.6, 27.3, 34.2, 122.2, 126.2, 126.6, 127.0, 127.6, 127.8, 127.9, 128.0, 128.2, 128.3, 128.5, 129.7, 131.9, 140.0, 148.1, 171.6; Mass spectrum (ESI), *m*/*z* 447.3267 $(M+H)^+$ ($C_{31}H_{42}O_2 + H^+$ requires 447.3263).

2.2.4. Compound 4: 2-cyclohexylphenyl-docosahexaenoate (CHP–DHA)

The CHP–DHA conjugate was purified by chromatography on a preparative thin layer silica gel. The compound was eluted with 95:5 hexane/ethyl acetate as a colorless oil. The yield was 81%. ^{1}H NMR ($CDCl_3$) δ 0.99 (tri, 3H, J = 7.4 Hz), 1.31 (m, 2H), 1.39 (m, 4H), 1.80 (m, 5H), 2.09 (m, 2H), 2.58 (m, 2H), 2.67 (m, 2H), 2.83–2.91 (m, 10H), 5.32–5.45 (m, 10H), 5.50 (m, 2H,), 6.99 (m, 1H), 7.20 (m, 2H), 7.31 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 14.2, 20.5, 22.8, 25.5, 25.6, 26.1, 26.9, 29.6, 30.2, 33.2, 34.2, 37.7, 122.2, 126.1, 126.5, 126.9, 127.1, 127.5, 127.8, 127.9, 128.0, 128.2, 128.3, 128.5, 129.7, 132.0, 139.2, 148.1, 171.7; Mass spectrum (ESI), *m*/*z* 487.3758$(M+H)^+$ ($C_{34}H_{46}O_2$ requires 487.3751).

2.2.5. Compound 5: phenyl–docosahexaenoate (P–DHA)

The P–DHA conjugate was purified by chromatography on a preparative thin layer silica gel. The compound was eluted with 95:5 hexane/ethyl acetate as a colorless oil. The yield was 92%. ^{1}H NMR ($CDCl_3$) δ 0.98 (tri, 3H, J = 7.5 Hz), 2.08 (m, 2H), 2.55 (m, 2H), 2.64 (m, 2H), 2.82–2.86 (br., m, 10H), 5.30–5.40 (br, m, 10H), 5.48 (m, 2H,), 7.09 (m, 2H), 7.25 (m, 1H), 7.38 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.2, 20.5, 22.7, 25.5, 25.6, 34.2, 121.5, 124.8, 125.7, 126.9, 127.5, 127.8, 127.9, 128.0, 128.2, 128.3, 128.5, 129.3, 129.6, 132.0, 150.6, 171.5; Mass spectrum (ESI), *m*/*z* 404.2718 $(M+H)^+$ ($C_{28}H_{36}O_2 + H^+$ requires 404.2715).

2.2.6. Compound 6: 2,6-diisopropylphenyl–docosahexaenoamide (DIPA–DHA)

The DIPA–DHA conjugate was purified by chromatography on a preparative thin layer silica gel. The compound was eluted with 92:8 hexane/ethyl acetate as a colorless oil. The yield was 67%. ^{1}H NMR ($CDCl_3$) δ 0.98 (tri, 3H, J = 7.6 Hz), 1.19 (m, 12H), 2.09 (m, 2H), 2.48 (m, 2H), 2.52 (m, 2H), 2.82–2.89 (m, 10H), 3.05–3.08 (m, 2), 5.37–5.46 (m, 12H), 6.91(s, 1H), 7.18 (d, 2H, J = 7.7 Hz), 7.29 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 14.2, 20.5, 22.3, 22.5, 23.5, 23.6, 25.6, 25.4, 25.5, 25.6, 28.3, 28.6, 36.3, 123.3, 123.8, 126.9, 127.8, 127.9, 128.0, 128.1, 128.2, 128.5, 128.8, 129.0, 129.5, 131.1, 131.9, 146.2, 171.7; Mass spectrum (HR ESI), *m*/*z* 488.3888 $(M+H)^+$ ($C_{34}H_{49}O_2$ requires 488.3892).

2.2.7. Compound 7: 2-isopropylphenyl–docosahexaenoamide (IPA–DHA)

The IPA–DHA conjugate was purified by chromatography on a preparative thin layer silica gel. The compound was eluted with 92:8 hexane/ethyl acetate as a colorless oil. The yield was 66%. ^{1}H NMR ($CDCl_3$) δ 0.98 (tri, 3H, J = 7.5 Hz), 1.23 (d, 6H, J = 6.9 Hz), 2.08 (m, 2H), 2.45 (m, 2H), 2.51 (m, 2H), 2.81–2.87 (br., m, 10H), 3.01 (m, 1H), 5.31–5.41 (br, m, 10H), 5.46 (m, 2H), 7.18 (m, 3H), 7.28 (m, 1H), 7.61 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 14.2, 20.5, 23.0, 23.4, 25.4, 25.5, 27.8, 37.1, 125.1, 125.5, 126.1, 126.2, 126.9, 127.8, 127.9, 128.0, 128.2, 128.3, 128.5, 128.7, 129.6, 131.9, 133.9, 140.7, 171.0; Mass spectrum (HR ESI), *m*/*z* 446.3428 $(M+H)^+$ ($C_{31}H_{43}O_2$ requires 446.3423).

