Interactive Effects of Flaxseed and Flaxseed Oil and Trastuzumab on the Growth of Breast Tumors Overexpressing HER2

by

Julie Kathleen Mason

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Nutritional Sciences
University of Toronto

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Abstract

Trastuzumab (TRAS), a first line therapy for human epidermal growth factor receptor 2 (HER2)-overexpressing breast cancer, is limited by innate and acquired resistance. Approaches to enhance TRAS effectiveness and prevent resistance are needed. Flaxseed (FS) contains high levels of lignans and oil (FSO) rich in the n-3 polyunsaturated fatty acid α-linolenic acid (ALA). FS is commonly consumed by breast cancer patients and has demonstrated anticancer effects. This thesis determined whether FS, FSO and ALA, alone and combined with TRAS, could reduce the growth of HER2-overexpressing breast cancer and explored potential mechanisms with focus on HER2 signaling. Dietary FS and FSO alone did not affect growth of HER2-overexpressing, estrogen receptor (ER) positive breast tumors (BT-474) in athymic mice; however, FSO enhanced TRAS effectiveness in reducing tumor growth and cell proliferation and increasing apoptosis. Dietary FSO reduced biomarkers of HER2 signaling (pHER2, pAkt/Akt, pMAPK/MAPK). Tumor levels of ALA and its metabolites eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were elevated in FS and FSO-fed mice while conversion of ALA to EPA and DHA did not occur in BT-474 cells in vitro. Using concentrations derived from in vivo studies, ALA and DHA, alone and combined with TRAS, reduced BT-474 cell growth in...
vitro but only DHA reduced biomarkers of HER2 signaling (pAkt/Akt and pMAPK/MAPK). To further understand ALA mechanisms, it was tested in MCF-7 cells (ER+, low HER2) and was shown to affect the expression of ER-related signaling biomarkers. ALA did not prevent the development of TRAS resistance but reduced the growth of TRAS-resistant BT-474 derivatives and the TRAS-resistant UACC-732 cells. Together, these findings improve the understanding of the effects and mechanisms of FS and its components, particularly ALA in breast cancer and suggest that ALA-rich FSO may be a cost-effective complementary treatment for women with breast cancer being treated with TRAS.
Acknowledgments

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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-AAD</td>
<td>7-aminoactinomycin D</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>ALA</td>
<td>α-linolenic acid, 18:3n-3</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Cell Culture</td>
</tr>
<tr>
<td>B2M</td>
<td>β-2 microglobulin</td>
</tr>
<tr>
<td>BCIRG</td>
<td>Breast Cancer International Research Group</td>
</tr>
<tr>
<td>BD</td>
<td>Basal diet</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CO</td>
<td>Corn oil</td>
</tr>
<tr>
<td>CS-FBS</td>
<td>Charcoal-stripped fetal bovine serum</td>
</tr>
<tr>
<td>C_T</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid, 22:6n-3</td>
</tr>
<tr>
<td>DMBA</td>
<td>Dimethylbenz(a)anthrazene</td>
</tr>
<tr>
<td>E2</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>ED</td>
<td>Enterodiol</td>
</tr>
<tr>
<td>EL</td>
<td>Enterolactone</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid, 20:5n-3</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen response element</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen-binding fragment</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fc</td>
<td>Crystallizable fragment</td>
</tr>
<tr>
<td>FO</td>
<td>Fish oil</td>
</tr>
<tr>
<td>FS</td>
<td>Flaxseed</td>
</tr>
<tr>
<td>FSO</td>
<td>Flaxseed oil</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GRB2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5’-triphosphate</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HERA</td>
<td>Herceptin Adjuvant</td>
</tr>
<tr>
<td>HPRT1</td>
<td>Hypoxanthine-guanine phosphoribosyltransferase 1</td>
</tr>
<tr>
<td>IGFIR</td>
<td>Insulin-like growth factor I receptor</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>LA</td>
<td>Linoleic acid, 18:2n-6</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
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<td>MTT</td>
<td>3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NCCN</td>
<td>National Comprehensive Cancer Network</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NMU</td>
<td>N-nitromethyl-urea</td>
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<tr>
<td>OA</td>
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<td>OD</td>
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<td>Hydroxyestrone</td>
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<td>Ovariectomized</td>
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<td>Progesterone receptor</td>
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<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
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<td>PH</td>
<td>Pleckstrin-homology</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol (4,5)-bisphosphate</td>
</tr>
<tr>
<td>PIP₃</td>
<td>Phosphatidylinositol (3,4,5)-triphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RPLP0</td>
<td>Ribosomal protein P0</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SDG</td>
<td>Secoisolariciresinol diglucoside</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SERD</td>
<td>Selective estrogen receptor downregulator</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective estrogen receptor modulator</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of sevenless</td>
</tr>
<tr>
<td>TAM</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>TRAS</td>
<td>Trastuzumab</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WST-1</td>
<td>Water soluble tetrazolium salt-1</td>
</tr>
<tr>
<td>XTT</td>
<td>2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide</td>
</tr>
</tbody>
</table>
CHAPTER 1
Introduction
In the Western world, breast cancer is the most commonly diagnosed type of cancer in women (World Cancer Research Fund/American Institute for Cancer Research 2007; American Cancer Society 2013; Canadian Cancer Society's Advisory Committee on Cancer Statistics 2014). Breast cancer mortality rates have been steadily declining over the past two decades and the most recent 5-year relative survival ratio in Canada is 88% (Canadian Cancer Society's Advisory Committee on Cancer Statistics 2014). Despite this promising trend in survival, breast cancer remains the second-leading cause of cancer-related death in women in North America. The high personal, societal and economic burden of breast cancer renders research into strategies for prevention and treatment a high priority.

Breast cancer is a heterogeneous disease with distinct molecular subtypes that differ in prognosis and treatment approaches. Breast tumors that overexpress the human epidermal growth factor receptor 2 (HER2, encoded by ERBB2) are aggressive, highly metastatic and show a high risk of relapse (Nahta and Esteva 2006). Trastuzumab (TRAS, Herceptin™) was the first HER2-targeted therapy approved by the United States Food and Drug Administration in 1998 to be used as both a single agent and adjuvant therapy with chemotherapy. TRAS revolutionized HER2+ breast cancer treatment; however, limitations include innate and acquired resistance as the initial response rate is low and responders develop resistance within one year of treatment (Hudis 2007). Approaches to enhance TRAS effectiveness and prevent the development of resistance are being explored.

Many breast cancer patients make dietary changes following diagnosis and consume supplements and functional foods including flaxseed (FS) (Boon et al. 2007; Boucher et al. 2012). FS is the richest dietary source of phytoestrogens called lignans, predominantly secoisolariciresinol diglucoside (SDG) (Thompson et al. 2006). Approximately 40% of FS is
comprised of oil (FSO) and this oil is exceptionally rich in the n-3 polyunsaturated fatty acid (PUFA) α-linolenic acid (ALA, 18:3n-3). FS and its lignan and oil components have anticancer effects in various experimental models (reviewed in Mason and Thompson 2014). Similarities have been shown between the observed effects of TRAS, FS and FSO. These include reducing HER2 signaling through pathways including the mitogen-activated protein kinase (MAPK) and the Akt pathways (Sliwkowski et al. 1999; Bergman Jungestrom et al. 2007; Saggar et al. 2010a; Saggar et al. 2010b; Truan et al. 2010). In addition, ALA-rich FSO or FS and n-3 PUFAs have been shown to exert further anti-cancer effects including reducing the expressions of other growth factor receptors (i.e. epidermal growth factor receptor (EGFR), insulin-like growth factor I receptor (IGFIR)) (Saggar et al. 2010a; Saggar et al. 2010b; Truan et al. 2010) and increasing the expression of the tumor suppressor phosphatase and tensin homologue (PTEN) (Ghosh-Choudhury et al. 2009). The mechanistic studies of FS and its components on breast cancer growth biomarkers have been conducted in MCF-7 xenografts which have low HER2 expression. Nevertheless, when taken together, these findings suggest that FS and FSO may be capable of reducing tumorigenesis and modulating HER2 expression and growth factor receptor signaling pathways and enhance TRAS effectiveness in HER2+ cancer.

The question of which specific component(s) in a functional food, such as FS, contribute to its biological effects is of interest to nutritionists and basic scientists. Whether ALA has effects independent of its metabolites, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), particularly in carcinogenesis is unclear (Anderson and Ma 2009). This thesis aims to isolate the component responsible for the effect of FS on HER2 pathways and to distinguish effects of ALA from the long-chain n-3 PUFA DHA. Preclinical experimental models including animal and cell culture models are used. While these are limited in their ability to directly mimic the complex physiology of human breast cancer, their use allows
for a greater mechanistic understanding of FS components and paves the way for subsequent studies in humans.

The work presented in this thesis builds on my Master’s thesis which showed that a diet with high levels of FSO (8%) enhanced the effectiveness of TRAS in reducing the growth and cell proliferation and increasing apoptosis of HER2-overexpressing breast tumors (BT-474) in the athymic mouse model. My Doctoral thesis tests the hypotheses that ALA-rich FS and FSO will enhance TRAS effectiveness at reducing the growth of HER2 overexpressing tumors through a combination of mechanisms including altered membrane fatty acid profile and reduced signaling through HER2. Additionally, the hypotheses that ALA will prevent the development of TRAS resistance and will also enhance TRAS effectiveness in resistant cells are tested. The overall objectives are several fold and include the following: (i) to determine the effects of FS and FSO, alone and in combination with TRAS on the growth of established breast cancer with amplified HER2 expression using the athymic mouse model; (ii) to determine, using tumor biomarker expression analysis whether the effects of FS and FSO involve the downregulation of growth factor receptors, particularly HER2, and their signaling pathways; (iii) to determine whether ALA is the component in FS and FSO responsible for effects seen in vivo by using in vitro methods; (iv) to explore other potential mechanisms that contribute to ALA effect; (v) to determine the effect of ALA on the development of TRAS resistance using in vitro methods; and (vi) to determine the effect of ALA on the growth of TRAS resistant cells. If FS, FSO and ALA are shown to reduce HER2 overexpressing breast cancer growth and enhance TRAS effectiveness it will support further testing in clinical settings and ultimately may lead to recommendations for their use as cost-effective, complementary treatments for breast cancer patients treated with TRAS.
CHAPTER 2
Literature Review

Published in part in:


2.1 Breast Cancer

2.1.1 Breast Cancer Statistics and Risk Factors

Breast cancer continues to top the list as cancer site most commonly diagnosed in women in the Western world (World Cancer Research Fund/American Institute for Cancer Research 2007; American Cancer Society 2013; Canadian Cancer Society's Advisory Committee on Cancer Statistics 2014). With major improvements in screening, early detection and treatment, breast cancer mortality rates have seen a steady decline in Canada, the United States, the United Kingdom and Australia and the most recent 5-year relative survival ratio in Canada is 88% (Canadian Cancer Society's Advisory Committee on Cancer Statistics 2014). Despite this promising trend in survival, breast cancer remains the second-leading cause of a cancer-related death in women in the Western world. The high personal, societal and economic burden of breast cancer renders research into strategies for prevention and treatment a high priority.

Like all cancers, breast cancer is a multifactorial disease. Well established non-modifiable risk factors include those that increase exposure to estrogens such as early menarche and late menopause. Of the estrogens 17β-estradiol (E2) is the most biologically active. Family history and genetics (e.g. BRCA1 and BRCA2 mutations) also play a role; however, it has been suggested that heritable factors contribute less than 30% to breast cancer causation and that modifiable/environmental factors play the largest role in breast cancer risk (Lichtenstein et al. 2000). The World Cancer Research Fund estimates that 40% of breast cancer can be prevented through food, nutrition, and physical activity (World Cancer Research Fund/American Institute for Cancer Research 2007). Several dietary factors, alcohol consumption, hormone replacement therapy use, radiation exposure, physical activity and lactation have all been suggested to
influence breast cancer risk. Evidently, modifiable factors such as nutrition play an important role in breast cancer prevention.

2.1.2 Breast Cancer Pathophysiology

Breast cancer is characterized by uncontrolled proliferation of breast epithelial cells, a loss of cellular differentiation and the ability of cells to migrate/metastasize to other parts of the body. This complex process consists of three stages: initiation, promotion and progression/metastasis and involves multiple cell signaling pathways (Pitot 1993; Russo et al. 2000; Welsh 2007). The initiation stage is characterized by irreversible DNA damage in an epithelial cell in the duct or lobule of the mammary gland. Genetic mutations occur spontaneously in the cells of healthy individuals and many simply undergo apoptosis or remain dormant; therefore not all initiated cells progress to the promotion stage. When the initiated cell is mutated such that it acquires a growth or survival advantage and can evade the apoptotic signals, it undergoes clonal expansion and moves into the promotion stage of carcinogenesis. The promotion stage of carcinogenesis reflects dysregulation in the balance between cell proliferation and apoptosis, processes which will be described in greater detail in sections 2.1.2.1 and 2.1.2.2. During clonal expansion, mutated cells proliferate before DNA repair mechanisms are activated and mutations are passed on to daughter cells. The clonally expanding cells form clusters resulting in a localized cancer known as ductal or lobular carcinoma in situ depending on its site of origin. If the in situ cancer is not removed, cells will continue to divide and acquire additional genetic and epigenetic changes and will eventually escape the capsule surrounding the in situ mass. The cancerous cells can then invade the surrounding breast tissue and migrate through the blood stream to distant organs thus reaching the final stage of carcinogenesis: progression/metastasis. Breast cancers typically metastasize to the bone, lung and liver.
2.1.2.1 Cell Proliferation

Cell proliferation is a process initiated by a variety of stimuli that transmit instructions via a number of signaling pathways telling the cell to enter the tightly regulated cell cycle (Malumbres and Barbacid 2009). Examples of stimuli that initiate the process of cell proliferation include growth factors, cytokines and mitogens. The pathways by which these stimuli transmit signals to the nucleus telling the cell to proliferate are complex and the understanding of them continues to evolve. The major pathways implicated in cell proliferation include the protein kinase C (PKC) pathway, the JAK/STAT pathway and the MAPK pathway which will be described in greater detail in Section 2.1.5.2. These pathways ultimately affect transcription of modulators of the cell cycle. Progression through the four phases of the cell cycle, G1, S, G2 and M is tightly regulated with important checkpoints at G1/S and G2/M to ensure the fidelity of cell division. Cyclin-dependent kinases (CDK) are important regulators of the cell cycle. CDKs are constitutively expressed and become active when they associate with cyclins, proteins that are periodically expressed and abruptly destroyed during the cell cycle. CDKs phosphorylate and activate substrates, for example, protein products of the retinoblastoma gene that regulate the progression through the different phases. In addition to the time-dependent expression of cyclins, CDK activity is regulated through (i) phosphorylation by CDK-activating kinases or dephosphorylation by the phosphatase CDC25, (ii) inhibitory proteins such as p27kip1, (iii) proteolysis and (iv) the subcellular localization of kinase and substrate.

