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Targeting triple negative human breast cancer with omega-3 docosahexaenoic acid (DHA) and tocotrienol

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by

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Dedication

This dissertation is dedicated to my parents, my husband and two lovely sons for their unconditional love, support and encouragements.

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My greatest thanks to my supervisors Dr. Kimberly Kline and Dr. Bob Sanders for their giving me this valuable opportunity to work in their lab. They always give me the strongest encouragement, support, help and understanding in my research and life! At this point, I thought of the long road of life in graduate school as ships sailing in the ocean. It will not always be smooth, bright and there are flowers, thorns, laughter and tears. They are like a lamp and "Hope" to direct me along the road, light my life and lead me into the ideal and happiness. I also want to sincerely thank all of my dissertation committee members: Dr. Christopher Jolly, Dr. Susan Fischer and Dr. Linda deGraffenried for their timely and helpful suggestions and support. Special thanks to Dr. Weiping Yu for her wonderful friendship in these years and mentorship in these projects. She is always the one who stands by me unwaveringly, shares up and downs in our life and gives me great help and encouragements. My thanks also extend to previous members in Drs. Kline and Sanders laboratories Archana, Teddy, Marla, Richa, Na and Wenbin Chen for their help in research. I am grateful for my parents-in-law for their unconditional support and help take care of two kids these years. Great great thanks to my parents for their love and they are always smiling to me in my heart and give me the greatest support no matter where they are. Last, I always appreciate the support from my husband and two lovely sons. They are my inspiration and I couldnot walk so long on this way without them.

Targeting triple negative human breast cancer with omega-3 docosahexaenoic acid (DHA) and tocotrienol

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Triple negative breast cancers (TNBCs) account for ~15-20% of human breast cancers in Western countries. TNBCs are associated with poor prognosis and a low five year survival rate due, in part, to high rates of tumor recurrence, multi-drug resistance, metastasis, and therapeutic toxicity. Tumor initiating cells (TICs) or cancer stem cells (CSCs) are proposed to be responsible for the origin and maintenance of tumors as well as cancer recurrence, metastasis and drug resistance. Nutritionally-based low- to non-toxic therapeutic nutrients that eliminate both bulk tumor cells (non-TICs) and TICs have potential for prevention and treatment of primary and metastatic cancers, including TNBCs. Omega-3 fatty acid-docosahexaenoic acid (DHA) and certain vitamin E compounds [gamma- and delta- tocopherols (γT and δT) and tocotrienols (γT3 and δT3)], separately and in combination, were investigated for their ability to eliminate non-TICs and TICs in human TNBCs and the mechanisms of action were studied. DHA induced apoptosis in several human TNBC cell lines via activation

of endoplasmic-reticulum stress (ER stress) mediated C/EBP (CCAAT/enhancer binding protein) homologous protein (CHOP)/death receptor-5 (DR5) pro-apoptotic signaling involving caspases-8 and 9. DHA eliminated TICs as measured by elimination of aldehyde dehydrogenase active ($ALDH^+$) population and inhibition of mammosphere formation. DHA eliminated TICs via suppression of phosphorylated Signal transducers and activators of transcription 3 (pStat-3) as well as downstream mediators cellular myelocytomatosis oncogene (c-Myc) and cyclin D1. SiRNA to Stat-3 reduced the number of $ALDH^+$ TNBCs cells and reduced pStat-3, c-Myc, and cyclin D1 mediators, showing that Stat-3 is necessary for maintaining $ALDH^+$ population and that c-Myc and cyclin D1 are downstream mediators of Stat-3. Studies also demonstrated that vitamin E compounds possess distinct anticancer activities. For example, RRR-α-tocopherol (αT) and synthetic vitamin E (*all-rac*-αT*)* did not exhibit pro-apoptotic properties, but instead increased numbers of TICs as determined by increased $ALDH^+$ population and enhanced numbers of mammospheres, as well as induced increased, rather than decreased, Stat-3 signaling; whereas, γT, δT, γT3 and δT3 exhibited both pro-apoptotic and anti-TIC properties. In summary, studies provide novel insights into therapeutic potential of DHA and certain vitamin E compounds for treatment of TNBCs.

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Abbreviation

ALA	Alpha-linolenic acid
ALDH+	Aldehyde dehydrogenase active
all-rac-αT	*All-racemic-alpha*-tocopherol
AML	Acute myeloid leukaemia
ASK1	Apoptosis-signal-regulating kinase 1
αT	RRR-alpha-tocopherol
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
α-TTP	Alpha-tocopherol transfer protein
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
CAD	Caspase-activated DNase
CD44	Cluster of differentiation 44
cFLIP	Cellular FADD (Fas-Associated Death Domain Protein) like interleukin-1beta-converting enzyme) –inhibitory protein
CHOP	C/EBP(CCAAT/enhancer binding protein) homologous protein
c-Myc	Cellular myelocytomatosis oncogene
COX	Cyclooxygenases

CSCs	Cancer stem cells
DHA	Docosahexaenoic acid
DISC	Death-inducing signal complex
DR5	Death receptor-5
δT	Delta- tocopherol
δT3	Delta- tocotrienol
EMT	Epithelial–mesenchymal transition
ER	Estrogen receptor
ER stress	Endoplasmic-reticulum stress
ESA	Epithelial surface antigen
FACS	Fluorescence-activated cell sorting
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GRP78	Chaperone glucose regulated protein
γT	Gamma tocopherol
γT3	Gamma tocotrienol
HER2	Human Epidermal Growth Factor Receptor 2
HUFAs	Highly unsaturated fatty acids
IRE1	Inositol-requiring enzyme 1
JNK	c-Jun N-terminal kinase
LA	Linoleic acid
LOX	Lipoxygenases

NAC	N-acetyl cysteine
NOD/SCID	Non-obese diabetic mice with severe combined immunodeficiency
PARP	Poly (adenosine diphosphate-ribose) polymerase
PERK	Type 1 transmembrane Endoplasmic Reticulum-resident protein kinase
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor
pStat-3	phosphorylated Signal transducers and activators of transcription 3
PUFA	polyunsaturated fatty acids
siRNAs	Small interfering ribonucleic acids
SHP-1	sarcoma (Src) homology phosphatase-1
TICs	Tumor initiating cells
TNBCs	Triple negative breast cancers
TRAF2	Receptor-associated factor-2
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
VEH	Vehicle control
VLDL	Very low density lipoproteins

XBP-1	X-box binding protein 1
XIAP	X-linked inhibitor of apoptosis protein

Chapter 1 Introduction

1.1 Triple negative breast cancer (TNBC)

Breast cancer is the most common type of cancer (excluding skin cancers) and the second leading cause of cancer death in American women [1]. 234,580 new cases and 40,030 deaths from breast cancer are estimated in the United States for 2013 [1].

Breast cancer is a heterogeneous disease which is divided into 6 subtypes [2]: i) luminal subtype A [estrogen receptor+ (ER+) and/or progesterone receptor (PR+), Human Epidermal Growth Factor Receptor 2 (HER2-)]; ii) luminal subtype B (ER+ and/or PR+, HER2+), iii) HER2+ subtype (ER-, PR-, HER2+); iv) normal like subtype (similar expression pattern to normal breast tissue); v) basal like subtype (ER-, PR-, HER2-), also called triple negative breast cancer (TNBC) and vi) claudin Low subtype (ER-, PR-, HER2-, claudin-, E-cadherin-, high expression of Epithelial–mesenchymal transition (EMT) [3]. Current treatments are surgery and radiation accompanied with chemotherapies and/or targeted therapies as an adjuvant. The treatments are effective at the beginning of therapy in 90% of primary breast cancers; however, tumor recurrence which is commonly associated with metastasis and drug resistance may occur [4]. Secondary tumors and some breast cancer subtypes such as TNBC remain challenges for curing breast cancer.

ER and HER2 levels are used as predictive markers to select specific targeted therapies [5]. TNBC, classified within the basal like breast cancer subtype [6], is defined

by a lack of expression of both estrogen and progesterone receptors as well as human epidermal growth factor receptor 2 [6]. TNBC has a poor prognosis with a low 5-year survival rate [6] due to its aggressive clinical course and lack of specifically targeted therapy. It comprises 15-20% human breast cancer in Western countries [6]. Due to lack of molecular targets, chemotherapy remains the only choice for these patients [6]. DNA damaging drugs such as doxorubicin, cisplatin and taxol are used as standards-of-care. Although TNBC has been reported to be sensitive to chemotherapy initially, prognosis for this subtype of breast cancer remains poor due to tumor recurrence, drug resistance and chemotherapy toxicity [7, 8]. Therefore, novel therapies are badly needed for this type of cancer.

1.2 Tumor initiating cells (TICs)/cancer stem cell (CSCs)

TICs/CSCs are hypothesized to be derived from a small population of stem cells or early progenitor cells in tumor tissue, and are capable of self-renewal and differentiation as well as tumorigenesis, metastasis and drug resistance [9]. This special population of cancer cells is thought to be responsible for the origin and maintenance of tumors. The first evidence to support the TIC concept came from the human leukemia studies by Bonnet and Dick [10]. They isolated a specific small population of cells from acute myeloid leukaemia (AML) patients and showed that this population could initiate AML following injection into non-obese diabetic mice with severe combined

immunodeficiency (NOD/SCID). M.S. Wicha and co-workers isolated a population of cells using fluorescence-activated cell sorting (FACS) based on the phenotype epithelial surface antigen (ESA+)/ Cluster of Differentiation 44 (CD44+)/ Cluster of Differentiation 24 (CD24-) from primary human breast tumors and found that less than 200 cells formed tumors 100% of the time when injected into NOD/SCID mice; whereas, 10,000 unsorted cells achieved 25% tumor take [11]. These studies support the concept that a small population of tumor cells (TICs/CSCs) is responsible for development, maintenance and differentiation into heterogeneous "bulk" tumor cells in several cancer types, including breast cancer.

Although chemotherapy and radiotherapy reduce breast tumor bulk (non-TIC), the majority of patients receiving such treatments develop tumor relapse or distant metastatic tumors [12]. Local tumor relapse and distant metastases following treatment are thought to be associated with breast TICs that are resistant to these treatments. TICs have two important features that contribute to tumor progression: First, TICs have acquired EMT for enhanced metastatic capacity, and second they are resistant to chemotherapeutic drugs and radiation therapy. Accumulating evidence supports the notion that both chemotherapy and radiotherapy only kill bulk cancer cells (non-TICs), thereby enriching TICs [9], thus, potentially promoting tumor recurrence, drug resistance and metastasis. Numbers of TICs, based on ESA^{+} /$CD44^{+}$/$CD24^{-}$ staining, were reported to increase 30 fold following treatment with Taxol or 5-flurouracil *in vitro* using human breast cancer cell lines (SUM 159 and MDA-MB-231 cells) [13]. In addition, Ginestier and co-workers

reported that docetaxel treatment increased the number of ALDH+ population, an established TIC marker, in xenograft mouse models of human breast cancer when compared to control mice [14]. Such data may explain, at least partially, how chemo-resistance or radio-resistance is developed [16]. Since many cancer therapies target only non-TICs that make up the bulk of a tumor, tragically the outcome of treatments is tumor relapse [9]. Thus, development of anticancer agents that can target both non-TICs and TICs provide promise of being an effective strategy for prevention and treatment of breast tumors.

Rapid advances in our understanding of TICs, including development of technologies for identifying TICs, are currently being made. The method of serial transplantation of a small number of sorted TIC-enriched human breast tumor cells to form tumors in NOD/SCID mice serves as an *in vivo* "gold standard" for showing the presence of TICs. Although there are no universal cell surface markers for the identification of TICs, which is a major limitation for TIC studies, several breast TIC molecular markers have been identified, including $ESA^{+}/CD44^{+}/CD24^{-/low}$, intracellular enzyme-ALDH+, and $ALDH+/CD44^{+}/CD24^{-/low}$. Markers $ESA^{+}/CD44^{+}/CD24^{-/low}$ have been used by Al-Hajj et al. and others to identify TICs from primary breast tumors and human breast cancer cell lines [15, 16 and 17]. Increased $ALDH^{+}$ has been associated with TICs in breast as well as other cancer types [14]. ALDH positive activity was shown to be expressed in 577 human breast carcinomas and to serve as a stem/progenitor cell marker *in vitro* and *in vivo* in certain breast cancer cell lines including SUM 159,

SUM 149, and MDA-MB-453 breast cancer cells [18]. The $ALDH^{+}/CD44^{+}/CD24^{-/low}$ population has been found to have TIC properties in MDA-MB-231 breast cancer cells [19]. The unique feature of TICs grown in non-differentiation media to form tumor spheres provides an *in vitro* mammosphere formation assay for identification of breast TICs. Cells isolated from mammospheres were shown to be more stem-like based on their epithelial-like morphology and expression of marker cytokeratin14 (CK14) which is associated with myoepithelial cells, and expression of cytokeratin18 (CK18), a marker for luminal cells when grown in differentiating conditions but not expressed in non-differentiating conditions [15]. TICs isolated from mammospheres also have been shown to express the self-renewal undifferentiated stem cell marker octamer-binding transcription factor 4 (Oct-4), and to generate tumors when as few as 1,000 cells were transplanted into the mammary fat pad of SCID mice [15]. Data reported in this dissertation used an ALDH activity assay and standard *in vitro* serial mammosphere assays for identifying TIC populations in human breast SUM 159 and MDA-MB-231 TNBCs.

A better understanding of molecular signaling pathways that regulate TIC survival, renewal and differentiation is necessary for the development of targeted therapy to eliminate TICs. Wnt/β-catenin, Notch, Hedgehog, Her-2/PI3K/AKT, PI3K/mTOR/Stat-3 and PTEN/Akt have been identified as regulators of TIC self-renewal [20, 21, 22 and 23]. Therefore, identification of agents that target one or more of these pathways provides potential for development of novel strategies for eliminating TICs.

Signal transducers and activators of transcription 3 (Stat-3) is a transcription factor [24] involved in cell proliferation, metastasis, and cell survival [25]. In response to cytokines and growth factors, Stat-3 family members are phosphorylated by receptor-associated kinases followed by formation of homo- or heterodimers that translocate to the cell nucleus, where they function as transcription activators or repressors [24]. Reports show that 50% of breast cancers express phosphorylated Stat-3 (pStat-3), with basal-like breast cancer cells expressing even higher levels [26, 27]. pStat-3 has been shown to be highly expressed in $CD44^{+}/CD24^{-}$ populations, to be necessary for tumor cell proliferation [27], and to be involved in maintaining TICs in breast cancer cell populations, including SUM 159 cells [27, 28 and 29]. Thus, promising *in vitro* and *in vivo* data point to Stat-3 signaling as a potential target for TIC elimination in basal-like breast cancer, including TNBC. In this study, for the first time, we demonstrated that DHA and DHA + γT3 possess the ability to eliminate TICs and to suppress Stat-3 signaling.

1.3 The anticancer actions of natural active compounds, vitamin E and docosahexaenoic acid

Accumulating evidence supports the notion that certain natural compounds possess anticancer activity via targeting both bulk tumor cells and TICs. Food derived compounds that have been identified to possess the ability to target TICs include curcumin, a natural compound extracted from curry and piperine from black and long

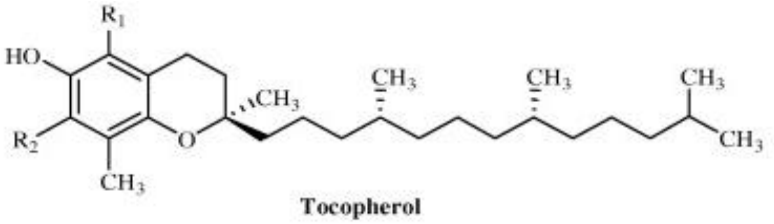
peppers singly and in combination. Curcumin and piperine have been shown to inhibit TIC self-renewal [30]. Several other natural compounds also have been shown to possess *in vitro* and/or *in vivo* anti-TIC properties including sulforaphane and piperine [31], galiellalactone [32], sesquiterpene lactone parthenolide [33], soy isoflavone genistein [34, 35], blueberry [35], γT3 [36] and DHA [37]. Since most food-derived compounds show no or low toxicity these compounds possess great potential as anticancer agents for cancer prevention and treatment. Studies described in this dissertation are focused on two of these natural source dietary compounds, vitamin E and DHA.

1.3.1 Vitamin E

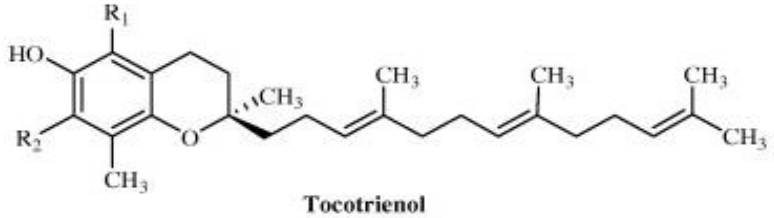
1.3.1.1 Structures and sources of Vitamin E

Vitamin E is a general term used indiscriminately to refer to a group of eight different naturally occurring compounds known as tocopherols and tocotrienols, as well as synthetic vitamin E (*all-rac*-α-tocopherol) and analogs of vitamin E such as (α-TEA/2,5,7,8-tetramethyl-2*R*-(4′*R*,8′*R*,12′-trimethyltridecyl) chroman-6-yloxy acetic acid and *RRR*-α-tocopheryl succinate/vitamin E succinate (VES)/α-TOS] [38]. Vitamin E is a family of fat soluble compounds consisting of a functional phenolic group on a chroman head and an attached phytyl tail [38]. There are eight naturally occurring vitamin Es, which are divided into two categories based on the degree of saturation in the

phytyl tail; tocopherols or tocotrienols. Both tocopherols and tocotrienols can be further divided into four forms (α, β, γ or δ) based on the numbers and location of methyl groups on the chroman head [38] (Fig 1.1). All tocopherols and tocotrienols found naturally in foods exhibit 2R stereochemistry. Among them, RRR-α-tocopherol (αT) is considered the most biologically active form since it is retained in human tissues at the highest amount [38].



Tocopherol



Tocotrienol

R_1	R_2	
CH_3	CH_3	α
CH_3	H	β
H	CH_3	γ
H	H	δ

Fig 1.1 Structures of various forms of tocopherols and tocotrienols of Vitamin E. Tocopherols and tocotrienols differ due to their saturated or unsaturated phytyl tails,

respectively. Based on the numbers and location of the methyl group on the chroman head, they are named α, β, γ and δ forms (adapted from [39]).

Different naturally occurring vitamin E forms are present in some plants [38]. Plant products such as nuts and soybean oils are enriched for tocopherols and palm oil, cereal grains, wheat germ and rice bran are enriched for tocotrienols [38]. Both RRR-α-tocopherol and *all-rac*-α-tocopherol can be purchased as acetate or succinate derivatives and are used as FDA approved vitamin E supplements and food additives [38]. Recently, gamma-tocotrienol enriched fraction extracted from rice bran and palm oil, and delta-tocotrienol enriched fraction extracted from annatto beans became available commercially [40].

1.3.1.2 Digestion, absorption and metabolism of Vitamin E

Tocopherols are absorbed directly from the diet; however tocotrienols are usually esterified and have to be hydrolyzed before absorption [41]. Similarly, synthetic ester forms of tocopherol have to be hydrolyzed before absorption. Pancreatic and duodenal mucosal esterases participate in hydrolyzation of ester forms of tocopherols and tocotrienols for absorption [41].

All vitamin E forms are thought to be absorbed equally in micelles along with the absorption of other fat soluble products [41]. Next, the various vitamin E forms are incorporated into chylomicrons which are the major lipoproteins for transporting exogenous fat to cells and tissues via the circulatory system [42].Vitamin E remaining in

the chylomicrons remanents are incorporated into the liver for re-packaging into very low density lipoproteins (VLDL) and then secreted back into circulation [42]. A specific protein in the liver called alpha-tocopherol transfer protein (α-TTP) is necessary for the selective transfer of RRR-α-tocopherol into VLDLs [43]. Because of the high selectivity of α-TTP for RRR-α-tocopherol, other vitamin E forms are poorly secreted back into the circulation [41]. Although γT is the predominant form of vitamin E in the American diet, RRR-α-tocopherol is the dominant form of vitamin E in the body.

Elimination of excess vitamin E involves ω-hydroxylation of the phytyl side chain, followed by several β-oxidations. Cytochrome P450 (CYP450) enzymes especially CYP4F2 and CYP3A4 catalyze ω-oxidation. Oxidation of the side chain results in the formation of carboxyethyl-hydroxychromane (CEHC) which may be conjugated with glucuronic acid and eventually excreted in the urine [44]. Both tocotrienols and tocopherols have been shown to activate a reporter gene driven by pregnane-X-receptor (PXR), a member of the family of nuclear receptors [45]. CYPs are induced by the activation of PXR [45]. Therefore, vitamin E can activate CYPs via activation of PXR, leading to elimination of vitamin E. The metabolism of vitamin E is tightly controlled.

1.3.1.3 Functions of natural forms of Vitamin E

Vitamin E was first discovered in 1922 by H. Evans and K. Bishop as a micronutrient to maintain reproduction in rats [46]. Following the initial finding, many

studies focused on the benefits of vitamin E leading to the discovery of eight vitamin E isomers. Vitamin E is best known as an antioxidant [38]. The antioxidant property of vitamin E results from the ability to donate hydrogen ions to free radicals from the hydroxyl group on the phenolic ring of vitamin E [41]. Most of the vitamin E antioxidant investigations have focused on αT, the major form in the body [47]. Supplementation with natural (RRR-αT) or synthetic (*all-rac*-αT) vitamin E has been associated with reductions in risk of chronic diseases like cardiovascular disorders [48], atherosclerosis and neurodegenerative diseases [49]. Besides these antioxidants properties, vitamin E's anti-cancer activities have provided mixed results [38]. Although many efforts have been made to prove the chemo-preventive role for natural or synthetic vitamin E in cancer, no evidence has been provided to support a beneficial effect of αT, αT derivative (*R,R,R*-α-tocopheryl acetate) or synthetic vitamin E acetate derivative (*all-rac*-α-tocopheryl acetate) on human cancer prevention [38]. A randomized, placebo-controlled trial (Selenium and Vitamin E Cancer Prevention Trial [SELECT]) on prostate cancer conducted between 2001 and 2004 did not find any benefit of *all-rac*-*α*-tocopheryl acetate for cancer prevention. In this study 35,533 men from 427 participating sites in the United States, Canada, and Puerto Rico were tested for the effect of selenium (200 μg/d from *L*-selenomethionine), vitamin E (400 IU/d of *all-rac*-α-tocopheryl acetate) and combination of selenium and vitamin E for prevention of prostate cancer [50]. Recent SELECT follow-up studies showed higher prostate cancer incidence in subjects who were given *all-rac*-α-tocopheryl acetate supplementation [51]. However, accumulating

data suggest that other forms of vitamin E such as gamma- and delta-tocopherols and -tocotrienols possess anticancer activities against breast cancer [52], prostate cancer [53], skin cancer [54] and hepatocarcinogenesis [55].

1.3.1.4 Anticancer activities of tocopherols and tocotrienols

With the disappointment of the findings in SELECT, studies have focused on the anticancer properties of other forms of vitamin E. Several *in vitro* and *in vivo* studies show that both gamma- and delta-tocopherols and -tocotrienols possess anticancer actions [56, 57 and58].

γT is the most common vitamin E form in the American diet and is found enriched in soybean and corn oils [57]. δT is primarily found in soybean and castor oils [57]. Studies show that γ and δT induce apoptosis in a variety of cancer cells including breast, colon and prostate cancer [57]. Both tocopherols have been shown to inhibit tumor growth in a lung xenograft model, but αT did not [59]. *In vitro* studies showed that δT was more effective than γT in inhibiting cell growth, whereas αT was not effective [60]. Several mechanisms have been proposed to account for the anti-cancer activities of tocopherols. One study showed that tocopherols arrest the cell cycle at the S phase as well as suppressed cyclin D1 and cyclin E protein levels [61]. Tocopherols also have been shown to induce apoptosis, involving activation of caspases-9 and -3, as well as interruption of the synthesis of sphingolipids [57]. γT or the combination of γT plus δT have been shown to induce apoptosis by induction of cytochrome c release, activation of

caspases-9 and -3, and cleavage of Poly (ADP-ribose) polymerase (PARP) in human prostate LNCaP cells. A study from our lab showed that γT induces human breast cancer cells to undergo apoptosis via DR5 pathway [62].