2.2.8. Compound 8: characterization of phenyl–docosahexaenoamide (A–DHA)

The A–DHA conjugate was purified by chromatography on a preparative thin layer silica gel. The compound was eluted with 90:10 hexane/ethyl acetate as a colorless oil. The yield was 90%. ^{1}H NMR ($CDCl_3$) δ 0.98 (tri, 3H, J = 7.5 Hz), 2.08 (m, 2H), 2.41 (m, 2H), 2.49 (m, 2H), 2.81–2.86 (br., m, 10H), 5.33–5.44 (br, m, 12H), 7.08 (m, 1H), 7.27 (m, 2H), 7.51 (m, 2H), 7.74 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.2, 20.5, 23.3, 25.5, 25.6, 37.3, 119.9, 124.1, 126.9, 127.8, 127.9, 128.0, 128.2, 128.3, 128.5, 128.8, 129.5, 131.9, 137.9, 170.9; Mass spectrum (HR ESI), *m*/*z* 404.2955 $(M+H)^+$ ($C_{28}H_{37}O_2$ requires 404.2953).

3. Results

3.1. Characterization of 2,6-diisopropylphenol–docosahexaenoate and its analogues

All the proton and carbon signals from both moiety of fatty acid and aromatic ring in the conjugate come up in the ^{1}H and ^{13}C NMR spectrum, and all the HR MS spectrum data show the target

conjugates were formed. Furthermore, when NMR signal in 2,6-diisopropylphenol–docosahexaenoate was compared with the parent compounds, the hydroxyl proton signal in 2,6-diisopropylphenol at about 4.82 ppm was disappeared, and the ^{13}C signal of the C-1 was shifted upfield from 149.9 ppm to 145.5 ppm, while its ^{13}C signal of the C-2 and C-6 were shifted downfield from 133.6 ppm to 140.2 ppm, indicating that the ester bond was formed at the C-1 hydroxyl group of the propofol.

3.2. Conjugation of 2,6-diisopropylphenol and its derivatives with DHA induce cellular toxicity to MDA-MB-231 breast cancer cells

Consistent with previous reports, MDA-MB-231 cell growth was inhibited by DHA in a dose-dependent manner (Fig. 1a), while significant cell loss was not observed at concentrations less than 25 μM. Figure 1b depicts the effect of the individual compounds on growth inhibition and cell loss. Only one compound, CHP, had a limited reduction in growth at the highest concentration screened (25 μM), which showed no indication of any cell loss. The effect of the DHA conjugates varied dramatically as shown in Figure 1c. Data points near "0" indicate overall viable cell activity neither increased nor decreased from treatment initiation; however, data points falling below 0 denote a loss of cell viability prior to treatment initiation. P–DHA conjugate demonstrated no significant deviation from the vehicle-treated cell population. In stark contrast, PP–DHA conjugate invoked remarkable growth inhibition and cell loss in a dose-dependent manner. IPP–DHA, DIPP–DHA and CHP–DHA conjugates were effective in inhibiting MDA-MB-231 cell growth and inducing cell loss in a dose-dependent manner; nonetheless, the effectiveness of these three conjugates was intermediate.

In order to determine if the conjugate effectiveness was simply a result of their hydrophobicity to permit more efficient cellular incorporation, we assessed the conjugates' ability to inhibit growth (IC_{50} and total growth inhibition) in relationship to their hydrophobicity values. A summary of these findings can be found in Table 1. If hydrophobicity alone was responsible for the dramatic effect on MDA-MB-231 cells, then the DIPP–DHA and CHP–DHA conjugates should mirror the observations produced upon PP–DHA conjugate-treated cells. Clearly, these findings are not consistent with this hypothesis.

We utilized this growth assay to determine if the decrease in MDA-MB-231 cell growth and the increase in cell cytotoxicity were a result of an additive effect of DHA in combination with either PP or IPA (Fig. 2). DHA, PP, IPP, and the combination of these compounds (5 μM each) resulted in no significant alterations in growth characteristics compared to vehicle treatment, while both PP–DHA and IPP–DHA conjugates (10 μM) displayed significant cell toxicity. This observation excludes the possibility of an enhanced DHA combinatorial effect on growth inhibition and cell cytotoxicity; furthermore, the DHA conjugates exert a novel mechanism thereby inhibiting MDA-MB-231 cell growth and inducing cytotoxicity.

In addition to inhibiting MDA-MB-231 cell growth, cell viability appeared to be significantly compromised in the DHA conjugate-treated cells. We investigated whether or not these treatments induced apoptosis by labeling the cells with Annexin V FLUOS in combination with propidium iodide to ascertain the extent of apoptosis and/or necrosis following 24 h of treatment. Healthy cells do not stain positive for Annexin V or incorporate propidium iodide into their nuclei. Apoptotic cells, which accumulate phosphatidylserine on the outer leaflet of the plasma membrane, do bind Annexin V in the presence of excess calcium. Propidium iodide serves as a discriminate to differentiate between apoptotic and necrotic cells. Table 2 summarizes the apoptotic classification of MDA-MB-231 cells following a 24 h treatment with the DHA