A hallmark of carcinogenesis is deregulated cell proliferation. This deregulation can occur due to mutations leading to overexpression or loss of any of the factors mentioned above. Examples include an overexpression of a growth factor receptor which is the case in HER2-overexpressing breast cancer (Murphy and Modi 2009) or the loss of a tumor suppressor such as p27kip1. Antineoplastic agents often target proliferative pathways and result in cell cycle arrest.
There is a wide array of methods that can be used to measure cell proliferation \textit{in vivo} and \textit{in vitro}. Clinical and \textit{in vivo} studies generally assess formalin-fixed paraffin embedded tumor tissue for expression of proteins expressed specifically in proliferating cells, for example Ki-67 or proliferating cell nuclear antigen. Measurement of proliferation \textit{in vitro} is generally indirectly based on cell viability or directly based on DNA synthesis. Cell viability assays measure the number of healthy cells in a population; if cell number is initially similar, fewer viable cells at the end of a treatment period compared to control indicate reduced cell growth caused by the treatment. Viable cells can either be measured directly through counting with the use of a hemocytometer or automated cell counter and a dye such as trypan blue. Trypan blue is excluded from viable cells with intact cell membranes. Alternatively, cell viability can be determined based on metabolic activity using assays based on tetrazolium salts [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), water soluble tetrazolium salt-1 (WST-1)] (Riss et al. 2013). These salts are cleaved into a coloured formazan product by metabolic activity. This coloured product is measured spectrophotometrically. Cell proliferation assays based on DNA synthesis, use labeled DNA precursors such as $^3$H-thymidine or bromodeoxyuridine (BrdU) and quantify their incorporation into DNA following incubation which is proportional to cell proliferation. Finally, cell proliferation can be assessed using more indirect measures such as determining the level of expression (mRNA or protein) or activity of regulators of the cell cycle. The selection of which proliferation assay to use should take into consideration the possibility of interference with treatment agents (Huang et al. 2004; Wang et al. 2010).
2.1.2.2 Apoptosis

Apoptosis is a natural process of programmed cell death in which unwanted cells are eliminated and is important in physiological processes such as development (Cotter 2009). Apoptosis is the results of a highly regulated sequence of events involving multiple signaling pathways. Two main pathways of apoptosis have been proposed: (i) the extrinsic pathway which is also referred to as the caspase 8 or death receptor pathway and (ii) the intrinsic pathway which is sometimes referred to as the mitochondrial pathway (Brenner and Mak 2009). The pathways are initiated through different mechanisms but both ultimately lead to activation of the executioner caspase 3, membrane alterations and DNA fragmentation.

There are three main approaches to measuring apoptosis which target different stages in the intrinsic and extrinsic apoptotic pathways: (1) caspase activity, (2) membrane alterations, (3) DNA fragmentation (Martinez et al. 2010). Caspases, or cysteiny1-aspartic acid proteases, are regulatory proteases that drive the progression of apoptosis through a cascade of activation. Caspases are classified into 3 groups: initiators (2, 8, 9, 10), executioners (3, 6, 7) and inflammatory (1, 4, 5). The relative abundance/activation of the different classes of caspases changes through early and late in the apoptosis process. An early feature of apoptosis is fragmentation of the plasma membrane. Methods to detect membrane fragmentation are based on the phenomenon in which phosphatidylserine (PS) which is found only in the inner membrane of healthy cells, moves to the outer leaflet of the plasma membrane. Annexin-V has a high affinity for PS and therefore is used to detect cells undergoing apoptosis. Co-detection of annexin-V with a vital dye such as 7-aminoactinomycin D (7-AAD) allows for differentiation between early apoptotic cells (annexin V positive, 7-AAD negative) and late apoptotic or necrotic cells (annexin V positive, 7-AAD positive). As apoptosis progresses, endonucleases break down genomic DNA between nucleosomes. This is an irreversible event that generates mono- and
oligonucleosomal DNA fragments of typical banding pattern. Methods to detect DNA fragmentation include DNA stains and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). Finally, microscopic examination can be used for a qualitative evaluation of the hallmark features of apoptosis including membrane blebbing and chromatin condensation.

2.1.3 Molecular Subtypes of Breast Cancer

While the carcinogenic process is similar among breast cancer subtypes, breast cancer is not one disease. To understand the biological features of a tumor, pathological assessment is used to determine expression levels of the estrogen receptor (ER), progesterone receptor (PGR) and HER2. ER and PGR are hormone receptors known to influence cell growth through transcriptional regulation of genes involved in cell cycle regulation and through activation of signaling pathways both directly and indirectly through cross-talk with growth factor signaling pathways. HER2, a member of the epidermal growth factor family, is a tyrosine kinase receptor that signals through the pro-proliferative and anti-apoptotic PI3K-Akt and MAPK signaling pathways. Each of these receptors will be discussed in greater detail in Sections 2.1.4 and 2.1.5, respectively.

On the basis of ER, PGR and HER2 status, a number of molecular subtype classifications for breast cancer have been proposed (Sorlie et al. 2003; Yang et al. 2007; Carlson et al. 2011; Goldhirsch et al. 2011). The St. Gallen Consensus panel of experts from Europe and North America also includes Ki-67 as a marker of proliferation and uses the following subtype classification: Luminal A (ER+/PGR+/HER2-/Ki-67 low), Luminal B (ER+/PGR+/HER2-/Ki-67 high), HER2 overexpressing (ER + or -/PGR+ or -/HER2+), basal (ER-/PGR-/HER2-) and normal breast-like (have characteristics similar to normal breast tissue) (Carlson et al. 2011;
Goldhirsch et al. 2011; Feeley et al. 2014). Recent investigations in breast tumor samples suggest that there are in fact up to 10 distinct forms of breast cancer (Curtis et al. 2012). A number of additional proteins and mutations are being explored for prognostic value and as therapeutic targets including PTEN (Singh et al. 2014), p53 (Chen et al. 2012) and caveolin-1 (Mercier and Lisanti 2012). Progression beyond the “one size fits all” approach to treatment has led to major improvements in care. As research continues to further characterize breast cancer, more novel treatment strategies can be developed. Like targeted pharmaceuticals, dietary agents may behave differently depending on the molecular subtype of breast cancer.

2.1.4 Hormone Receptors in Breast Cancer

Luminal breast cancer and some HER2-overexpressing breast cancer have the ER which can influence cell growth through multiple routes. These can be generally classified as genomic and non-genomic and are outlined in Figure 2-1. In the classical genomic mechanism of E2 action, E2 diffuses through the cell membrane to bind to the ER, of which there are two subtypes ERα and ERβ. The E2-ER complexes homo- or heterodimerizes and binds to a DNA segment called the estrogen response element (ERE) located in the promoter region of many target genes. This E2-ER binding causes conformational changes and the recruitment of transcription factors and co-activators which leads to the transcription of E2-sensitive genes. The ER can exert genomic effects in the absence of E2 through ligand-independent activation by various proteins including growth factor receptors and by caveolin-1 (Schlegel et al. 2001). Finally, ER can exert genomic effects through an ERE-independent mechanism by interacting with transcription factor complexes such as activator protein-1 (AP-1). There are an expansive number of E2-sensitive genes including those that code for PGR, cyclin D1 and pS2/TFF1. It is estimated that a third of E2-sensitive genes do not have an ERE (Bjornstrom and Sjoberg 2005). Non-genomic effects are mediated by membrane-associated ERs which activate various protein-kinase signaling cascades.
Figure 2-1. Molecular effects of 17β-estradiol (E2) and the estrogen receptor (ER) in breast cancer. E2 and the ER act through a number of mechanisms. (1) E2 that diffuses through the cell membrane can bind to the ER which dimerizes and binds to the estrogen response element (ERE) to induce gene transcription. (2) The ER can be activated/phosphorylated in the absence of E2 by various proteins including growth factor receptors and caveolin-1. (3) E2-bound ER interacts with transcription factor complexes such as fos and jun that can bind to DNA at regions other than the ERE. (4) Various transcription factors that activate the ERE can be activated by signaling through ER found in the plasma membrane and through cross-talk with growth factor receptors.

E2 = 17β-estradiol; ER = estrogen receptor; ERE = estrogen response element; PGR = progesterone receptor
ER are promiscuous receptors which have been shown to bind a wide range of molecules with varying affinity. Several compounds including phytoestrogens have been studied for estrogenic effects. In breast cancer, PGR acts in a similar manner to ER and the expression of the two receptors are closely linked.

### 2.1.5 HER2 Structure, Function and Signaling

The human epidermal growth factor receptor family of receptors share common structure with an intracellular domain with kinase activity, a transmembrane domain and an extracellular domain with four subdomains (Figure 2-2) (Pohlmann et al. 2009). HER2 is a 185 kD orphan receptor that is activated by homo- or heterodimerization with another member of the HER family. HER2 signals through a number of pathways including the Akt and MAPK pathways.

#### 2.1.5.1 Akt Signaling

The Akt signaling pathway, depicted in Figure 2-3, is known to influence cell survival and proliferation and is regulated by a number of receptors including HER2 and the tumor suppressor PTEN. The signaling cascade is activated when PI3K binds the tyrosine kinase domain of HER2. This causes the dissociation of the two subunits that make up PI3K: the 85 kDa regulatory (p85) subunit and 110 kDa catalytic subunit (p110α). p110α then migrates to the plasma membrane, where it phosphorylates and converts phosphatidylinositol (4,5)-bisphosphate (PIP₂) to phosphatidylinositol (3,4,5)-triphosphate (PIP₃). PIP₃ binds pleckstrin-homology (PH)-containing proteins such as Akt and phosphatidylinositol-dependent kinase 1 (PDK1). When bound to PIP₃, Akt is phosphorylated with the help of PDK1. Phosphorylated Akt (p-Akt) is the active form of the protein and it has been shown to have cellular effects including interaction
Figure 2-2. Structure of the Human Epidermal Growth Factor Receptor 2. (A) Human epidermal growth factor receptor family structure and (B) HER2 activation.

Figure 2-3. Akt signaling pathway regulation. PIP$_2$: phosphatidylinositol (4,5)-bisphosphate, PIP$_3$: phosphatidylinositol (3,4,5)-triphosphate, PDK1: phosphatidylinositol-dependent kinase 1, PTEN: phosphatase and tensin homologue.
with transcription factors, reducing apoptosis, increasing cell proliferation, invasion and survival as well as angiogenesis (Pohlmann et al. 2009).

2.1.5.2 MAPK Signaling

The MAPK signaling cascade, depicted in Figure 2-4, is known to influence a number of cellular processes including differentiation, cell cycle control, apoptosis and angiogenesis. Activation of the MAPK signaling cascade occurs through the adaptor protein growth factor receptor-bound protein 2 (GRB2) which recognizes the phosphorylated site on HER2. GRB2 also binds to the guanine nucleotide exchange factor Son of Sevenless (SOS). Activated HER2 binds to the GRB2/SOS complex resulting in SOS activation. SOS then removes guanosine diphosphate (GDP) from inactive RAS. The free RAS can then accept guanosine-5’-triphosphate (GTP) and become activated starting a cascade of activation. Activated RAS binds and activates Raf-1 (MAP3K), Raf-1 activates MEK1 (MAP2K1) and MEK2 (MAP2K2). These two forms of MEK phosphorylate and activate the MAPK1 and MAPK2. MAPK activation has several downstream effects including cell cycle control, differentiation and migration as well as apoptosis and angiogenesis.

2.1.6 Caveolin-1

Caveolin-1, encoded by CAV1, is structural protein localized in caveolae within lipid raft domains of cell membranes. The biological role of caveolin-1 is not yet fully understood. It plays a crucial role in normal mammary development; however, its role in tumorigenesis is contentious with both tumor suppressor and oncogenic potential (Patani et al. 2012). Experimental evidence suggests that through its caveolin scaffolding domain caveolin-1 negatively regulates membrane-bound components of signaling pathways. Examples include growth factor receptors, members of the MAPK signaling pathway, G-proteins and Src-family tyrosine kinases.
Figure 2-4. MAPK Signaling. GRB2: growth factor receptor-bound protein 2; GDP: guanosine diphosphate; GTP: guanosine-5’-triphosphate; SOS: Son of Sevenless
(Patani et al. 2012). As such, caveolin-1 has been proposed as a potential tumor suppressor. In contrast, caveolin-1 has been shown to promote steroid receptor signaling including pathways activated by the ER (Schlegel et al. 2001). These different effects on regulators of breast cancer growth suggest that caveolin-1 may have complex influence on breast cancer development and growth which requires further elucidation.

2.1.7 Treatment of Breast Cancer

Treatment approaches for breast cancer are complex and vary greatly based on tumor stage and molecular subtype and are outlined in the National Comprehensive Cancer Network (NCCN) guidelines (Carlson et al. 2011; Theriault et al. 2013). The general approaches considered are local treatments including surgery and/or radiation along with or followed by single agent or combination systemic therapies (i.e. chemotherapy, endocrine therapy and biologic agents). Cancer characteristics (example: stage, node status, histology, receptor expression, metastasis), patient characteristics (example: age, menopausal status, comorbidities) and patient preference are important considerations when determining which local and/or systemic treatments are employed.

If systemic treatments are employed, molecular subtype dictates which therapies are selected. ER+ tumors are treated with the ER antagonist tamoxifen (TAM) and, in the postmenopausal setting, with an aromatase inhibitor in combination with chemotherapeutic agents. First line treatment for HER2+ breast cancer, which is the focus of my doctoral work, includes the HER2-targeted agents TRAS and pertuzumab paired with chemotherapy. TRAS will be reviewed in more detail below. Novel approaches to treat HER2+ breast cancer including the tyrosine kinase inhibitor, lapatinib, and the antibody-drug conjugate, ado-trastuzumab (T-DM1)
are being investigated in clinical studies (Singh et al. 2014; Wong and Hurvitz 2014). How and if dietary agents interact with these systemic therapies is an area of research interest.

2.1.7.1 Experimental Models used in the Study of Breast Cancer Treatment

To study the effects of various treatments including diet and novel drugs on breast cancer growth, several useful animal models have been developed. Animal models are important tools in cancer research as they allow for the control of various factors that are difficult to regulate in a clinical setting such as E2, diet and environment. An overview of preclinical models used in studies of breast cancer treatment and prevention is found in Figure 2-5.

Tumors are established in animal models through a number of approaches. Murine tumors are developed in carcinogen-induced or transgenic rat or mouse models. Commonly used carcinogens used in the study of mammary carcinogenesis include dimethylbenz(a)anthracene (DMBA) and N-nitrosomethyl-urea (NMU). Transgenic models contain genetic alterations that result in spontaneous tumor development. A commonly used transgenic model used in the study of mammary carcinogenesis is the mouse mammary tumor virus (MMTV)/c-neu model of HER2-overexpressing breast cancer (Bouchard et al. 1989). These models can be used in the study of breast cancer treatment when interventions are provided once tumors have established and tumor growth is monitored. In addition, these models are useful in the study of breast cancer prevention as interventions can be provided prior to carcinogen-treatment or throughout the life cycle in transgenic animals and tumor development can be monitored. Disadvantages of these models relate to the fact that developed tumors are not of human origin and therefore do not accurately represent the molecular characteristics of human tumors.

To investigate the effect of interventions in the treatment of established tumors immunodeficient xenograft rodent models are also commonly used. This immunodeficiency
Figure 2-5. **Experimental models of breast cancer treatment.** DMBA= dimethylbenz(a)anthrazene; MMTV= mouse mammary tumor virus; NK= natural killer; NMU= N-nitromethyl-urea; SCID= severe combined immunodeficiency

allows for human cancer cells to be injected without rejection (Kavanaugh et al. 2002). Athymic and severe combined immunodeficiency (SCID) mice are the two most commonly employed models. The athymic mouse model is beneficial for studying human cancer as they have a nu/nu genetic mutation which results in the lack of a functional thymus gland. There is therefore a suppression of T-cell production, although B-cell and natural killer (NK) cells levels are normal (Charles River 2010). SCID mice have mutated protein kinase, DNA activated catalytic polypeptide (Prkdc), and therefore lack all immune cells (T cells, B cells and NK cells). Athymic mice are the preferred model when testing agents that may work through induction of an immune response through antibody-dependent cellular cytotoxicity (ADCC), a process that involves B cells and NK cells. One limitation of the xenograft model is that the injected cells are already transformed which does not allow for the study of the early stages of carcinogenesis.
In vitro studies are useful for investigating the role of potential anti-cancer agents as they provide information on specific effects and mechanisms of action to build upon in future in vivo and clinical studies. This involves the propagation of human breast cancer cells in vitro and varying the conditions of the culture medium. A number of important outcomes evaluated in vitro include cell growth, cell proliferation, apoptosis and changes in mRNA and protein expression.

2.1.8 Trastuzumab

2.1.8.1 Overview and Clinical Studies

TRAS was the first HER2-targeted therapy approved by the Food and Drug Administration in 1998 to be used as both a single agent and adjuvant therapy with chemotherapy for the treatment of HER2-overexpressing breast cancer (Hudis 2007). It is a recombinant humanized monoclonal antibody that specifically binds to domain IV of HER2. Many properties of HER2 in breast cancer have rendered the receptor an effective therapeutic target (Nahta and Esteva 2006). Firstly, its high expression levels correlate strongly with cancer development and poor prognosis. Secondly, HER2 is present in a high proportion in tumor cells and tumors have been shown to have uniform, intense immunohistochemical staining for HER2, which indicates that targeting HER2 could have effects on most cancer cells in a patient. Finally, HER2 has been shown to be expressed both in primary tumor sites as well as in metastatic sites, which suggests that targeting HER2 may be effective in all disease sites.