Tocotrienols exhibit antitumor actions *in vitro*, including induction of apoptosis [52], inhibition of cell proliferation and inhibition of angiogenesis [63]. Furthermore, γT3 has been shown to possess anticancer actions in preclinical animal models [52], and reported to inhibit NF-kB, a transcription factor regulating anti-apoptotic, inflammatory and angiogenic factors [64]. Ahn, et al. reported that γT3 downregulates NF-kB in pancreatic cancer cells as well as down-stream anti-survival factors [B-cell lymphoma-extra large (Bcl-xL), B-cell lymphoma 2 (Bcl-2), Cellular caspase-8 (FLICE)-like inhibitory protein (cFLIP), X-linked inhibitor of apoptosis protein (XIAP) and survivin, anti-proliferation factors (cyclin D, cMyc), and anti-angiogenesis factors-Vascular endothelial growth factor (VEGF) [65]. Shah et al. showed that γT3 suppressed the PI3K/AKT survival signaling pathway in malignant mouse mammary cells [66]. Moreover, suppressed PI3K/AKT was associated with inactivation of anti-apoptotic factor c-Flip and activation of pro-apoptotic factor caspase 8 [66]. Our lab has reported that γT3 induces apoptosis in MCF-7 and MDA-MB-231 human breast cancer cells *in vitro* via activation of a c-Jun N-terminal kinases (JNK)/ CCAAT-enhancer-binding protein homologous protein (CHOP)/DR5 pro-apoptotic pathway and suppression of c-FLIP and Survivin anti-apoptotic factors, and inhibits tumor growth in a transplantable syngeneic BALB/c mouse 66cl-4-GFP mammary cancer model [52]. A recent *in vivo*

and *in vitro* study firstly reported that γT3 targets prostate TICs [67]. It has also been reported that γT3 and δT3 levels specifically accumulated in tumor tissue although they were not detected in liver and lung, further supporting the application potential of tocotrienols for cancer prevention and treatment [68]. A recent pilot clinical trial has been conducted to test the effect of the combination of tocotrienol rich fraction with tamoxifen in women with early diagnosed breast cancer. Although the study did not achieve a statistically significant benefit in a 5 year survival rate, there was a positive trend in that the combination of tocotrienol rich fraction plus tamoxifen had higher survival rates [69]. A recent phase I clinical trial has been initiated to study the effects of δT3 on pancreatic cancer patients [70]. This study will be the first study to test different doses of a single tocotrienol in patients [71]. It has been reported from this study that a dosage of 800 mg/day were well tolerated by patients. Accumulated promising data suggest that γT3 may be an effective anti-cancer agent alone as well as with other anticancer agent to enhance its anticancer effects and reduce toxicity [71].

1.3.2 Docosahexaenoic acid (DHA)

DHA is a long-chain (22 carbon) omega-3 highly unsaturated fatty acid (n-3 HUFA). It has six cis double bonds with the first double bond located at the third carbon from the omega (methyl) end [72]. DHA is highly enriched in cold water fishes such as salmon, herring, mackerel and sardines and their oils are widely used as dietary supplements [73].

1.3.2.1 Synthesis and metabolism of polyunsaturated fatty acids (PUFAs)

PUFAs can be divided into different families based on the location of the first double bond from the methyl end. They are n-3 and n-6 families. Linoleic acid (LA; 18:2 n-6) and α-linolenic acid (ALA; 18:3 n-3) are precursors of n-6 and n-3 highly unsaturated fatty acids (HUFAs) containing more than 20 carbons, respectively [74]. Linoleic and linolenic fatty acids are essential for humans since humans do not possess enzymes capable of inserting double bonds at the omega-3 and 6 positions [74]. Both linoleic and α-linolenic fatty acids can be metabolized to 20 or 22-carbon long-chain fatty acids by delta-4,5,6 desaturases and elongases [75]. LA is metabolized to arachidonic acid (AA; 20:4 n-6), and ALA to eicosapentaenoic acid (EPA 20:5 n-3) and DHA (22:6 n-3) (Fig 1.2) [75]. The efficiency of conversion from ALA to EPA and DHA is small and variable [76]. It has been shown that 8% to 20% of ALA is converted to EPA in humans and only 0.5% to 9% of ALA is converted to DHA [76]. Burgee and co-workers compared the apparent conversion efficiency of ALA to DHA in men and women. There was no detectable conversion found in men whereas an efficiency of 9% was observed in women [77]. Therefore, the conversion of ALA to DHA is limited and variable among humans.

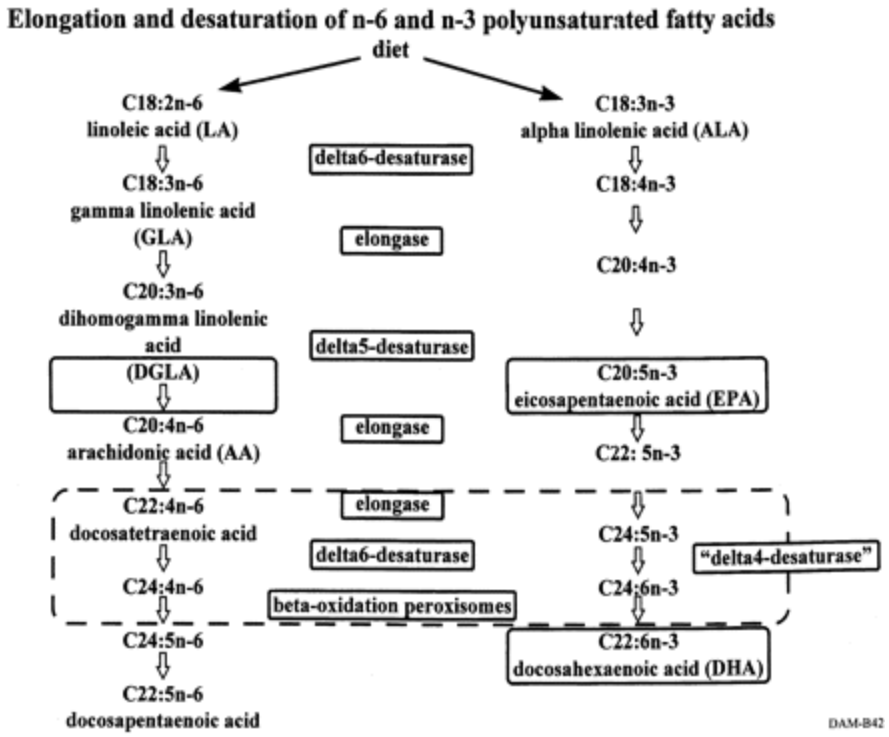


Fig 1.2 metabolism of omega-3 and omega-6 PUFAs Metabolisms of PUFAs begins from their precursors ALA n-3 and LA n-6 found in diets. ALA is metabolized by Δ6desaturation, elongation and Δ5 desaturation, further elongation and Δ6 desaturation and beta oxidation in peroxisomes and resulting in the production of DHA. With the participation of same enzymes in the conversion of ALA to EPA and DHA, LA is converted to docosapentaenoic acid (DPA). (adapted from [75]).

Among these two families, n-3 and n-6 are competing for the desaturation enzymes. Although both Δ-5 and Δ-6 desaturases prefer omega-3 to omega-6 fatty acids, a high LA intake interferes with the desaturation and elongation of ALA to n-3 FAs [75]. These two categories of HUFAs have important opposing physiological functions because of different oxidized metabolites; namely different eicosanoid products produced by n-3 and n-6 FAs. AA and DHA or EPA are parent compounds of eicosanoids (Fig 1.3). Both AA

and DHA/EPA are metabolized by cyclooxygenases (COX) and lipoxygenases (LOX) enzymes. AA serves as a substrate of COX to produce pro-inflammatory products like prostaglandins (PGE2), prostacyclins (PGI2) and thromboxanes (TXA2) [78]. AA also can be oxidized by LOX for the production of Leukotriene E4 (LTE4). Generally, eicosanoids produced from AA are more pro-inflammatory [78]. DHA/EPA produce eicosanoids such as prostaglandin 3 (PGE3), resolvins, leukotriene 5 (LTE5) and protection Ds which have less pro-inflammatory or anti-inflammatory effects (Fig 1.3) [79]. Cell culture and animal studies have shown that n-3 PUFAs are protective; whereas, n-6 PUFAs promote inflammation and cancer [80]. It is hypothesized that a very high omega-6/omega-3 ratio, as is found in today's Western diets, promotes the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune diseases [80]. Thus, the ratio of dietary n-6/n-3 taken is very important for human health, with a recommendation that the ratio of n-6/n-3 is close to 1 [75]. However, studies [75] show that the ratio of n-6/n-3 is 15/1–16.7/1 in Western diets. This review of omega-3 and omega-6 highlights an urgent need for Americans as well as people in many other countries to address the lop-sided n-3/ n-6 ratio. Studies in this dissertation focus on the anticancer beneficial effects of n-3 DHA alone and in combination with γT3.

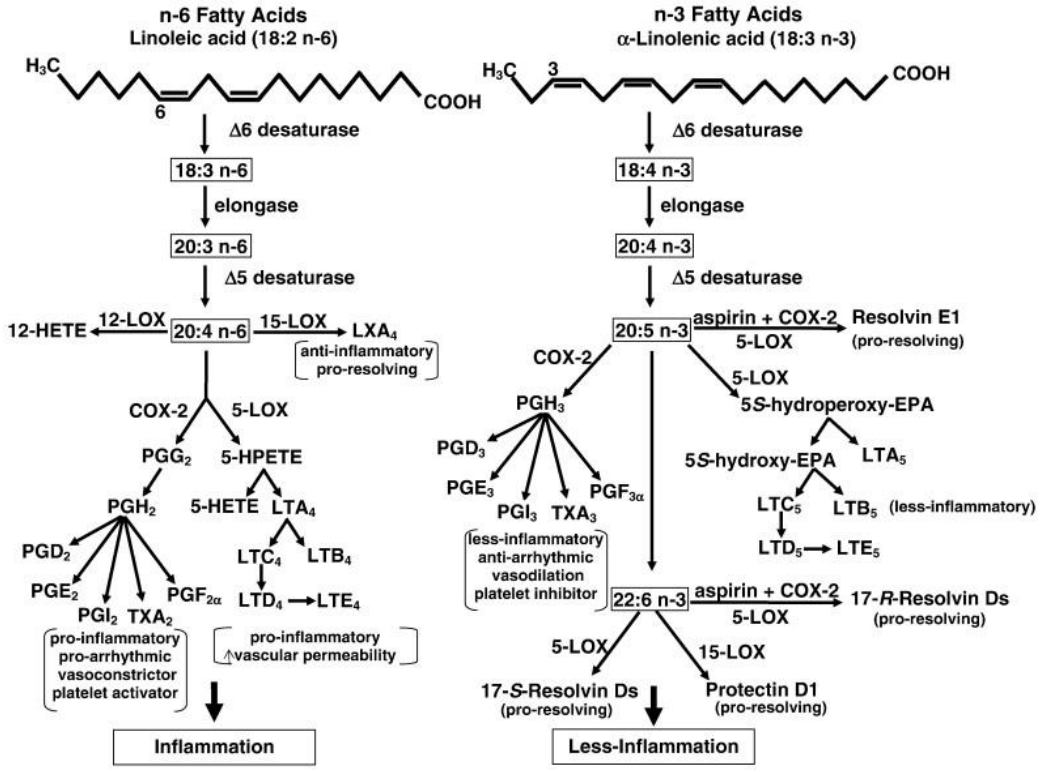


Fig 1.3 Oxidized metabolism of n-3 and n-6 PUFAs to produce their respective eicosinoids and pro-resolving products Eicosanoids such as LTE4, 5-HETE, PGI2, PGE2 produced from AA are more pro-inflammatory. PGI3, PGE3, LTE5 produced from DHA/EPA are less inflammatory and resolving, E1 is anti-inflammatory. (Adapted from [74]).

1.3.2.2 Anticancer functions of DHA

The health benefits from the consumption of n-3 HUFAs are evident in the Greenland Eskimos [81]. They consume a high seafood diet rich in n-3 and have low rates of coronary heart disease, asthma and diabetes mellitus. Furthermore, there is sufficient data to show that the n-3 FAs provide benefits in prevention of cancer, inflammatory disease, immune system disease and psoriasis. However, too much DHA

intake could cause side effects such as nausea, gas or prolonged bleeding . The incidence of hemorrhagic stroke is higher among Greenland Eskimos [82]. It has been documented that n-3 PUFAs can reduce platelet aggregation, inhibiting blood clotting [83]. A study by Yongsoon Park et al. showed that intake of DHA+EPA increase the risk of hemorrhagic stroke in a rat model [84].

The anti-inflammation and cardioprotection functions of DHA have been well documented [85, 86]. Accumulating data from cell culture, animal studies and human clinical trials suggest that DHA can reduce tumorigenesis in a variety of cancers, including breast cancer [87]. The effects of DHA on inhibition of proliferation, metastasis and angiogenesis, as well as induction of apoptosis in MDA-MB-231 cells have been reported [88]. *In vitro* data show DHA to induce apoptosis and inhibit metastasis via multiple mechanisms, including inhibition of biosynthesis of proinflammatory molecules such as COX-2 [89], activation of PPAR-γ [89], suppression of NF-kB [90], generation of reactive oxygen species (ROS) [91], and ER-stress [92]. n-3 PUFA's are readily incorporated into the lipid micro-domains of cellular membranes, which may enhance membrane-associated signaling proteins such as Ras, Akt, EGFR and lipids such as ceramide [93, 94]. Recently, Kent et al. reported that DHA significantly inhibited secondary mammosphere formation in cells isolated from polyoma virus middle T virus spontaneous mammary cancer mouse model [95], an *in vitro* method for testing self-renewal of TICs [96] and induces apoptosis of TIC-enriched colon cancer cells [37], suggesting the potential of DHA to eliminate TICs. Taken together, data suggest that

DHA is a promising nutrient-based anticancer agent to prevent metastasis and tumor recurrence, or eradicate cancers since DHA possesses the potential to target both TICs and non-TICs.

1.4. Apoptosis and Endoplasmic Reticulum stress (ER stress)

1.4.1 Introduction of Apoptosis and ER stress

Apoptosis is one form of programmed cell death (PCD) [97]. It is characterized by cell morphology changes including blebbing, shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. In contrast to apoptosis, necrosis is a nonspecific form of cell death, characterized by rupture of the plasma membrane with inflammatory responses and damage to surrounding cells and tissues [98]. Apoptosis often provides beneficial effects to the organism, while necrosis is almost always detrimental and can be fatal [99] due to the inflammatory responses to surrounding cells and tissues. Apoptotic cells are engulfed and removed by phagocytic cells when they receive the “eat-me” signals from the apoptotic cells [98]. This characteristic of apoptosis provides the opportunity for clinical use to selectively target tumor cells but not damage normal cells.

Two major signaling pathways are involved in triggering apoptosis, the extrinsic pathway (death receptor pathway) and intrinsic pathway (mitochondrial pathway) [100] (Fig 1.4). Cysteine-aspartic proteases (caspases) are a family of cysteine proteases that play essential roles in apoptosis [101]. Caspases are divided into two categories: Initiator

caspases such as caspases 2, 8, 9 and 10 which act in cleaving inactive pro-forms of effector caspases to activate them, and effector caspases, also named executioner caspases such as caspases 3, 6 and 7, which function in cleavage and activation of their protein substrates to trigger apoptosis processes [101].

Intrinsic stresses, such as oncogene dysregulation, direct DNA damage, hypoxia, and survival factor deprivation, can activate the intrinsic apoptotic pathway [100] (Fig 1.4). The intrinsic pathway is characterized by changes in the mitochondria membrane permeability and release of two main groups of pro-apoptotic proteins [102]. One group of the pro-apoptotic proteins includes cytochrome c and Smac/DIABLO in group 2 [103]. Cytochrome *c* forms an apoptosome via binding of Apaf-1 and procaspase-9 [104]. This binding results in the activation of caspase-9 and caspase-3. Smac/DIABLO induce apoptosis via inhibiting IAP (inhibitors of apoptosis proteins) activity. The other group of pro-apoptotic proteins includes Apoptosis inducing factor (AIF), endonuclease G and Caspase-Activated DNase (CAD). AIF causes DNA fragmentation [105] and endonuclease G cleaves nuclear chromatin to cause DNA fragmentation [106]. AIF and endonuclease G both function in a caspase-independent manner, whereas CAD induces apoptosis via activation of caspase-3 [107]. Bcl-2 family proteins control and regulate mitochondrial dependent apoptotic events [108]. These proteins can be divided into pro-survival and anti-apoptotic proteins. Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG function as anti-apoptotic (pro-survival) proteins, while Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk function as pro-apoptotic (death) proteins [100]. The balance of these anti- and pro-

apoptotic Bcl-2 proteins decides the fate of cells; death or survival. The main action of Bcl-2 family members in controlling apoptosis is to regulate cytochrome c release from the mitochondria. The extrinsic and intrinsic pathways cross-talk [100]. In both cases, if a cell is initiated by either pathway, the mitochondria membranes become permeable to release cytochrome c to form apoptosome to activate executioner caspase 3 [101].

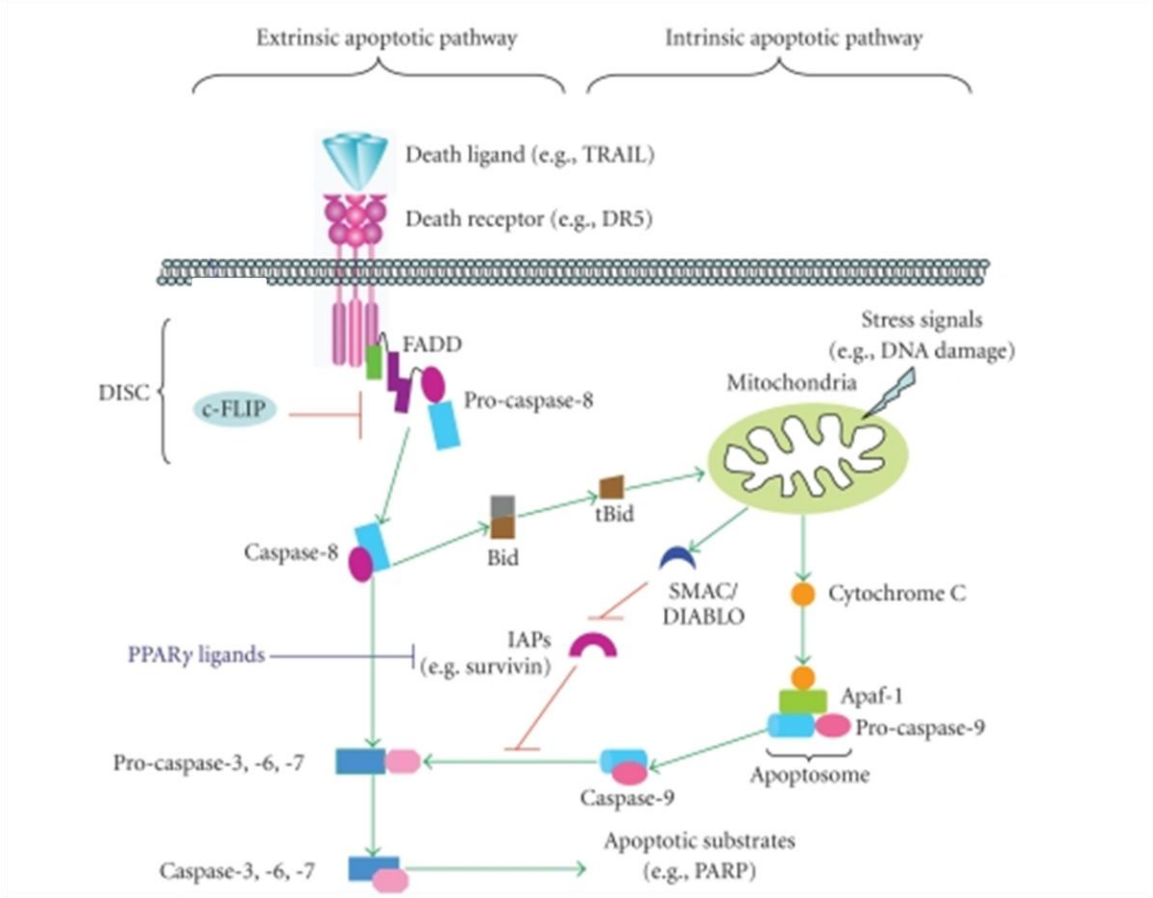


Fig 1.4 extrinsic and intrinsic pathways of apoptosis extrinsic pathway starts by activating death receptors on cell membranes. Adaptor proteins initiate DISC which activates caspase 8 and then caspase 3 to trigger apoptosis. The intrinsic pathway is characterized by changes in the mitochondria membrane permeability and release of two

main groups of pro-apoptotic proteins-Cytochrome *c*, Smac/DIABLO, and endonuclease G. Smac/DIABLO and Endo G induce apoptosis via inhibiting IAPs activities. Released cytochrome c form apoptosome to activate executioner caspase 3 to trigger apoptosis.(adapted from [109])

The extrinsic apoptotic pathway is initiated by activation of death receptors via their ligands such as Fas receptor and Fas ligand, and death receptor 4/5 (DR4/DR5) and their TNF-related apoptosis-inducing ligand (TRAIL). Once the cell receives the death stimuli, death receptors and their ligand complexes recruit death-containing protein (FADD) and caspase-8 to form the death-inducing signal complex (DISC) [100], leading to cleavage and activation of caspase-8. Activation of caspase-8 can be blocked by FLICE inhibitory protein (FLIP) via competition with caspase-8. Caspase 8 triggers apoptosis via activation of effector caspase, caspase-3.

Triggering cells to undergo stress is another mechanism to induce apoptosis, such as oxidative stress and ER stress. ER is the organelle which is responsible for synthesis and folding of proteins, synthesis of steroids, cholesterol and some other lipids [110], and also a calcium storage and signaling site. ER functions in an oxidative environment with chaperone proteins and high levels of calcium. All secretary proteins enter the ER before they enter the Golgi complex where they undergo post-translational modifications and folding to form disulfide bonds [111]. Correctly folded proteins are transported out of the ER and down to their secretory pathways; whereas, misfolded proteins are degraded by cytoplasmic proteasomes. Disruption of any of these processes causes ER stress [111].

ER stress is associated with activation of specific signaling pathways including the unfolded protein response (UPR) and some diseases including cancer [112]. In the lumen of the ER, chaperone glucose regulated protein (GRP78) (also named BIP) expression is increased in a variety of cancers including breast, lung and colon cancers [113]. In cells under non-stressed condition, GRP78 binds to transmembrane sensor proteins such as pancreatic ER kinase-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) where they remain in an inactive form [114] (Fig 1.5). Whereas, cells under stress accumulate unfolded proteins and GRP78 pulls away from the unfolded proteins and these sensor proteins are released and activated, leading to UPR [114]. UPR is involved in restoring or repairing the normal functions of ER via activation of signaling pathways to produce factors involved in folding proteins. If ER stress damage cannot be repaired within a certain period of time, UPR is involved in the initiation of apoptosis.

IRE1 oligomerizes in ER membranes when released by GRP78. It contains a Ser/Thr kinase domain and an endoribonuclease domain. The endoribonuclease domain processes X box protein-1 (XBP-1) mRNA [116]. IRE1 plays a key role in the removal of an intron from XBP-1, leading to the production of XBP-1 protein, a transcription factor that regulates several genes involved in the degrading and transport of misfolded proteins to the cytosol [116]. The Ser/Thr kinase domain of IRE1 is self-activated and autophosphorylated upon release from GRP78 [116]. IRE1 then binds to receptor-

associated factor-2 (TRAF2) and apoptosis-signal-regulating kinase 1(ASK1). ASK1 activates cell death signaling pathways via JNK [116].

PERK is a Ser/Thr protein kinase. Once detached from the GRP 78 complex, it is activated via autophosphorylation. It phosphorylates and inactivates the eukaryotic initiation factor 2α (eIF2α) family and thereby shuts off mRNA translation and reduces the protein load on the ER [116]. Certain genes gain advantage for translation from inactivation of eIF2a like activating transcription factor 4 (ATF4). ATF4 is a member of the bZIP family of transcription factors which regulates several genes involved in the UPR [116]. Release of GRP78 also releases ATF6 which translocates to the golgi apparatus where it is cleaved by resident proteases at a juxtamembrane site, thereby, releasing it into the cytosol where it is to transferred to the nucleus where it is involved in the regulation of gene expression, including genes like XBP1 and GRP78 which are involved in ER homeostasis [116].

1.4.2 C/EBP homologous protein (CHOP)/Gadd153, an ER stress-induced cell death modulator

CHOP is also called growth arrest and DNA damage-inducible gene 153. It is a member of the C/EBP family of transcription factors which is involved in ER stress-mediated apoptosis [52]. CHOP is regulated by XBP1, ATF6 and ATF4 that bind to cis-acting elements in the promoter region [116] (Fig 1.5). Several reports have shown that CHOP negatively regulates cell growth and promotes ER stress-induced apoptosis [115]. CHOP is constitutively expressed at low levels in cells, but increases when cells

are under ER stress [116]. Several studies have investigated the role of overexpression of CHOP in ER stress induced apoptosis [117,118]. Reduced apoptosis under ER stress was observed in chop-/- mice [119]. Targeted genes that are regulated by CHOP includes GADD34 and Bcl-2 family members such as anti-apoptotic Bcl-2 and pro-apoptotic Bim, Bak and Bax, as well as DR5.