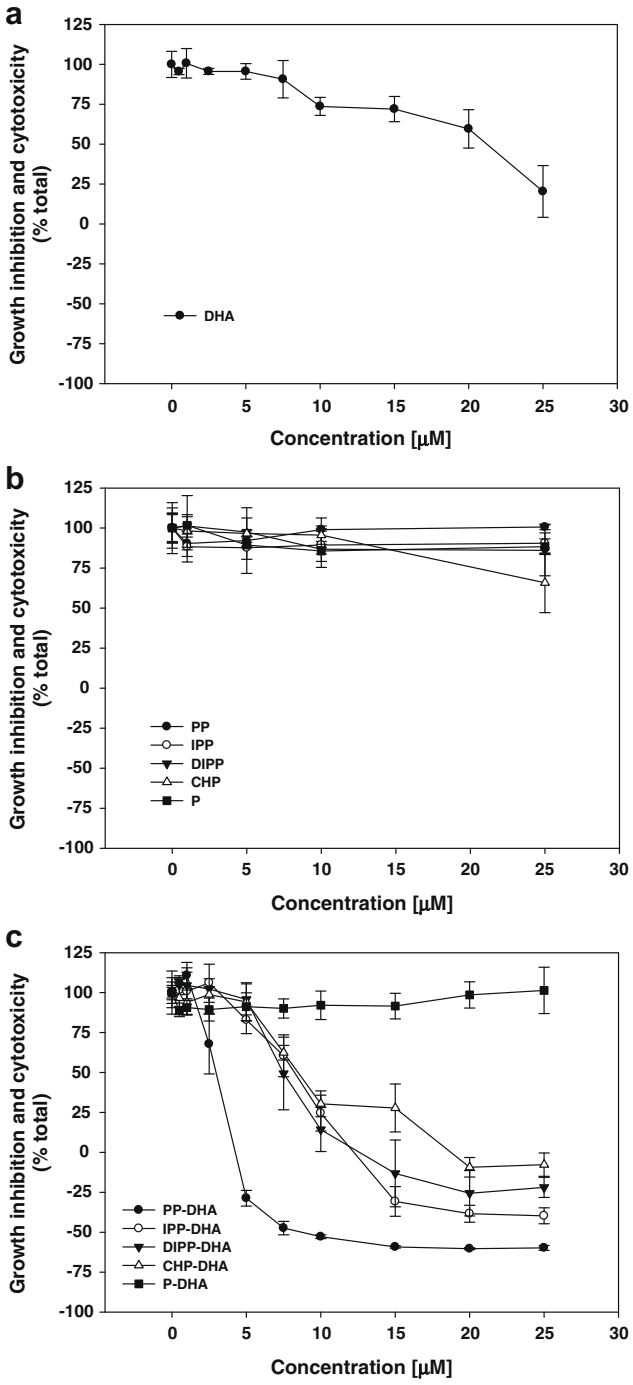


Figure 1. Conjugation of 2,6-diisopropylphenol and its derivatives with DHA induces cellular toxicity to MDA-MB-231 breast cancer cells. Breast cancer MDA-MB-231 cells (5000 cells/well) were cultured for 18 h in DMEM complete medium in 96 well flat bottom tissue culture plates to establish a linear growth rate. At this stage a subset of established wells were used to determine cellular growth (X) (baseline at day 0) by administering WST-1 reagent (10 μL/well), whereas medium in other wells was replaced with DMEM supplemented with 2% FBS and treatment conditions (100 μL total volume/well) for growth inhibition assessment. Cell cultures were maintained for 48 h at 37 °C in a humidified atmosphere containing 5% CO_2. WST-1 reagent was added and readings were used to calculate growth inhibition or cell loss based on baseline readings. Vehicle controls (ethanol) served as the non-treated control was used for total cell growth (Y). The cell growth (Z) was normalized to 100% using formula $[(Y - X/X) * 100]$. Effects of conjugates were determined in comparison to Z values. Values from 100 to 0 indicate cell growth inhibition whereas 0 to −100 indicates cell death. Results are expressed as mean ± SD for at least four experiments. Compounds tested included 2,6-diisopropylphenol–docosahexaenoate (PP–DHA), 2,4-isopropylphenol–docosahexaenoate (IPP–DHA), 2,4-diisopropylphenol–docosahexaenoate (DIPP–DHA), 2-cyclohexylphenol-docosahexaenoate (CHP) and phenol–docosahexaenoate (P–DHA).

Table 1
Relation of the hydrophobicity of the conjugates to growth inhibition of MDA-MB-231 cells

Conjugates	Hydrophobicity (Log *p*)	Growth inhibition (IC_{50}, μM)	Growth inhibition (total, μM)
PP–DHA	11.3 ± 0.6	2.9	4.2
DIPP–DHA	11.3 ± 0.6	7.3	12.6
CHP–DHA	11.1 ± 0.6	8.4	18.7
IPP–DHA	10.0 ± 0.6	8.2	12.1
P–DHA	8.6 ± 0.6	>25	>25

Hydrophobicity values (Log *p*) of the compounds were calculated using ChemDraw 10.0 (CambridgeSoft, Cambridge, MA, USA). Values for growth inhibition were derived from the data presented in Figure 1.

Table 2
Analysis of apoptotic and necrotic effects of the compounds

		Viable	Apoptotic	Necrotic
Vehicle (1% EtOH)		89.0 ± 1.0	1.8 ± 0.3	9.2 ± 0.8
DHA	1 μM	90.3 ± 2.2	2.1 ± 0.3	7.6 ± 1.9
	10 μM	87.6 ± 1.0	1.8 ± 0.4	10.7 ± 0.8
PP	1 μM	90.9 ± 1.6	2.0 ± 0.4	7.1 ± 1.3
	10 μM	88.2 ± 3.4	2.4 ± 0.6	9.4 ± 2.9
PP–DHA	1 μM	90.5 ± 1.6	1.8 ± 0.2	7.7 ± 1.4
	10 μM	37.4 ± 0.1*	22.2 ± 0.1*	40.4 ± 0.1*
IPP	1 μM	93.7 ± 1.1*	1.5 ± 0.7	4.9 ± 0.8*
	10 μM	88.8 ± 1.1	2.2 ± 0.3	9.0 ± 0.9
IPP–DHA	1 μM	89.6 ± 1.5	1.8 ± 0.3	8.6 ± 1.8
	10 μM	36.2 ± 0.6*	26.4 ± 1.1*	37.5 ± 0.5*

Cells (1.5×10^5) were treated with DHA, PP, IPP, PP–DHA and IPP–DHA as described in the legends of Figure 1. After treatment, cell pellets were resuspended in Annexin V FLUOS/Propidium iodide labeling solution, which was prepared as described by the manufacturer. Cell analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with an air-cooled argon laser emitting at a 488 nm wavelength. Fluorescence was detected through a 530 nm band pass filter and quantified using CellQuest Software (Becton Dickinson, San Jose, CA).

conjugates or the individual compounds. DHA, PP, and IPP demonstrated very limited variation in the percentage of apoptotic and necrotic cells in comparison to vehicle-treated cells. Both PP–DHA and IPP–DHA conjugates (10 μM) significantly induced a population shift by a majority of the cells into apoptosis and necrosis within the 24 h treatment. This confirms not only are the MDA-MB-231 cells growth inhibited by 10 μM DHA conjugate treatment, cell loss is emanate within 24 h of treatment.