TRAS has been studied in a number of clinical trials (Table 2-1). TRAS efficacy and safety as a monotherapy were evaluated in a number of single-armed clinical trials with overall response rates ranging from 12-26% and minimal toxicity (Baselga et al. 1996; Cobleigh et al. 1999; Vogel et al. 2002). Four pivotal phase III clinical trials have been conducted thus far. The
Table 2-1. Clinical trials of TRAS treatment

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Treatment(s)</th>
<th>Results</th>
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<tr>
<td><strong>Phase II Trials (monotherapy)</strong></td>
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<tr>
<td>Baselga et al, 1996</td>
<td>46 MBC patients</td>
<td>• LD: 250mg; MD: 100mg</td>
<td>• Overall RR: 11.6%</td>
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<td></td>
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<td></td>
<td>• 37% of patients had minimal responses or stable disease lasting 5.1 months</td>
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<tr>
<td>Cobleigh et al, 1999</td>
<td>222 MBC patients who had previous chemotherapy</td>
<td>• LD: 4mg/kg; MD: 2mg/kg weekly</td>
<td>• Objective RR: 15% (intent to treat)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Median duration =9.1 months;</td>
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<td></td>
<td></td>
<td></td>
<td>• Median survival=13 months</td>
</tr>
<tr>
<td>Vogel et al, 2002</td>
<td>114 previously untreated MBC patients</td>
<td>• LD: 4mg/kg, MD: 2mg/kg weekly OR</td>
<td>• Overall RR: 26% (no difference between groups)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• LD: 8mg/kg, MD: 4mg/kg weekly</td>
<td></td>
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<tr>
<td><strong>Pivotal Phase III Trials</strong></td>
<td></td>
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<tr>
<td>Slamon et al, 2001</td>
<td>3222 patients with early stage breast cancer</td>
<td>• Control: AC - T (n = 1073)</td>
<td>• Estimated disease-free survival at 5 years: AC - T = 75%, TC+TRAS = 84%, AC – T+TRAS= 81%</td>
</tr>
<tr>
<td>(BCIRG 006)</td>
<td></td>
<td>• TC+TRAS (n = 1075)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>• AC – T+TRAS (n = 1074)</td>
<td></td>
</tr>
<tr>
<td>Goldhirsch et al., 2013</td>
<td>5102 patients with early stage breast cancer</td>
<td>• Control: Observation only (n=1553)</td>
<td>• At 8 years follow up:</td>
</tr>
<tr>
<td>(HERA)</td>
<td></td>
<td>• TRAS (1 year) (n=1552)</td>
<td>• Overall survival: Control vs. 1 yr TRAS: HR = 0.76 (0.67-0.86)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• TRAS (2 years) (n=1697)</td>
<td>• Control vs. 2 yr TRAS: HR = 0.76 (0.65-0.88)</td>
</tr>
<tr>
<td>Perez et al., 2011</td>
<td>4046 patients with early operable breast cancer</td>
<td>• Control: AC - T (n = 1880)</td>
<td>• Overall survival: HR = 0.63 (0.54-0.73)</td>
</tr>
<tr>
<td>(NSABP B31 &amp; NCCTG N9831 jointly analyzed)</td>
<td></td>
<td>• AC – T+TRAS (n = 1872)</td>
<td></td>
</tr>
</tbody>
</table>

AC – T = anthracycline (doxorubicin) + cyclophosphamide followed by taxel (docetaxel or paclitaxel); LD= loading dose; MBC= metastatic breast cancer; MD= maintenance dose; HR= hazard ratio; OS= overall survival; T= taxel (docetaxel or paclitaxel); TC = taxel + carboplatin; TRAS= trastuzumab
Breast Cancer International Research Group trial (BCIRG 006) involved 3222 women with HER2+ early stage breast cancer and showed a significant benefit of incorporating TRAS into common adjuvant chemotherapy regimens with lower cardiac side effects observed when TRAS was combined with a nonanthracycline-based chemotherapy regime (Slamon et al. 2011). The Herceptin Adjuvant (HERA) trial involved 5102 breast cancer patients with HER2+ early stage breast cancer and showed a significant benefit of TRAS (1 and 2 years) treatment following the standard treatment regimen in North Americans (surgery, chemotherapy and radiation (if indicated)) compared to observation at a median follow-up of 8 years. There was no additional benefit of 2 years compared to 1 year of TRAS treatment (Goldhirsch et al. 2013). The National Surgical Adjuvant Breast and Bowel Project B-31 and North Central Cancer Treatment Group N9831 trials were analyzed together and showed an overall benefit of adding TRAS treatment to anthracycline-based chemotherapy following surgical removal of HER2+ breast tumors after a median of 8.4 years on the study (Perez et al. 2014). The development and approval of TRAS was a breakthrough in the treatment of HER2-overexpressing breast cancer, however, limitations include a low response rate, the development of resistance (i.e. tumors regrow) within one year of treatment and cardiotoxicity (i.e. reduced left ventricular ejection fraction).

2.1.8.2 Mechanism of Action

The exact mechanisms of TRAS action have yet to be fully elucidated; however, \textit{in vitro} and \textit{in vivo} models have revealed many molecular and cellular effects which are outlined in Figure 2-6. Effects can be classified as those related to the antigen-binding fragment (Fab) and those related to the crystallizable fragment (Fc) of the TRAS antibody. The Fab binds domain IV of HER2 which is suggested to cause: (1) a reduction in HER2 signaling through MAPK and PI3K/Akt pathways and subsequent accumulation of p27kip1, a CDK inhibitor that promotes cell cycle arrest and apoptosis (Sliwkowski et al. 1999; Baselga et al. 2001); (2) inhibition of
Figure 2-6. Proposed mechanisms of TRAS effect. ADCC = antibody dependent cellular cytotoxicity; EGFR = epidermal growth factor receptor; HER2 = human epidermal growth factor receptor 2; MAPK = mitogen-activated protein kinase; PTEN = phosphatase and tensin homologue; TRAS = trastuzumab
angiogenesis by reducing the expression of pro-angiogenic factors, including vascular endothelial growth factor (VEGF), and increasing the expression of anti-angiogenic factors (Izumi et al. 2002); (3) inhibition of the shedding of the extracellular domain of HER2 (Pohlmann et al. 2009); (4) reduction of HER2 expression caused by internalization and degradation, although this mechanism has not been consistently observed (Sliwkowski et al. 1999; Baselga et al. 2001). Fc-related mechanisms include the induction of an immune response via ADCC (Lewis et al. 1993; Clynes et al. 2000).

2.1.8.3 Trastuzumab Resistance

Resistance to TRAS treatment is a significant clinical barrier and research is being done to better understand the molecular mechanisms driving this issue. Many HER2+ tumors fail to initially respond to treatment and those that do eventually become resistant to therapy (Pohlmann et al. 2009). A number of hypothesized mechanisms of action have been developed from preclinical studies which are suggested to either (1) prevent TRAS binding to HER2, (2) activate signaling pathways downstream from HER2, (3) increase signaling through alternative pathways and/or (4) reduce the ADCC-response to TRAS (Pohlmann et al. 2009). TRAS binding to HER2 may be reduced because of loss of or masking of the antigen binding site. A truncated form of HER2 (p95HER2) has been detected in TRAS resistant tumors (Scaltriti et al. 2007). Membrane proteins such as the glycoprotein mucin-4 and CD44 are suggested to mask the HER2 antigen binding site in the HER2-overexpressing JIMT-1 cell line contributing to the cells innate TRAS resistance (Nagy et al. 2005; Palyi-Krekk et al. 2007). Signaling pathways activated by HER2, namely the Akt pathway, have been shown to be reactivated in TRAS resistant cells and this may be related to either a loss of the tumor suppressor PTEN which acts by inhibiting PI3K or by an activating mutation to the catalytic site of PI3K. Following treatment with TRAS, other pathways are suggested to be upregulated including those activated by IGFIR, EGFR and HER3.
A number of pharmacological approaches to counteract these potential mechanisms of action are being explored. For example, lapatinib targets the tyrosine kinase domain of both HER2 and EGFR. Dietary approaches to reduce TRAS resistance have not been fully explored.

2.1.9 Diet and Breast Cancer Treatment

No specific dietary guidelines have been established in Canada specifically for women with breast cancer. The World Cancer Research Fund’s Continuous Update Project suggests that the following nutrition-related factors may improve breast cancer survival: healthy body weight, consumption of fibre-containing foods, consumption of soy-containing foods, lower intake of total fat and in particular saturated fat (World Cancer Research Fund/American Institute for Cancer Research 2014). A great deal of research is being done to explore the effectiveness of various dietary compounds, including dietary FS, and breast cancer treatment outcomes. With more and well-designed studies, it is hoped that dietary guidelines can be established.

2.2 Flaxseed

2.2.1 Composition

Cultivated FS varies in composition depending on cultivar, and growth location and environmental conditions but it typically contains approximately 30% dietary fiber, 20% protein, 40% oil (Daun et al. 2003; Hall et al. 2006). Of interest and the main reason for its use as dietary supplement or ingredient for health benefits is its high amount of oil rich in the n-3 fatty acid, ALA, the high amount of dietary fiber, and the phytoestrogens called lignans.

FS is the richest dietary source of lignans with approximately 820 to 1,050 µmol lignan per 100g of FS (Thompson et al. 2006). Lignans are diphenolic compounds formed through the coupling of two cinnamic alcohol residues. Many types of lignans are found in plant foods. The predominant lignan in FS is SDG (approximately 95%) although matairesinol, pinoresinol and
lariciresinol are also found. Ethanol extraction can isolate SDG from FS and methods are available for producing products containing about 40% SDG (Hall et al. 2006). Some of these extracts are now commercially sold for use as dietary supplements or food ingredients. Upon consumption, SDG is metabolized by colonic bacteria to the enterolignans, enterodiol (ED) and enterolactone (EL). Since the conversion of plant lignans is dependent on microbiota activity, the conversion to enterolignans differs with subjects’ dietary habits and lifestyle, and is reduced by antibiotic intake (Adlercreutz 2007). The enterolignans are either absorbed in the colon where they undergo enterohepatic circulation and are excreted in the urine or are directly excreted in the feces. Urinary excretion is primarily in the form of glucuronide conjugates with a small amount as sulfate conjugates or free enterolignans. Urinary and serum levels of enterolignans (or their conjugates) are related to dietary plant food intake and have been used in epidemiological studies as indicators of lignan intake (Buck et al. 2010; Zaineddin et al. 2012). Lignans have a structural similarity to E2 and are suggested to have estrogenic or anti-estrogenic effects in the body and have been studied in hormone-related diseases such as breast cancer.

FSO contributes to approximately 40% of FS by weight. The majority (~96%) of lipids in FSO is neutral lipids including acylglycerols and free fatty acids and there is a small content of polar lipids. The most abundant type of lipid is triacylglycerols with 30-35% shown to be trilinolenate (Ayorinide 2000; Holcapek et al. 2003). The approximate fatty acid profile of FSO is 9% saturated fatty acids, 18% monounsaturated fatty acids and 73% PUFA. The predominant PUFAs are ALA which makes up about 57% of the total fatty acids, followed by the n-6 PUFA linoleic acid (LA) which makes up approximately 16% of the total fatty acids. Both ALA and LA are essential fatty acids meaning that they cannot be synthesized de novo in the body and therefore must be consumed in the diet. The metabolism of ALA and LA to long chain PUFAs is explained in greater detail in Section 2.2.4.
FS is an important source of other nutrients. FS is a source of both soluble and insoluble fiber. The soluble:insoluble fiber ratio varies between 20:80 and 40:60. The major type of insoluble fiber in FS is mucilage gums. FS also contains many other phytochemicals (Daun et al. 2003). Phenolic compounds which are suggested to have antioxidant effects may contribute to the beneficial health effects of FS. Phenolic acids found in FS include ferulic, coumaric, caffeic, chlorogenic, gallic, protocatechuic, p-hydroxybenzoic acid, sinapic and vanillic. Of some concern is the presence of antinutritional compounds such as cyanogenic glycosides, phytic acid, linatine and cadmium. Cyanogenic glycosides found in FS include linustatin (213-352 mg/100 g), neolinustatin (91-203 mg/100 g) and linamarin (<32 mg/100 g). These can produce toxic hydrogen cyanide when hydrolyzed, however, they can be removed by solvent extraction or with heating. Phytic acid has been shown to reduce the bioavailability of minerals. Linatine is a vitamin B6 antagonist that can be removed with alcohol extraction. Finally, cadmium can be absorbed from the soil by the flax plant and end up in the seed. The levels of cadmium measured in FS vary greatly, likely because of differences in growth conditions.

2.2.2 Flaxseed in the Diet of Breast Cancer Patients

Research suggests that breast cancer patients change their diet and lifestyle upon diagnosis (Boon et al. 2007; Boucher et al. 2012). Several studies have assessed FS intake in breast cancer patients and found that 12-50% of breast cancer patients consume FS (Boon et al. 2007; Greenlee et al. 2009; Rausch et al. 2011; Boucher et al. 2012). One study showed that 70% of recently diagnosed breast cancer patients consume high lignan foods, 52% consume flax bread and 33% consume 1 tablespoon of FS at least once per week (Boucher et al. 2012). Hence there is a large interest in finding the scientific evidence for the use of FS and its components in the treatment of breast cancer and whether they affect drug response (Patterson 2011).
2.2.3 Effects of Flaxseed and its Components in Breast/mammary Cancer

Many studies have investigated the effect of FS and its components for the prevention and treatment of breast cancer. My research is on the role of FS and FSO in the treatment of breast cancer, therefore, this review will be limited to studies of the effect of FS and its components on treatment. Results from available studies on each of FS and its lignan and oil components from various designs including animal, observational and clinical studies will be described. Studies conducted in animals are summarized in Table 2-2. The major focus of my work is on the effect of ALA-rich FSO; therefore, in vitro studies investigating the specific effect of ALA will also be reviewed.