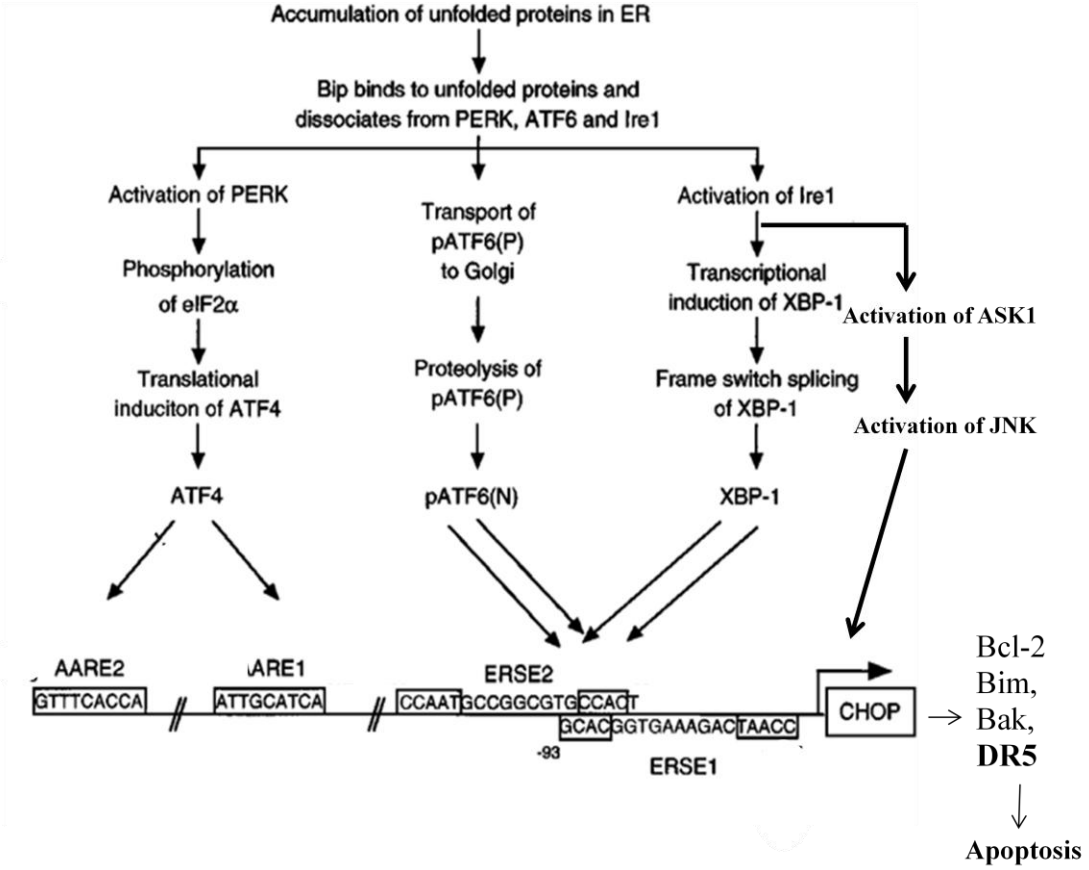


Fig 1.5 Mechanisms involved in regulation of ER stress mediators. PERK, ATF6 and IRE1 are released from chaperones upon UPR. followed by activation of a series of events resulting in activation of CHOP. CHOP, a transcription factor, regulates downstream factors Bcl-2, Bim, Bak and DR5 to induce apoptosis. (modified and adapted from [120])

1.5 Specific Aims and Objectives

Breast cancer is the most frequently diagnosed cancer in women in the United States. Triple negative breast cancer (TNBC), which constitutes the majority of the basal-like subtype of breast cancer, represents 15-20 % of breast cancers in women. Due to lack of both estrogen and progestrone receptors as well as epidermal growth factor recetor-2 (HER-2), the only available treatment for TNBC is chemotherapeutics. Although TNBC patients are initially sensitive to chemotherapeutics, recurrent tumors often occur, which are associated with multi-drug resistance and metastasis. Patients with TNBC have a poor prognosis and low 5 year survival rate. Due to the lack of effective therapeutics for TNBCs, there is a great need for development of new therapeutics with low to no toxicity that eliminate breast tumors, especially TNBC tumors. Can dietary nutrients serve as therapeutics for certain cancers? Accumulating data show that certain natural compounds, separately and in combination, possess anticancer activity via targeting both bulk tumor cells as well as TICs, also referred to as CSCs. Since most natural compounds exhibit no or low toxicity, these compounds possess great potential as anticancer agents for cancer prevention and treatment.

Objective:

Investigate the ability of two low to no toxic nutrients, DHA and γT3, alone and in combination, to eliminate both human TNBC breast bulk cancer cells and TICs in culture. DHA, a long-chain (22 carbon) omega-3 poly unsaturated fatty acid enriched in

cold water fishes, has been reported to have the ability to reduce tumorigenesis in a variety of cancers including breast cancer. Vitamin E describes a family of eight compounds, four tocopherols and four tocotrienols found primarily in vegetable oils, seeds and nuts. γT3 has been shown to target several cancers, including breast cancer as well as prostate TICs *in vitro* and in animal models.

Specific Aims:

1) Identify the distinct roles of different forms of vitamin E in combination with DHA in TNBCs *in vitro*, and establish an understanding of the underlying mechanisms of action.

2) Determine the ability of DHA alone and in combination with γT3 to eliminate TNBC TICs, and examine mechanisms of action.

3) Evaluate the anti-tumor actions of γ- and δ– (delta) tocopherols, γ- and δ-tocotrienols in induction of apoptosis in bulk TNBCs and for elimination of TICs; and the absence of anti-tumor actions of natural vitamin E (RRR-α-tocopherol, αT) and synthetic vitamin E (*all-rac*-α-tocopherol).

Dissertation Outline:

Chapter 1 provides a review of the literature for establishing pertinent background information relevant to a better understanding of the anti-cancer actions of DHA and γT3. Chapter 2 studies investigate the pro-apoptotic effects of DHA and mechanisms of action, including the involvement of reactive oxygen species and endoplasmic reticulum stress signaling pathways. Furthermore, data highlight the distinct roles of two forms of

vitamin E, αT and γT3, in DHA-induced apoptotic events. Chapter 3 documents DHA's ability to eliminate TICs in human TNBC and the cooperative effects of γT3. Chapter 4 addresses the anti-cancer actions of different forms of vitamin E in human breast cancer cell lines. Chapter 5 summarizes the studies presented in this dissertation and proposes future research directions.

Chapter 2 Distinct roles of different forms of vitamin E in DHA-induced apoptosis in triple negative breast cancer cells

2.1 Abstract

Scope: DHA has been shown to exhibit anticancer actions *in vitro* and *in vivo* in a variety of cancers. Here, we investigated the role for DHA in inducing apoptosis in TNBC and studied the mechanisms of action.

Methods and results: DHA induces apoptosis as detected by Annexin V-FITC/PI assay; as well as, induces cleavage of caspase-8 and -9, ER stress and elevated levels of death receptor-5 (DR5) protein expression as detected by western blot assays. Chemical inhibitors of caspase-8 and -9, and small interfering RNAs (siRNAs) show DHA to induce ER stress/CHOP/DR5 mediated caspase-8 and -9 dependent apoptosis. Furthermore, DHA induces elevated cellular levels of ROS, and antioxidant αT blocked DHA-induced apoptotic events. In contrast to the antagonistic impact of αT, γT3 was demonstrated to cooperate with DHA in inducing apoptotic events in TNBC cells.

Conclusion: Data, for the first time, demonstrate that DHA induces apoptosis in TNBC cells via activation of ER stress/CHOP/DR5 mediated caspase-8 and -9 dependent pro-apoptotic events, and that different forms of vitamin E exhibit distinct effects on DHA-induced apoptosis; namely, inhibition by αT and enhancement by γT3.

2.2 Introduction

TNBC is defined by a lack of expression of both estrogen and progesterone receptors; as well as, lack of human (Her-2) [6], and it has a poor prognosis due to lack of specific targeting therapy. TNBCs comprise 15-20% human breast cancer in Western countries [6]. The only therapeutic option currently available for TNBC is chemotherapy. However, prognosis for TNBC remains poor due to drug resistance and toxicity [7, 8].

DHA, a long-chain (22 carbon) polyunsaturated fatty acid (n-3 PUFA), is highly enriched in oils from cold water fishes (salmon, herring, mackerel and sardines) and widely used as a dietary supplement. DHA has a wide range of biological activities. Its anti-inflammation and cardioprotection functions have been well documented [85, 86]. Data from cell culture and animal studies, as well as human clinical trials suggest that DHA can reduce tumorigenesis and tumor development in a variety of cancers, including breast cancer [87]. DHA anticancer mechanisms include inhibition of proliferation, metastasis, and angiogenesis; as well as, induction of apoptosis [88]. *In vitro* data show DHA to induce apoptosis via multiple factors, including inhibition of biosynthesis of proinflammatory molecules such as COX-2 [89], activation of peroxisome proliferator-

activated receptor (PPAR) [89], suppression of NF-kB [90], induction of ROS [91] and ER stress [92]. However, a complete understanding of the mechanisms whereby DHA induces apoptosis is not fully understood. Further insights into the signaling networks modulated by DHA may provide key insights into how DHA may be used for prevention and treatment of human breast cancer.

Vitamin E is a general term used to refer to a group of eight naturally occurring compounds known as tocopherols (α, β, γ and δ) and tocotrienols (α, β, γ and δ) and synthetic vitamin E (*all-rac*-α-tocopherol); as well as, vitamin E analogs such as RRR-α-tocopherol ether-linked acetic acid analogue (α-TEA) [38]. Both RRR-α-tocopherol and *all-rac*-α-tocopherol can be purchased as acetate or succinate forms in traditional vitamin E supplements and as food additives to protect lipids from pro-oxidation. Tocotrienol forms of vitamin E are found in palm oil, cereal grains and rice bran [38]. To date, γT3, δT3 and Tocotrienol Rich Fraction (TRF) have been marketed as vitamin E supplements. Despite many research efforts, the anticancer functions of natural and synthetic vitamin E compounds remain to be clearly defined [38]. Pre-clinical studies suggest promise of a subset of vitamin E forms as anticancer agents; namely, gamma- and delta-forms of tocopherol and tocotrienol [56, 57 and 58]. However, there is no evidence that vitamin E supplements (αT or *all–rac*-αTAc) conferred any protection against breast cancer [38]. Instead, recent findings suggest that high dosage vitamin E supplements enhance the incidence of prostate cancer [50]. Thus, it is important to distinguish among the different vitamin E forms as to their anticancer actions.

In this study, we studied the mechanisms of DHA action on inducing apoptosis in TNBC and evaluated the effects of vitamin E compounds; αT and γT3, on DHA's pro-apoptotic actions. Data show that DHA induces apoptosis in TNBC cells via activation of ERS-mediated CHOP/DR5, and that αT blocks and γT3 enhances DHA pro-apoptotic actions.

2.3 Materials and methods

2.3.1 Cell Culture and Reagents

MDA-MB-231, SUM159 and SUM149 human TNBC cell lines [121] were used in this study. MDA-MB-231 cells were cultured in MEM media with 10% FBS as described previously [122]. SUM 159 and SUM 149 human breast cancer cells (Asterand, Inc. Detroit, MI) were cultured in Ham's F12 medium (Invitrogen, Carlsbad, CA) with 5% fetal bovine serum, 5 μg/ml insulin, 1μg/ml hydrocortisone (Sigma-Aldrich, St Louis, MO), and 10 mM HEPES buffer. For experiments, FBS was reduced to 2% to all of media and cells were allowed to attach overnight before treatments. αT (TAMA Biochemical Company, LTD, Tokyo, Japan) and γT3 (a gift from Malaysian Palm Oil Board, Kuala Lumpur, Malaysia) were dissolved in 1:4 DMSO/ethanol at 40 mM as stock solution. Equivalent levels of 1:4 DMSO/ethanol was used as vehicle control (VEH). N-acetyl cysteine (NAC, Sigma) was dissolved in H_2O. Dichlorodihydrofluorescein diacetate (H_2DCF-DA) was obtained from Invitrogen (Carlsbad, CA). Caspase 8 and 9 inhibitors: Z-IETD-FMK and Z-LEHD-FMK were

obtained from Biovision (San Francisco, CA). DHA was purchased from Sigma-Aldrich (St. Louis, MO).

2.3.2 Western Blot Analyses

Whole cell protein extracts were prepared and examined by western blotting as described previously [122]. Proteins at 15-30 μg/lane were separated by SDS-PAGE and transferred to nitrocellulose (Optitran BA-S supported nitrocellulose, Schleicher and Schuell, Keene, NH). Antibodies to the following proteins were used: poly (ADP-ribose) polymerase (PARP), CHOP, and GRP-78 (Santa Cruz Biotechnology, Santa Cruz, CA), Caspase-8, Caspase-9, DR5, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Beverly, MA). Horseradish peroxidase conjugated goat-anti-rabbit or goat-anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories, Rockford, IL) were used and followed by detection with ECL PLUS solution kit (PerkinElmer, Waltham, MA).

2.3.3 RT-PCR

mRNA levels of spliced X-box binding protein 1 (XBP-1) was determined by RT-PCR following a previously published protocol [123]. Briefly, total RNA was extracted using RNA isolation kit (Qiagen Inc. Valencia, CA). Semi-quantitative analyses were conducted to detect spliced XBP-1 mRNA form by reverse transcriptase-polymerase chain reaction (RT-PCR) using the housekeeping gene β-actin as a loading control. 4 μg

of total RNA was reverse transcribed to cDNA using 1 µl Superscript RTase (250 U, Invitrogen) following the manufacture's instructions. 1 µl cDNA was used per PCR reaction with 15 µl Taq PCR Master Mix Kit (Qiagen Inc) plus 10 µM oligonucleotide primer pairs (Invitrogen).

2.3.4 RNA Interference

A scrambled RNA duplex that does not target any of the known genes was used as the nonspecific negative control for RNAi (referred to as control siRNA). Transfection of MDA-MB-231 cells with siRNA to DR5 and CHOP (Ambion, Austin, TX) were performed in 100 mm cell culture dishes at a density of 1.5×10^6 cells/dish using Lipofectamine 2000 (Invitrogen) and siRNA duplex. After one day exposure to transfection conditions, the cells were re-cultured in 100 mm dish at 1.5×10^6 cells/dish and incubated for one day followed by treatments.

2.3.5 Quantification of apoptosis

Apoptosis was quantified by Annexin V-FITC/PI assays following the manufacturer's instructions (Invitrogen, Carlsbad, CA) and published procedure [123]. The Annexin V-FITC/PI assay measures amount of phosphatidylserine on the outer surface of the plasma membrane (a biochemical alteration unique to membranes of apoptotic cells) and amount of propidium iodide (PI), a DNA binding dye that does not cross the plasma membrane of viable cells but readily enters dead cells or cells in the late

stages of apoptosis. Fluorescence was measured using FACS analyses with a FACS Fortessa flow cytometer (BD Bioscience). Cells displaying phosphatidylserine on their surface (positive for Annexin-V fluorescence) were considered to be apoptotic. Annexin V/FITC was a gift from Dr. Shawn Bratton (University of Texas at Austin).

2.3.6 Detection of Reactive Oxygen Species (ROS)

Intracellular ROS generation was detected by FACS analyses after staining the cells with H_2DCF-DA, a cell-permeant ROS sensing probe for 30 minutes. H_2DCF-DA is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation. Staining procedures followed the manufacturer's instructions and Fluorescence was detected by a FACS Fortessa flow cytometer (BD Bioscience).

2.3.7 Statistical Analyses

Apoptosis data were analyzed using a one-way analysis of variance (ANOVA) followed by Turkey test for comparison of more than two treatments or a two-tailed student *t*-test for comparison between two treatments to determine statistical differences. Differences were considered statistical significant at $p<0.05$.

2.4 Results

2.4.1 DHA induces apoptosis in human TNBC cell lines

DHA has been reported to induce apoptosis in estrogen receptor positive, PR+ and Her-2 low-expressed MCF-7 cells and estrogen receptor negative [124], PR- and Her-2 over-expressed SK-BR-3 [124] human breast cancer cells [125]. Here, we evaluated the effect of DHA on inducing apoptosis in TNBC cells. Treatment of cells with DHA at physiologically relevant concentrations [126] for 24 hrs induced apoptosis in a dose-dependent manner as detected by Annexin V/PI analyses (Fig 2.1A) and PARP cleavage, a biomarker for caspase dependent apoptosis (Fig 2.1B). It has been reported that DHA is incorporated into the cellular surface membrane within 5.2-17 minutes [127]. Western blot analyses reveal that DHA induces cleavage of both caspase-8 and -9 (Fig 2.1B). Caspase-8 (Z-IETD-FMK) and -9 (Z-LEHD-FMK) inhibitors significantly reduced DHA-induced apoptosis as detected by Annexin V/PI analyses (Fig 2.1C) demonstrating that DHA induces casapse-8 and -9 dependent apoptosis.

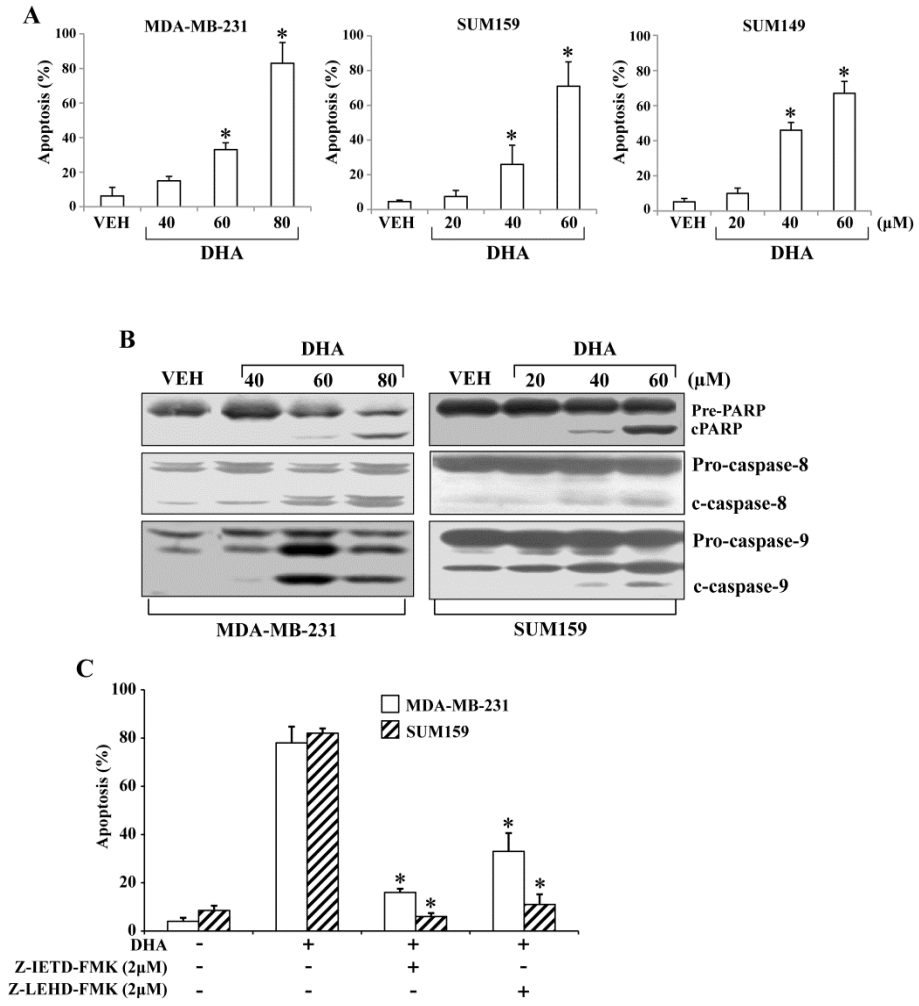


Fig 2.1 DHA induces apoptosis in TNBC cells. TNBC cells were treated with DHA at different concentrations for 1 day followed by Annexin V/PI analyses to determine percentage of cells undergoing apoptosis (A) and western blot to determine protein levels of biomarkers of apoptosis; cleaved PARP (pre-PARP = full length PARP and cPARP= cleaved PARP) (B). TNBC cells pre-treated with Z-IETD-FMK (caspase-8 inhibitor) or Z-LEHD-FMK (casapse-9 inhibitor) for 3 hours followed by treatment of the cells with DHA at 60 μM and 80 μM for SUM 159 and MDA-MB-231 cells, respectively, for 1 day were analyzed for apoptosis using Annexin V/PI (C). Data in A and C are depicted as

mean ± SD of three individual experiments. Data in B represents two separate experiments. * Statistically different from vehicle control (A) or DHA treatment alone (C), $p<0.05$.

2.4.2 DHA induces ER stress.

DHA has been reported to induce ER stress in human colon cancer cells [128]. To see if DHA induces ER stress in TNBC cells we determined the effect of DHA on the expression of ER stress biomarkers. Treatment of cells with increasing doses of DHA for 1 day induced elevated levels of ER stress markers GRP78 and CHOP protein expression as detected by western blot analyses (Fig 2.2A), as well as, elevated levels of spliced XBP-1 mRNA expression as detected by RT-PCR (Fig 2.2B), indicating that DHA induces ER stress in TNBC cells. γT3, a known ER stress inducer [52], was used as a positive control in this study (Fig 2.2A).

2.4.3 CHOP is involved in DHA-induced apoptosis

Since CHOP has been reported to be involved in ER stress mediated apoptosis, we examined if CHOP is involved in DHA-induced apoptosis. Knockdown of CHOP using siRNA significantly reduced the ability of DHA to induce apoptosis as detected by Annexin V assay (Fig 2.2C) and cleaved PARP as detected by western blotting (Fig 2.2D), suggesting that DHA induces apoptosis via ER stress-mediated CHOP.

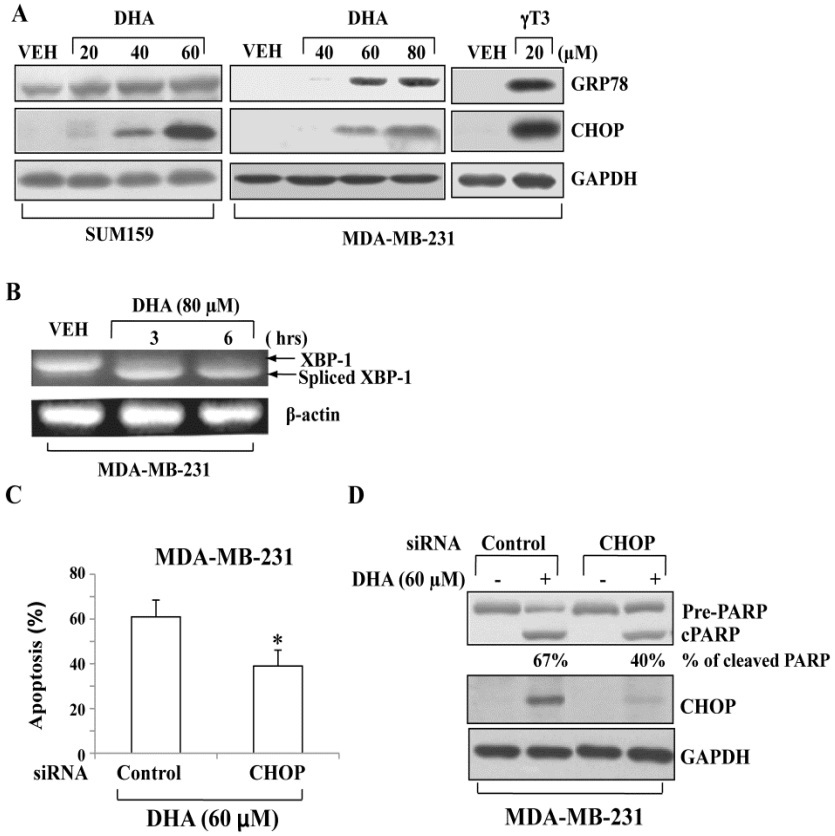


Fig 2.2 DHA induces endoplasmic reticulum stress (ER stress). Cells were treated with DHA at different concentrations for 1 day followed by western blotting (A) or by RT-PCR (B) to determine presence of ER stress associated markers (B). MDA-MB-231 cells treated with γT3 for 1 day were used as positive control for ER stress markers (A). Cells transfected with siRNAs to CHOP or control were treated with DHA for 1 day followed by Annexin V/PI analyses to determine apoptosis induction (C) or western blot analyses to determine protein levels (D). Data in A, B, and D are representative of at least two separate experiments. Data in C are depicted as mean $\pm$ SD of three individual experiments. Pre-PARP = full length PARP and cPARP= cleaved PARP. The % of cleaved PARP(c PARP) is determined by densitometry analyses using Scion Image Software (Scion Corporation, Frederick, MD) and calculated using the formula [% of

cleaved PARP = cPARP/(pre-PARP + cPARP) x 100%]. * Statistically different from control siRNA treated with DHA, $p<0.05$.

2.4.4 DR5 is upregulated and involved in DHA-induced apoptosis via CHOP

Since several studies have shown that protein expression of the death receptor for DR5 is increased following ER stress and elevation of CHOP protein expression in induction of apoptosis by different agents, such as α-TEA, γT3, and synthetic triterpenoid methyl-2-cyano-3,12-dioxoolean-1,9-dien-28-oate (CDDO-Me) [123, 52 and 129], we investigated if DR5 is regulated by DHA and involved in DHA-induced apoptosis. Treatment of cells with increasing doses of DHA for 1 day induced elevated levels of DR5 protein expression (Fig 2.3A). γT3 was used as positive control in this study (Fig 2.3A). Knockdown of DR5 using siRNA resulted in a significant decrease in DHA induced apoptosis as detected by Annexin V analyses (Fig 2.3B) and cleaved PARP as detected by western blot analyses (Fig 2.3C), indicating that DHA induces DR5 dependent apoptosis. Furthermore, data show that knockdown of CHOP using siRNA blocked the ability of DHA to upregulate DR5 (Fig 2.3C), demonstrating that DHA upregulation of DR5 is CHOP dependent.