3.3. Propylphenolic–DHA conjugates are stable during treatment to MDA-MB-231 cells

In order to check the stability of the conjugates in blood, we incubated the conjugates with human and mouse serum at 37 °C for 2 h. Presence of DHA and 2,6-diisopropylphenol from the hydrolysis of conjugates were then determined in the lipid extract by HPLC. When comparing the retention time of the DHA and the conjugates with the corresponding reference standard, there were no detectable DHA or 2,6-diiospropylphenol peaks from PP–DHA or IPP–DHA in both mouse and human serum (Supplementary data).

In order to further insure that the PP–DHA is not readily hydrolyzed, we synthesized DHA conjugates using aniline-derivatives of PP, IPP and P. The aniline-conjugates 2,6-diisopropyl aniline–DHA (DIPA–DHA), 2-isopropyl aniline–DHA (IPA–DHA) and aniline–DHA (A–DHA) responded similarly to their phenolic-ester counterparts in MDA-MB-231 growth inhibition and cell loss (Fig. 3). DIPA–DHA conjugate was the most effective amide conjugate, which inhibited 50% of the cell growth at 3.7 μM and completely inhibited cell growth at 5.5 μM. IPA–DHA conjugate significantly impaired cell growth of MDA-MB-231 cells as well (IC_{50}—6.5 μM and total growth inhibition—8.5 μM). Importantly, the A–DHA conjugate did not induce a growth inhibitory effect. Clearly, these non-hydrolyzable aniline-conjugates closely mimicked their phenolic-ester counterparts, which further suggest a certain degree of stability within the conjugates.

3.4. Effect of propylphenolic–DHA conjugates on different breast cancer cell lines

As an extension of the findings with the MDA-MB-231 (ER-negative, Her-2 negative) cell line, representative breast cancer cell lines, based on estrogen α receptor (ER) and oncogene Her-2 expression, were chosen to determine the efficacy of the DHA conjugates, PP–DHA and IPP–DHA, in comparison to the individual

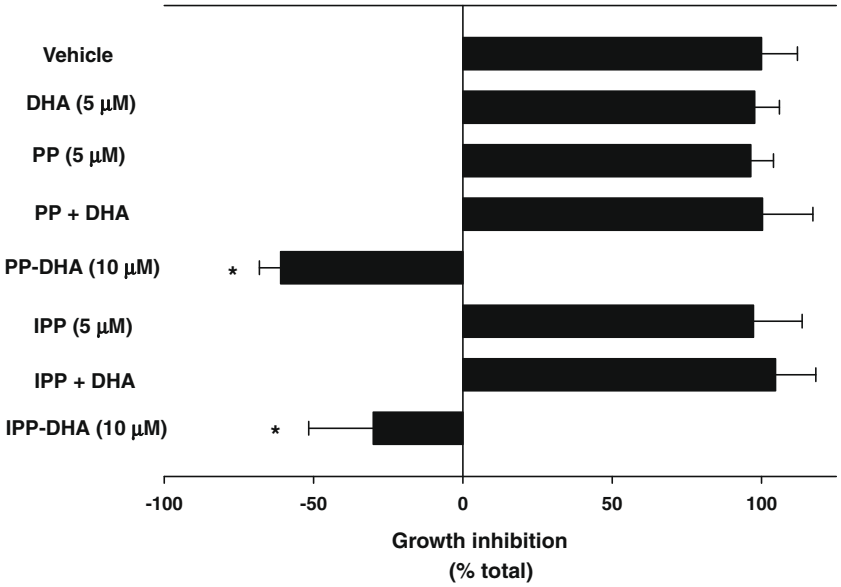


Figure 2. Propylphenolic and DHA compounds alone or combined are less potent then conjugated propylphenolic–DHA derivatives. Effect of DHA, PP, IPP alone and in combination along with PP–DHA and IPP–DHA conjugate were determined on breast cancer MDA-MB-231 cells are as described in the legend of Figure 1. Results are expressed as the mean ± SD four experiments and analyzed using Student *t* tests. An '*' represents significance at $p < 0.05$.

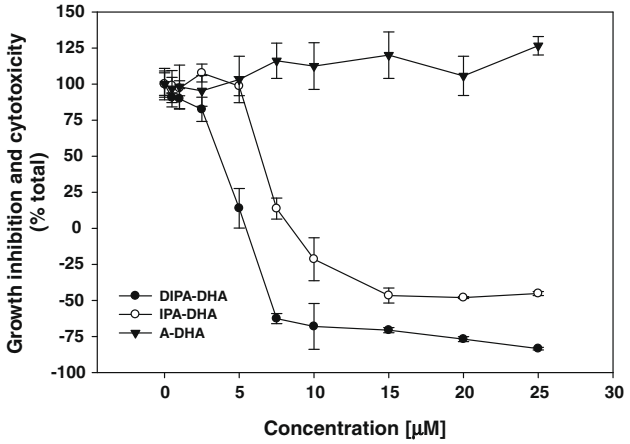


Figure 3. Non-hydrolyzable propylphenolic–DHA conjugates induce similar effects on MDA-MB-231cells. The amide derivatives of PP, IPP and P were used to synthesize 2,6-diisopropyl aniline–DHA (DIPA–DHA), 2-isopropyl aniline–DHA (IPA–DHA) and aniline–DHA (A–DHA). Growth inhibition effect on MDA-MB-231 cells was determined as described in the legends of Figure 1. Results are expressed as the mean ± SD of four experiments.