2.2.3.1 Effects of flaxseed in breast/mammary cancer

Animal studies

Several studies in animal models have been conducted that evaluate the effect of whole ground FS on the growth of established breast tumors. First, the effect of 2.5% and 5% FS diets fed starting 13 weeks after DMBA administration during the tumor development and progression stage of carcinogenesis was determined (Thompson et al. 1996). Tumors that were already established at the start of the dietary treatment significantly regressed in the FS groups compared to control and there was significantly lower total tumor volume (established at start of treatment + newly developed) with FS treatment. Interestingly, there were no effects of the FS diets on the incidence or volume of newly developed tumors (Thompson et al. 1996). Subsequent experiments evaluated the effect of FS on the growth of established human breast tumors using the ovariectomized (OVX) athymic mouse model. One advantage of this model is that ovariectomy eliminates the production of endogenous E2 and allows for experimental
**Table 2-2. In vivo** preclinical studies examining the effects of FS, lignan and FSO in the treatment of breast cancer

<table>
<thead>
<tr>
<th>Model</th>
<th>Treatments</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVX athymic mice; MCF-7 xenografts</td>
<td>• BD, FSO (38.5 g/kg), SDG (1 g/kg) and FSO+SDG</td>
<td>• ↑ tumor regression rate in all groups vs. control</td>
<td>Saggar et al., 2010b</td>
</tr>
<tr>
<td></td>
<td>• Low E2</td>
<td>• ↓ cell proliferation in all groups compared to control</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• No effect on apoptosis</td>
<td></td>
</tr>
<tr>
<td>OVX athymic mice; MCF-7 xenografts</td>
<td>• BD, 10% FS</td>
<td>• ↓ tumor growth, cell proliferation and ↑ apoptosis in FS vs. control</td>
<td>Chen et al., 2009</td>
</tr>
<tr>
<td></td>
<td>• Low E2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVX athymic mice; MCF-7 xenografts</td>
<td>• BD, 10% FS diet</td>
<td>• ↓ tumor growth in all treatment groups compared to +E2 control</td>
<td>Power et al., 2008; Saarinen et al., 2006</td>
</tr>
<tr>
<td></td>
<td>• Low E2</td>
<td>• No difference in tumor area, cell proliferation or apoptosis in FS vs–E2 control</td>
<td></td>
</tr>
<tr>
<td>OVX athymic mice; MCF-7 xenografts</td>
<td>• BD, 4% FSO</td>
<td>• ↓ tumor growth, cell proliferation and ↑ apoptosis in FSO vs. control</td>
<td>Truan et al., 2010</td>
</tr>
<tr>
<td></td>
<td>• High E2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVX athymic mice; MCF-7 xenografts</td>
<td>• BD, ED (15 mg/kg), EL (15 mg/kg) or 10% FS</td>
<td>• ↓ tumor growth and angiogenesis in all treatments vs. control</td>
<td>Bergman Jungestrom et al., 2007</td>
</tr>
<tr>
<td></td>
<td>• High E2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sprague-Dawley rats with DMBA-induced tumours (progression and tumour development stages)</td>
<td>• BD, 2.5% or 5% FS diet or FSO or SDG at levels present in 5% FS</td>
<td>• ↓ established tumor growth in 2.5% and 5% FS and FSO compared to control; no effect of SDG</td>
<td>Thompson et al., 1996a</td>
</tr>
<tr>
<td></td>
<td>• Diet treatment started 13 weeks post DMBA</td>
<td>• ↓ new tumor volume in SDG vs. control; no effect of 2.5% or 5% FS or FSO</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>• ↔ tumor incidence and number between groups</td>
<td></td>
</tr>
<tr>
<td>Athymic mice; 410 and 410.4 xenografts</td>
<td>• BD, FSO or 4:1 fish oil (FO):corn oil (CO) fed (i) before implantation, (ii)</td>
<td>• (i) ↔ tumor incidence or tumor size</td>
<td>Fritsche 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• (ii) primary tumors grew faster and were larger in</td>
<td></td>
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before implantation with removal of primary tumor, (iii) after implantation

<table>
<thead>
<tr>
<th>In Vivo Animal Studies: Drug-Diet Interaction</th>
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<tbody>
<tr>
<td>OVX athymic mice; BT-474 xenografts</td>
</tr>
<tr>
<td>• TRAS ± FSO (80 g/kg)</td>
</tr>
<tr>
<td>• ↓ tumor area, cell proliferation and ↑</td>
</tr>
<tr>
<td>apoptosis in FSO +TRAS2.5 vs. TRAS2.5</td>
</tr>
<tr>
<td>ε Mason et al., 2010</td>
</tr>
<tr>
<td>OVX athymic mice; MCF-7 xenografts</td>
</tr>
<tr>
<td>• BD, FSO (38.5 g/kg), SDG (1g/kg) and FSO</td>
</tr>
<tr>
<td>+SDG ± TAM</td>
</tr>
<tr>
<td>• Low E2</td>
</tr>
<tr>
<td>• ↓ tumor growth, cell proliferation and ↑</td>
</tr>
<tr>
<td>apoptosis in all treatment groups vs</td>
</tr>
<tr>
<td>control</td>
</tr>
<tr>
<td>ε Saggar et al., 2010a</td>
</tr>
<tr>
<td>OVX athymic mice; MCF-7 xenografts</td>
</tr>
<tr>
<td>• BD± TAM, ± 5%, 10% FS</td>
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<tr>
<td>• Low E2</td>
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<tr>
<td>• ↓ tumor regrowth, cell proliferation and</td>
</tr>
<tr>
<td>↑ apoptosis in TAM+10% FS vs TAM alone</td>
</tr>
<tr>
<td>ε Chen et al., 2007b</td>
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<tr>
<td>OVX athymic mice; MCF-7 xenografts</td>
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<tr>
<td>• BD± TAM, ± 5%, 10% FS</td>
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<tr>
<td>• High E2</td>
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<tr>
<td>• ↓ tumor growth, cell proliferation and ↑</td>
</tr>
<tr>
<td>apoptosis in all groups vs control</td>
</tr>
<tr>
<td>ε Chen et al., 2007a</td>
</tr>
<tr>
<td>OVX athymic mice; MCF-7 xenografts</td>
</tr>
<tr>
<td>• BD± TAM, ± 10% FS</td>
</tr>
<tr>
<td>• Low and high E2</td>
</tr>
<tr>
<td>• Low E2: ↓ tumor growth, cell proliferation</td>
</tr>
<tr>
<td>and ↑ apoptosis in FS and FS+TAM vs. TAM</td>
</tr>
<tr>
<td>and control</td>
</tr>
<tr>
<td>ε Chen et al., 2004</td>
</tr>
</tbody>
</table>

Abbreviations: ALA= α-linolenic acid, BD= basal diet, CO= corn oil, DMBA= dimethylbenz(a)anthracene, E2= 17β-estradiol, ED= enterodiol, EL= enterolactone, FO= fish oil, FS= flaxseed, FSO= flaxseed oil, HER2= human epidermal growth factor receptor 2, OVX= ovariectomized, SDG= secoisolariciresinol diglucoside, TAM= tamoxifen, TRAS= trastuzumab
manipulation of E2 levels to achieve levels that fall within the range of pre- and post-menopausal women (Rosenberg et al. 1994). A series of experiments has shown that 10% dietary FS reduces the growth of established MCF-7 tumors, representative of luminal breast cancer (ER+/PGR+/low HER2), when fed for 5-8 weeks with an E2 implant so circulating levels of E2 are high (Chen et al. 2004; Bergman Jungestrom et al. 2007; Chen et al. 2007b). Established MCF-7 tumors normally regress when circulating levels of E2 drop unless an estrogenic compound is introduced. After E2 pellet removal, dietary FS resulted in a greater regression compared to control in the short term (7 weeks) (Chen et al. 2009b) although no differences in regression were observed between control and FS groups in longer term studies where tumor area was followed for 16 weeks (Chen et al. 2007a) and 25 weeks (Saarinen et al. 2006; Power et al. 2008). This indicates that FS has no estrogenic tumor promoting effect under low E2 conditions typical of postmenopausal situations. Together these data suggest that FS has no estrogenic effect and can reduce the growth of human breast cancer.

**Clinical Trials**

Only one randomized double blind placebo-controlled trial has investigated the role of FS on tumor biological markers in postmenopausal women (Thompson et al. 2005). Patients diagnosed with breast cancer for the first time were divided between two arms, 13 women consumed a placebo muffin daily for 39 days and 19 women consumed a muffin containing 25 g of ground flax (contains approximately 50 mg of SDG and 10 g of FSO) for 32 days. At the beginning and end of the treatment, tumor biopsy tissue was taken and analyzed for tumor growth biomarkers. Cell proliferation, as measured by Ki-67 labelling index, and HER2 protein expression were significantly lower by 34% and 71%, respectively, and apoptosis was significantly higher by 31% at the end of the treatment in the FS group. There were no changes
in the placebo group for any of these biomarkers. These results indicate that tumor growth is slowed by dietary FS in agreement with the results from animal models.

2.2.3.2 Effects of flaxseed lignans in breast/mammary cancer

Animal Studies

Animal studies suggest that FS lignan contributes to the antitumor effect of FS. Studies using the carcinogen-treated rodent model suggest that SDG has antitumor effects. Daily gavage of 1.5 mg SDG (equivalent intake levels from 5% FS) starting 13 weeks after DMBA-administration during the tumor development and progression stage of carcinogenesis significantly reduced the volume of new tumors that grew after the start of the treatment (Thompson et al. 1996). However, SDG had no effect on tumor incidence or multiplicity or on the growth of tumors that were already established at the start of the dietary treatment. The SDG metabolite EL has also been tested for its effects in reducing the promotion stage of carcinogenesis: treatment with 10 mg/kg body weight of EL (similar to intake levels in a 10% FS diet) daily for 7 weeks commencing 9 weeks after DMBA administration resulted in significantly lower tumor volume compared to control with more pronounced reductions in the newly formed tumor growth (Saarinen et al. 2002). The tested lower dose of EL (1 mg/kg; similar to levels in a 1% FS diet) did not result in any significant differences in tumorigenesis highlighting the importance of dose. Together these studies suggest that SDG can attenuate the development of tumors but has less effect in reducing established tumors.

The athymic mouse model has been useful in exploring the effect of SDG on the growth of established human breast tumors. Compared to control, 0.1% SDG diets (level present in 10% FS diet) fed for 7-8 weeks caused a 54% greater regression of ER+ MCF-7 tumors, representative of luminal breast cancer, with low circulating levels of E2 (Chen et al. 2009b;
Saggar et al. 2010b). In contrast, compared to control, FS hull which is rich in SDG did not significantly reduce the growth of MCF-7 tumors at low circulating levels of E2 (Chen et al. 2009b). As the level of SDG (0.076%) in the FS hull diet in this study was lower than in the SDG alone diet (0.1%), this suggests that the level of SDG in the diet is important to achieve maximal tumor reducing effect. At high circulating levels of E2, a 0.1% SDG diet had no effect on tumor growth (Truan et al. 2012) while direct injection with ED or EL (15 mg/kg body weight; similar to intake levels in a 15% FS diet) reduced tumor growth (Bergman Jungestrom et al. 2007). Together these data support a role of FS lignans in reducing the growth of ER+ breast cancer and suggest that dose plays an important role.

Observational Studies

Several observational studies relating lignan exposure to breast cancer survival have been conducted. Lignan exposures have been estimated from the intake of dietary plant lignan or the equivalent amount of enterolignans produced from those plant lignans recorded in dietary questionnaires, and measurements of enterolignan concentration in the urine or blood. Five studies published between 2010 and 2011 that followed breast cancer patients from 6.1 to 10 years, have all shown significant reductions in mortality (40-53% reduction in all-cause mortality, 33-70% reduction in breast cancer mortality) with increased lignan exposure measured by diet record or serum lignan level in postmenopausal women (McCann et al. 2010; Buck et al. 2011a; Buck et al. 2011b; Olsen et al. 2011; Guglielmini et al. 2012). Importantly, the protective association between lignan intake and survival was observed in populations with comparatively low (e.g. McCann et al. 2010; mean intakes approximately 250 µg/day) and high (e.g. Olsen et al. 2011; mean intakes approximately 730 µg/day) lignan intakes. Of particular interest is that in the study by Guglielmi and colleagues (2012) no interaction or interference was observed
between lignans and TAM effect. Together, the consistent and strong associations suggest that dietary lignans are safe and beneficial for breast cancer patients.

There is a high level of heterogeneity in the observational studies. Sources of heterogeneity include exposure measurement (reported intakes, urine and blood biomarkers), menopausal status and tumor ER status. Urinary and blood enterolignan levels have been measured by different methods (chromatographic vs. time-resolved fluoroimmunoassay) with different sensitivities across the studies (Saarinen et al. 2010). The level of exposure in the different populations studied varies widely and the range of intake/biomarker within a population is often very narrow. Moreover, an important limitation noted in the observational studies to date is the potential for confounding. Although lignans are the richest in FS, they are also found in many plant foods including nuts, grains, fruits and vegetables (Thompson et al. 2006); therefore it cannot be concluded that the significant association between lignans and breast cancer survival is specifically due to FS. Rather, high lignan intake may be closely related to high plant food intake and the overall dietary pattern of high lignan consumers may be related to reduced risk and increased survival. The lignans may be acting synergistically with other healthful plant food components.

2.2.3.3 Effects of flaxseed oil in breast/mammary cancer

Animal Studies

Animal studies suggest that FSO-rich diets may inhibit breast/mammary tumor growth (Table 2-2). Thompson and colleagues (1996a) showed that a 2% FSO diet (level found in 5% FS diet) fed 13 weeks after DMBA was administered to Sprague-Dawley rats reduced the growth of established tumors but had no effect on new tumor formation. Studies using mouse models with implanted tumors have been valuable in determining the effect of FSO in reducing
established tumors. One study compared the effect of diets rich in various oils (from corn, FS and fish) at 10% level on the growth of implanted tumors derived from mouse mammary tumors (410 and 410.1) (Fritsche and Johnston 1990). No differences were observed among diets on the growth of 410 tumors; however, FSO had the greatest effect at reducing the growth and metastasis of 410.1 tumors. Xenograft studies have also determined the effect of ALA-rich FSO diets on the growth of human breast tumors. 4% FSO diets (level in 10% FS diet) fed for 7-8 weeks reduced the growth of ER+ MCF-7 tumors under both low (Saggar et al. 2010b) and high (Truan et al. 2010) E2 conditions. These results are further supported by a study which showed that diets rich in FS cotyledon (level in 10% FS diet) which is the primary location of FSO in the seed, reduced the growth of MCF-7 tumors at low circulating levels of E2 (Chen et al. 2011b). Together these data suggest that FSO is effective in reducing the growth of ER+ human breast tumors with low expression of HER2. In my Master’s thesis work, I showed that a diet with high levels of FSO (8%) did not affect the growth of HER2-overexpressing breast tumors. These findings were surprising considering the beneficial effects seen in MCF-7 xenografts with a 4% FSO diet. It is therefore of interest to evaluate the effect of FSO at levels that have been previously shown to be effective in MCF-7 xenografts (4%).

In Vitro Studies

In vitro studies have provided greater insight into the role of ALA on breast cancer growth. Several breast cancer cell lines are unable to convert ALA to EPA and DHA allowing for the study of the independent effect of ALA (Grammatikos et al. 1994; Bardon et al. 1996; Wiggins et al. 2015). Regardless of the lack of conversion to EPA and DHA, ALA has been shown to reduce the growth of several breast cancer cell lines in vitro (Table 2-3) (Gore et al., 1994; Grammatikos et al. 1994; Chajes et al. 1995; Menendez et al. 2006; Kim et al. 2009; Tran
### Table 2-3. *In vitro* studies examining the effects of ALA in the treatment of breast cancer

<table>
<thead>
<tr>
<th>Model</th>
<th>Treatments</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 cells</td>
<td>• 25-100 µM ALA</td>
<td>• ↓ cell proliferation with 100 µM ALA</td>
<td>Vanden Heuvel, 2012</td>
</tr>
<tr>
<td>MCF-7 cells</td>
<td>• 50 µM ALA + 1nM E2 for 5 days</td>
<td>• ↓ cell proliferation by 33%</td>
<td>Truan et al, 2010</td>
</tr>
<tr>
<td>MCF-7 cells</td>
<td>• Up to 100µM ALA for 24, 48, 72 hours</td>
<td>• ↓ cell growth dose and time dependently</td>
<td>Kim et al, 2009</td>
</tr>
<tr>
<td>MCF-7 cells</td>
<td>• Up to 30 µM ALA for 7 days</td>
<td>• ↓ cell growth from 18-30 µM</td>
<td>Grammatikos et al., 1994</td>
</tr>
<tr>
<td>MCF-7 cells</td>
<td>• 102.7 µM ALA methyl ester) for up to 7 days</td>
<td>• ↓ cell growth</td>
<td>Gore et al., 1994</td>
</tr>
<tr>
<td>MCF-7, MDA MB 231 cells</td>
<td>• 50 and 100 µM ALA for 5 days</td>
<td>• ↓ cell growth with 100 µM ALA in MCF-7 but not MDA-MB-231</td>
<td>Tran et al., 2010</td>
</tr>
<tr>
<td>MCF-7, MDA MB 231 cells</td>
<td>• 71.83µM ALA, 5 days</td>
<td>• ↓ cell growth in MDA MB 231 but not MCF-7</td>
<td>Chajes et al, 1995</td>
</tr>
<tr>
<td>MDA MB 231</td>
<td>• 150uM ALA + 40uM OA + 40uM LA, 48 hours.</td>
<td>• ↔ cell viability</td>
<td>Yu et al., 2010</td>
</tr>
<tr>
<td>MDA MB 231</td>
<td>• 10-200µM ALA, 24 hours</td>
<td>• ↓ cell number</td>
<td>Horia et al, 2005</td>
</tr>
<tr>
<td>SKBr3, BT 474 cells</td>
<td>• 10-20 µM ALA + TRAS, 48 hours</td>
<td>• ↓ HER2 expression and dose dependently ↓ cell proliferation when ALA combined with TRAS</td>
<td>Menendez et al, 2006</td>
</tr>
</tbody>
</table>