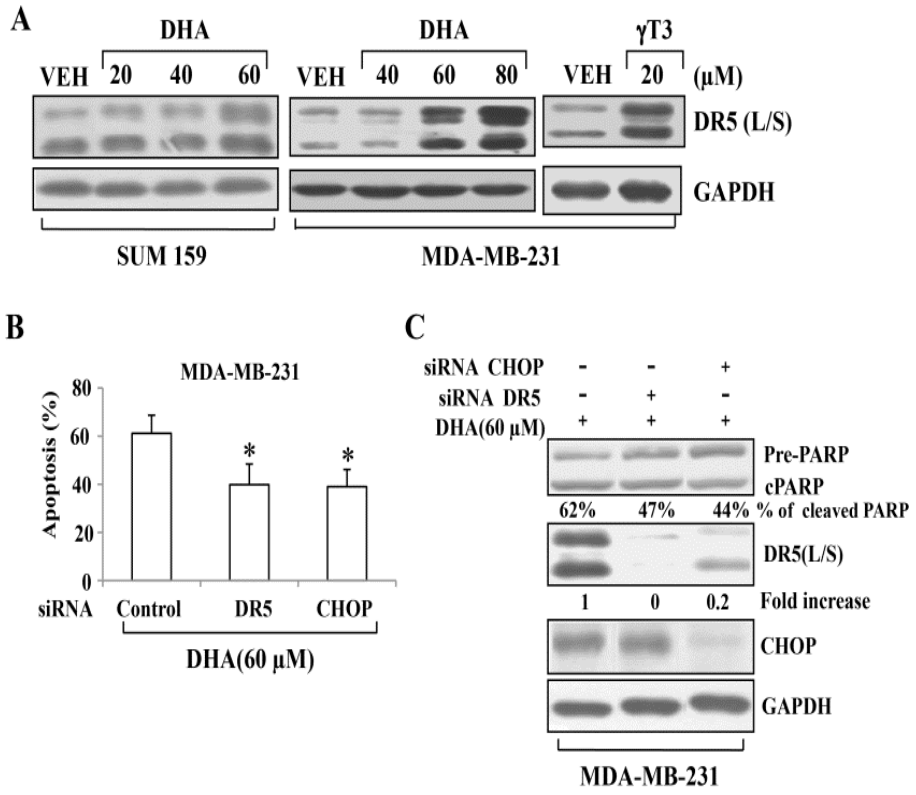


Fig 2.3 DR5 is involved in DHA-induced apoptosis and is regulated by CHOP. MDA-MB-231 and SUM159 cells were treated with DHA in different concentrations for 1 day followed by western blot analyses to determine protein levels (A). MDA-MB-231 cells treated with γT3 for 1 day were used as positive control for DR5 (A). MDA-MB-231 cells transfected with siRNAs to DR5, CHOP or control were treated with DHA 60 μM for 1 day followed by Annexin V/PI analyses to determine apoptosis induction (B) or western blot analyses to determine protein levels (C). Pre-PARP = full length PARP and cPARP= cleaved PARP. The % of cleaved PARP(c PARP) is determined by densitometry analyses using Scion Image Software (Scion Corporation, Frederick, MD) and calculated using the formula [% of cleaved PARP = cPARP/(pre-PARP + cPARP) x 100%]. DR5(L/S) refers to long and short forms of DR5. Fold increase of DR5 protein expression is determined by densitometry analyses using image software and normalized by GAPDH with the value for control as 1. Data in A and C are representative of at least

two separate experiments. Data in B are depicted as mean ± SD of three individual experiments. * Statistically different from control siRNA treated with DHA, $p<0.05$.

2.4.5 Antioxidants block DHA-induced apoptotic events.

Since ROS plays an important role in DHA-induced apoptosis [130] and ROS can trigger ER stress [131] it was of interest to see if ROS is involved in DHA-induced apoptotic events in TNBC cells. Thus, we evaluated the effect of antioxidants; fat soluble αT and water soluble NAC, on DHA-induced apoptotic events. Pre-treatment of MDA-MB-231 cells with αT at different concentrations for 2 hrs followed by DHA treatment for one day significantly reduced the ability of DHA to induce apoptosis in a dose dependent manner as detected by Annexin V (Fig 2.4A). Furthermore, pre-treatment of MDA-MB-231 and SUM159 cells with αT at 20 μM or NAC at 10 mM for 2 hrs followed by DHA treatment for one day significantly reduced the ability of DHA to induce apoptosis as detected by Annexin V (Fig 2.4B and C) and PARP cleavage as detected by western blot analyses (Fig 2.4D and E). Although treatment of MDA-MB-231 cells with αT showed an increased percentage of apoptotic cells (Fig 2.4 C) it is not a statistically significant difference from vehicle control. Both antioxidants markedly reduced the ER stress associated biomarkers GRP78 and CHOP, as well as DR5 (L/S) protein expression (Fig 2.4 D and E). Although western blot data show that DR5 protein level is increased following αT treatment it is not different from the VEH control based on densitometric analyses performed using GAPDH levels to normalize lane loads.

These data demonstrate that ROS is an upstream mediator in DHA-induced apoptotic events. Importantly, these data indicate that both water soluble and fat soluble antioxidants are antagonistic to DHA's anticancer pro-apoptotic properties.

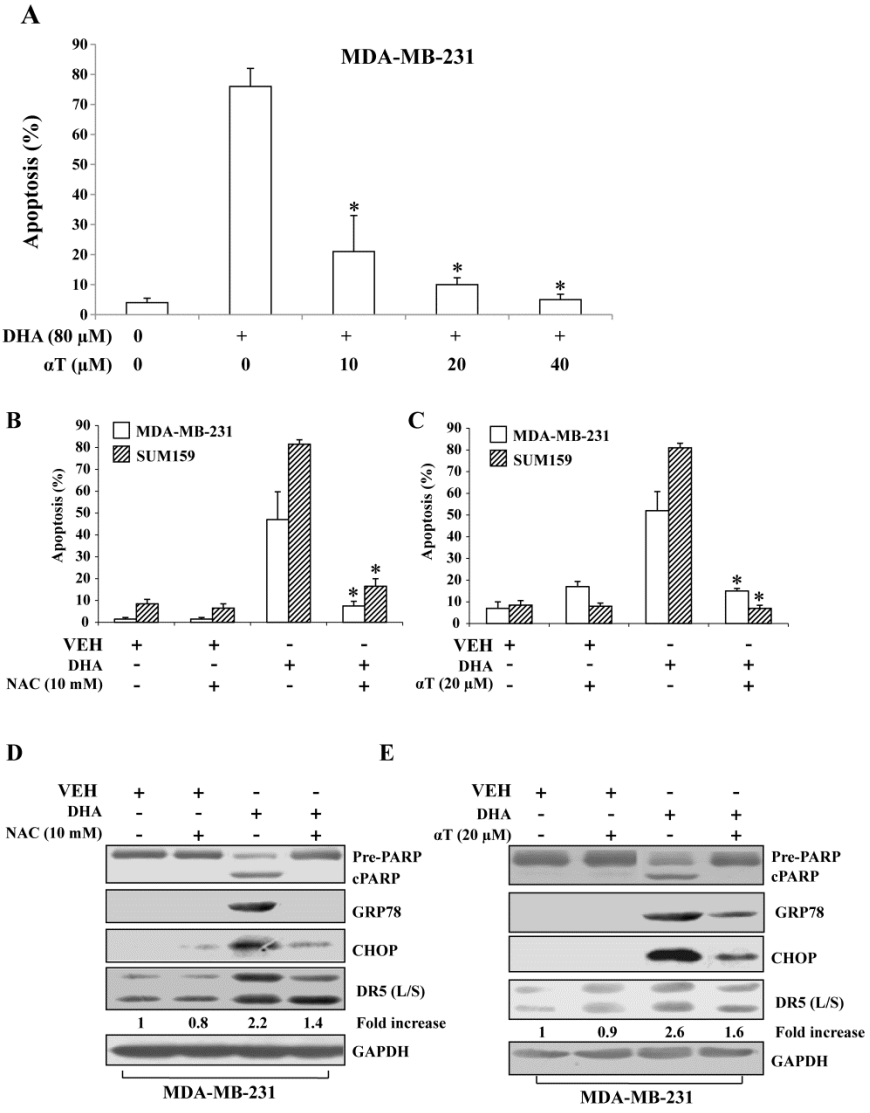


Fig 2.4 Antioxidants block DHA-induced apoptotic events. Pre-treatment of MBA-MB-231 cells with αT at indicated concentrations for 2 hrs followed by DHA treatment for 1 day significantly reduced the ability of DHA to induce apoptosis as detected by

Annexin V/PI analyses (A). MDA-MB-231 and SUM159 Cells pre-treated with αT or NAC for 2 hrs were treated with DHA 80 μM and 60μM, respectively, for 1day followed by Annexin V/PI analyses to determine apoptosis induction (B and C) and western blot analyses to determine protein levels (D and E). Pre-PARP = full length PARP and cPARP= cleaved PARP. DR5 (L/S) refers to long and short forms of DR5. Fold increase of DR5 protein expression is determined by densitometry analyses using Scion Image Software (Scion Corporation, Frederick, MD) and normalized by GAPDH with the value for control as 1. Data in A, B and C are depicted as mean $\pm$ SD of three individual experiments. Data in D and E are from two separate experiments.* Statistically different from DHA treatment alone, $p<0.05$.

2.4.6 γT3 and DHA work cooperatively to induce apoptotic events.

Since previous data show that γT3 induces apoptosis via activation of ER stress mediated CHOP/DR5 pro-apoptotic signaling events in MDA-MB-231 and MCF-7 human breast cancer cells [52], it was of interest to see the effect of this form of vitamin E on DHA induced apoptosis. γT3 induced apoptosis in both cell lines in a dose-dependent manner at levels of 10 μM and above (Fig 2.5A and B). Thus, when cells were co-treated with sub-apoptotic doses of γT3 (namely, treated with a level of γT3 that does not induce apoptosis within one day) plus DHA for one day to evaluate cooperative apoptotic effects, data show that the combination of γT3 + DHA acts cooperatively to induce apoptosis as detected by Annexin V (Fig 2.5 C and D) and cleaved caspases 8, 9 and PARP (Fig 2.5 E). Furthermore, data in Fig 2.5 F and G show that γT3 at different concentrations enhanced DHA-induced apoptosis in both cell lines in comparison with

γT3 and DHA alone. Moreover, data from western blot analyses show that combination of DHA + γT3 enhanced ER stress as detected by ER stress biomarkers, GRP78 and CHOP, and ER stress associated DR5(L/S) protein expression (Fig 2.6 A). siRNAs to CHOP or DR5 reduced the combination pro-apoptotic effect of DHA + γT3 (Fig 2.6 B), and siRNA to CHOP reduced combination (DHA + γT3) upregulation of DR5 protein expression (Fig 2.6 C), demonstrating that ER stress mediated CHOP/DR5 pro-apoptotic pathway is involved in apoptosis induced by the DHA + γT3 combination.

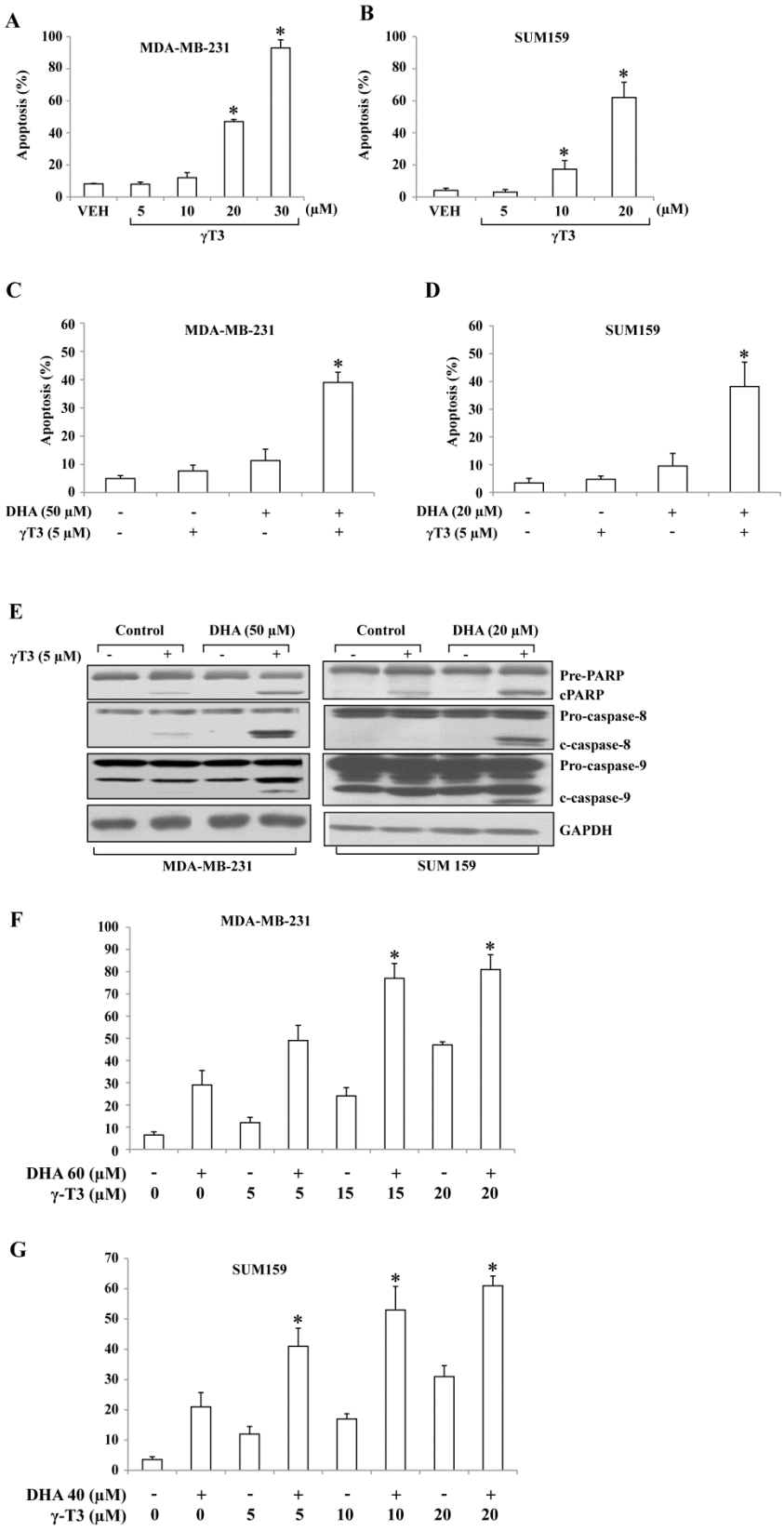

A
MDA-MB-231
Apoptosis (%)
VEH
5
10
20
30
(μM)
γT3
B
SUM159
C
DHA (50 μM)
γT3 (5 μM)
D
DHA (20 μM)
E
Control
DHA (50 μM)
DHA (20 μM)
Pre-PARP
cPARP
Pro-caspase-8
c-caspase-8
Pro-caspase-9
c-caspase-9
GAPDH
F
DHA 60 (μM)
γ-T3 (μM)
G
DHA 40 (μM)

Fig 2.5 γT3 and DHA work cooperatively to induce apoptosis MDA-MB-231 and SUM 159 cells were treated with different doses of γT3 as indicated for 1 day followed by Annexin V/PI analyses to determine apoptosis induction (A and B). The cells were treated with sub-apoptotic doses of γT3 and DHA for 1 day followed by Annexin V/PI analyses to determine apoptosis induction (C and D) or western blot analyses to determine protein levels (E). MDA-MB-231 and SUM159 cells were co-treated with different doses of γT3 + apoptotic doses of DHA as indicated for 1 day followed by Annexin V/PI analyses to determine apoptosis induction (F and G). Pre-PARP = full length PARP and cPARP= cleaved PARP. Data in A, B, C, D, F and G are depicted as mean $\pm$ SD of three individual experiments. Data in E are representative of data generated in at least two separate experiments. * Statistically different from vehicle control (A and B) or from single treatments (C, D, F and G), $p<0.05$.

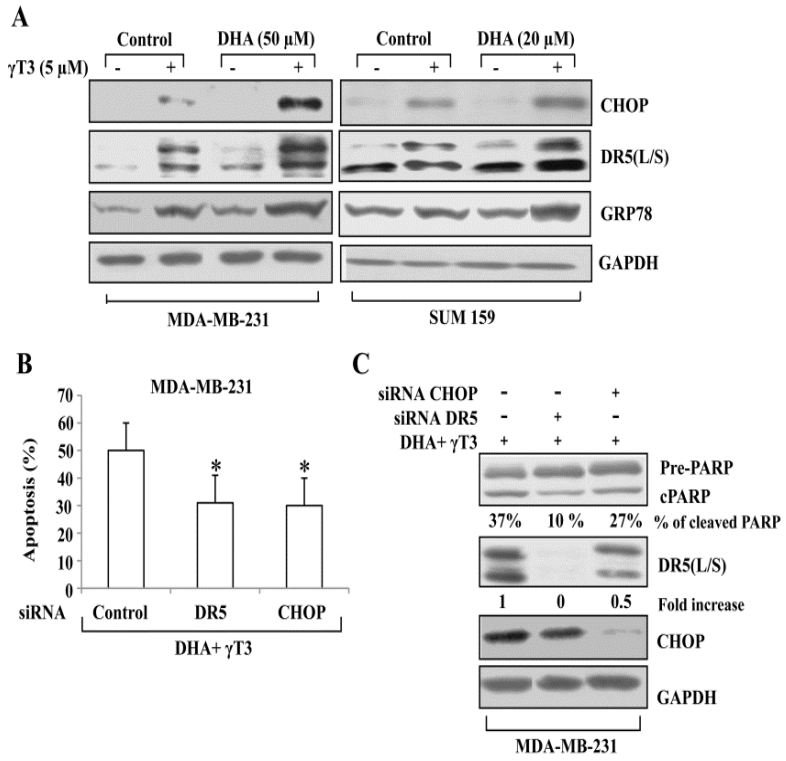


Fig 2.6 ER stress is involved in γT3 + DHA induced apoptosis MDA-MB-231 and SUM159 cells were co-treated with γT3 + DHA for 1 day followed by western blot analyses to determine the ER stress marker protein expression (A). Cells transfected with siRNAs to DR5, CHOP or control were treated with a combination of γT3 + DHA for 1 day followed by Annexin V/PI analyses to determine apoptosis induction (B) and western blot analyses to determine protein levels (C). Pre-PARP = full length PARP and cPARP= cleaved PARP. The % of cleaved PARP(c PARP) is determined by densitometry analyses using image software and calculated using the formula [% of cleaved PARP = cPARP/(pre-PARP + cPARP) x 100]. DR5(L/S) refers to long and short forms of DR5. Fold increase of DR5 protein expression is determined by densitometry analyses using image software and normalized by GAPDH. Data in B are depicted as mean $\pm$ SD of three individual experiments. Data in A and C are representative of data generated in at least two separate experiments. * Statistically different from control siRNA transfected cells treated with γT3 + DHA, $p<0.05$.

2.4.7 The effects of αT and γT3 on DHA induction of ROS

Since αT and γT3 exhibited completely opposite effects on DHA-induced ROS dependent apoptosis, it was of interest to determine the impact of γT3 and αT on DHA induction of ROS.

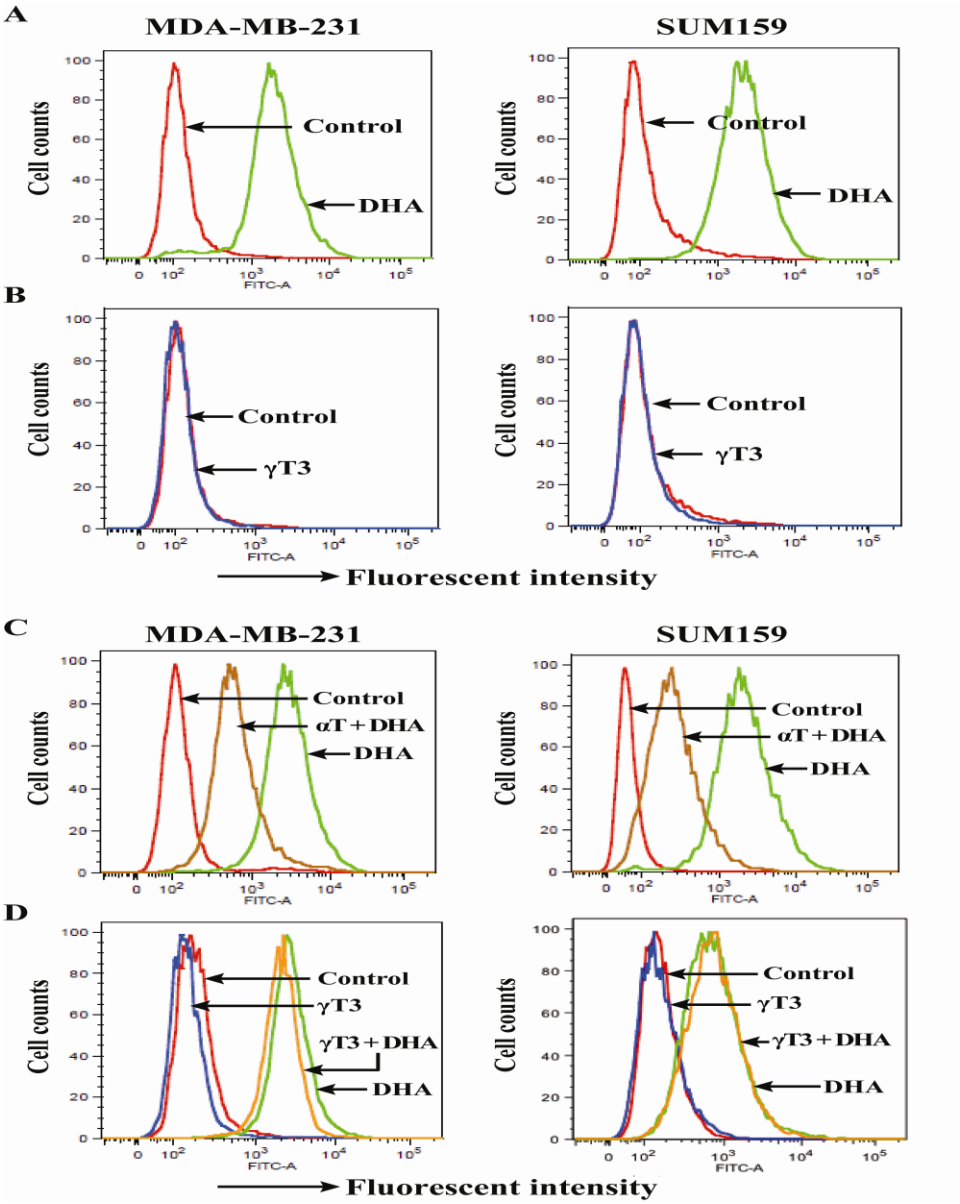


Fig 2.7 DHA induces ROS. MDA-MB-231 and SUM159 cells were treated with DHA at 60 and 40 μM, respectively (A), γT3 at 5 μM (B), αT at 20 μM + DHA at 60 and 40 μM, respectively (C), and γT3 at 5 μM + DHA 50μM and 20μM, respectively (D) for 3 hrs.

ROS intensity was determined by FACS analyses after staining the cells with special ROS indicator dye- H2DCF-DA.

Treatment of both cell lines with DHA for 3 hours induced elevated levels of ROS (Fig 2.7A). In contrast, treatment of both cell lines with γT3 for 3 hours had no effect on ROS induction (Fig 2.7B). Treatment of cells simultaneously with αT + DHA for 3 hours reduced ROS induction in comparison with DHA alone (Fig 2.7C). While treatment of cells with γT3 + DHA for 3 hours showed neither enhancement nor diminution of DHA-induced ROS generation (Fig 2.7D). These data demonstrated that; (i) γT3 is not acting as either a pro-oxidant or antioxidant in DHA-induced apoptosis, (ii) αT is acting as an antioxidant capable of blocking DHA's induction of ROS, which is critical for its pro-apoptotic effects on TNBC cells, and (iii) γT3 enhances DHA-induced apoptosis via a ROS independent mechanism.

2.5 Discussion

DHA, a dietary component with known anti-inflammatory and cardioprotective benefits, is a widely used supplement. Data show that DHA at physiologically relevant levels induces apoptosis in human breast cancer cells (Fig 2.1 A), and DHA did not induce apoptosis in normal human mammary epithelial cells (data not shown), using the same conditions as described in Fig 2.1A. Those data indicate that DHA exhibits a

selective anticancer property and hold as a nontoxic promising anticancer agent. The novel findings in this study are: (i) DHA induces apoptosis in TNBC cells via activation of ER stress mediated CHOP/DR5 pro-apoptotic events involving both caspase-8 and 9, (ii) ROS is a necessary upstream mediator of DHA-induced apoptosis, (iii) vitamin E in the form of αT blocks DHA-induced ROS formation and apoptotic events, indicating a potential antagonistic effect of supplemental αT on DHA's anticancer action, and (iv) γT3, a form of vitamin E that exhibits anticancer activity, enhances DHA-induced apoptotic events. Taken together, our data, for the first time, demonstrate that ERS-mediated CHOP/DR5 pro-apoptotic signaling events are involved in DHA-induced apoptosis, and that two different vitamin E forms have the potential to either block or enhance the anticancer actions of DHA.