compounds in terms of inhibiting growth and/or invoking cell loss. Importantly, primary normal human mammary epithelial cells (HMEC) were utilized as a control to insure specificity of the DHA conjugate cancer cell inhibition. Figure 4 is a compilation of the growth inhibition and cytotoxicity assay results for (a) MCF-7 (ER-positive, Her-2 negative); (b) MDA-MB-361 (ER-positive, Her-2 positive); (c) AU565 (ER-negative, Her-2 positive); (d) HMEC. The individual compounds, PP and IPP, did not dramatically effect the growth of any cell line or provide any evidence of cytotoxicity at concentrations up to 25 μM. Conversely, the DHA conjugates, PP–DHA and IPP–DHA, significantly inhibited the growth of MCF-7, MDA-MB-361 and AU565 cell lines and demonstrated signs of cytotoxicity within each cell line. While both DHA conjugates were effective, the PP–DHA conjugate was more efficient in all breast cancer cell lines tested. Notably, the normal epithelial cells were virtually unaffected by the individual compounds or the DHA conjugates at concentrations up to 25 μM.

3.5. Fatty acid analysis of cells-treated with DHA conjugates

In order to further evaluate if DHA is hydrolyzed from conjugates, we determined the concentration of DHA in MDA-MB-231 cells after treating them with DHA, PP–DHA or DIPA–DHA conjugates alone or DHA with PP–DHA or DIPA–DHA conjugates. The results shown in Figure 5a,b indicate that the similar concentration of DHA was found in cell on either DHA alone or DHA + PP–DHA or DIPA–DHA treatments. Furthermore, the concentration of DHA in cell does not increase over controls when cells were treated with conjugates alone.

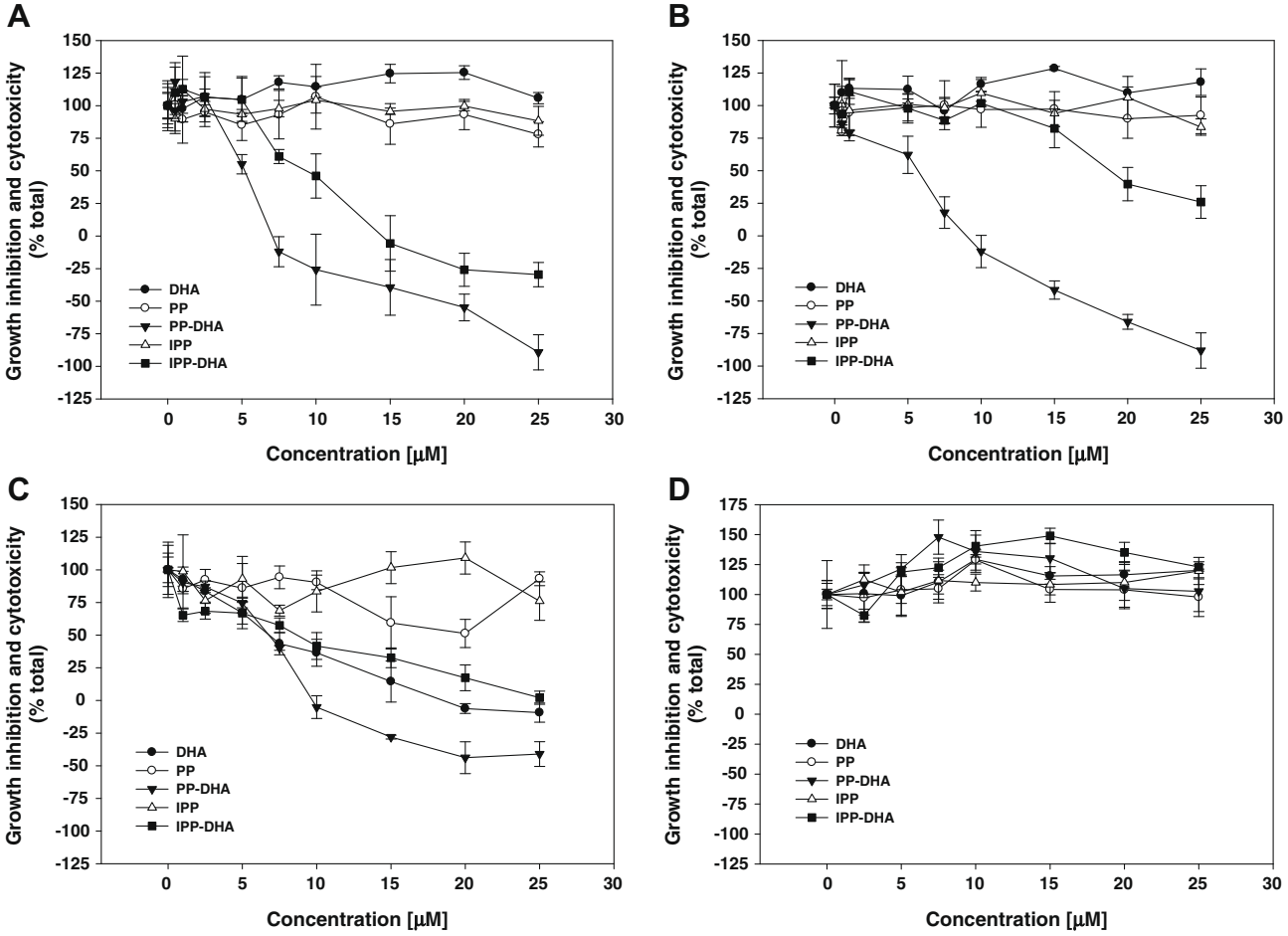


Figure 4. Effect of propylphenolic–DHA conjugates on different breast cancer cell lines. PP–DHA and IPP–DHA conjugates along with free DHA, PP and IPP were tested on (A) MCF-7, (B) MDA-MB-361, (C) AU565, and (D) normal mammary epithelial cells as described in the Figure 1 legend. Results are expressed as the mean ± SD of four experiments.