ALA= α-linolenic acid; HER2= human epidermal growth factor receptor 2; LA= linoleic acid; OA= oleic acid; TRAS= trastzumab
This reduction of cell growth with ALA treatment has been shown in most, but not all studies. Different outcomes have been observed with ALA treatment between and within cell lines. The inconsistency in the ALA effect between cell lines is illustrated by the findings of three studies that used similar concentrations of ALA (50-100 µM) in both ER+ MCF-7 and ER- MDA-MD-231 cells. Each produced somewhat different results: one only saw a significant growth reduction of ALA in MCF-7 cells (Tran et al. 2010), one only saw significant growth reduction of ALA in MDA-MB-231 cells (Chajes et al. 1995) and one saw a growth reduction of ALA in both cell lines (Wiggins et al. 2015). In addition to these studies, others have looked at the effect of ALA in ER+ MCF-7 cells (Gore et al. 1994; Grammatikos et al. 1994; Kim et al. 2009; Truan et al. 2010; Vanden Heuvel et al. 2012) and all have shown significant growth reduction. The additional results in the ER- MDA-MB-231 cell line have been less consistent with one study showing a growth reduction with ALA treatment (Horia and Watkins 2005) and another showing no effect (Yu et al. 2011). Overall, these findings suggest that ALA may reduce the growth of both ER+ and ER-breast cancer growth, although the effect is more consistent in ER+ MCF-7 cells. Only two studies have looked at effects on HER2-overexpressing BT-474 cells. ALA alone (Menendez et al. 2006; Wiggins et al. 2015) and combined with TRAS (5 µg/ml) (Menendez et al. 2006) reduced BT-474 cell growth. Menendez et al. (2006) saw significant reductions in HER2 mRNA and protein following treatment with 20 µM ALA in study. Overall the data suggests that ALA reduces the growth of breast cancer cells in vitro; however, additional work is needed to clarify mechanisms of effect.
2.2.3.4 Flaxseed drug interactions

Rodent studies have also been useful for studying potential interactions between TAM and FS or its components. Several studies have shown that combining TAM treatment (5 mg, 60 day release pellet; 80 mg/day) with dietary 10% FS significantly enhances the anti-tumor effect of TAM in MCF-7 xenografts under both low and high circulating levels of E2 (Chen et al. 2004; Chen et al. 2007a; Chen et al. 2007b). These data suggest that FS does not interfere with TAM effect, it rather enhances TAM effect. Similarly findings have been observed for the combinations of FS lignans and FSO with TAM in the athymic mouse model with MCF-7 xenografts. Under low circulating levels of E2, when TAM treatment (80 mg/day) was combined with either 0.1% SDG diet or 4% FSO, tumor area and cell proliferation were significantly reduced and apoptosis was significantly increased compared to TAM treatment alone (Saggar et al. 2010a). Similarly, the combination of TAM with FSO-rich cotyledon at level equivalent to amount present in 10% FS diet, resulted in greater reduction in tumor cell proliferation compared to TAM treatment alone (Chen et al. 2011b). In my Masters work, we found that a high FSO diet (8%) combined with TRAS (2.5 mg/kg) enhanced TRAS effect in reducing tumor size and cell proliferation and increasing apoptosis (Mason et al. 2010). It is not known if a lower level of FSO will produce similar effect. Together these studies support the role of FS and/or its components in enhancing TAM and TRAS effect and the safety of their consumption FS alongside cancer drug therapies. However, further studies are needed in breast cancer patients to confirm these findings.

2.2.4 Potential Mechanisms of Effect

A number of potential mechanisms of effect have been proposed for the anti-cancer effects of FS and its components (Figure 2-7). In vitro and animal studies have provided a great deal of insight into potential mechanisms of FS, SDG and its metabolites ED and EL as well as
Figure 2-7. Potential mechanisms of FS, lignan and FSO effect. E2 = 17β-estradiol; ER = estrogen receptor; HER2 = human epidermal growth factor receptor 2; MAPK = mitogen-activated protein kinase; PGR = progesterone receptor
ALA. As the majority of my work will focus on the effects of FSO and ALA, greater attention is focused on potential mechanisms of ALA and n-3 PUFAs.

Tumor analysis from the xenograft studies outlined above has suggested that the anticancer effects of FS and its components are related to reduced cell proliferation and increased apoptosis (Chen et al. 2004; Chen et al. 2007a; Chen et al. 2007b; Chen et al. 2009b; Saggar et al. 2010a; Saggar et al. 2010b; Truan et al. 2010; Chen et al. 2011b) and reduced angiogenesis (Dabrosin et al. 2002; Bergman Jungestrom et al. 2007). While it is likely that the effect of FS is related to both the lignan and oil components, studies comparing the effect of SDG and FSO suggest that the component most responsible for the observed tumor growth reduction may depend on the E2 environment and presence or absence of drugs. For example, SDG is more effective than FSO in reducing the growth of ER+ MCF-7 tumors at low levels of E2 (Saggar et al. 2010b) while FSO is more effective than SDG in reducing MCF-7 tumor growth at high levels of E2 (Thompson et al. 2010) and when fed in combination with TAM treatment (Saggar et al. 2010a). These findings can be interpreted a number of ways. First, it is likely that E2-related mechanisms contribute largely to SDG effect since it is most effective in conditions where there is less E2 to compete for the ER. Second, FSO may be acting in part through modulation of growth factor receptor signaling pathways such as through HER2. Prolonged treatment with TAM has been shown to result in upregulation of HER2 (Dowsett 2001). That FSO was more effective when fed in combination with TAM treatment suggests that it may be reducing HER2 expression or signaling. These potential mechanisms, the growth-factor signaling reducing effects, the antiestrogenic effects and others are further discussed below.

FS may exert its anticancer effect in part through modulation of growth factor receptor signaling pathways. Dietary FS has been shown to reduce the expression of HER2 in breast
cancer patients (Thompson et al. 2005). Furthermore, reduced HER2 expression was seen in MCF-7 xenografts in TAM-treated mice fed FS (Chen et al. 2007a) and FSO (Saggar et al. 2010a) diets. Reduction in HER2 was also seen following FSO (Truan et al. 2010) and SDG (Truan et al. 2012) feeding in mice with MCF-7 tumors with high circulating levels of E2. Growth factor signaling was also involved in the effect of lignans as indicated by reduced expression of growth factor receptors such as EGFR, IGFIR and HER2 (Chen et al. 2007a; Chen et al. 2009; Saggar et al. 2010a; Saggar et al. 2010b; Truan et al. 2012). FSO has been shown to reduce the expression of biomarkers of growth factor signaling pathways including phosphorylated or total levels of MAPK and Akt in MCF-7 xenografts at low and high circulating levels of E2 (Saggar et al. 2010b; Truan et al. 2010). SDG diets did not affect these biomarkers in various studies using the MCF-7 xenograft model at low or high levels of E2 (Saggar et al. 2010a; Saggar et al. 2010b; Truan et al. 2012). Together, this suggests that FS, FSO and SDG may influence breast cancer risk and progression through the modulation of growth factor receptors and their signaling pathways, though FSO may be the most effective FS component. No studies have looked at the effect of FS or its components on growth factor receptor signaling in models of HER2-overexpressing breast cancer.

FS may affect breast cancer growth through its modulation of hormone levels, metabolism and activity. FS lignans have been shown in vitro to inhibit the activity of several enzymes involved in hormone regulation and metabolism including aromatase, 5α reductase and 17β-hydroxysteroid dehydrogenase suggesting that they may lower the levels of serum hormones and their metabolites (Adlercreutz et al. 1993; Wang et al. 1994; Evans et al. 1995; Brooks and Thompson 2005). Several clinical trials have determined the effect of FS consumption (5-25 g/day) on serum hormone and hormone metabolite levels. The majority of studies found no changes in hormone levels (Frische et al. 2003; Brooks et al. 2004; Sturgeon et al. 2008) with
only one study showing a reduction in serum E2 and estrone sulfate but no changes in other hormone measures with dietary FS fed for 7 weeks (Hutchins et al. 2001). E2 metabolites including the hydroxyestrone (OHE1), 2OHE1 and 16αOHE1 are more sensitive to dietary FS. 2OHE1 has little biological activity with some antiestrogenic activity (Schneider et al. 1982; Bradlow et al. 1996) and 16αOHE1 has estrogenic agonistic and proliferative activities (Bradlow et al. 1996; Gupta et al. 1998), thus higher 2OHE1:16αOHE1 is suggested to be protective against the development of breast cancer (Schneider et al. 1982; Osborne et al. 1993). Daily FS consumption by both premenopausal (Haggans et al. 2000) and postmenopausal women (Haggans et al. 1999; Brooks et al. 2004; McCann et al. 2007) for 10 days to 3 months resulted in elevated 2OHE1:16αOHE1 in three studies while one study where FS consumption (7.5 g/day for 6 weeks followed by 15 g/day for 6 weeks) by postmenopausal women resulted in a reduction in 2OHE1:16αOHE1 (Sturgeon et al. 2010). In vitro work suggests that the FS lignan EL (Penttinen et al. 2007) and ALA (Tran et al. 2010) can both bind to ERα and ERβ although with lower affinity compared to E2. As observed in animal studies, this has led to a number of downstream effects including modulation of E2-sensitive genes including cyclin D1, pS2, PGR, ERα and ERβ (Chen et al. 2007b; Chen et al. 2009b; Saggar et al. 2010b; Saggar et al. 2010a).

Together, these findings suggest that E2-related mechanisms may contribute to the effects of FS, FS lignans and FSO.

A number of potential mechanisms have been suggested for the anticancer effects of FSO and ALA. ALA can be metabolized to a limited extent to the long-chain n-3 PUFA EPA and DHA as outlined in Figure 2-8 (Brenna et al. 2009). Therefore, the anti-tumor effects of FSO may be related directly to ALA or indirectly to its metabolites EPA and DHA (Anderson and Ma 2009). A number of reviews have focused on the potential mechanisms of n-3 PUFA in breast cancer (Calder 2012; Larsson et al. 2004; Wiggins et al. 2013). Proposed mechanisms of effect
Figure 2-8. n-3 and n-6 PUFA metabolic pathways. ALA = α-linolenic acid; ARA = arachidonic acid; COX = cyclooxygenase; CYP450 = cytochrome P450; DGLA = dihomo-γ-linolenic acid; DHA = docosahexaenoic acid; DPA = docosapentaenoic acid; EPA = eicosapentaenoic acid; GLA = γ-linolenic acid; HETE = hydroxyeicosatetraenoic acid; LA = linoleic acid; LOX = lipoxygenase; LT = leukotrienes; PG = prostaglandins; RV = resolvin; TX = thromboxanes. Figure used with permission from: (Mason et al. 2013b)
relate to (1) regulation of transcription factors, (2) alterations in the membrane phospholipid fatty acid profile and the downstream effects on growth factor receptor expression and activity and on eicosanoid biosynthesis, and (3) lipid peroxidation.

Altered activity of transcription factors including peroxisome proliferator-activated receptors (PPAR) and nuclear factor-kappa B (NF-κB) has been shown following treatment with fatty acids including ALA (Jump 2004). PPARs regulate the expression of genes implicated in cancer processes such as cellular differentiation, proliferation and inflammation and as such has been studied as a target for ALA (Berger and Moller 2002; Larsson et al. 2004). Expression of NF-κB, a regulator of cell proliferation, apoptosis, inflammation and angiogenesis, has been shown to be reduced with ALA treatment in a colitis model with a concomitant reduction in tumor necrosis factor-α and cyclooxygenase 2 expressions (Hassan et al. 2010). Few studies have evaluated the role of ALA and FSO on regulation of transcription factors in breast cancer and research is warranted in this area.

Alterations in the phospholipid fatty acid profile influence cellular properties and functions including growth factor signaling pathways and eicosanoid biosynthesis (Wang and Dubois 2010). Dietary FSO has been shown to increase serum ALA, EPA and DHA and reduce n-6 PUFA in the athymic mouse model (Truan et al. 2010). Changes in the membrane phospholipid fatty acid profile, specifically within the lipid raft microdomain, have been shown to alter the expression, localization and activity of membrane bound receptors such as EGFR (Schley et al. 2007). As highlighted previously, in in vitro and in vivo models of breast cancer ALA and ALA-rich FS and FSO have been shown to decrease the total expression of membrane-bound growth factor receptors including HER2, IGFIR and EGFR (Chen et al. 2002; Menendez et al. 2006; Saggar et al. 2010a; Saggar et al. 2010b; Truan et al. 2010). These changes are
suggested to affect downstream signaling pathways as indicated by changes in total or phosphorylated Akt and MAPK (Saggar et al. 2010b; Truan et al. 2010). Additionally, membrane fatty acid profile changes are suggested to alter the synthesis of eicosanoids which are synthesized from PUFA cleaved from membrane phospholipids. n-3-derived eicosanoids have anti-inflammatory or less potent inflammatory actions compared to n-6-derived eicosanoids (Wang and Dubois 2010). Few studies have directly linked dietary FS and FSO to eicosanoid production; however, an early study demonstrated that n-6-derived prostaglandin-E2 was suppressed in rats fed a diet rich in FSO (Marshall and Johnston 1982). This provides support for the idea that the enrichment of tumors with n-3 PUFA seen with FS suppresses n-6-derived and may increase n-3 derived eicosanoid synthesis.

ALA is unsaturated and therefore is susceptible to oxidation. Oxidation of ALA produces free radicals and reactive oxygen species (Larsson et al. 2004). It has been suggested that the anticancer effect of ALA and n-3 PUFA is related to lipid peroxidation (Gonzalez et al. 1991; Cognault et al. 2000). The tumor-reducing effect of FSO is lower in the presence of antioxidants and greater in the presence of pro-oxidants (Cognault et al. 2000) further supporting this idea.

Evidently, there are a number of potential mechanisms that may contribute to the anticancer effects of FS and its components. Further research is needed to more completely understand these mechanisms in order to support the use of FS and FSO as anticancer agents.

2.3 Summary and Questions

Breast cancer is a highly prevalent disease. A number of therapies are available for the treatment of breast cancer, including the subtype in which HER2 is overexpressed. TRAS is a first line therapy in the treatment of HER2+ breast cancer and while very beneficial, it is limited by innate and acquired resistance. Approaches to enhance its effectiveness are being explored
and previous research from our laboratory and others suggest that FS and FSO may be effective in this breast cancer subtype. The studies conducted to date suggest that FS and FSO reduce tumorigenesis in various models of breast cancer and this effect may in part be due to reduced HER2 expression and signaling. However, few studies have looked at the effects of FS and FSO on HER2 signaling in models of HER2-overexpressing breast cancer. My Master’s thesis work suggests that high levels of FSO (8% diet) combined with TRAS treatment significantly enhances the effect of TRAS in reducing tumor growth but, a number of questions remain to be addressed including: 1) Does dietary FS show similar results to dietary FSO? 2) Does the lower level of FSO (4%) typically used in studies in the Thompson lab show a similar TRAS-enhancing effect to the 8% diet used in my MSc work? 3) Is a beneficial interaction observed with an even lower dose of TRAS (1 mg/kg) and dietary FSO?; 4) Does the modulation of HER2 and biomarkers of its signaling pathways (Akt and MAPK) contribute to the effect of FSO as shown in models of breast cancer with low HER2?; 5) Is ALA the component responsible for FSO effect observed in vivo?; and 6) Since FSO has been shown to reduce the expression of growth factor receptors other than HER2 and these growth factor receptors are known to be upregulated in TRAS resistance, can ALA prevent the development of TRAS resistance and affect the growth of TRAS resistant cells?
CHAPTER 3
Hypotheses, Objectives and Thesis Organization
3.1 Hypotheses

ALA-rich FS (10%) and FSO (4%) will enhance TRAS effectiveness at reducing the growth of HER2 overexpressing tumors (BT-474) through a combination of mechanisms including altered membrane fatty acid profile and reduced signaling through HER2. ALA will prevent the development of TRAS resistance and will also enhance TRAS effectiveness in resistant cells.