Targeting the homeostatic status of the endoplasmic reticulum has been reported to be an effective way to trigger cancer cells to undergo apoptosis [132]. Several natural compounds have been reported to induce apoptosis via induction of ER stress, such as curcumin [133], γT3 [52], and epigallocatechin gallate (EGCG) [134]. DHA has been reported to induce ER stress in colon cancer cells [128]. However, whether or not ER stress is critical for DHA-induced apoptosis has not been addressed. CHOP, as a transcription factor for promoting DR5 transcription [135], has been reported to be a critical mediator in ER stress mediated apoptosis, in part, via increased levels of DR5 [136]. Here, for the first time, we report that DHA induces ER stress in TNBC cells and ER stress-mediated increased expression of CHOP is involved in DHA-induced apoptosis

via upregulation of DR5, leading to activation of death receptor dependent caspase-8 and -9 apoptotic cascades.

DHA is the longest, most unsaturated, and hence, most oxidizable fatty acid commonly found in nature [137]. Its pro-oxidant property is one of the mechanisms of DHA action in its ability to induce apoptosis [138]. However, how ROS mediates DHA-induced apoptosis has not been elucidated. ROS has been reported to trigger ER stress, leading to apoptosis [139]. Here, for the first time, we report that ROS mediates DHA-induced apoptosis via upregulation of ER stress-mediated CHOP/DR5 in TNBC cells. Evidence supporting this conclusion include: (i) DHA induces increased levels of ROS, (ii) antioxidants αT and NAC blocked the DHA's ability to induce apoptosis and blocked DHA's ability to increase levels of ER stress biomarkers GRP78 and CHOP, and DR5, and (iii) αT blocked DHA's ability to increase levels of ROS.

An additional important finding in this study is that different vitamin E forms exhibit opposite roles in DHA-induced apoptosis. αT functions as an antioxidant and directly antagonizes DHA's anticancer actions. In striking contrast, γT3 exhibits neither pro-oxidant nor antioxidant properties, and exhibits a marked beneficial combination effect on DHA's pro-apoptotic actions. These findings further support the notion that different forms of vitamin E possess distinctly different anticancer properties, and secondly, they provide important insights into how DHA's application in the clinic for cancer prevention or treatment will need to take into account other dietary influences. Further studies will be required to ascertain if limitation of αT consumed from

supplements and food sources, and/or supplement with γT3 impacts DHA's anticancer efficacy.

It appears that the pro- or anti-oxidant properties of vitamin E depend on not only the form of vitamin E, but also the cell type and experimental conditions. For example, Kannappan et al. reported that γT3 induced upregulation of DR5 via ROS in human colon cancer cells [140], and Nowak et al. reported that γT3 protected against oxidant injury in renal proximal tubular cell [141]. Data show that γT3 exhibited neither pro- nor anti-oxidant properties in TNBC (Fig 2.7 B), even when apoptotic doses were used (up to 20 μM, data not shown).

How γT3 cooperates with DHA to induce apoptosis is not entirely clear. Data showing that γT3 did not enhance DHA-induced ROS (Fig 2.7 D) rules out the possibility that γT3 enhances DHA-induced apoptosis via a pro-oxidation impact. Recently, we have studied the role of ceramide in γT3-induced ER stress mediated apoptosis. Data show that γT3 induces elevated intracellular levels of ceramide and that the chemical inhibitors of *de novo* ceramide synthesis pathways were capable of blocking γT3-induced apoptosis and ER stress-mediated CHOP/DR5 pro-apoptotic events [56], indicating that ceramide generated from *de novo* ceramide synthesis pathway are involved in γT3-induced apoptosis. Since both ceramide generated from *de novo* ceramide synthesis pathway [142] and ROS can trigger ER stress [139], it is possible that γT3 cooperates with DHA to induce apoptosis via a combination of increased ROS and increased ceramide. Further

studies will be needed to address this possibility. Based on the data presented here and our unpublished studies, a schematic diagram of proposed signaling events in DHA induced apoptosis of TNBC and the distinct role of αT and γT3 on DHA pro-apoptotic actions are depicted in Fig 2.8.

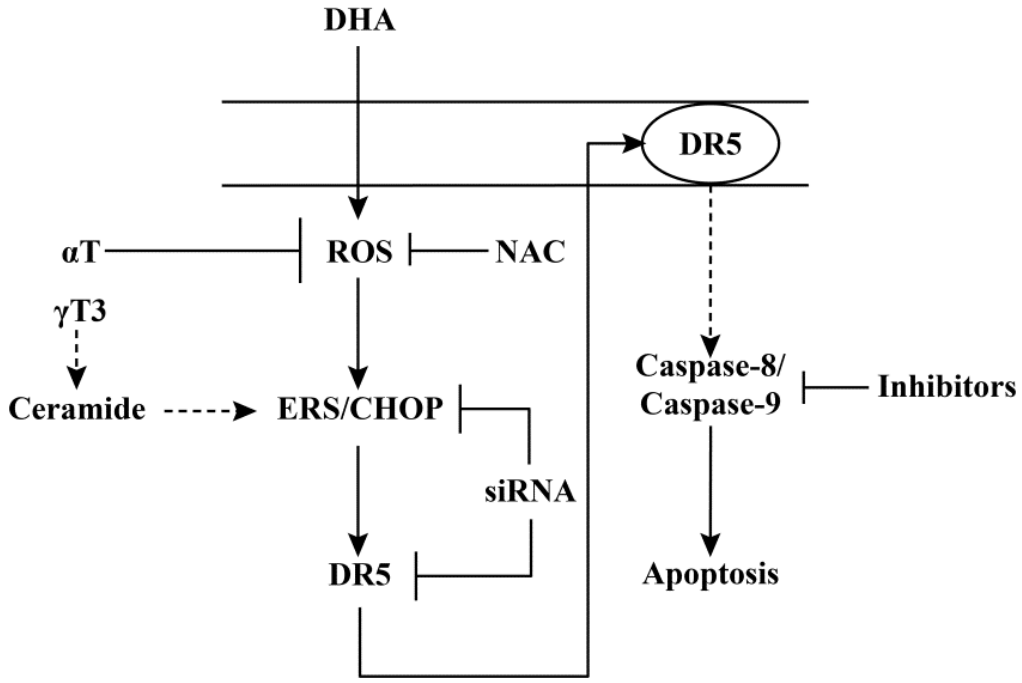


Fig 2.8 Proposed signaling events in DHA induced apoptosis of TNBC and the role of αT and γT3 on DHA pro-apoptotic actions. Based on data presented here and other data generated by our lab (unpublished), we propose the following signaling events in DHA-induced apoptosis and the role of αT and γT3 in DHA pro-apoptotic actions: (i) DHA alone induces elevated levels of ROS, leading to induction of ER stress, (ii) DHA upregulates DR5 protein expression via ER stress mediated CHOP, (iii) DHA

upregulation of DR5 triggers caspase-8 and caspase-9 dependent apoptotic cascade, (iv) αT blocks DHA's ability to induce ROS thus blocking DHA's pro-apoptotic actions via its antioxidant property, and (v) γT3 cooperates with DHA to produce ER stress and downstream pro-apoptotic actions. Unbroken arrows indicate data generated in the present study; broken arrows indicate postulated signaling pathways.

In summary, our data show that DHA induces apoptosis in TNBC cells via activation of ER stress mediated CHOP/DR5 pro-apoptotic events, in which ROS is an upstream mediator. αT, acting as a classic antioxidant, blocks DHA pro-apoptotic action, while γT3 enhances DHA pro-apoptotic action via a ROS-independent mechanism. These studies not only provide novel insights into a better understanding of the mechanisms underlining DHA-induced apoptosis but also provide *in vitro* evidence to support the use of γT3, and avoidance of αT, to improve DHA anticancer actions. These data provide a sound rationale for further *in vivo* study.

Chapter 3 Gamma-tocotrienol cooperates with docosahexaenoic acid to eliminate human triple negative breast tumor initiating cells

3.1 Abstract

DHA eliminates ALDH+ cells and inhibits mammosphere formation, two indicators of tumor initiating cells (TICs), as well as suppresses pStat-3 and its downstream mediators c-Myc, and cyclin D1 in human TNBC cell lines. γT3 cooperatively enhances DHA's actions. siRNA to Stat-3 reduced ALDH+ cells as well as suppressed pStat-3/total Stat-3, c-Myc and cyclin D1, suggesting that DHA and γT3 eliminate TICs by suppressing Stat-3 signaling.

3.2 Introduction

Despite progress in treatment of breast cancer, TNBC, a sub-type of breast cancers that lack expression of ER, PR and Her-2, present a special therapeutic challenge due to their aggressive clinical course and lack of targeted therapy [2]. TNBCs comprise 15-20% of human breast cancers in Western countries and are associated with a low 5-year survival rate [2]. Due to lack of molecular targets, the only therapeutic option currently available for TNBC is chemotherapy. DNA damaging drugs such as doxorubicin, cisplatin and taxol are used as standard-of-care. Although TNBC is initially sensitive to chemotherapy, prognosis remains poor due to tumor recurrence, drug resistance, and toxicity associated with chemotherapy [2].

TICs or CSCs are a minor population of tumor cells that exhibit both self-renewal and differentiation capabilities as well as play key roles in metastasis, drug resistance and tumorigenic properties [9]. Transplantation of limiting numbers of TICs has been shown to regenerate breast tumor in NOD/SCID mice [11], demonstrating that TICs possess tumorgenic properties. Standard therapies, such as chemotherapy and radiation therapy, reduce tumor size via targeting bulk tumor cells, but not TICs, leading to tumor recurrence associated with metastasis and treatment resistance [143]. Thus, targeting TICs is considered a promising strategy for cancer prevention and treatment.

DHA is a long chain polyunsaturated omega-3 fatty acid (22:6 n-3), obtained from cold water fishes. DHA is being investigated as a potential dietary-based agent for cancer prevention including breast cancer [91]. DHA has been shown to exhibit multiple anticancer mechanisms of action, including inhibition of cell proliferation [144], metastasis [145], blood vessel formation [146], inflammation [147], as well as induction of cell cycle arrest [148], inhibition of differentiation [149] and induction of apoptosis [144]. Recent data show that DHA possesses the ability to inhibit mammary tumor formation in a mouse polyoma virus middle T antigen induced mammary cancer mouse model [95], to inhibit formation of mammospheres and to induce apoptosis of TIC-enriched human colon cancer cells [37], suggesting potential for eliminating TICs.

The term vitamin E encompasses a family of structurally distinct chemicals that are classified as tocopherols and tocotrienols in which there are four sub-forms, α, β, γ and δ [38]. Their anticancer actions have been widely studied [38]. Accumulating data show that gamma-tocotrienol (γT3), found in palm oil, cereal grains and rice bran as well as tocotrienol supplements, possess anticancer actions *in vitro* and *in vivo* [52]. Recent data show that γT3 possesses anti-TIC properties in human prostate and breast cancer cells [67, 150].

Published data from our lab showed that DHA induced apoptosis and that γT3 enhanced DHA pro-apoptotic actions in TNBC cells [72]. Objectives of this study are to evaluate the ability of DHA alone and in combination with γT3 to eliminate TICs and to investigate the mechanisms of action involved using human TNBC cells in culture.

3.3 Materials and Methods

3.3.1 Cell Culture and Reagents

Human TNBC cell lines MDA-MB-231, SUM159 and SUM149 [121] were used in this study. The sources and culture conditions of these cell lines were previously described for MDA-MB-231 human breast cancer cells [122], as well as SUM159 and SUM149 human breast cancer cells [151]. For experiments, fetal bovine serum (FBS) was reduced to 2% in all media to mimic *in vivo* conditions and cells were allowed to attach overnight before treatment initiation. DHA was purchased from Sigma-Aldrich. γT3 was a gift from the Malaysian Palm Oil Board (Kuala Lumpur, Malaysia). Both DHA and γT3 were dissolved in 1:4 DMSO/ethanol at 40 mM as stock solutions. Equivalent levels of 1:4 DMSO/ethanol was used as vehicle control (VEH).

3.3.2 Western blot analyses

Western blot analyses were conducted as described previously [122]. Primary antibodies to the following proteins were used in this study: c-Myc, and Cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA), pStat-3 (Tyr705) and Stat-3 (Cell Signaling), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, made in house). Secondary antibodies used included: horseradish peroxidase conjugated goat-anti-rabbit and rabbit-anti-mouse (Jackson Immunoresearch, Rockford, IL).

3.3.3 Analysis of aldehyde dehydrogenase activity and sorting of ALDH+ population

ALDH activity was determined using an Aldefluor assay kit (Stem Cell Technologies, Inc, Vancouver, BC, Canada) according to the manufacturer's instructions. Aldefluor fluorescence was detected by fluorescence activated cell sorting (FACS) using a FL1 detector in a LSR Fortessa flow cytometer and analyzed using BD FACSDiVa software (version 6.1.3). ALDH+ cells were sorted using a FACS Aria cell sorter (Becton Dickinson, Palo Alto CA).

3.3.4 Mammosphere formation assay

Unsorted and sorted ALDH+ cells were assessed for ability to form mammospheres using established mammosphere formation conditions [152, 153]. Briefly, cells were plated in 6-well ultra-low attachment plates at 100,000 cells/well under mammosphere formation conditions described in [153] followed by different treatments for 7 days. After counting the number of primary mammospheres, the mammospheres were disassociated into single cell suspensions and re-cultured for another 7 days without treatments to determine the number of secondary mammospheres. Then, the secondary mammosphere were disassociated and seeded back for another 7 days to determine numbers of tertiary mammospheres. Counting mammospheres of three generations were performed manually under 10 X microscopic examination.

3.3.5 Statistical analyses

Data were analyzed using one-way analysis of variance followed by the Turkey test for comparison of more than two treatments or a two-tailed Student *t*-test for comparison between two treatments to determine statistical differences. $p < 0.05$ was considered as statistically significantly different.

3.4 Results

3.4.1 DHA eliminates ALDH+ population in TNBC cells

ALDH+ has been shown to be a TIC marker in certain breast cancer cell lines [14]. Ginestier et al. have demonstrated that only ALDH+ cells from human breast cancer tumors can be xenotransplanted into NOD/SCID mice and serially passaged *in vivo* [14], whereas ALDH negative cells can not be serially passaged [14]. FACS analyses based on ALDH+ marker show that TNBC cell lines SUM 159, SUM 149 and MDA-MB-231 contain 4.7 ± 1.11%, 7.1 ± 0.56% and 2.0 + 0.4% ALDH+ cells, respectively (Fig 4.1a). Treatment of the ALDH+ cells from these three cell lines with different concentrations of DHA for 18 hrs induced a dose-dependent reduction of the ALDH+ cells (Fig 4.1b). Since ALDH+ is an established TIC marker in SUM 159 cells [14] and has been studied in SUM 149 cells as TIC marker [18], as well as in MDA-MB-231 cells as one of several TIC markers (CD44+/CD24-/ALDH+) [154], the data presented in Fig 1b suggest that DHA has potential to eliminate human breast TICs.

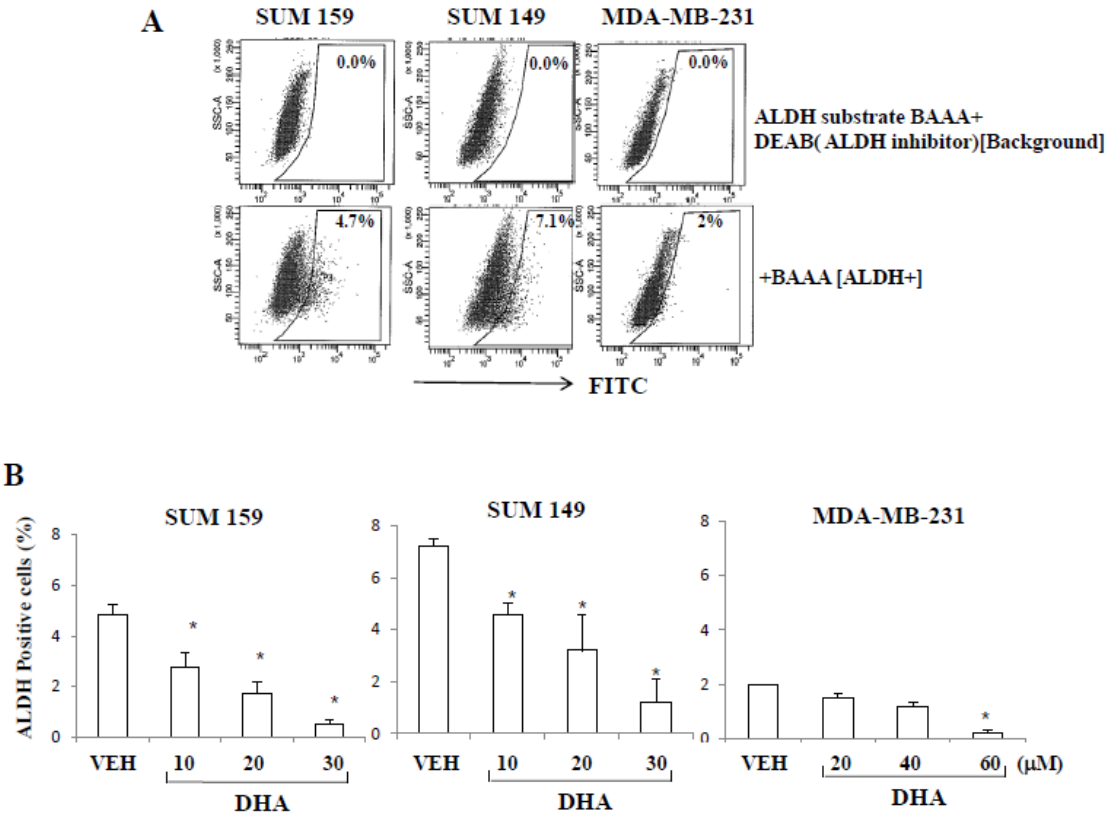


Fig 3.1 DHA eliminates ALDH+ population in TNBC cells. A. Percentages of ALDH+ population in three TNBC cell lines were determined using ALDH activity assay. Data are representative of one of three repeats. B. Percentages of ALDH+ population in cells treated with different concentrations of DHA for 18 hrs were determined by ALDH activity assay. Data are depicted as mean ± S.D. of three independent experiments. *significantly different from VEH, $p<0.05$.

3.4.2 DHA inhibits mammosphere formation

To further determine if DHA eliminates breast TICs we evaluated the effects of DHA on mammosphere formation, an established functional characteristic of TICs, using

serial mammosphere formation assays. Unsorted SUM 159 and MDA-MB-231 cells were treated with different concentrations of DHA for 7 days followed by counting the number of primary mammosphere. The numbers of secondary and tertiary mammospheres were determined after the primary and secondary mammospheres were diassociated and re-cultured for 7 days, respectively (Note: cells were treated with DHA for 7 days only in primary mammosphere formation). Vehicle treated cells served as control. DHA induced a dose-dependent reduction of the number of mammospheres in three generation in both cell lines (Fig 3.2A and 3.2B). Since the ALDH+ population has been established as TIC-enriched population in SUM 159 cells, we further determine if DHA inhibits serial mammosphere formation in the ALDH+ population sorted from SUM 159 cells. Data show that DHA induced a dose-dependent reduction of numbers of primary mammospheres of ALDH+ cells (Fig 3.2C). These data demonstrated that DHA has a potential to eliminate TNBC TICs.

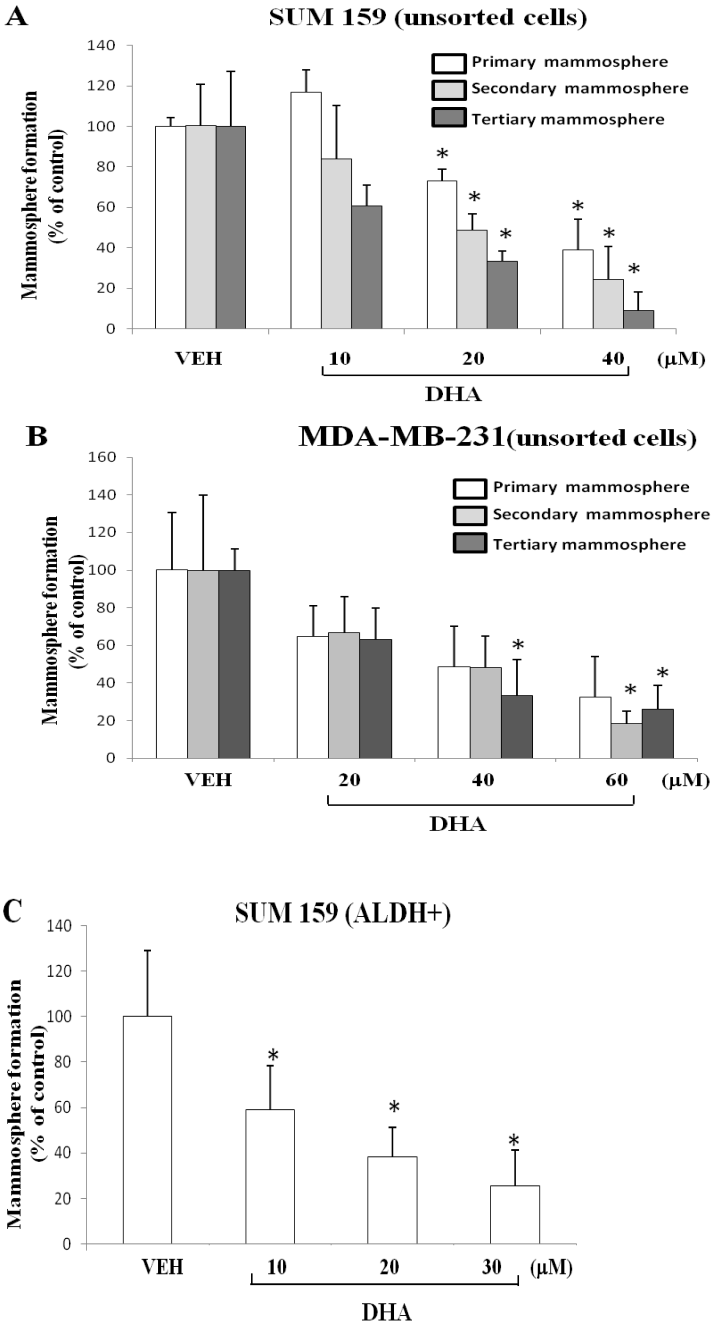

A
SUM 159 (unsorted cells)
Primary mammosphere
Secondary mammosphere
Tertiary mammosphere
Mammosphere formation (% of control)
VEH
10
20
40
(μM)
DHA
B
MDA-MB-231(unsorted cells)
Primary mammosphere
Secondary mammosphere
Tertiary mammosphere
Mammosphere formation (% of control)
VEH
20
40
60
(μM)
DHA
C
SUM 159 (ALDH+)
Mammosphere formation (% of control)
VEH
10
20
30
(μM)
DHA

Fig 3.2 DHA inhibits serial mammosphere formation. (A, B) Serial mammosphere formation assays were performed using unsorted SUM 159 and MDA-MB-231 TNBC cells cultured with different concentrations of DHA in the 7-day primary mammosphere formation assay. Secondary and tertiary mammosphere formation assays were conducted as described in the Materials and Methods section without treatment. (C) Primary mammosphere formation assay was performed using sorted ALDH+ cells treated with different concentrations of DHA for 7 days. The numbers of mammospheres are presented as mean ± S.D. of three individual experiments. *significantly different from VEH treatment, $p<0.05$.

3.4.3 DHA suppresses Stat-3 signaling

Stat-3 has been reported to contribute to maintenance of TICs in breast cancers [155]. To gain an understanding of the molecular mechanisms by which DHA eliminates TICs, we determined the effects of DHA on protein levels of pStat-3 and total Stat-3 (tStat-3) as well as the status of two Stat-3 target proteins: c-Myc, and cyclin D1. GAPDH served as sample loading controls. DHA suppressed levels of pStat-3, c-Myc, and cyclin D1 with no effect on total Stat-3 protein expression in both SUM 159 and MDA-MB-231 cell lines (Fig 3.3 A). Furthermore, DHA at 30 μM suppressed pStat-3, c-Myc and cyclin D1 protein levels in ALDH+ cells sorted from SUM 159 cells (Fig 3.3 B). These data suggest that DHA suppression of cyclin D1 and c-Myc via Stat-3 signaling may contribute to DHA elimination of ALDH+ population.