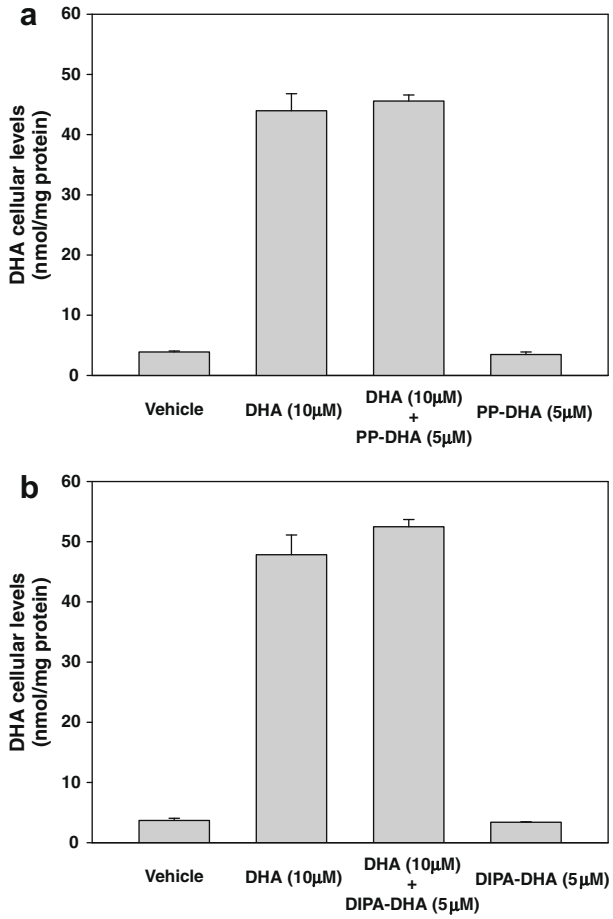


Figure 5. Concentration of DHA in cells on conjugate treatment. Effect on DHA concentrations in cell after treatment with (a) PP–DHA or (b) DIPA–DHA conjugates were determined by treating cells vehicle alone, DHA (10 μM) or DHA (10 μM) + PP–DHA (5 μM), or DHA (10 μM) + DIPA–DHA (5 μM) along with PP–DHA (5 μM), and DIPA–DHA (5 μM) as described in the legend of Figure 1. Lipids were extracted and fatty acids concentrations were determined as methyl ester by Gas Chromatography. Results are expressed as the mean ± SD of four experiments.

3.6. 2,6-Diisopropylphenol–DHA conjugate targets histone deacetylase activity

To determine the effect of DHA, PP, and PP–DHA conjugate-treated MDA-MB-231 cell lysates on HDAC activity, we employed a non-specific HDAC assay kit to measure the overall effect of these compounds in respect to total HDAC activity. The PP–DHA conjugate inhibited HDAC activity in a dose-dependent manner (Fig. 6). Maximum HDAC inhibition was observed between 10–25 μM, which depleted HDAC activity by approximately 30% compared to vehicle-treated samples. Neither DHA nor PP alone inhibited total HDAC activity at the highest 25 μM concentration, suggesting conjugate specificity.

4. Discussion

The epidemiological studies, animal studies and cell culture experiments strongly suggest that omega-3 polyunsaturated fatty acids (n-3 PUFAs), namely docosahexaenoic acid and eicosapentaenoic acid, possess anticancer properties. A dose of 2–6 g of n-3 PUFAs/day is recommended using for their cancer effects. This amount is achievable by taking 4–12 fish oil capsules/day. Although this is achievable, taking high amounts of fish oil may lead to some undesirable effects including fishy burps and sometimes diarrhea. Previous attempts have been made to increase bio-efficacy of n-3 PUFAs by making their ethyl esters. These fatty acid esters are effective against cardiovascular diseases[18] and have been commercially marketed under various brand names. We designed n-3 PUFAs-esters with phenolic derivatives. Our previous study indicates that phenolic esters are far more efficacious than free n-3 PUFAs against breast cancer. The present study is an elaboration of our previous work to demonstrate stabilities and cellular effects of various analogues of phenolic–DHA conjugates.

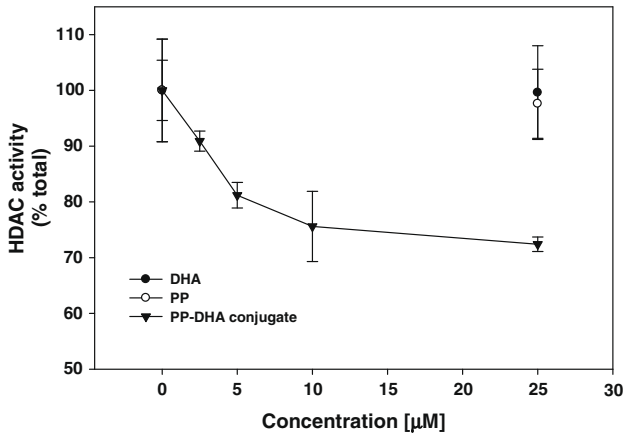


Figure 6. 2,6-Diisopropylphenol–DHA target histone deacetylase activity. MDA-MB-231 breast cancer cells were treated for 2 h at 37 °C with varying concentrations of the PP–DHA conjugate, DHA alone or PP alone in DMEM supplemented with 2% FBS. The histone deacetylase (HDAC) activity in the cell lysates was performed as described by the manufacturer. An inhibitor of HDAC activity, trichostatin A, was utilized to determine maximum inhibition in order to adjust for non-specific assay readings. Data was expressed as the percentage of total HDAC activity.