3.2 Objectives

The overall objectives of my doctoral thesis are:

- to determine the effect of 10% FS, alone and in combination with TRAS (2.5 mg/kg) on the growth of established breast cancer with amplified HER2 expression using the athymic mouse model;

- to determine the effect of 4% FSO, alone and in combination with TRAS (1 and 2.5 mg/kg) on the growth of established breast cancer with amplified HER2 expression using the athymic mouse model;

- to determine using tumor biomarker expression analysis whether the effects of FS and FSO involve the modulation of HER2 and biomarkers of its signaling pathways (Akt and MAPK)

- to determine whether ALA is the component in FS and FSO responsible for effects seen in vivo by using in vitro methods;

- to explore other potential mechanisms that contribute to ALA effect with the use of qRT-PCR array methods;
• to determine the effect of ALA on the development of TRAS resistance using *in vitro* methods;

• to determine the effect of ALA on the growth of TRAS resistant cells.

### 3.3 Thesis Organization

To achieve these objectives, a series of experiments have been conducted each included in a separate chapter. The studies progress from determining the effect of whole ground FS (Chapter 4, Study 1), then more specifically the effect of FSO (Chapter 5, Study 2) a major component of FS, and the major fatty acid found in FSO, ALA (Chapter 6-8, Studies 3-5), and its metabolite DHA (Chapter 6, Study 3). Studies 1-3 investigate the interaction of FS and its components alone and combined with TRAS in TRAS-sensitive ER+ HER2-overexpressing BT-474 cells. Study 4 moves to the ER+ MCF-7 breast cancer that expresses low level of HER2 in order to explore potential mechanisms of ALA action in a cell line that is less driven by HER2. Study 5 focuses on TRAS resistance and involves two sub-studies. The first involves the long-term culture of BT-474 cells in the presence of TRAS alone or combined with ALA to determine whether or not ALA can prevent the development of TRAS resistance. The second determines whether ALA affects the growth of TRAS resistant cells and sensitizes these cells to TRAS effect. Chapter 9 gives an overall thesis summary and discusses limitations, future research directions, implications and conclusions.

Some studies have already been published or submitted in peer reviewed journals and are adapted as separate chapters.
CHAPTER 4
Study 1: Dietary flaxseed-trastuzumab interactive effects on the growth of HER2-overexpressing human breast tumors (BT-474)

Adapted from:

4.1 Introduction

FS is rich in SDG which can be metabolized by colonic bacteria to the enterolignans ED and EL (Thompson and Mason 2010). FS contains approximately 40% oil, 57% of which is the n-3 PUFA ALA (18:3n-3) (Cunnane 2003; Thompson and Mason 2010). As enterolignans and n-3 PUFA have demonstrated anti-cancer effects (Thompson and Mason 2010), FS is a food with the potential to reduce cancer risk and hence is commonly consumed by breast cancer patients (Boon et al. 2007; Boucher et al. 2012).

Preclinical work shows that dietary FS, FSO and SDG can inhibit the growth of established ER+ (MCF-7) human breast tumors in the athymic mouse model (Bergman Jungestrom et al. 2007; Chen et al. 2007a; Saggar et al. 2010b; Truan et al. 2010). Furthermore, dietary FS reduces MCF-7 tumor growth in athymic mice which have undergone prolonged treatment with TAM and may be progressing towards TAM resistance (Chen et al. 2007a; Chen et al. 2007b). Importantly, in a randomized control trial in postmenopausal breast cancer patients, dietary FS reduced tumor cell proliferation and increased apoptosis (Thompson et al. 2005). One commonly observed mechanism is the reduction in the expression and activity of HER2 (Thompson et al. 2005; Chen et al. 2009b; Saggar et al. 2010a; Truan et al. 2010). Together, this indicates that dietary FS can reduce carcinogenesis and this may be related to the reduction in HER2.

HER2 is a member of the epidermal growth factor receptor family (Ravdin and Chamness 1995) and is overexpressed in approximately 25% of breast cancer resulting in aggressive and highly metastatic tumors and poor prognosis (Hurvitz et al. 2012). Hence, HER2 has become an important therapeutic target. TRAS, a monoclonal antibody targeted to HER2, is a first line therapy in the treatment of HER2-overexpressing breast cancer and is effective as a monotherapy (Cobleigh et al. 1999; Vogel et al. 2002; Baselga et al. 2005) and in combination with
chemotherapy (Gianni et al. 2011; Slamon et al. 2011). However, response rate is quite low, patients develop resistance and many suffer cardiotoxicity. Thus approaches to enhance TRAS effectiveness are being sought.

The exact mechanism of TRAS action is not fully understood, however, several effects have been observed some of which are similar to mechanisms of FS effect. For example, TRAS has been shown to downregulate the expression and activity of HER2 (Baselga et al. 2001). Reduced HER2 expression has also been shown with ALA treatment in vitro (Menendez et al. 2006), with dietary FS and FSO in athymic mice (Chen et al. 2009b; Saggar et al. 2010a; Truan et al. 2010), with lignan precursors in vitro and in vivo (Youngren et al. 2005; Menendez et al. 2008) and with FS consumption in postmenopausal patients (Thompson et al. 2005). TRAS treatment reduces HER2 signaling through the MAPK and Akt pathways which are regulators of cell proliferation and apoptosis (Ripple et al. 2005); these effects have also been shown with FS and FSO in athymic mice (Chen et al. 2009b; Saggar et al. 2010a; Truan et al. 2010). TRAS reduces angiogenesis (Izumi et al. 2002), an effect seen with dietary FS in athymic mice (Bergman Jungestrom et al. 2007) and n-3 PUFA in vitro (Szyczewski et al. 2008). Because of these similarities, we investigated the interactive effect of dietary FSO and TRAS in the treatment of HER2-overexpressing BT-474 tumors in the athymic mouse model and found that FSO significantly enhanced the tumor-reducing effect of TRAS (2.5 mg/kg) (Mason et al. 2010). It is unknown how dietary FS, the source of FSO which also contains lignans, acts in HER2-overexpressing breast cancer and whether similar TRAS-enhancing effects will be observed. Hence our objective was to determine the effect of dietary FS alone and combined with TRAS treatment on the growth of established BT-474 tumors. If FS can increase TRAS effectiveness, it could lead to a simple, inexpensive, complementary treatment of breast cancer and will also help
understand any harmful or beneficial interactions of such supplement with a widely used breast cancer drug.

4.2 Materials and Methods

4.2.1 Experimental Diets

Macronutrient, ingredient, and fatty acid composition of experimental diets are presented in Table 4-1. The basal diet (BD) composition was based on the AIN-93G diet (Reeves et al. 1993) with modification such that 20% fat was provided by corn oil (CO) due to its low phytosterol and n-3 PUFA content to minimize confounding. FS was provided by Glanbia Nutritionals (Angusville, MB, Canada) and contained 1.28% SDG as analyzed by high performance liquid chromatography-mass spectrometry (LC-MS) and 38.43% oil 55.1% of which is ALA as analyzed by gas chromatography (GC). The FS diet contained 10% of freshly ground FS as previously described (Chen et al. 2007a), corrected for the contribution of FS to fat, fiber, available carbohydrate and protein and, therefore, the energy densities were similar between the test diets. Diets were prepared by Dyets Inc. (Bethlehem, PA), sterilized by gamma irradiation by Isomedix Corp. (Whitby, ON) and stored at 4°C until used. Fresh diet was provided every 2-3 days.

4.2.2 Animal Housing and Conditions

Animal care was conducted according to the Guide to the Care and Use of Experimental Animals and protocol was approved by the University Animal Care Committee (University of Toronto; protocol number: 20008100). OVX athymic mice (BALB/c nu/nu, 4-5 weeks old) were purchased from Charles River (Senneville, QC, Canada) and housed in micro-isolator cages (4 per cage; 15-16 per group) in a pathogen-free isolation facility with a 12:12-hr light-dark cycle at 22-24°C and 50% humidity. Animals were fed the diets and sterilized water ad libitum.
Table 4-1. Macronutrient, ingredient, and fatty acid composition of experimental diets.

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<th>Component</th>
<th>Basal Diet</th>
<th>Flaxseed Diet</th>
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<td></td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>Fat</td>
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<td>20.0</td>
</tr>
<tr>
<td><strong>Component (g/kg)</strong></td>
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<td></td>
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<tr>
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<td>2.9</td>
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<tr>
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<td>% composition</td>
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<td><strong>18:2n-6:18:3n-3</strong></td>
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4.2.3 BT-474 Cell Culture

BT-474 human breast cancer cells (ER+) were obtained from American Type Cell Culture (ATCC, Manassas, VA). BT-474 cells were selected for this study because they exhibit amplified HER2 and have been used in preclinical studies investigating the effect of TRAS (Baselga et al. 1998) and dietary components including ALA (Menendez et al. 2006). Hence, it is suitable for the study of FS-TRAS interaction. Cells were maintained in RPMI-1640 medium (GIBCO, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% antibiotics at 37°C in a humidified atmosphere of 5% CO₂ in air. Before injection, cells were harvested by routine trypsinization and resuspended in serum free medium with Matrigel (1:1 vol) at 1x10⁸ cells/mL as previously described (Chen et al. 2007b; Saggar et al. 2010a)[9, 11] and kept on ice. Cell viability after cell injections was confirmed to be at least 85% by trypan blue exclusion assay.

4.2.4 Trastuzumab

TRAS (Genentech Inc., San Francisco, CA) was purchased from University Health Network Pharmacy (Toronto, ON). A stock solution (20 mg/ml) was prepared every 3 weeks using bacteriostatic water (with 1.1% benzyl alcohol) supplied with the powdered TRAS. The 2.5 mg/kg body weight dose was prepared fresh from the stock solution for each injection.

4.2.5 Study Design

After one week of acclimatization while being fed the BD, mice were injected with BT-474 cells (1x10⁷ in 50µl Matrigel solution) in four sites of mammary fat pad subcutaneously as previously described (Chen et al. 2007a) and implanted with a sterilized E2 pellet (0.36mg, 60-day release; producing 0.5-0.7 nmol/L E2 blood level; Innovative Research of America, Sarasota, FL). Tumors were palpated weekly after injection and surface area was calculated as
After 4 weeks when tumors had reached an average area of 20.7 mm², mice were separated into 4 treatment groups such that tumor size and body weight were similar among groups: (1) Control group fed the BD (n=16); (2) FS group fed the FS diet (n=15); (3) TRAS group received 2.5 mg/kg body weight TRAS intraperitoneal injections twice weekly and fed the BD (n=16); (4) FS+TRAS group received the same treatment as the TRAS group but fed FS diet (n=15). Mice with no TRAS treatment (control and FS groups) received intraperitoneal injections with phosphate buffered saline twice weekly. Body weight and palpable tumor area were measured weekly. Food intake was measured for the first 3 weeks of treatment; however, mice fed the BD were losing weight. Therefore, pellets were broken up to a powder starting at week 3 in an effort to make it more easily consumed at the expense of the accuracy of food intake measurement. Mice were sacrificed at week 5 by CO₂ asphyxiation. Uteri and livers were weighed and examined for gross pathological changes as indicators of toxicity and estrogenicity in the tissues. A portion of 1-2 tumors per mouse were preserved in 10% buffered formalin for analysis by immunohistochemistry (IHC). Tumors to be used for quantitative real-time polymerase chain reaction (qRT-PCR), western blot and fatty acid analyses were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

4.2.6 Ki-67 Labeling Index and Apoptosis

The Ki-67 labeling index, a marker of cell proliferation, was measured using IHC as described previously (n=8-13/group) (Truan et al. 2010). Apoptosis was determined by in-situ TUNEL assay (n=8-13/group) (ApopTag Detection Kit, Millipore, Temecula, CA) according to manufacturer’s instructions. All slides were read blindly under a light microscope at 400X magnification by two independent readers (JC, JM) with results in agreement. The Ki-67 labeling index was determined by counting the number of positive cells over total cell number
counted from 5-10 fields. The number of apoptotic cancer cells was counted and expressed as apoptotic cell number/field.

4.2.7 Preparation of RNA

Total RNA was extracted from frozen tumors (n=8-14/group) using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The concentration and purity of the RNA were determined using a NanoDrop2000 (Thermo Scientific, Wilmington DE). Measurements were done in duplicate and had an average CV of ~10%. The purity (optical density (OD)260/OD280) for each of the samples was ~1.9. RNA integrity was assessed in a randomly chosen subset of samples by agarose gel electrophoresis, and the OD ratio of 28S to 18S rRNA was consistently 1, indicating high-quality RNA.

4.2.8 cDNA Synthesis and TaqMan Quantitative Real-Time PCR

One-step cDNA synthesis and qRT-PCR were performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) with One-Step TaqMan RT-PCR Master Mix Reagents (Roche, Branchburg, NJ) according to the manufacturer’s protocol and as previously described (Fu et al. 2009). Multiplex RT-PCR was employed; target gene primers and probe and internal standard gene primers and probe were run in the same reaction. Samples were run in duplicate in separate wells simultaneously with RNA- and RT-negative controls. The following mRNA-specific primers and probes were designed based on the cDNA sequence in GenBank (National Center for Biotechnology Information (NCBI), Bethesda, MD) with Primer3 designer (Rozen and Skaletsky 2000): (i) glyceraldehyde 3-phosphate dehydrogenase (GAPDH; internal control) forward 5’gaaggggtgacagatctctgt-3’, reverse 5’-caggtctgtgtggtctca-3’, probe 5’accaatatccagaagccagctctcttt-3’; (ii) HER2 (ERBB2) forward 5’-ccagctctgacacacct-3’, reverse 5’-acgtccagagaccctactc-3’, probe 5’-tcaagggacacctcgagag-3’; (iii) MAPK (ERK1/2)
forward 5'-tgctgaactcaaggctatac-3', reverse 5'-ggttgagctgatccg-3', probe 5'-
tgcattctggctgagatctcctact-3', (iv) Akt1 forward 5'-tcggagaagaacctcgg-3', reverse 5'-
acagcccaagtctcttcttt-3', probe 5'-acctcaagctggagac-3'; (v) Akt2 forward 5'-
gctctctgacgtgggtttggag-3', reverse 5'-tgactcttggttcgatgttcaag-3', probe 5'-
acctcaagctggagac-3'; (vi) ERα forward 5'-tgcaggattgttgtggctact-3', reverse 5'-ggttgagctgatccg-3', probe 5'-
tttctgccctactttccccctttgttctct-3'. Primer and probe specificity were checked with Basic Local
Alignment Search Tool (NCBI) and thermal dynamic were manipulated by calculating delta G
with Analyzer of Oligo (Integrated DNA Technologies, Coralville Iowa). All target gene probes
were fluorescently labeled with FAM/BHQ dyes while the GAPDH housekeeping gene was
labeled with VIC/MGB dyes (Applied Biosystems, Foster City, CA, USA). Fluorescence
emission was detected through a filter corresponding to the reporter dye at the 5’ end of each
probe and threshold cycle (C_T) was automatically calculated and displayed. C_T values of the
target gene were averaged and the GAPDH C_T was subtracted to obtain ∆C_T. Quantitation of
target gene expression was done by measuring the threshold cycle (C_T). For each sample, C_T
values of the target gene from technical replicates were averaged and the average GAPDH C_T
was subtracted to obtain ∆C_T. Statistical analyses on relative mRNA expression were performed
on 2^-∆CT. Data are presented graphically as relative units, calculated by dividing each sample’s 2^-
∆CT value by the mean 2^-∆CT of the control group.