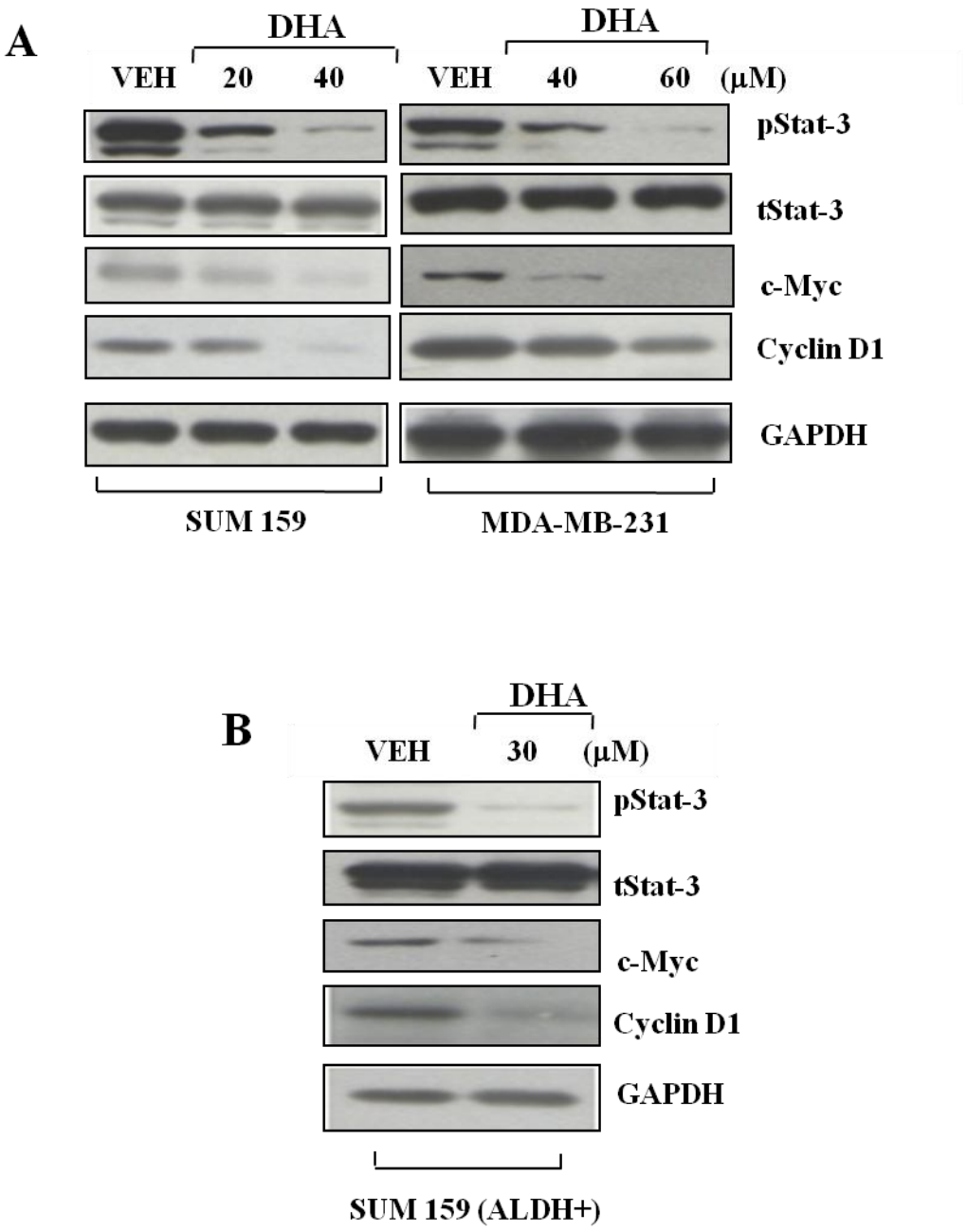


Fig 3.3 DHA suppresses pStat-3, cyclin D1 and c-Myc protein levels in TNBCs. (A and B) Western blot analyses were conducted to determine the effect of DHA on pStat-3, tStat-3, c-Myc and cyclin D1 protein levels in unsorted cells (A) and in ALDH+ sorted

SUM 159 cells (B). GAPDH was used as loading control. Data are representative of two or more independent experiments.

3.4.4 Stat-3 signaling contributes to maintenance of ALDH+ population and expression of cyclin D1 and c-Myc

To determine if Stat-3 contributes to the expression of c-Myc and cyclin D1 proteins, knock down studies were conducted using SUM 159 cells. SUM 159 cells were transfected with siRNA to Stat-3 or irrelevant siRNA for 18 hours, followed by determining numbers of ALDH+ cells. siRNA to Stat-3 reduced the number of ALDH+ cells from 5.1% (in irrelevant siRNA treated cells) to 0.8% (Fig 3.4A & 3.4B), clearly showing a role for Stat-3 in survival of ALDH+ cells. Western blot analyses show that siRNA to Stat-3 knocks down total Stat-3 and pStat-3, as well as c-Myc and cyclin D1 protein levels (Fig 3.4 C), confirming that c-Myc and cyclin D1 are downstream mediator of Stat-3.

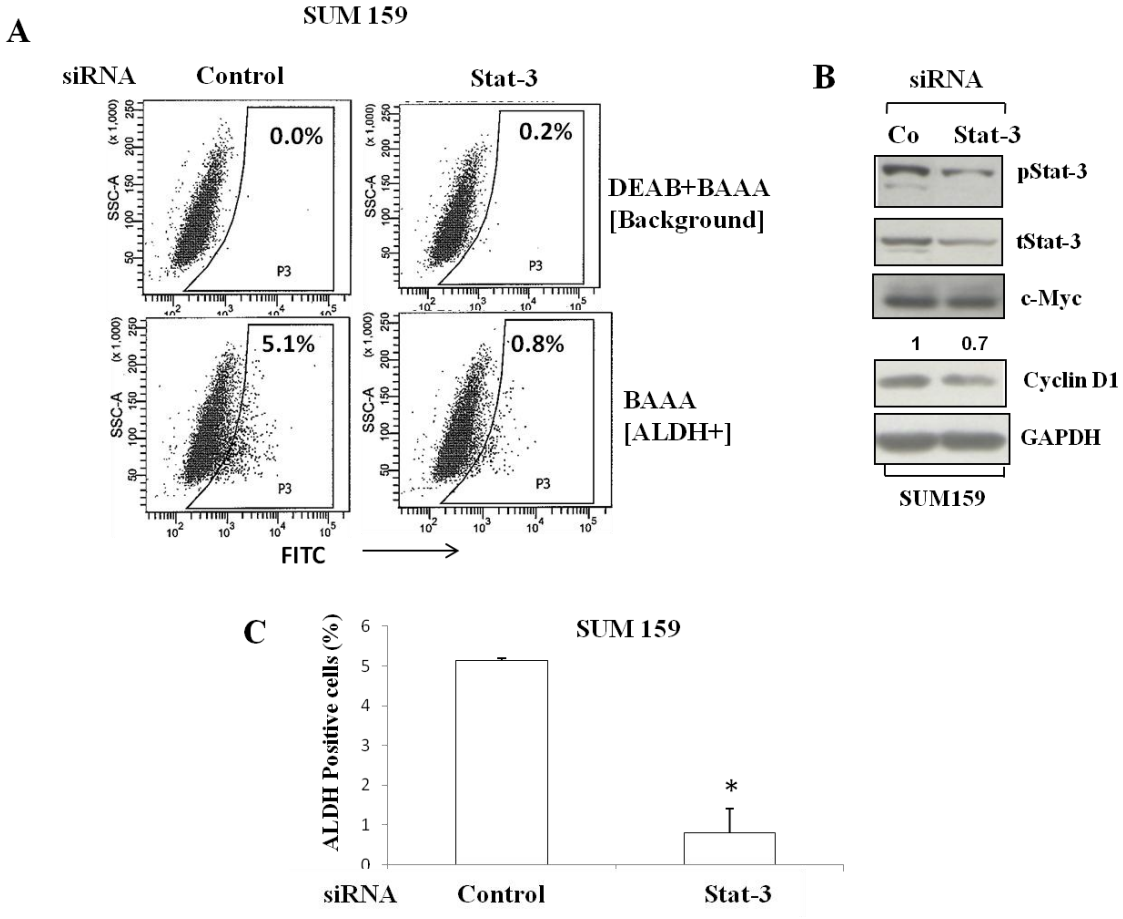
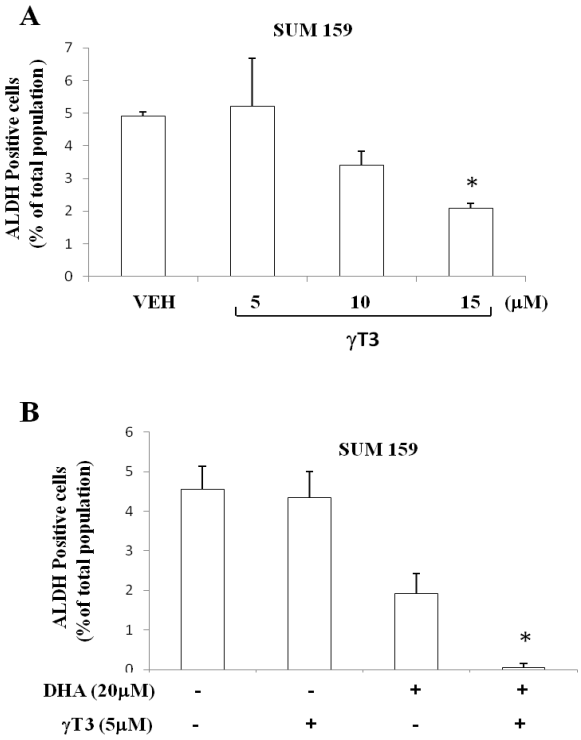
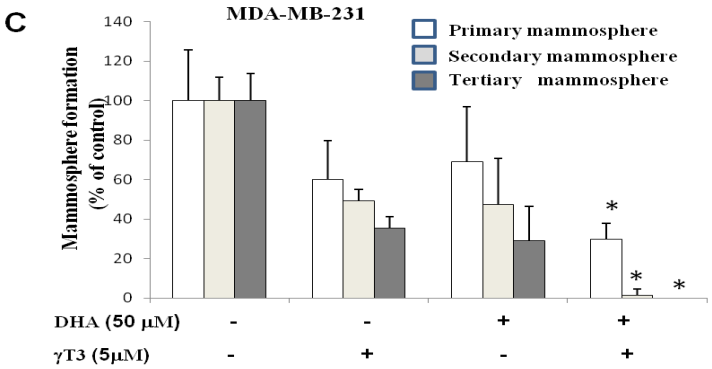


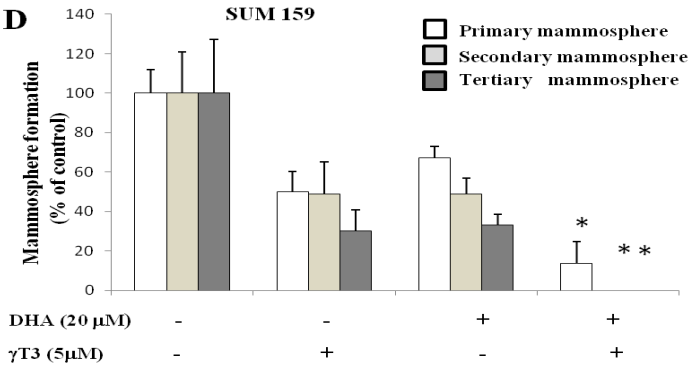
Fig 3.4 Stat-3 signaling contributes to numbers of ALDH+ cells as well as levels of cyclin D1 and c-Myc protein. (A and B) The percentages of ALDH+ population in SUM 159 cells transfected with siRNAs to Stat-3 or control siRNA were determined by ALDH activity assay. Data are depicted as mean ± S.D. of three independent experiments. (C) Western blot analyses were performed to determine pStat-3, tStat-3, c-Myc, and Cyclin D1 protein levels. Fold decrease of c-Myc protein expression was determined by densitometry analyses using Scion image software and normalized to GAPDH with the value for control as 1. GAPDH served as control. Data are representative of two or more independent experiments.

3.4.5 γT3 acts cooperatively with DHA to eliminate TICs and suppress Stat-3 signaling

Previously, we reported that γT3 possesses the ability to inhibit mammosphere formation and reduce ALDH+ population as well as suppress Stat-3 signaling in tamoxifen and doxorubicin drug resistant breast cancer cell lines [36]. Since γT3 has been reported to cooperate with DHA to induce apoptosis in TNBC cell lines [72], it was of interest to determine if γT3 cooperatively enhances DHA elimination of TICs and suppression of Stat-3 signaling. First, we evaluated the ability of γT3 to eliminate the ALDH+ population in SUM 159 cells. Data show that γT3 at 15 μM significantly reduced the numbers of ALDH+ cells (Fig 3.5A). Next, SUM 159 cells were treated with DHA and γT3 separately and in combination for 18 hours, followed by assessing numbers of ALDH+ cells. The combination of DHA at 20 μM and γT3 at 5 μM significantly reduced the number of ALDH+ cells in comparison to vehicle control and single treatments with DHA and γT3 (Fig 3.5 B). Treatment of SUM 159 and MDA-MB-231 cells with DHA plus a sub-optimal dose of γT3 significantly reduced mammosphere formation (Fig 3.5 C and D). Taken together these data show that DHA and γT3 act cooperatively to reduce the numbers of ALDH+ cells as well as mammosphere formation in TNBC cells. Furthermore, western blot analyses show that the combination of DHA + γT3 cooperatively reduced levels of pStat-3, c-Myc, and cyclin D1 (Fig 3.5 E), demonstrating that Stat-3 signaling is impacted by the combination of these two nutrients.


A
SUM 159
ALDH Positive cells
(% of total population)
7
6
5
4
3
2
1
0
*
VEH
5
10
15
(μM)
γT3
B
SUM 159
ALDH Positive cells
(%of total population)
6
5
4
3
2
1
0
*
DHA (20μM) - - + +
γT3 (5μM) - + - +


C
MDA-MB-231
Primary mammosphere
Secondary mammosphere
Tertiary mammosphere
Mammosphere formation (% of control)
140
120
100
80
60
40
20
0
*
*
*
DHA (50 μM) - - + +
γT3 (5μM) - + - +


D
SUM 159
Primary mammosphere
Secondary mammosphere
Tertiary mammosphere
Mammosphere formation (% of control)
140
120
100
80
60
40
20
0
*
* *
DHA (20 μM) - - + +
γT3 (5μM) - + - +

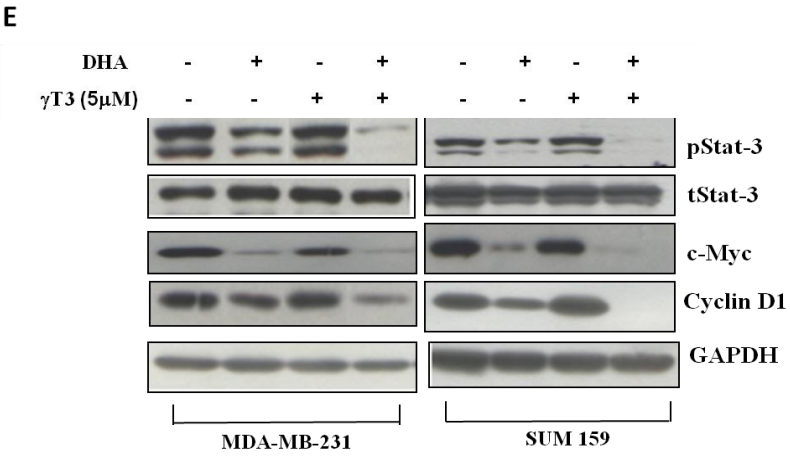

E
DHA - + - + - + - +
γT3 (5μM) - - + + - - + +
pStat-3
tStat-3
c-Myc
Cyclin D1
GAPDH
MDA-MB-231
SUM 159

Fig 3.5 DHA and γT3 cooperatively eliminate ALDH+ population, inhibit mammosphere formation, and suppress Stat-3 signaling. (A) The percentages of ALDH+ population in SUM 159 cells treated with different concentrations of γT3 for 18 hrs were determined by ALDH activity assay. (B) The percentages of ALDH+ population in SUM 159 cells treated singly with 5 μM γT3 or 20 μM DHA and in combination for 18 hrs were determined by ALDH activity assay. (C and D) Serial mammosphere formation assays were performed using unsorted MDA-MB-231 cells treated singly with γT3 or DHA alone and in combination in 7 day primary mammosphere formation assays. Secondary and tertiary mammosphere assays were conducted without further treatments as described in the Materials and Methods section. (E) Western blot analyses were performed to determine pStat-3, tStat-3, c-Myc, and Cyclin D1 protein levels in lysates from MDA-MB-231and SUM 159 cells treated with 5 μM γT3, 20 μM DHA, or with combination for 18 hours. Data in a-d are depicted as mean ± S.D. of three independent experiments. *significantly different from single treatments, $p<0.05$. Data in E are representative of two or more independent experiments.

3.5 Discussion

Previously, we reported that γT3 possesses the ability to eliminate TICs in tamoxifen and doxorubicin resistant human breast cancer cell lines [36] and can cooperatively enhance DHA-induced apoptosis in TNBC cells [72]. In this study, we investigated the ability of DHA alone and in combination with γT3 to eliminate TICs using human TNBC cells, and explored their molecular mechanisms of action. Novel findings from this study include: (i) DHA eliminates TICs as determined by the ability of

DHA to reduce the ALDH+ population and to inhibit mammosphere formation, (ii) the ability of DHA to eliminate TICs is correlated with the ability of DHA to reduce levels of pStat-3 as well as c-Myc and cyclin D1, (iii) siRNA to Stat-3 reduced the number of SUM159 ALDH+ cells and reduced pStat-3, c-Myc, and cyclin D1, suggesting that Stat-3 is necessary for maintaining ALDH+ population and contributes to the expression of c-Myc and cyclin D 1, and (iv) γT3 cooperatively enhances DHA elimination of TICs and suppression of Stat-3 signaling. Taken together, these data provide *in vitro* evidence that DHA and DHA + γT3 possess potential to eliminate TICs of human TNBC via suppression of Stat-3 signaling.

Accumulating *in vitro* and *in vivo* data support the notion that traditional cancer therapeutics such as chemotherapy and radiotherapy mainly target non-TICs (bulk of the tumor cells) and leave behind small numbers of TICs, which account, in part, for tumor recurrence, multi-drug resistance, and metastasis [152, 156 and 157]. Thus, targeting TICs is an emerging novel strategy for those challenged breast cancers including TNBC. Several natural compounds have been shown to possess *in vitro* and/or *in vivo* anti-TIC properties including curcumin [158], sulforaphane and piperine [31], galiellalactone [32], sesquiterpene lactone parthenolide [159], soy isoflavone genistein [160], blueberry [161], γT3 [36] and DHA [37], proving new opportunity as anticancer agent for cancer prevention and treatment. The present study further confirms that DHA possesses the potential to eliminate TICs, and for the first time, suggest that a combination of two

generally recognized as safe food based agents can act cooperatively to eliminate TICs, offering a potential treatment.

Stat-3 is a transcription factor [24] that is involved in cell proliferation, metastasis, and cell survival [25]. In response to cytokines and growth factors, Stat-3 family members are phosphorylated by receptor-associated kinases followed by forming homo- or heterodimers that translocate to the cell nucleus, where they function as transcription factors [24]. It has been reported that 50% of breast cancers express phosphorylated Stat-3, in which basal-like breast cancer cells express even higher levels [26, 27]. pStat-3 has been shown to be highly expressed in CD44+/CD24- populations and required for tumor cell growth [27], as well as involved in maintaining TICs in breast cancer cells, including SUM 159 cells [27]. Importantly, normal and benign breast cells do not express pStat-3 [26]. Thus, promising *in vitro* and *in vivo* data point to Stat-3 signaling as a target for TIC elimination in basal-like breast cancer, including TNBC.

Some of the natural compounds that have been shown to possess *in vitro* and/or *in vivo* anti-TIC properties exhibit the ability to suppress Stat-3 signaling [36, 158 and 161]. In this study, for the first time, we demonstrated that DHA and DHA + γT3 possess the ability to suppress Stat-3 signaling. How these two nutrients suppress Stat-3 signaling is not known and is worthy of further study.

Although pre-clinical studies provide evidence supporting omega-3 fatty acids obtained from cold water fishes, flax, walnuts and soybeans, as a potential dietary-based agent for breast cancer prevention, the epidemiologic studies have been inconclusive due,

at least in part, to the complex interactions among dietary lipids [162]. Clinical studies show that high level intake of EPA+DHA (at least 2.52 g /d) is necessary for anticancer efficacy [163]. However, studies report that high intakes of DHA/EPA have the risk to extend bleeding time [164]. Thus, a strategy that can enhance DHA anticancer efficiency is needed to increase the potential of omega-3 use as an anticancer agent. γT3 possesses low *in vivo* bioavailability and the maximal concentration that can be transiently reached is considered to be approximately 5 μM [165], which is a non-effective dose when tested *in vitro* for its anticancer actions. γT3, at the achievable concentration (5 μM), was shown to cooperate with DHA *in vitro* to induce apoptosis in TNBC cells in previous study [72], and to eliminate TICs in a recent study, providing a novel strategy that can maximize the anticancer efficacy of DHA and γT3 as an effective dietary cancer therapeutic. These studies also provide a sound rationale for further investigation of this nutrient-based combination for clinic use.

In summary, present data showing that DHA alone and the combination of DHA + γT3 eliminated human TNBC TICs and suppressed Stat3 signaling provides *in vitro* evidence to support the use of this nutritional combination for treatment of TNBC.

Chapter 4 Evaluation of the anticancer properties of vitamin E compounds

4.1 Abstract

Gamma- and delta-tocopherols (γT and δT) and -tocotrienols (γT3 and δT3) decrease the number of tumor initiating cells (TICs) in human SUM 159 triple negative breast cancer cells (TNBCs) *in vitro*. In striking contrast, RRR-α-tocopherol (αT) and synthetic vitamin E [*all-racemic*-α-tocopherol (*all-rac*-αT)] enhance the number of TNBC TICs. TICs were identified as the aldehyde dehydrogenase activity positive (ALDH+) sub-population and by their ability to form mammospheres *in vitro*. All of the anti-TIC vitamin E compounds (i.e. γT and δT as well as γT3 and δT3) suppressed pStat-3 and downstream mediators Cyclin D1 and c-Myc; whereas, αT and *all-rac*-αT enhanced pStat-3, Cyclin D1 and c-Myc. Taken together, the data show that vitamin E compounds exhibit different effects on TNBC TICs and suggest that Stat-3 signaling plays an important role in these events.

4.2 Introduction

One of the daunting challenges for treatment of breast cancer is triple negative breast cancers (TNBCs), a sub-type of breast cancer that accounts for ~15-20% of breast cancers in Western countries [2]. Due to a lack of effective targeted therapy, the only treatment available for this type of cancer is chemotherapy [2]. TNBCs are associated with poor prognosis and a low five year survival rate due, in part, to a high rate of tumor recurrence, multi-drug resistance, metastasis, and therapeutic toxicity [2]. It is urgent to identify low to non-toxic anticancer agents that successfully treat TNBCs. Accumulating

data suggest that successful treatment regimens need to eliminate both tumor bulk and tumor initiating cells (TICs)/cancer stem cells (CSCs) [143].

TICs/CSCs are defined as a small population in cancer cells, that exhibit normal stem cell characteristics of self-renewal and differentiation; as well as, the properties of metastasis, tumorigenesis and drug resistance [9]. Based on the TIC/CSC hypothesis, TICs are responsible for cancer initiation, metastasis, drug resistance and cancer recurrence [11]. Most current cancer therapies such as chemotherapy or radiation therapy, eliminate differentiated cancer cells that make up the bulk of tumor cells, also called non-TICs, but not TICs [12]. Accumulating data show that chemotherapy and radiation can increase the number of TICs [16]. This may explain the failure of current cancer therapies in some subtypes of breast cancer, including TNBCs. Thus, identification of non-toxic agents that eliminate TICs as well as non-TICs holds potential for successful treatment of the breast cancers, where current treatments are limited.

Certain naturally occurring bioactive compounds, such as curcumin [30], sulforaphane and piperine [31], galiellalactone [32], sesquiterpene lactone parthenolide [33], soy isoflavone genistein [35], blueberry [35], and docosahexaenoic acid (DHA) [95], have become attractive for both prevention and therapy of cancers due to their low toxicity and potential to eliminate both bulk of tumor cells and TICs. γT3, one form of vitamin E, has been reported to eliminate TICs in prostate and breast cancer cell lines [36, 67].

There are eight naturally occurring vitamin E compounds in plants: four tocopherols (α, β, γ, and δ) and four tocotrienols (α, β, γ, and δ) [38]. Various vitamin E forms have been studied for their anticancer properties [38]. Early studies were focused on αT due to its highest serum levels over other vitamin E forms in humans [38]. However, little to no effect on either cancer prevention or therapy has been observed [38, 50]. Instead, the recently published follow-up study of prospective clinic trail using selenium and synthetic vitamin E for prostate cancer prevention, namely SELECT, showed higher prostate cancer incidence in subjects given the *all-rac*-αT supplement [50]. More recent studies have focused on the γ- and δ-forms of tocopherol and tocotrienol. Accumulating data show that these forms of vitamin E possess anticancer actions both *in vitro* and *in vivo* [38]. γT3 and δT3 are enriched in cereal grains, wheat germ, rice bran and palm oil [38]. The unique anticancer features of γT3 and δT3 include: induction of cell cycle arrest [47], down-regulation of telomerase activity [166] and induction of apoptosis [47], as well as anti-angiogenic and anti-metastasis activities [47]. γT and δT are enriched in soybean oil, corn oil and castor oil [57]. These two tocopherols have been shown to inhibit tumor growth in a lung xenograft model, whereas αT did not [59]. These two forms of tocopherol exhibit the ability to inhibit cell growth by arresting the cell cycle at the S phase as well as suppressing cyclin D1 and cyclin E protein levels [61]. Tocopherols have been shown to induce apoptosis, involving activation of caspases-9 and -3, as well as interruption of the synthesis of sphingolipids [57]. A study from our lab showed that γT induces human breast cancer cells to undergo apoptosis via a death

receptor 5 (DR5) pathway [56]. Several *in vivo* studies have shown that both γ and δ forms of tocopherols and tocotrienols possess the ability to inhibit tumor formation and growth in preclinical xenograft mouse models [52, 59].