We conjugated phenolic analogues with DHA using both ester and amide bonds. The esterified phenolic–DHA conjugate include PP–DHA, DIPP–DHA, CHP–DHA, IPP–DHA and P–DHA, whereas the amidified conjugates include DIPA–DHA, IPA–DHA and A–DHA. These conjugates were positively identified and characterized using ^{1}H and ^{13}C NMR and HR MS spectrum. We assayed the effect of these conjugates on growth inhibition and cell death. The effect of phenolic–DHA conjugates was similar in different breast cancer cell lines irrespective of their expression of ER and Her2; however, the conjugate was not effective against normal mammary epithelial cells. Our data demonstrate that the effect of various phenolic–conjugates were in PP–DHA > IPP–DHA > DIPP–DHA > CHP–DHA >> P–DHA order. This rank order of their effect was not correlated with their hydophobicity values suggesting that PP–DHA simply did not have greater access to pass through the cell membrane as compared to other analogues. PP–DHA conjugate remarkably affected growth inhibition and cell loss in a dose-dependent manner, whereas the effectiveness of IPP–DHA, DIPP–DHA and CHP–DHA conjugates was intermediate and P–DHA was not effective. This implies that these conjugates are variably stable in media and hydrolyzed by serum esterases. PP–DHA was most stable because of the structural hindrance due to the presence of two bulky propyl groups around the ester linkages. IPP–DHA, DIPP–DHA, and CHP have only one bulky group around the ester linkage and are therefore relatively less stable. P–DHA has no protective group around the ester linkage and therefore was quickly cleaved off and was not effective. Since we plan to use these conjugates for in vivo studies in mice and eventually for clinical trials, we tested the stability of these compounds in mouse and human serum. Our data demonstrated that both PP–DHA as well as IPP–DHA conjugates were stable in both serum as evident by the absence of 2,6-diisopropylphenol and DHA peaks. We further confirmed the effect of phenolic–DHA conjugates on

cellular growth by testing DIPA–DHA, IPA–DHA and A–DHA, which are conjugated through amide linkages and represent non-hydrolyzable analogues of PP–DHA, IPP–DHA, and P–DHA, respectively. The effect of non-hydrolyzable amide-linked conjugates is remarkably similar to the ester-linked conjugates. This data clearly suggest that ester-linked phenolic–DHA conjugates are as stable as non-hydrolyzable amide-linked aniline–DHA conjugates. Stability of the conjugates was further tested by analyzing the concentration of DHA in cells. It is clear from data in Figure 5a,b that concentration of DHA in cell does not increase over controls when cells were treated with conjugates alone, as well as similar concentrations of DHA was found in cell on either DHA alone or DHA + PP–DHA or DIPA–DHA treatments. These observations suggest that both ester and amide-linked conjugates were non-hydrolyable in cells. Furthermore, this data suggest that the presence of side chains around ester or amide linkage may have resulted in structural hindrance and played an important part in their cellular effects. Compounds are most effective when both side chains are present. Removal of one side chain decreases their effectiveness and the removal of both side chains results in loss of their activity. It is possible that these side chains play an essential role in binding of these conjugates to the target molecule.

Next we looked for the possible target molecule for PP–DHA conjugate cellular effects. DHA and other fatty acids binding to nuclear receptors such as peroxisome proliferator-activated receptor (PPAR) and retinoid X receptors (RXRs) have been reported.[19] DHA is a general ligand of PPARs, but binds more selectively to RXR transcription factors. RXRs form homo- or heterodimers with PPAR and other nuclear hormone receptor super-families that include receptors for steroids, thyroid hormones, retinoic acid and vitamin D. In order to elucidate a role of PPARs effect in PP–DHA cellular effects, we used GW9662 as an inhibitor for PPARα and PPARγ. However, our results clearly demonstrated that PP–DHA activates neither PPARα nor PPARγ (data not shown). Furthermore, PP–DHA appears to have structural resemblance to trichostatin A (TSA), which selectively inhibits the class I and II mammalian histone deacetylase (HDAC) families of enzymes.[20] Our data demonstrated that PP–DHA conjugate 10–25 μm concentration partially inhibited HDAC activity (30%) whereas PP or DHA alone has no effect. This observation suggests that inhibition of HDAC activity is perhaps one possible contributory mechanism of action of these conjugates. Further experimentation is needed to elucidate the mechanism of action of these potent anticancer phenolic conjugates.

In conclusion, our results demonstrated that PP–DHA or DIPA–DHA conjugates effectively inhibited breast cancer growth irrespective of their ER or Her-2 expression. The presence of both side chains is essential for their maximum effects of these conjugates. Removal of one side chain results in reduction in their effects; whereas, complete removal of both side chains results in loss of their effects. Inhibition of HDAC activity may be one possible mechanism through which PP–DHA exerts its effects on breast cancer cells.