4.2.9 Western Blot Analysis

Western blotting was performed on cell lysates from homogenized tumor tissue (n=8-
10/group) as described previously (Truan et al. 2010). Equal amount of total protein were
separated by electrophoresis on 7.5-10% polyacrylamide gels and transferred to polyvinylidene
difluoride membranes. Membranes were blocked for 1 hour at room temperature in blocking
buffer (5% non-fat dry milk or 5% bovine serum albumin) and incubated overnight at 4°C with
primary antibody in 1% blocking buffer. Primary antibodies included: HER2 (1:2000), phosphorylated HER2 (pHER2; 1:2000), Akt1 (1:2000), pAkt1 (1:2000), p44/42 MAPK; (1:2000), and pMAPK (1:2000), ERα (1:1000), pERα (1:1000) and GAPDH (1:2000) (Cell Signaling Technology, Beverley, MA). Anti-rabbit-HRP-conjugated secondary antibody (1:4000, BioShop, Canada, Burlington, ON) was used and proteins were visualized using Immobilon chemiluminescent-horse radish peroxidase kit (Millipore). Chemiluminiscence was detected on X-ray film (Clonex Corporation, Markham, ON, Canada) and the intensity of bands was quantified using FluorChem Imaging System (AlphaInnotech, San Leandro, CA). The “relative intensity unit” of each biomarker was calculated by dividing each sample’s biomarker band intensity by its GAPDH band intensity.

4.2.10 Tumor Fatty Acid Analysis

Total lipids were extracted from homogenized tumor tissue (n=6/group) according to the method of Folch et al. (Folch et al. 1957) using chloroform:methanol:0.88% potassium chloride (2:1:0.75 by volume). Extracts were collected into test tubes containing a known amount of heptadecanoic acid (17:0), converted into fatty acid methyl esters and analyzed by GC as described in Chen et al. (Chen et al. 2009a) using a Varian-430 gas chromatograph (Varian, Lake Forest, CA, USA) equipped with a Varian Factor Four capillary column and a flame ionization detector. Peaks were identified by retention times of fatty acid methyl ester standards (Nu-Chek Prep, Elysian, MN). Fatty acids concentrations were estimated by proportional comparison of GC peak areas with that of the 17:0 internal standard and were expressed as percent of total fatty acids.
4.2.11 Serum Lignan Analysis

Equal volumes of serum from two mice from the same treatment were pooled for lignan analysis. A total of 6 pooled samples/group for the FS and FS+TRAS groups and 2 pooled samples/group for the control and TRAS groups were analyzed. Samples were analyzed using LC-MS/MS (Clark et al. 2011) at the Roswell Park Cancer Institute (Buffalo, NY).

4.2.12 In Vitro Cell Growth Assay

BT-474 cells in the log phase of growth were plated into 24 well plates (3.6 x 10^4 cells/well) in complete RPMI medium complete. After approximately 72 hours, the medium was removed and replaced with treatment medium. Treatment solutions were prepared using phenol red-free RPMI 1640 medium (Gibco) containing 1% antibiotics-antimycotics, 5% charcoal stripped FBS (CS-FBS) and 1 nM 17β-estradiol (E2; Sigma-Aldrich) dissolved in ethanol. ALA, ED and EL (>99% pure) were obtained from Sigma-Aldrich. Stock solutions were prepared in 100% ethanol, aliquoted and stored at -20°C. In preparation for treatment, ethanol was evaporated from the ALA stock with nitrogen gas and a 4 mM solution was prepared in CS-FBS and incubated for 1 hour at 37°C to allow for the fatty acids to complex with albumin in the CS-FBS. The control treatment medium contained no ALA, lignans or TRAS. TRAS treatment was at 10 µg/ml, a dose commonly used in vitro (Wang et al. 2005a; Pfeiler et al. 2007) and a target serum trough concentration in clinical studies (Cobleigh et al. 1999). ALA treatment was at a 100 µM ALA which is the upper range seen in athymic mice fed 10% FS (Mason et al. 2014). ED and EL treatment were each at 0.7 µM doses which are within the range measured in the serum of FS-fed mice in this study. Treatments included ALA, ED+EL, ALA+ED+EL alone and combined with TRAS. The treatment medium was refreshed after 48 hours.
After a total treatment period of 96 hours, cells grown in 24 well plates were harvested in 0.25% trypsin-EDTA (Sigma-Aldrich). Cell number was counted using the TC20 automated cell counter (Bio-Rad, Hercules, CA, USA) based on the trypan blue exclusion assay. The total live cell number for each well was recorded and the average of the 3 technical replicates was taken. The experiments were repeated 3-5 times. Results are presented as a percentage of the untreated control cell number.

4.2.13 Statistical Analysis

Statistical analyses were performed using SigmaPlot (version 12.0; Systat Software Inc., San Jose, CA, USA). Data are expressed as mean ± standard error of mean (SEM). Non-parametric testing was used to analyze body weight, relative organ weights, relative tumor area and fatty acids as they did not fit criteria for parametric testing. Mann-Whitney Rank Sum tests were used to analyze the difference within group in palpable tumor area between week 0 and week 5. To analyze organ weights, tumor size and fatty acid levels, Kruskal-Wallis test with Tukey’s post hoc test was used. Parametric testing was used to analyze the difference in tumor growth biomarkers (cell proliferation, apoptosis, protein and mRNA expressions) and for difference between the FS and FS+TRAS groups for serum lignans. Two-way analysis of variance (ANOVA) was used to test for TRAS and FS effects and FSxTRAS interaction. If there was a significant interaction Tukey’s post hoc test was used to determine differences between groups. Student’s t-tests were used to determine differences in serum lignans between FS-fed groups. Kaplan-Meier survival analysis with Log Rank comparison was used to determine treatment differences on survival as commonly used in xenograft studies (Sugar et al., 2012, Wu et al., 2013). Three-way ANOVA was used to test for main effects of ALA, lignans and TRAS and their interactions on the growth of BT-474 cells in vitro. Significance was set at P<0.05 except the interaction effect where p<0.10 is considered acceptable.
4.3 Results

4.3.1 Body Weight and Food Intake

At treatment randomization (week 0), there was no difference in body weight among treatment groups; the mean body weight in the BD-fed mice (control and TRAS groups) was 16.4 ± 3.2 g and in the FS-fed mice (FS and FS+TRAS) was 15.9 ± 3.9 g. At the end of the treatment period, BD-fed mice had lost 2.5 ± 0.4 g while FS-fed mice gained 4.0 ± 0.4 g. Average food intake per day per mouse over the first 3 weeks of treatment was 2.14 ± 0.15 g in the control, 2.4 ± 0.1 g in the TRAS, 2.8 ± 0.1 g in the FS and 2.9 ± 0.1 g in the FS+TRAS groups. Mice in the FS and FS+TRAS groups consumed significantly more food compared to control and TRAS groups. There was no difference in average daily food intake/mouse between the FS and FS+TRAS groups and between the control and TRAS groups.

4.3.2 Uterus and Liver Weights

There were no differences between groups in uterine or liver weights relative to body weight and in gross pathological appearance (data not shown).

4.3.3 Tumor Growth

Since body weights differed between groups, tumor area was expressed relative to body weight (Figure 4-1). Relative tumor area at week 0 and week 5 were compared within each treatment group. Tumor area at week 5 was 148% higher compared to week 0 in the control group (P<0.001) and 117% higher compared to week 0 in the FS group (P<0.001). There was no significant difference in tumor area between week 0 and week 5 in TRAS and FS+TRAS treated mice. Two-way ANOVA was used to determine the effects of TRAS and FS and their interaction at week 2 and week 5 as indicators of short-term and long-term effects, respectively. At 2 weeks, tumor area was 38% lower in the FS group, 46% lower in the TRAS group and 43% lower in the
Figure 4.1. Effect of FS and TRAS, alone and in combination on (A) relative palpable tumor area (n = 39-47 tumors/group), (B) cell proliferation (Ki-67 labeling index; n=7-13/group) and (C) apoptosis (n= 9/group) of BT-474 breast tumors in athymic mice. In (A), different letters indicate significantly different at that treatment week at P<0.05.
FS+TRAS group compared to control. There were significant effects of both TRAS (P<0.001) and FS (P<0.001) and a FSxTRAS interaction (P=0.065). At 5 weeks, tumor area was 8% lower in the FS group, 61% lower in the TRAS group and 53% lower in the FS+TRAS group compared to control. There was a significant TRAS effect (P<0.001), no effect of FS and no significant interaction.

4.3.4 Survival

There was a significant difference in survival among treatment groups (P<0.001; Figure 4-2). Survival was highest in mice fed the FS diet with 100% of mice in the FS and 93% of mice in the FS+TRAS groups surviving to week 5. 81% of mice in the TRAS groups survived to week 5 while only 50% of mice in the control group survived to week 5.

4.3.5 Cell Proliferation and Apoptosis

Figure 4-1 shows no significant main effect of TRAS or FS and no significant FSxTRAS interaction on cell proliferation while there was a significant effect of TRAS (P=0.01), a trend toward a significant FS effect (P=0.06) and no significant FSxTRAS interaction on apoptosis.

4.3.6 Growth Factor Receptor and Estrogen Receptor Signaling Biomarkers (mRNA and Protein)

There were no significant effects of TRAS or FS on HER2 mRNA and protein, Akt1 protein, MAPK protein, ERα and pERα protein (Figure 4-3). However, there was significant TRAS effect on pHER2 protein (P=.048), Akt1 mRNA (p=0.043), Akt2 mRNA (P=0.042; data not shown), MAPK mRNA (P=0.041), pMAPK protein (P=0.002), ERα mRNA (P=0.004) and significant FS effect in pAkt1 protein (P=0.021). Significant FSxTRAS interactions were seen in
Figure 4-2. Effect of FS and TRAS, alone and in combination on the survival of mice with BT-474 tumors. Survival curves are significantly different between treatment groups by Kaplan-Meier analysis with Log-Rank comparison (P<0.001)
Figure 4-3. Effect of FS and TRAS, alone and in combination on signaling pathway biomarkers including the mRNA and protein expressions of total and phosphorylated HER2, Akt1, MAPK and ERα in the BT-474 tumors. (n= 8-13/group)
HER2 mRNA (P=0.036) and pAkt1 protein (P=0.072). Compared to control, Akt1 mRNA expression was reduced by 43% with TRAS treatment and 42% with FS+TRAS treatment while Akt2 mRNA expression was reduced by 51% with TRAS treatment, 27% with FS and 37% with FS+TRAS treatment (data not shown). Compared to control, pAkt1 protein expression was significantly reduced by 41% in the TRAS group, 60% in the FS group and 48% in the FS+TRAS group; Erk1/2 (MAPK) mRNA expression was reduced by 56% in the TRAS group and 38% in the FS+TRAS group; pMAPK protein expression was reduced by 16% in the TRAS group and 25% in the FS+TRAS group; ERα mRNA expression was reduced by 51% in the TRAS and 35% in the FS+TRAS groups and increased by 39% in the FS group.

4.3.7 Tumor Fatty Acids

Figure 4-4 shows the percent of total fatty acids in the tumors as ALA, EPA and DHA and the n-6:n-3 ratio. Expressed as mean concentrations, ALA, EPA and DHA in the tumors were: 35.7 ± 5.9, 32.4 ± 3.7 and 411.8 ± 28.7 nmol/g, respectively, in the control group; 41.8 ± 5.3, 36.7 ± 4.9 and 456.3 ± 80.7 nmol/g, respectively, in the TRAS group; 536.0 ± 54.5, 91.7 ± 14.6 and 750.6 ± 101.9 nmol/g, respectively, in the FS group; and 510.5 ± 91.6, 80.9 ± 17.3 and 730.8 ± 94.6 nmol/g, respectively, in the FS+TRAS group. Median concentrations of ALA (P<0.001), EPA (P<0.01) and DHA (P=0.03) were all different among treatment groups. Post-hoc testing showed that ALA, EPA and DHA levels were significantly higher in the FS and FS+TRAS groups compared to the control and TRAS groups (P<0.05). TRAS treatment with and without FS did not affect fatty acid concentrations. The n-6:n-3 ratio was significantly different among treatment groups (P<0.001) and was significantly lower in the FS and FS+TRAS groups compared to control and TRAS groups (P<0.05). Similar patterns of results were observed when statistical analysis was done on percent of total fatty acids.
Figure 4-4. Effect of FS and TRAS, alone and in combination on fatty acid composition and n-6:n-3 ratio in the BT-474 tumors. Bars with different letters are significantly different (n=6 tumors/group).
4.3.8 Serum Lignans

As expected, there were no detectable levels of ED or EL in the serum of BD-fed mice (control and TRAS group). Mean serum concentrations of ED were $685.7 \pm 138.2$ nM and $568.1 \pm 123.3$ nM in the FS and FS+TRAS groups respectively. Mean serum concentrations of EL were $876.0 \pm 103.4$ nM and $683.5 \pm 77.8$ nM in the FS and FS+TRAS groups respectively. The FS and FS+TRAS groups did not differ for concentrations of ED and EL.

4.3.9 In Vitro Cell Growth

ALA and TRAS both significantly reduced the growth of BT-474 cells in vitro (Figure 4-5). Lignans (ED+EL) did not affect cell growth. There were no significant interactions between any of the treatments.

4.4 Discussion

This study shows that dietary FS has early effects in reducing the size of established BT-474 tumors in the athymic mice but these effects are not sustained after 5 weeks. TRAS, a primary therapy for HER2-overexpressing breast cancer, effectively reduced relative tumor area in the short and long-term irrespective of diet treatment. FS did not interfere with the action of TRAS both in the short- and long-term. We have previously shown that dietary FSO combined with TRAS treatment (2.5 mg/kg) resulted in significantly lower tumor area compared to TRAS treatment alone (Mason et al. 2010). Considering this and the results of the current study, it is suggested that the effect of dietary FSO is dependent on the form of consumption and other components in dietary FS may interfere with the ability of FSO to increase the effectiveness of TRAS; however, additional work is needed to directly compare FS and FSO effects in parallel in order to draw this conclusions.
Three-way ANOVA: ALA: P<0.01
Lignans: P=0.840
TRAS: P<0.001

Interactions:
ALA x Lignans: P=0.545
ALA x TRAS: P=0.165
Lignans x TRAS: P=0.934
ALA x Lignans x TRAS: P=0.935

**Figure 4-5.** Effect of 100 µM ALA, lignans (0.7 µM ED + 0.7 µM EL), 10 µg/ml TRAS and their combinations on the growth of BT-474 cells *in vitro*. (n=3)
Cell proliferation and apoptosis in tumors excised at week 5 suggest that the effects on tumor size may be more related to apoptosis than cell proliferation. The lack of effect of TRAS on cell proliferation may relate to the fact that measurements were done only on tumors at the end of the study (week 5) when the rate of change in tumor size in the control group was already low suggesting that control tumors were no longer growing. Future studies should consider measurement of growth biomarkers at both early and late stages of treatment.

The body weight difference between the BD- and FS-fed groups was unexpected based on our previous work; however, this may be explained in part by the difference in food intake between groups. Palpable tumor area was expressed relative to the body weight in order to control for these differences. Interestingly, survival over the treatment period was significantly different between groups. Survival in xenograft studies using immunodeficient animals is multifactorial with tumor size and body weight being important factors. Survival was 81% in the TRAS group and 50% in the control group. Relative tumor area differed between these groups while body weight did not, suggesting that tumor burden played an important role and body weight was not the only important factor related to survival in this study. Survival was 93% in the FS group and relative tumor area did not differ between the control and FS groups suggesting that despite not affecting the tumor burden, FS was able to improve the survival. All mice in the FS+TRAS group survived. Recent epidemiological studies have shown a significant inverse association between lignan intake or serum level and all cause and breast cancer mortality (McCann et al. 2010; Olsen et al. 2011). Although the design of those studies is different from the present study, the similar effect of high lignan intake from FS on survival is of interest and merits further investigation.
To determine whether growth factor receptor-related signaling pathways are affected by FS, TRAS and their combination, mRNA and protein biomarkers of these pathways were measured. TRAS, FS and their combination did not affect HER2 mRNA and protein expressions. While some studies have shown that TRAS reduces HER2 expression (Baselga et al. 2001), this has not been consistently shown and more recent studies suggest that this is not a mechanism of TRAS action (Valabrega et al. 2007). HER2 activation through hetero- or homo-dimerization results in the indirect activation of growth-inducing signal transduction molecules including MAPK and Akt and their phosphorylation resulting in the formation of pMAPK and pAkt, respectively (Yarden and Sliwkowski 2001). Results suggest that the tumor reducing effect of TRAS is related to reduced HER2 signaling as indicated by reduced Akt and MAPK mRNA and reduced pHER2, pAkt and pMAPK protein expressions. FS did not have any effect on mRNA, total or phosphorylated MAPK or Akt1 mRNA and protein expression but did reduce the expression of pAkt1, an effect which has been observed in FSO in previous studies (Truan et al. 2010).