In this study, we evaluated the effects of the most studied forms of vitamin E on TICs in TNBC cells and found that different forms of vitamin E compounds exhibit differential effects on TICs and that the Stat-3 signaling pathway plays an important role in the anticancer effects.

4.3 Materials and Methods

4.3.1 Reagents

all-rac-αT was purchased from Sigma Chemical Co (St. Louis, MO, USA). αT and γT were gifts from TAMA Biochemical in Japan. δT was isolated from a natural mixed tocopherols concentrate (COVI-OX T-95, BASF, the chemical company, Illinois, USA). γT3 was a gift from the Malaysian Palm Oil Board (Kuala Lumpur, Malaysia) and δT3 was a gift from American River Nutrition, Inc (Hadley, MA).

4.3.2 Cell culture

Human breast cancer cell line SUM 159 cells were used in this study. The sources and culture conditions were described in Chapter 2 [72]. For experiments, FBS was reduced to 2%. For short term treatment (18 hrs), cells were plated at 1.5 x 10^5 cells/12 well for apoptosis, 3 x 10^5/6 well for ALDH activity and 3 x 10^6/100 mm dish for western blot analyses, respectively, referred as high cell density. For long term treatment (3 day),

cells were plated at 1 x 10^5/6 well for ALDH activity and 1 x 10^6/100 mm dish for western blot analyses, respectively, referred as low cell density. Cells were allowed to attach overnight before treatments. Vitamin E compounds were dissolved in 1:4 DMSO/Ethanol at 40 mM as stock solution, respectively. Equivalent levels of 1:4 DMSO/Ethanol was used as vehicle control (VEH).

4.3.3 Quantification of apoptosis

Apoptosis was quantified by Annexin V-FITC/PI assays following the manufacturer's instructions. Fluorescence was measured using Fluorescence Activated Cell Sorter (FACS) analyses with a LSR Fortessa flow cytometer, and data were analyzed using FACSDiVa software (version 6.1.3) (BD Biosciences, San Jose, CA). Cells displaying phosphatidylserine on their surface (i.e. positive for annexin-V fluorescence) were considered to be apoptotic.

4.3.4 Mammosphere formation assay

Unsorted and sorted ALDH+ cells were assessed for ability to form mammospheres using established mammosphere formation conditions [30]. Briefly, cells were plated in 6-well ultra-low attachment plates at 100,000 cells/well under mammosphere formation conditions described in [30] followed by different treatments for 7 days. After counting the number of primary mammospheres, the mammospheres were disassociated into single cell suspensions and re-cultured for another 7 days without treatments to determine the number of secondary mammospheres. Then, the secondary mammosphere were disassociated and seeded back for another 7 days to determine

numbers of tertiary mammospheres. Counting of mammospheres were performed manually under 10 X magnification on an Olympus DL71 microscope.

4.3.5 Analysis of aldehyde dehydrogenase activity and sorting of ALDH+ population

ALDH activity was determined using an Aldefluor assay kit (Stem Cell Technologies, Inc, Vancouver, BC, Canada) according to the manufacturer's instructions. Aldefluor fluorescence was detected by fluorescence activated cell sorting (FACS) using a FL1 detector in a LSR Fortessa flow cytometer and analyzed using BD FACSDiVa software (version 6.1.3). ALDH+ cells were sorted using a FACS Aria cell sorter (Becton Dickinson, Palo Alto CA).

4.3.6 Western blot analyses

Western blot analyses were conducted as described previously [72]. Primary antibodies to the following proteins were used in this study: pStat-3 and Stat-3 (Cell Signaling), c-Myc and Cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA), and glyceraldehyde-3-phosphate drogenase (GAPDH, made in house). Secondary antibodies used included: horseradish peroxidase conjugated goat-anti-rabbit and rabbit-anti-mouse (Jackson Immunoresearch, Rockford, IL).

4.3.7 Statistical Analysis

The student's *t*-test was used to determine statistical differences between treatment and control values. Differences were considered statistical significant at $p<0.05$.

4.4 Results

4.4.1 Both γT3 and δT3 induce apoptosis in SUM 159 TNBC cells.

Studies examined the ability of γT3 or δT3 to induce apoptosis in SUM 159 human TNBC cells using Annexin V-FITC-FACS apoptotic procedures. Treatment of the TNBCs for 18 hours with γT3 at 10 and 20 μM or δT3 at 5 and 7.5 μM significantly increased apoptotic percentages in comparison to vehicle control (VEH) (Fig 4.1). Treatment of human mammary epithelial cells (HMECs) with either 40 μM γT3 or δT3 did not induce apoptosis (data not shown), indicating that γT3 or δT3 selectively target cancer cells.

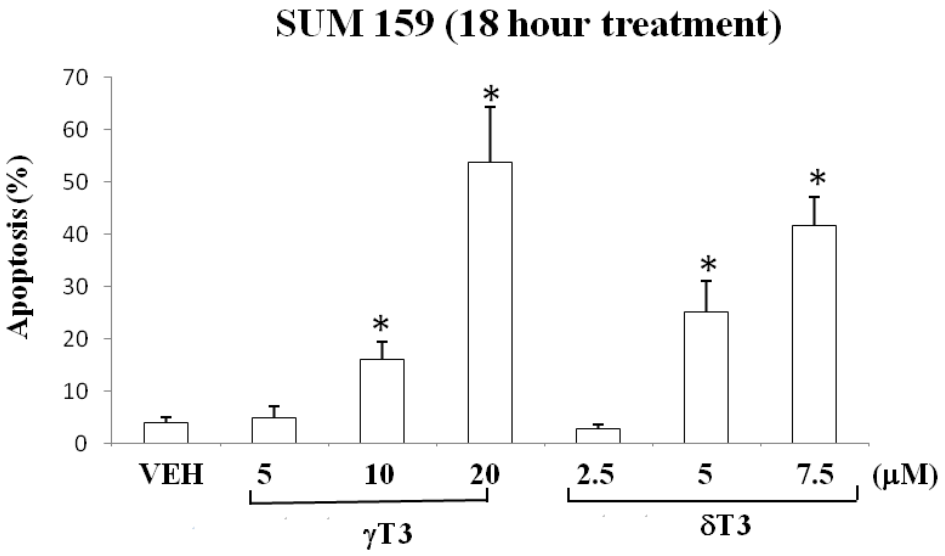


Fig 4.1 Both γT3 and δT3 induce apoptosis in SUM 159 TNBCs. Cells were treated with different concentrations of γT3 and δT3 for 18 hours, followed by Annexin V/PI analyses to determine percentage of cells undergoing apoptosis. Data are depicted as

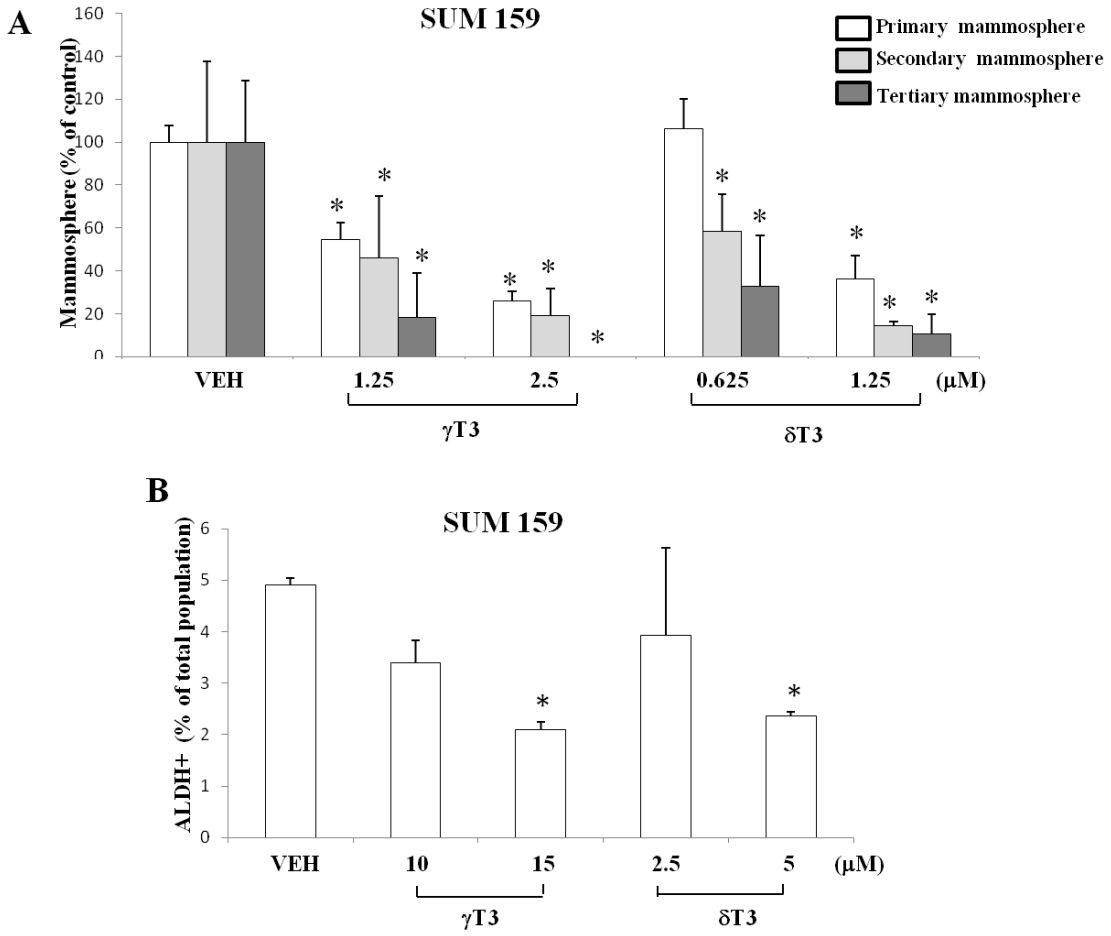
mean ± S.D. of three individual experiments. * Statistically different from vehicle control, $p<0.05$.

4.4.2 γT3 or δT3 reduce numbers of TICs in SUM 159 TNBC cells.

Studies investigated the ability of either γT3 or δT3 to reduce numbers of SUM 159 TICs by conducting three-generation (serial) mammosphere formation assays as well as analyzing the numbers of ALDH+ cells, two well established procedures for assessing the ability of treatments to reduce numbers of TICs [14, 15]. γT3 at 1.25 and 2.5μM significantly reduced the number of mammosphere forming cells in all three generations with γT3 at 2.5 μM eliminating all mammosphere forming cells by the third generation in comparison to vehicle control (Fig 4.2A). δT3 at 0.625 μM significantly reduced the number of mammosphere forming cells in the second and third mammosphere generations; whereas, γT3 at 1.25, significantly reduced the number of mammosphere forming cells in all three generations (Fig 4. 2A).

As an alternative approach, the ALDH activity assay was used to determine if γT3 or δT3 eliminated ALDH+ population, an established TIC marker in SUM159 cells [18]. Treatment of unsorted SUM 159 cells for 18 hours with γT3 at 15 μM or δT3 at 5 μM significantly reduced numbers of ALDH+ cells in comparison to vehicle control treated cells (Fig 4.2B). Furthermore, treatment of sorted ALDH+ TNBCs with low levels of

γT3 or δT3 eliminated the primary mammosphere forming cells in comparison to vehicle control (Fig 4.2C).



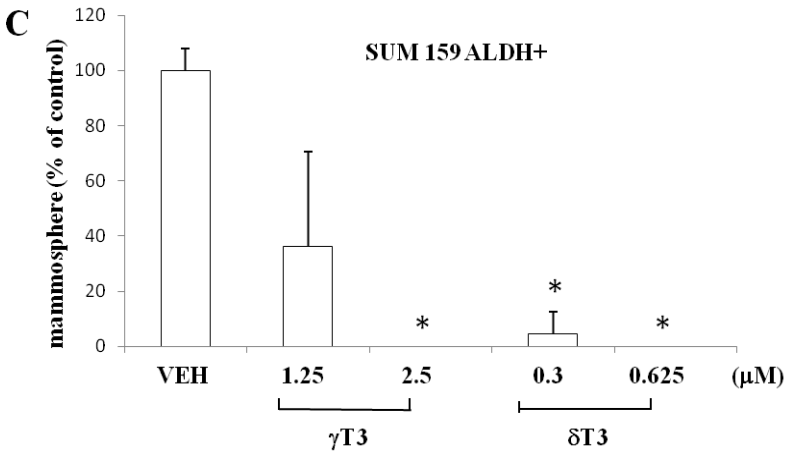


Fig 4.2 Both γT3 and δT3 eliminate TICs in SUM 159 TNBCs. Serial mammosphere formation assays were performed using unsorted SUM 159 cells cultured with different concentrations of γT3 and δT3 in the 7-day primary mammosphere formation assay. Secondary and tertiary mammosphere formation assays were conducted as described in the Materials and Methods section without further treatment. Mammospheres, using vehicle control as 100% are presented as mean ± S.D. of three individual experiments (A). Percentages of ALDH+ cells in unsorted population of SUM 159 cells treated for 18 hours with different concentrations of γT3 and δT3 were determined by ALDH activity assay (B). Primary mammosphere formation assay was performed using 100 sorted ALDH+ cells/24 well, treated with different concentrations of γT3 and δT3 for 7 days (C). A, B and C data are presented as mean ± S.D. of three individual experiments. *significantly different from VEH treatment, $p<0.05$.

4.4.3 γT3 or δT3 suppress pStat-3 as well as Cyclin D1 and c-Myc protein levels in SUM 159 TNBC cells

Treatment of SUM 159 cells for 18 hours with either γT3 or δT3 reduced levels of pStat-3 as well as pStat-3 downstream mediators Cyclin D1 and c-Myc (Fig 4.3).

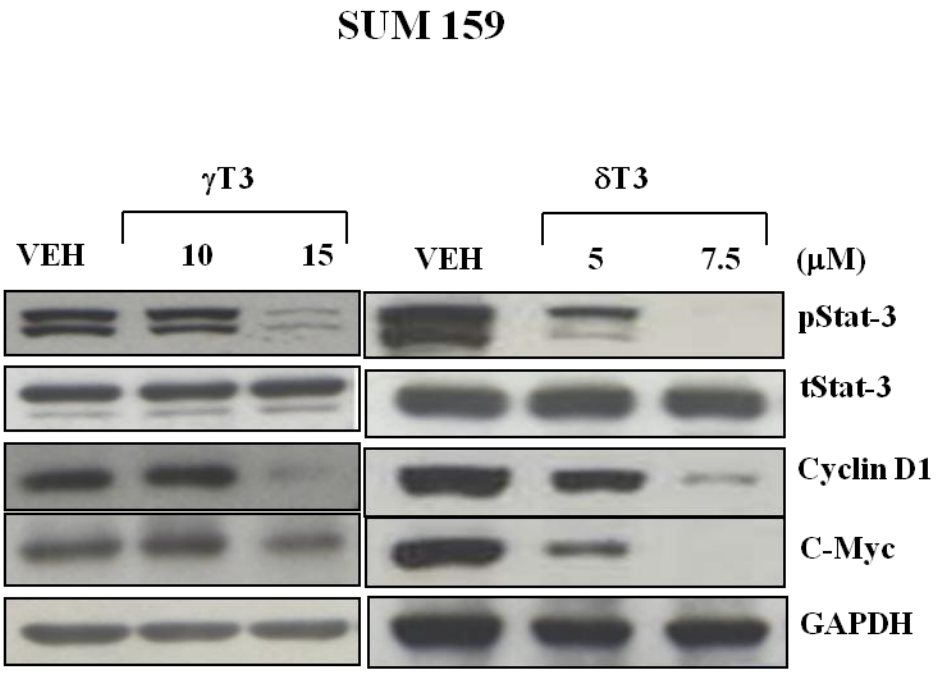


Fig 4.3 Both γT3 and δT3 suppress pStat-3, Cyclin D1 and c-Myc in SUM-159 TNBCs. Cells were treated with different concentrations of γT3 and δT3 for 18 hours, followed by western blot analyses to determine the protein levels pStat-3, tStat-3, Cyclin D1, and c-Myc in comparison with vehicle control. GAPDH was used as loading control. Data are representative of two or more independent experiments.

4.4.4 γT or δT induce apoptosis in SUM 159 TNBC cells.

Next, we evaluated the *in vitro* pro-apoptotic effects of γT and δT using SUM159 TNBC cells under both short term (18 hrs) and long term (3 days) treatment conditions**.** γT and δT treatments at different doses up-to 100 μM for 18 hours did not induce apoptosis (Data not shown). However, treatment with γT3 and δT3 for 3 days significantly induced apoptosis in comparison to control (Fig 4.4).

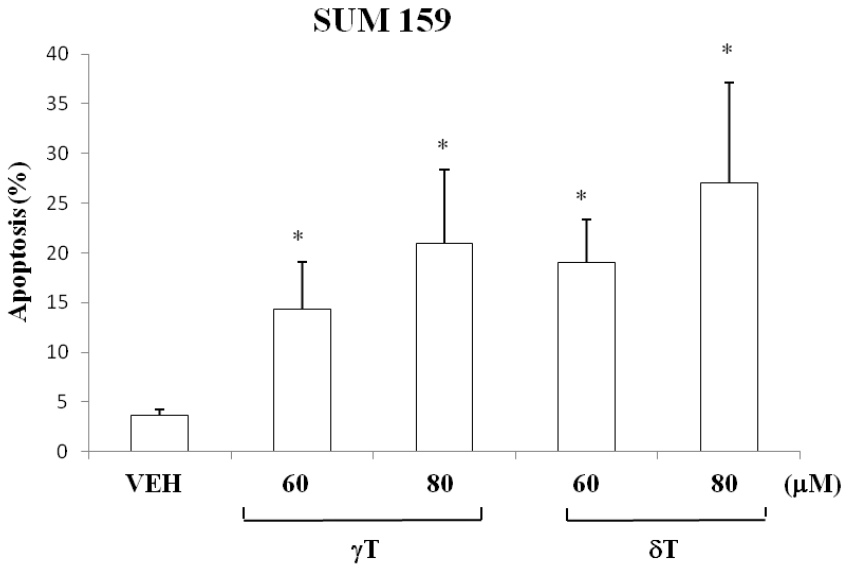
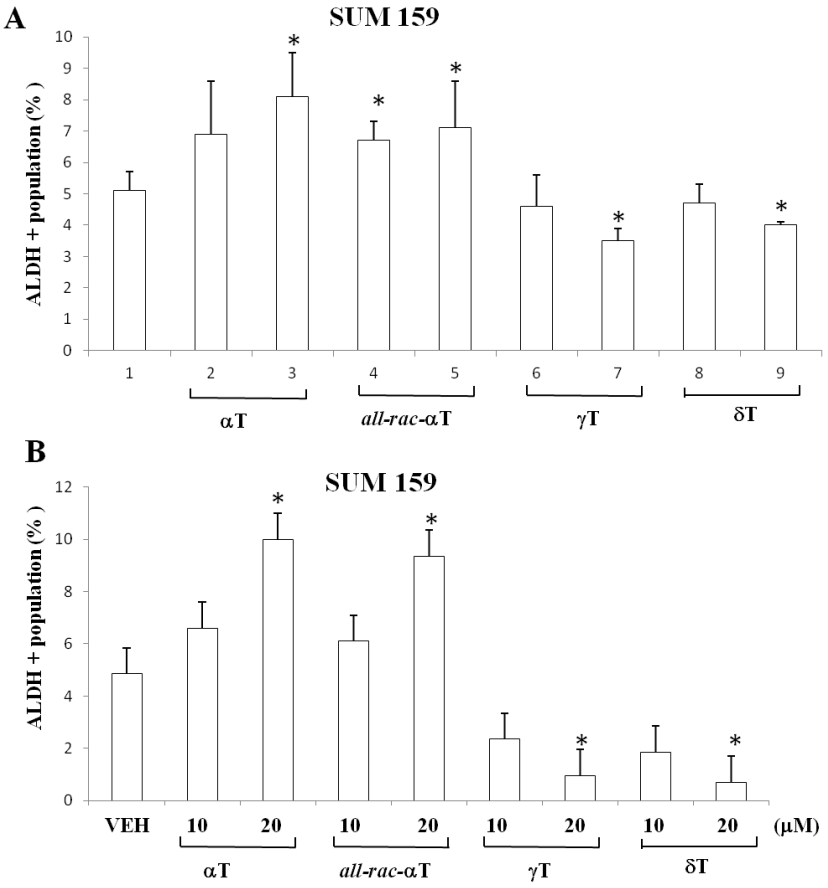


Fig 4.4 Both γT and δT exhibit effects on induction of apoptosis in SUM-159 TNBCs. Cells were treated with γT or δT at 60 and 80 μM for 3 days, followed by Annexin V/PI analyses to determine percentage of cells undergoing apoptosis. Data are depicted as mean ± S.D. of three individual experiments. * Statistically different from vehicle control, $p<0.05$.

4.4.5 Tocopherols exhibit different effects on TICs in SUM 159 TNBC cells.

The effects of αT, *all-rac*-αT, γT or δT on TNBC ALDH+ populations were investigated using 18 hour treatments (Fig 4.5A) as well as three day treatments (Fig 4.5B). γT or δT treatments of SUM 159 TNBCs at 40 μM for 18 hours, respectively, significantly reduced the numbers of ALDH+ cells; whereas, treatments with αT or *all-rac*-αT at 40 μM for the same time period significantly enhanced ALDH+ cells (Fig 4.5A). Three days treatment of the SUM 159 cells with the same vitamin E compounds at 10 or 20 μM gave similar results as observed with the 18 hour treatment time period, i.e., γT or δT significantly reduced the numbers of ALDH+ cells while αT or *all-rac*-αT significantly increased ALDH+ cells in comparison to vehicle control (Fig 5B). Furthermore, γT in a seven day mammosphere assay significantly reduced the number of primary mammosphere forming cells in unsortred SUM 159 cells (Fig 4.5C). αT at 20 μM significantly increased the number of mammosphere forming cells (Fig 4.5C), whereas, *all-rac*-αT treatments at 10 and 20 mM enhanced the percentages of mammospheres, but not significantly different from vehicle control (Fig 4.5C). Taken together, these data show that tocopherol compounds generate markedly different effects on TNBC TICs; namely, eliminated by γT and δT and increased by αT and *all-rac*-αT .


A
SUM 159
ALDH + population (%)
αT
all-rac-αT
γT
δT
B
SUM 159
ALDH + population (%)
VEH
10
20
(μM)
αT
all-rac-αT
γT
δT

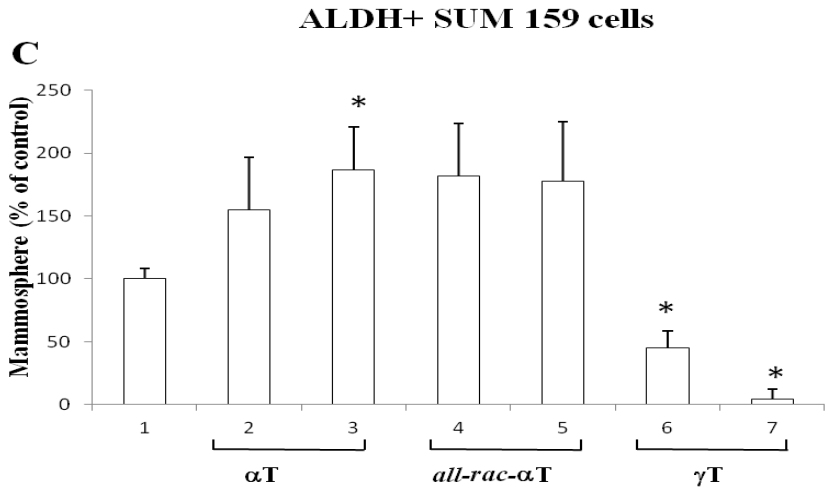

ALDH+ SUM 159 cells
C
Mammosphere (% of control)
αT
all-rac-αT
γT

Fig 4.5 Tocopherol compounds exhibit different effects on TICs in SUM 159 TNBCs SUM 159 Cells were treated for 18 hours with 20 and 40 μM αT, *all-rac*-αT, γT and δT, respectively (A). SUM 159 cells were treated for 3 days with these four vitamin E forms at 10 and 20 μM (B). Following treatment, the percentage of ALDH+ cells in unsorted SUM 159 cells were determined using ALDH activity assay (A and B). Sorted SUM 159 ALDH+ cells were treated with 10 and 20 μM αT, *all-rac*-αT or γT for 7 days during the primary mammosphere formation assay as described in the Materials and Methods section (C). Data are presented as mean ± S.D. of three individual experiments. *significantly different from VEH treatment, $p<0.05$.

4.4.6 αT and *all-rac*-αT enhanced pStat-3 protein levels, while γT and δT suppressed pStat-3 protein levels.

The effects of tocopherol treatments of SUM 159 cells on pStat-3, cyclin D1and c-myc proteins were evaluated by western blot analyses. Treatment of SUM 159 cells for three days with 10 or 20 μM αT or *all-rac*-αT increased pStat-3, c-Myc, and Cyclin D1 protein levels in comparison to vehicle control (Fig 6A). Treatment of SUM 159 cells with 60 or 80 μM γT or δT for three days suppressed pStat3, Cyclin D1 and c-Myc proteins levels (Fig 6B).