5. Experimental

5.1. Materials

All disposable tissue culture materials were obtained from Fisher Scientific (Pittsburgh, PA). Dulbecco's Modified Eagle's Medium (DMEM) and tissue culture supplements were purchased from Invitrogen Corporation (Carlsbad, CA). Normal human mammary epithelial cells (HMEC) and mammary epithelial basal medium bullet kits were acquired from Lonza Incorporation (Walkersville, MD). Breast cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). Docosahexaenoic acid was obtained from Nu-Chek Prep Incorporated (Elysian, MN). The cell proliferation reagent, WST-1, and the Annexin V FLUOS staining kits were purchased from Roche Applied Science (Indianapolis, IN). Histone deacetylation fluorometric cellular activity assay kits were purchased from BioVision (Mountain View, CA). Additional reagents were obtained from Sigma–Aldrich (St. Louis, MO), unless otherwise noted.

5.2. Cell culture maintenance

Human mammary epithelial cells (HMEC) were maintained in mammary epithelial basal media supplemented with bullet kit materials as specified by the manufacturer. All breast cancer cell lines were maintained in DMEM containing 5% fetal bovine serum (FBS) and a 1% antibiotic-antimycotic solution. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

5.3. Growth inhibition and toxicity assay

Epithelial cells (5000 cells/well) were cultured overnight in DMEM complete medium in 96 well flat bottom tissue culture plates to establish a linear growth rate. Spent medium was replaced with DMEM supplemented with 2% FBS and treatment conditions (100 μL total volume/well) upon initiation of the growth inhibition assessment. Vehicle controls (ethanol) served as the non-treated total growth potential. To obtain baseline values and an initial cell viability assessment, a subset of established wells were used for monitoring cellular growth using WST-1 assay as recommended by the manufacturer (Roche Applied Science, Indianapolis, IN). The cell cultures treated with different phenolic-fatty acids conjugates were continued to maintain for 48 h at 37 °C in a humidified atmosphere containing 5% CO_2. At the end of the incubation period, cellular growth (inhibition or cell loss) was determined using WST-1 assay reagent as described previously.[21]

5.4. Apoptosis detection

Cells (1.5×10^5) were plated in complete DMEM in six well tissue culture treated plates overnight to initiate a linear growth rate. Spent media was replaced with 3 mL of DMEM supplemented with 2% FBS with varying doses of DHA, 2,6-diisopropylphenol (PP), and the PP–DHA conjugate. An equal volume of ethanol served as the vehicle control. At the conclusion of the treatments, the cells were rinsed in phosphate buffered saline (PBS) and trypsinized from the wells. All spent medium, PBS washes and trypsinized cells were collected and combined to harvest both adherent and non-adherent cells for analysis. Cell pellets were resuspended in Annexin V FLUOS/Propidium iodide labeling solution, which was prepared as described by the manufacturer. Cell suspensions were labeled in the dark at room temperature for 20 min. Analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with an air-cooled argon laser emitting at a 488 nm wavelength. Fluorescence was detected through a 530 nm band pass filter and quantified using CellQuest Software (Becton Dickinson, San Jose, CA).

5.5. Determination of phenolic conjugate stabilities in serum

The phenolic–fatty acid conjugates (1 mM) suspended in ethanol were mixed with human and mouse serum (1 ml) and incubated at 37 °C for 2 h. At the end of incubation period, the serum was extracted using Folch method[22] and the dried lipid extracts were suspended in 200μL ethanol for HPLC analysis. The HPLC analysis was performed with reversed-phase high-performance liquid chromatography (RP-HPLC) method by using a Agilent 1100 HPLC system equipped with an Agilent 1100 Diode-Array detector, an Agilent 1100 Auto Sampler system and an Agilent ZORBAX SB-C18 column

(4.6 × 250 mm, 5 μm particle size), with an injection volume of 20 μL and a flow rate of 1.5 mL/min. The mobile phase is a mixture of water containing 0.01% trifluoroacetic acid (solvent A), acetonitrile containing 0.01% trifluoroacetic acid (solvent B). The elution gradient was from 80% (B), to 100% (B) in 40 min, followed by 100% (B) for 10 min to clean the column. The chromatograms were detected at 214 nm.

5.6. Histone deacetylase activity assay

MDA-MB-231 breast cancer cells were treated for 2 h at 37 °C with varying concentrations of the PP–DHA conjugate, DHA alone or PP alone in DMEM supplemented with 2% FBS. Spent media was removed and the cells were rinsed in PBS and harvested by trypsinization. Samples were normalized to 2×10^6 cells/mL in PBS containing 0.1% Triton X-100. Samples were kept on ice and sonicated to insure cell lysis. The HDAC activity assay was performed as described by the manufacturer. An inhibitor of HDAC activity, trichostatin A, was utilized to determine maximum inhibition in order to adjust for non-specific assay readings. Data was expressed as the percentage of total HDAC activity.

5.7. Lipid composition analysis

The effect of PP–DHA and DIPA–DHA conjugates on cellular lipid composition was determined using gas chromatography (GC). Briefly, MDA-MB-231 breast cancer cells were treated with DHA + conjugates and DHA or conjugate alone for 24 h. Cells were extracted using Folch method[22] and the fatty acid the extract were methylated using boron trifluoride-methanol solution as described.[23] The fatty acid methyl esters were analyzed on a gas chromatography system (Shimadzu GC2010) equipped with a Zebron ZB-WAX plus column (100 m, 0.25 mm ID, 0.25 μm film thickness). The oven temperature was programmed from 30 °C (2 min hold) to 180 °C @ 20 °C/min (2 min hold), then to 207 °C @ 4 °C/min (3 min hold), then to 220 °C @ 2 °C/min (2 min hold), and finally to 240 °C @ 2 °C/min (2 min hold). Detection was performed with a FID @ 250 °C to resolve fatty acids peaks, which were identified using authentic standards (Restek Corp., Bellefonte, PA). Data was analyzed with Shimadzu's GC solutions software.

5.8. Data analysis

Results are expressed as the mean ± SD four experiments and analyzed using Student *t* tests. An '*' represents significance at $p < 0.05$.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.01.045.

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