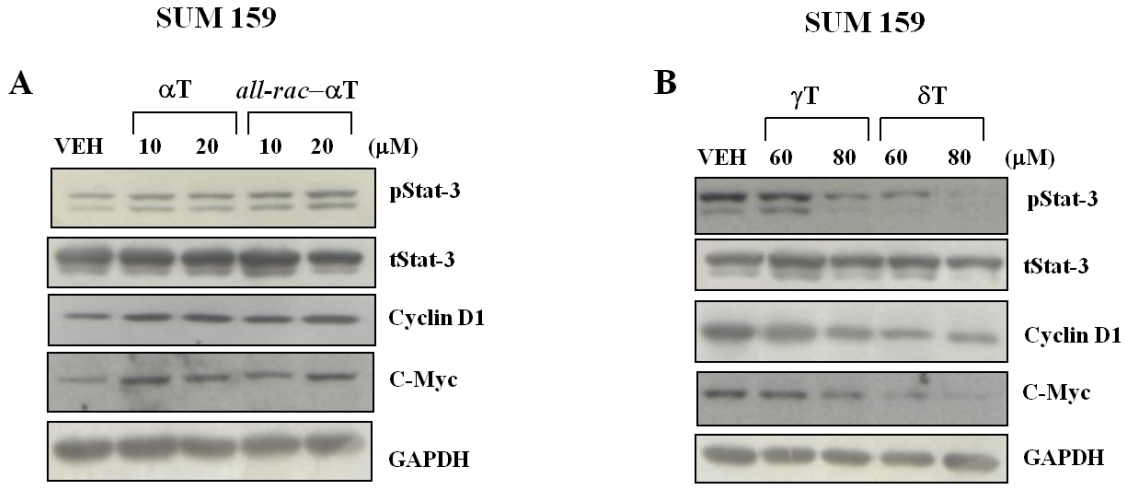


Fig 4.6 αT and *all-rac*-αT promote Stat-3 signaling in SUM 159 TNBCs while γT and δT suppress. SUM 159 Cells were treated for 3 days with 10 or 20 μM αT and *all-rac*-αT respectively (Fig A) or treated with 60 and 80 μM γT and δT for 3 days (B), followed by western blot analyses to determine protein levels of pStat-3, tStat-3, c-Myc and Cyclin D1. GAPDH was used as loading control. Data are representative of two or more independent experiments.

4.5 Discussion

There is growing evidence for the existence of TICs, which are a small subset of cells within a tumor, capable of self renewing and initiating/sustaining tumor growth [167,168]. TICs are a small subset of cells in tumors, i.e., 1% in colon cancer and leukemia and approximately 2% in breast cancer [169, 170 and 171]. Our study as well as other investigations, show the TIC sub-population of TNBCs to be approximately 5% [172].

The elimination of TICs provides a new opportunity for prevention and treatment of cancers, including human TNBC. In this regard, developing naturally occurring bioactive compounds that can target TICs as anticancer agent is highly significant for prevention of primary tumor and tumor recurrence. This study evaluated different vitamin E compounds for ability to eliminate TICs, and induce apoptosis in TNBCs. Novel findings are: (i) Tocotrienols (γT3 and δT3) induce apoptosis in TNBC cells, but not normal mammary epithelial cells, suggesting selective toxicity of γT3 and δT3 for breast cancer cells. Although tocopherols (γT and δT) induce apoptosis in TNBC cells, their pro-apoptotic potency is lower than tocotrienols; in marked contrast, αT or *all-rac*-αT did not exhibit pro-apoptotic properties. (ii) Both tocopherol and tocotrienol γ and δ compounds eliminated TICs, as demonstrated by their ability to reduce ALDH+ population and inhibit primary mammosphere formation of ALDH+ population. In contrast, αT and *all-rac*-αT increased the ALDH+ population, and enhanced the percentage of mammosphere forming cells (iii) Both tocopherol and tocotrienol forms of γ and δ suppressed pStat-3 as well as pStat-3 downstream targets c-Myc and Cyclin D1; whereas αT and *all-rac*-αT enhanced pStat-3 as well as Cyclin D1 and c-Myc protein levels. Taken together, our *in vitro* data show that γ- and δ-tocopherols or tocotrienols possess the *in vitro* ability to eliminate TICs, while αT and *all-rac*-αT promote TICs, thereby showing that some, but not all, vitamin E have anticancer properties, and most importantly showing that αT and *all-rac*-αT have potential to promote cancer. Data also

show that Stat-3 signaling is involved in the differential effects of these different forms of vitamin E on TICs.

Accumulating *in vitro* and *in vivo* data support the notion that γ- and δ-forms of tocopherols and tocotrienols are vitamin E forms that possess anticancer properties [56, 57 and 58]. Previous data from our lab showed that γT induced apoptosis in both MCF-7 and SUM 159 cells [56]. Jiang and co-workers reported that both γT and δT induced apoptosis in prostate cancer cells [173]. *In vitro* data showed that tocotrienols have better pro-apoptotic properties than tocopherols [56]. Here, for the first time, we show that not only γT3, but also δT3, as well as γT and δT possess *in vitro* anti-TIC properties. Data show that γT3 and δT3 not only induce TNBCs to undergo apoptosis, but selectively eliminate TICs. Although γT and δT show less pro-apoptotic property they eliminate TICs. However, due to the differences *in vivo* bioavailability of different forms of vitamin E [174], these *in vitro* data provide the rational for evaluation of select tocopherols and tocotrienols for their *in vivo* anticancer actions.

How γT and δT as well as γT3 and δT3 more selectively eliminate TICs is not known and worthy for further studies. TICs can be inhibited via different mechanisms, including inhibiting their self-renewal and differentiation capacity as well as triggering TICs to undergo apoptosis. TICs are highly resistant to apoptosis due to high expression of pro-survival pathway mediators [175]. Many targeted anticancer agents that activate pro-apoptotic pathways, but do not suppress pro-survival pathways, hardly have the ability to eliminate TICs [176]. While agents that can activate both pro-apoptotic

pathway and pro-survival pathways, such as chemotherapeutic drugs plus TRAIL, have the potential to stimulate TICs [176], suggesting that only agents that can both activate pro-apoptotic pathways and inhibit pro-survival pathways have the potential to eliminate TICs by apoptosis. Previous data from our lab showed that γT3 and δT3, as well as γT induce apoptosis via activation of ER-mediated DR5 pro-apoptotic pathway [56]. In this study, data show that both γ- and δ- forms of tocopherol and tocotrienol suppress Stat-3 survival pathway. Stat-3 has been shown to regulate survival mediators such as Bcl-xL, Bcl-2, Survivin, and c-FLIP. It has been reported that γT suppresses c-FLIP and Survivin [177]. In addition, γT3 has been reported to suppress Akt and NF-kB pro-survival mediators [65], suggesting that the ability vitamin E compounds, to activate pro-apoptotic pathways and to suppress pro survival mediators, may contribute to their ability to eliminate TICs via induction of apoptosis of TICs. Since c-Myc and Cyclin D1 are two important mediators for TIC renewal, we do not rule out the possibility that these vitamin E compounds eliminate TICs via inhibition of TIC renewal.

Importantly, these studies demonstrate that αT and *all-rac*-αT increase the numbers of TNBC TICs. Although the *in vitro* findings may not correlate with *in vivo* results, these studies may provide the first clue to explain why the recently finished follow-up of SELECT analyses showed higher prostate cancer incidence in subjects receiving *all-rac*-αT [51]. Our studies suggest further investigation of αT and *all-rac*-αT for its cancer risk potential, especially since both are widely used as dietary supplement and food additives.

How αT and *all-rac*-αT increase TICs is not known. Since our preliminary data show that the antioxidant N-acethyl cysteine (NAC) increased TNBC ALDH+ population as well as increased pState-3 protein levels (data not shown), our working hypothesis is that αT and *all-rac*-αT promote TICs via their antioxidant properties. It has been reported that TICs are enriched under redox conditions [178], suggesting that redox is essential for maintaining TICs.

To the best of our knowledge, this is the first report showing that αT and *all-rac*-αT increase Stat-3 protein levels in TNBCs, which is in conflict with previously published data showing that antioxidants such as NAC and diphenyleneiodonium (DPI) block Stat-3 activation in Hela cells [179]. Further studies are needed to resolve this conflicting data.

In summary, these data, for the first time, demonstrated that γT3, δT3, αT and δT, possess *in vitro* anti-TIC property in TNBCs, while αT and *all-rac*-αT exhibit the potential to promote TICs. Data also showed that suppression and activation of Stat-3 signaling are associated with elimination of TICs in γ- and δ-forms of tocopherol and tocotrienol treatments, and promotion of TICs in αT and *all-rac*-αT treatments, respectively.

Chapter 5 Summary and Future Directions

5.1 Summary

These studies investigated the anti-cancer properties of vitamin E compounds and DHA singly and in combination in human triple negative breast cancer cell lines.

Chapter 2 studies investigated the anti-TNBC properties of highly unsaturated omega-3 fatty acid docosahexaeonic acid, a dietary component widely used as a supplement with established anti-inflammatory, cardioprotective and anti-cancer properties. Data in this chapter showed that DHA at physiologically relevant levels induced apoptosis in human TNBCs, but did not induce apoptosis in normal human mammary epithelial cells under the same experimental conditions. Investigations of DHA's mechanisms of pro-apoptotic actions in TNBCs revealed triggering of endoplasmic-reticulum stress mediated CHOP/DR5 pro-apoptotic events involving both caspase-8 and caspase 9, suggesting both extrinsic and intrinsic apoptotic signaling. Data also showed that reactive oxygen species were a necessary upstream mediator of DHA-induced apoptosis. Vitamin E in the form of RRR-α-tocopherol blocked DHA-induced ROS formation as well as apoptotic events, indicating the potential for antagonistic effect of supplemental αT on DHA's anticancer actions. In contrast to αT, vitamin E in the form of γ-tocotrienol exhibited anticancer activity and enhanced DHA-

induced apoptosis. These data showed that DHA exhibited pro-apoptotic properties, αT blocked DHA-induced apoptosis, and γT3 enhanced DHA-induced apoptosis.

Chapter 3 studies investigated the ability of DHA to eliminate TICs and the cooperative effects of γT3. Novel findings in this study include: (i) DHA eliminated TICs as determined by the ability of DHA to reduce the ALDH+ population and to inhibit mammosphere formation, (ii) the ability of DHA to eliminate TICs was correlated with DHA's ability to reduce levels of pStat-3 as well as c-Myc and cyclin D1, (iii) siRNA to Stat-3 reduced the number of SUM 159 ALDH+ cells and reduced pStat-3, c-Myc, and cyclin D1, suggesting that Stat-3 is necessary for maintaining ALDH+ population and contributes to the expression of c-Myc and cyclin D 1, and (iv) γT3 cooperatively enhanced DHA elimination of TICs and suppression of Stat-3 signaling. Taken together, these data provide *in vitro* evidence that DHA and DHA + γT3 possess the potential to eliminate TICs from human TNBCs via suppression of Stat-3 signaling.

Chapter 4 studies investigated the anticancer effects of several vitamin E compounds for ability to eliminate TICs and induce apoptosis in TNBCs. γT3 and δT3 induced apoptosis in TNBCs, and importantly, had no adverse effects on normal human mammary epithelial cells, suggesting selective anti-cancer properties of these forms of vitamin E to TNBCs. In comparison with γT3 and δT3, γT and δT exhibited less pro-apoptotic activity; whereas, αT and synthetic vitamin E (*all-rac*-αT) did not exhibit pro-apoptotic properties. Furthermore, γT and δT as well as γT3 and δT3 eliminated TICs, as determined by their ability to reduce ALDH+ population and inhibit mammosphere

formation. In contrast, αT and *all-rac*-αT increased the ALDH+ population and promoted mammosphere formation, suggesting that these two forms of vitamin E enhance tumor progression rather than induce tumor suppression. γ- and δ-forms of tocopherols and tocotrienols suppressed pStat-3 and its downstream targets c-Myc and Cyclin D1; whereas, αT and *all-rac*-αT increased levels of Stat-3 signaling. In summary, these data showed that γ- and δ-forms of tocopherols and tocotrienols possess the *in vitro* ability to eliminate TICs; whereas, αT and *all-rac*-αT promote TICs, highlighting the fact that vitamin E forms exhibit quite different actions on cancer cells. Data also suggested that the Stat-3 signaling pathway is involved in the differential effects of different vitamin E compounds on TICs.

In summary, data presented in this dissertation showed that DHA and certain forms of vitamin E can induce apoptosis and eliminate TICs in TNBCs, and the combination of DHA + γT3 at physiological levels enhances the anticancer actions, thereby, providing a novel strategy for using those nutrient-based compounds singly or in combination as effective non-toxic dietary cancer therapeutics. In striking contrast, αT showed potential to promote TICs and block DHA anticancer action, suggesting a potential negative impact of αT on cancer prevention and treatment. These data provide a sound rationale for further investigations of these nutrient-based compounds for clinic use.

5.2 Future directions

Study #1 *In vivo* verification of the anticancer effects of DHA and γT3 singly as well as in combination. An important and necessary study before considering testing these two nutrients as anticancer agents in humans will be to test their anti-cancer properties *in vivo* by establishing TNBC tumors in the NOD/SCID pre-clinical xenograft mouse model and conducting a serial transplantation assay. End-points for efficacy evaluation would be to determine treatment ability to: (i) reduce primary tumor volume and metastases, (ii) eliminate TICs in primary tumor by *ex vivo* testing of tumors from treated mice for reduced ALDH+ population and reduced ability to form primary, secondary, and tertiary mammospheres, (iii) to reduce tumor incidence of secondary and tertiary transplants, and (iv) evidence that treatment activated ER stress/CHOP/DR5 and suppressed Stat-3 signaling in the primary tumor.

Study #2 *In vivo* verification of the effect of αT and *all-rac*-αT on promoting TICs and on inhibiting the anticancer action of DHA + γT3. *In vivo* evidence that the combination of γT3 and DHA are effective anticancer agents *in vivo* (i.e. expected outcome of Study #1) would provide the necessary data to test the ability of natural RRR-α-tocopherol) and synthetic vitamin E, the two most common forms of vitamin E taken as dietary supplements and used as food additives in the United States to promote TICs and to inhibit the anticancer actions of γT3 and DHA in the NOD/SCID mouse model using the same methods as study#1. This is a critical study that has important significance

for the beneficial use of DHA and γT3, and the harmful impact of RRR-α-tocopherol and synthetic vitamin E in the treatment of TNBC patients.

Study #3. Identification of the key role of Stat-3 signaling in regulation of breast cancer TICs and identification of upstream activators of Stat-3. Data showed that DHA alone or in combination with γT3 suppressed pStat3 pathway as a regulator of TIC in TNBCs, and that αT and *all-rac*-αT enhanced Stat3 signaling. Further studies are needed to determine if Stat-3 is necessary for the TIC blocking events and what up-stream initiators of Stat3 signaling are involved in either the beneficial (anti-TIC) versus harmful (pro-TIC) actions of various vitamin E compounds. Overexpression and knockdown assays will need to be used to determine the role of Stat-3 in elimination of TICs by DHA and γT3, as well as, by the combination of DHA + γT3, and in promotion of TICs by αT and *all-rac*-αT, respectively. Preliminary data are presented below as well as relevant references to focus future studies on identification of upstream mediators of Stat-3. It has been reported that γT3 suppresses pStat-3 via upregulation of protein-tyrosine phosphatase - sarcoma (Src) homology phosphatase-1 (SHP-1), a phosphatase that inhibits the phosphorylation of Stat-3 by Janus family kinases (JAK) [180]. Stat-3 also has been shown to cross-talk with NF-kB and PI3K/Akt pathways [181]. Preliminary data from our studies show that DHA and γT3 alone and in combination suppressed phospho-IkB (p-IKB) and downstream mediators of NF-kB signaling pathway in two TNBC cell lines. Furthermore, DHA prevented the translocation of NF-kB subunit p65 from the cytosol to the nucleus (Fig 5.1). Enhanced expression of SHP-1 has also been

observed in TNBCs treated with DHA and γT3 alone or in combination (Fig 5.2). Therefore it will be of interest to determine if NF-kB and SHP-1 are upstream mediators of Stat-3 in these events. A diagram of the proposed signaling pathways is presented in Fig 5.3.

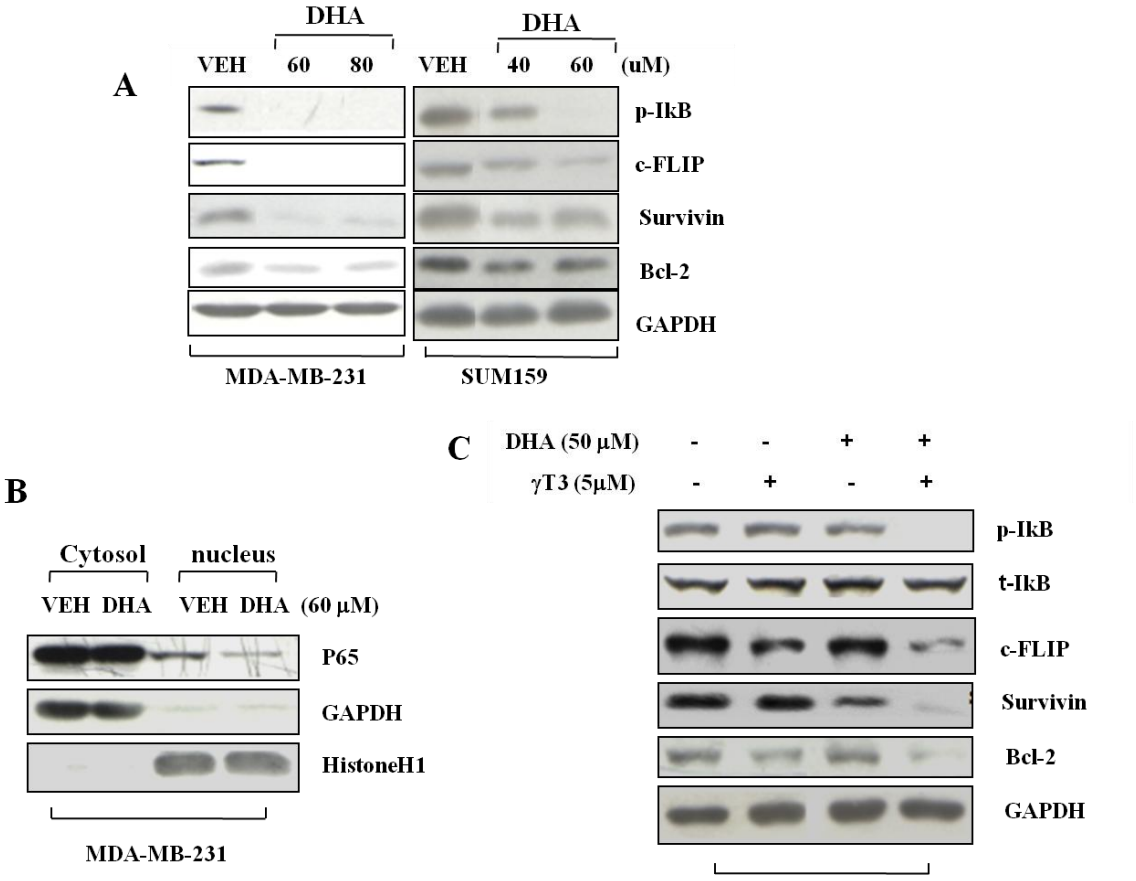


Fig 5.1 DHA and in combination of γT3 suppresses NF-kB pathway. A. Western blot data show that Phospho-IkB (p-IkB) and NF-kB downstream mediators, c-FLIP, Survivin and Bcl-2 are suppressed by DHA, suggesting that DHA inhibits NF-kB pathway. B. DHA suppresses P65 translocation of P65 from cytosol to nucleus, suggesting the ability of DHA to inhibit NF-kB activation. C. DHA in combination of

γT3 suppresses NF-kB as well as downstream mediators. Data are from two separate experiments.

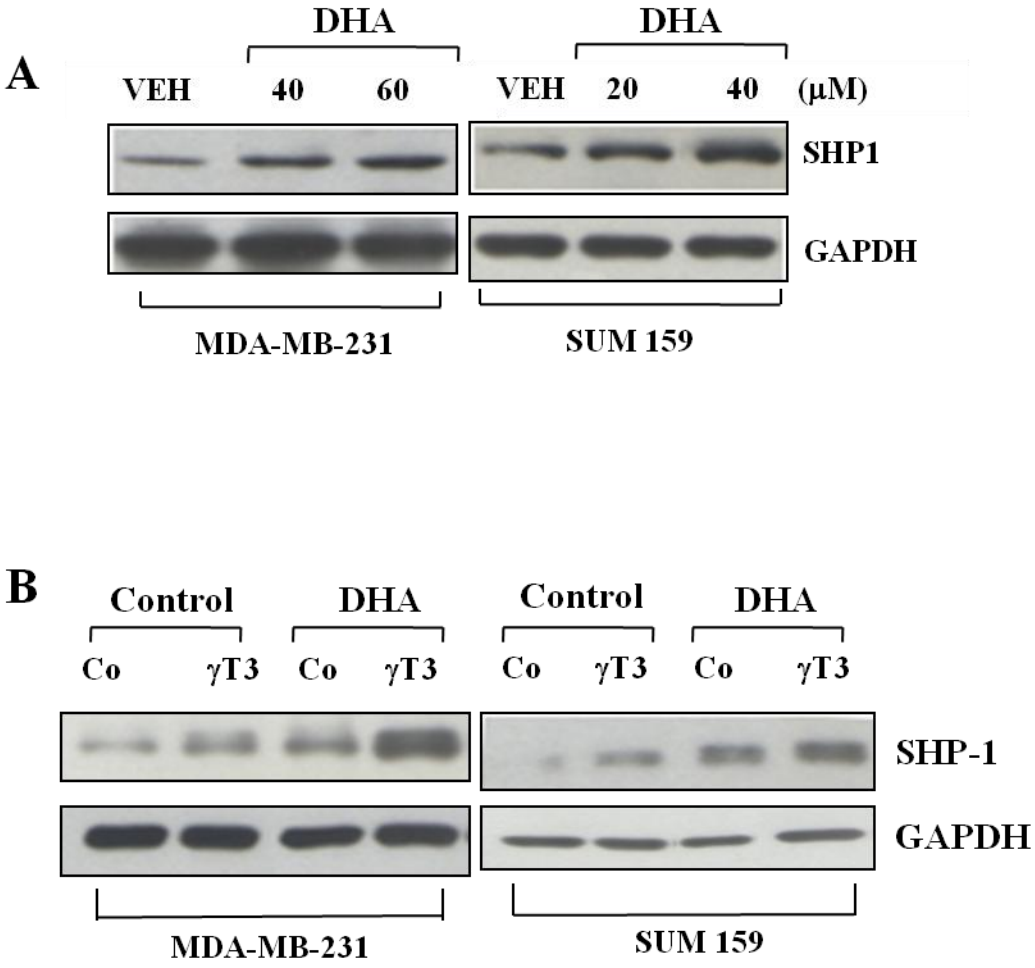


Fig 5.2 Upregulation of SHP-1 in MDA-MB-231 and SUM 159 cells Western blot show upregulation of SHP-1 with treatment of DHA alone (A) as well as in combination of γT3(B). Data are represented as two individual experiments.

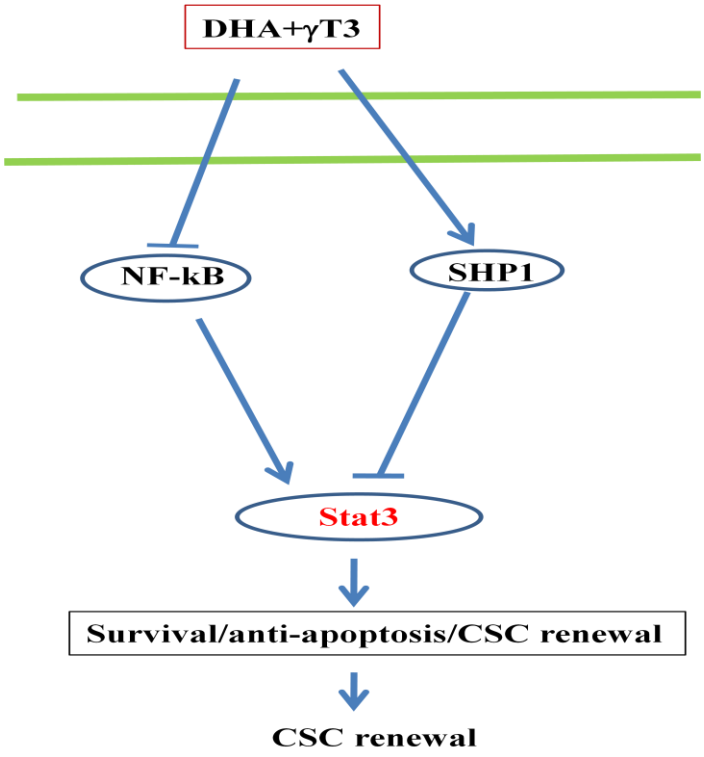


Fig 5.3 Proposed mechanisms of regulation of Stat-3 by NF-kB and SHP-1 Published and our present data show that DHA alone or in combination with γT3 suppress NF-kB as well as downstream factors c-FLIP, Bcl-2 and survivin. Therefore, we propose that DHA alone or in combination of γT3 eliminate TIC in TNBCs via suppressing CSC pro-survival mediator-pStat3 which is negatively regulated by SHP-1 and positively regulated by NF-kB.

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