E2 can also modulate the growth-factor related signal transduction pathway through the activity of the membrane-bound ER (Osborne et al. 2005). FS, SDG and FSO have been shown to modulate the expression and activity of ER in MCF-7 tumors which are ER+ but have low HER2 expression (Chen et al. 2009b; Saggar et al. 2010a; Saggar et al. 2010b); however, the effect seems dependent on the presence or absence of other components. For example, in the presence of TAM, SDG, FSO and their combination all reduced ERα protein expression (Saggar et al. 2010a), however, in the absence of TAM only SDG reduced ERα mRNA expression and the effect was abrogated when SDG was combined with FSO (Saggar et al. 2010b). Interestingly, in the present study, TRAS but not FS reduced the ERα mRNA expression. Previous work has demonstrated a cross-talk between the ER and HER2 pathways and a reduction in ERα
expression with TRAS treatment (Chang et al. 2006). Neither FS nor combined FS and TRAS treatments affected ERα mRNA expression. None of the treatments affected ERα or pERα protein expression. Together these data suggest that dietary FS alone and combined with TRAS is not affecting ER expression in HER2 overexpressing tumors but a potential mechanism by which TRAS may also be reducing tumor area is through reduced ER transcription.

The major components of FS that are suggested to exert biological effects are n-3 PUFA and lignans. Dietary FS with and without TRAS treatment increased the tumor level of ALA and its metabolites EPA and DHA indicating that ALA was absorbed, metabolized and reached the tumor tissue. Serum levels of the ED and EL were significantly higher in mice fed the FS diet than those fed the BD indicating that SDG was also metabolized and absorbed in the mouse model.

This study provides a greater understanding of how dietary FS acts in breast cancer with HER2 overexpression. There are, however, several limitations. First, while the athymic mouse model is commonly used in preclinical studies of anti-cancer agents including TRAS (Fendly et al. 1990), it may not be reflective of human biology as they are immunodeficient and lack T-cell function. Thus, it is not known how these results will translate to humans. However, our previous work on the effect of FS on MCF-7 tumors in the athymic mouse model (Chen et al. 2002; Chen et al. 2007a; Chen et al. 2007b) was reproduced in a randomized, placebo- controlled clinical trial (Thompson et al. 2005). Secondly, only one cell line (BT-474; ER+, HER2+) was selected for this study and it is of interest to repeat the experiment in other HER2 overexpressing cell lines (e.g. SkBr3; ER-, HER2+). Finally, signal transduction pathways in carcinogenesis involve numerous molecules but only select biomarkers were measured. Other mechanisms of action may be playing a role and should be explored in future research.
Many previous studies have shown beneficial effects of FS and its components on breast tumor growth and biomarker expressions in both animal models (Chen et al. 2009b; Saggar et al. 2010a; Saggar et al. 2010b; Truan et al. 2010) and humans (Thompson et al. 2005). Those animal studies have been conducted using cancer models with low levels of HER2 expression while BT-474 cells are a model of the HER2 overexpressing phenotype of breast cancer. The discrepancy in the effect of FS between the previous studies using MCF-7 cells and the present study using BT-474 cells, suggests that the FS effect depends largely on the molecular subtype (ER, HER2 expression) of cancer.

Despite the lignans and PUFA availabilities in this study, FS did not show any effect on tumor growth, cell proliferation and apoptosis at week 5, alone and combined with TRAS. Dietary FSO at 8% level has been previously shown to enhance the effectiveness of TRAS in reducing the growth of BT-474 tumors (Mason et al. 2010). This suggests that other components in FS may be interfering with FSO’s effect; however, since these two studies were not conducted in parallel, the influence of non-dietary factors cannot be ruled out. The results of the in vitro experiment suggest that ALA and TRAS reduce BT-474 cells growth but the lignans ED and EL have no significant effect on cell growth at the concentrations and treated regime used. Lignans combined with ALA showed no significant difference compared to ALA alone. These findings suggest that at the cellular level, lignans do not interfere with ALA effect. Taken together with the in vivo data, it is likely that other components in FS interfere with the effect seen with dietary FSO (Mason et al. 2010). While the mechanism for this effect is not yet understood, similar results have been seen in MCF-7 tumors where the effect of FSO combined with TAM was greater than when FSO was given in combination with both TAM and SDG (Saggar et al. 2010a). The 10% dietary FS used in this study equates to about 25-50g of FS per day in the
human diet and our results suggest that this level of intake does not seem to interfere with TRAS action.

In conclusion, TRAS reduced tumor growth and dietary FS did not further improve its tumor reducing effect. Compared to control, dietary FS alone reduced tumor size in the early stage of treatment but not in the long term. However, alone and combined with TRAS, dietary FS improved overall survival. Dietary FS increased the level of n-3 PUFA in the tumors and enterolignans in the serum, indicating that they are bioavailable in this model and may have played a role in the observed effects on early tumor growth reduction (ALA) and survival (ALA and lignans). The mechanisms of TRAS effect observed in the present study include modulation of HER2 signaling pathways.
CHAPTER 5

Study 2: Dietary flaxseed oil-trastuzumab interactive effects on the growth of HER2-overexpressing human breast tumors (BT-474)

Adapted from:

5.1 Introduction

HER2 (c-erbB2/neu) is overexpressed in approximately 20-25% of breast cancer and is associated with tumor aggressiveness, a high rate of metastasis, poor prognosis and difficulties in treatment (Nahta and Esteva 2006). TRAS (Herceptin™), a recombinant, humanized monoclonal antibody, is a first line therapy in HER2+ breast cancer (Hudis 2007). TRAS effectively regresses tumors, however, is limited by resistance and cardiotoxicity suffered by 5% of patients (Vogel et al. 2002; Baselga et al. 2005; Hudis 2007). Approaches to increase TRAS effectiveness are, therefore, being investigated.

Many breast cancer patients use complementary/alternative medicine including dietary components to treat their breast cancer and improve health. FS is a commonly consumed food and has noted anticancer effects (Boucher et al. 2006; Boon et al. 2007). FS contains approximately 40% oil (FSO), 57% of which is ALA (18:3n-3) (Cunnane 2003). FS is also the richest source of mammalian lignan precursors, namely SDG (Thompson et al. 2006). Studies conducted in postmenopausal breast cancer patients and xenograft models with ER+ MCF-7 cells suggest that dietary FS and its SDG and FSO components may reduce tumorigenesis through a reduction in HER2 expression and cell proliferation and increase tumor cell apoptosis (Thompson et al. 2005; Chen et al. 2007a; Chen et al. 2007b; Truan et al. 2010).

Similarities have been observed between the proposed independent mechanisms of TRAS and FSO. These include reducing HER2 signaling through pathways including the MAP kinase and the PI3k-Akt kinase cascades (Sliwkowski et al. 1999; Bergman Jungestrom et al. 2007; Saggar et al. 2010a; Saggar et al. 2010b; Truan et al. 2010). In addition, ALA-rich FSO or FS and n-3 fatty acids have been shown to exert further anti-cancer effects including reducing the expressions of other growth factor receptors (i.e. EGFR, IGFIR) (Saggar et al. 2010a; Saggar et
al. 2010b; Truan et al. 2010), reducing the expression and activity of fatty acid synthase (FAS) (Menendez et al. 2004) and increasing the expression of the tumor suppressor PTEN (Ghosh-Choudhury et al. 2009). Taken together, these findings suggest that FSO may be capable of reducing tumorigenesis and modulating HER2 expression and growth factor receptor signaling pathways and enhance TRAS effectiveness in HER2+ cancer.

The present study aims to determine the effect of FSO (4%) alone and combined with TRAS treatment (1 and 2.5 mg/kg) on the growth of established BT-474 tumors in the athymic mouse model and to explore potential mechanisms of effect with specific focus on the HER2, Akt and MAPK signaling pathways. This study builds on our previous work on the interactive effects of TRAS and ALA-rich diets on BT-474 tumor growth in athymic mice (Mason et al. 2010; Mason et al. 2013a). Study 1 (Chapter 4) showed that, despite a delay in tumor growth and improved survival, 10% dietary FS has no effect alone or when combined with TRAS treatment on BT-474 tumor growth, cell proliferation or apoptosis after 5 weeks (Mason et al. 2013a), whereas findings from my Master’s thesis showed that a diet with high levels of FSO (8%) significantly enhances TRAS effect (2.5 mg/kg) in reducing palpable tumor size and cell proliferation and increasing apoptosis after 4 weeks (Mason et al. 2010). The current study advances from the previous studies by demonstrating that in HER2-overexpressing tumors: (i) a lower level of FSO (4%) also enhances the effectiveness of TRAS for the same outcomes measured previously (i.e. palpable tumor area, cell proliferation, apoptosis); (ii) FSO alone has no effect on tumor growth, cell proliferation or apoptosis; (iii) FSO increases the levels of ALA, EPA and DHA in BT-474 tumors which may contribute to its antitumor effect when combined with TRAS; and (iv) HER2 signaling pathways may be involved in the mechanism of action as indicated by measurements of protein and mRNA biomarkers.
5.2 Materials and Methods

5.2.1 Experimental Diets

Macronutrient, ingredient, and fatty acid composition of experimental diets are presented in Table 5-1. Freshly cold pressed FSO provided by Pizzey Nutritionals (Angusville, MB, Canada), was analyzed by GC (AOAC Official Method 996.06). The BD composition was the same as that described in Study 1 (Section 4.2.1). The FSO diet was the BD with FSO (40 g/kg diet) in place of CO. FSO diet and BD were isocaloric. As in Study 1, diets were prepared by Dyets (Bethlehem, PA) and sterilized by gamma irradiation by Isomedix Corp. (Whitby, ON) and stored at 4°C until used.

Mice were fed respective treatment diets and sterilized water ad libitum and diets were replaced every 2-3 days. Peroxide values were measured in the BD and FSO diets both fresh and after two day exposure to air at room temperature as a marker of oxidative stability. BD has 1.2 mequiv/kg and FSO diet has 2.1 mequiv/kg and did not increase after exposure to air because of antioxidants in the diet (vitamin E), indicating stability.

5.2.2 Animal Housing and Conditions

Animals and housing conditions were the same as in Study 1. The experimental protocol for this study was approved by the University of Toronto Animal Care Committee (Protocol number: 200007630). 12 mice per group were used in this study.

5.2.3 BT-474 Cell Culture

BT-474 cells were cultured and prepared for injection as described in Section 4.2.3.

5.2.4 Trastuzumab

TRAS was obtained and prepared as described in Section 4.2.4.
Table 5-1. Macronutrient, ingredient, and fatty acid composition of experimental diets.

<table>
<thead>
<tr>
<th>Component</th>
<th>Basal Diet</th>
<th>Flaxseed Oil Diet</th>
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<tr>
<td><strong>Macronutrient (% w/w)</strong></td>
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<td></td>
</tr>
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<td>Protein</td>
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<td>% composition</td>
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<tr>
<td><strong>18:2n-6:18:3n-3</strong></td>
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</tr>
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</table>
5.2.5 Study Design

The experimental design was similar to that described in Section 4.2.5, with the following key differences. The E2 pellet provided a 0.36mg dose released over 90 days thereby producing 0.4-0.5 nmol/L E2 blood level. Separation into treatment groups occurred after 2 weeks of tumor growth when tumors had reached an average area of 32.7 mm². The treatment groups included:

1) Control group fed the BD, 2) FSO group fed the FSO diet, 3) TRAS1 group received 1 mg/kg body weight TRAS intraperitoneal injections twice weekly and fed the BD, 4) TRAS2.5 group received 2.5 mg/kg body weight TRAS intraperitoneal injections twice weekly and fed the BD, 5) FSO+TRAS1 group received the same treatment as TRAS1 group and fed the FSO diet, and 6) FSO+TRAS2.5 group received the same treatment as TRAS2.5 group and fed the FSO diet. Mice were sacrificed after 4 weeks in this study. During the study several mice died due to reasons unrelated to the treatments, leaving 10-12 mice/group. Mice that died were not included in the data analysis.

5.2.6 Ki-67 Labeling Index and Apoptosis

Ki-67 and apoptosis staining of formalin-fixed paraffin-embedded tumors was conducted as described in Section 4.2.6.

5.2.7 Preparation of RNA, cDNA Synthesis and TaqMan Quantitative Real-Time PCR

RNA was extracted and checked following the procedures outlined in Section 4.2.7. cDNA synthesis and RT-PCR analysis were conducted as described in Section 4.2.8. MAPK3 (Erk1) and MAPK1 (Erk2) transcripts were analyzed separately in this study with the following primers and probes: (i) MAPK3 forward 5’-tgctgaactcaagggctatac-3’, reverse 5’-ggttgagctcaggtagt-3’, probe 5’-ctgcatcaggtctcaaccg-3’; (ii) MAPK1 forward 5’-
ccatccccctctgggtacct-3', reverse 5' - gaccattctctgcccaagttac -3', probe 5' -
cagattggccatgaacaccacttcatcttc-3'. The following target genes were also analyzed in this study:
(i) FASN forward 5' - gaagagagcacttttgatgac-3', reverse 5' - catgcagctcaggtcta-3', probe 5'
ttgagcctgtgcatccatccagatag-3'; (ii) PTEN forward 5' - ctcggttaaagggtgaagata-3', reverse 5'
aggtacaaggtcagggaact-3', probe 5' - cacagcgggaagaagctcatgta-3'; (iii) IGFIR forward 5'
cattccagcagctccaggtatat-3', reverse 5' - acatctccaatgtgccttag-3', probe 5'
agctgcaacaggaacatataaa-3'; (iv) EGFR forward 5' - cacgtacagatgtgatac-3', reverse
5' - gcctgtatctgtcaccacata-3', probe 5' - caaatagcagtttggtgccacctgc-3'. All target gene probes were
fluorescently labeled with FAM/BHQ dyes while the GAPDH housekeeping gene was labeled
with VIC/MGB dyes (Applied Biosystems, Foster City, CA, USA). Quantification and analysis
of target gene expression was conducted as described in Section 4.2.8.

5.2.8 Protein Biomarker Analysis

Protein biomarker analysis was conducted following a similar protocol to that described
in Section 4.2.9, with the following key differences. LumiGLO (CST) was used in place of the
Immobilon chemiluminescent-horse radish peroxidase kit to visualize proteins as it was shown to
produce a cleaner signal. Densitometric analysis was performed using the ImageJ software
program (National Institutes of Health, Bethesda, MD, USA). The ratios of phosphorylated:total
Akt1 and MAPK were calculated as an indicator of signaling pathway activation. ERα and pERα
were not analyzed in this study.

IHC was used to measure phosphorylated HER2 (pHER2) expression according to
antibody manufacturer's instructions (CST). This method was used as optimal conditions for
western blot analysis were difficult to achieve and also due to insufficient protein lysates. All
slides were read blindly under a light microscope at magnification ×400 and pHER2 was scored
using the Allred method (Allred et al. 1998) that has been validated for measure of HER2 expression (O'Malley et al. 2001).

5.2.9 Tumor Fatty Acid Analysis

Tumor fatty acid analysis was conducted as described in Section 4.2.10.

5.2.10 Statistical Analysis

Statistical analyses were performed using SigmaPlot (version 12.0; Systat Software Inc., San Jose, CA, USA). Data are expressed as mean ± SEM. Data that were not normally distributed were log transformed prior to analysis. Paired t test was used to compare the difference in palpable tumor area between week 0 (pretreatment) and week 4 within treatment groups. A 2×2 factorial analysis was used to determine the main (overall) and interaction effects of FSO and TRAS on the extent of tumor growth (tumor area at week 4 minus tumor area at week 0), cell proliferation, apoptosis, tumor mRNA and protein biomarkers and fatty acid levels. A significant main effect of one factor (FSO or TRAS) indicates that the overall direction of effect (increase or decrease) of that factor is consistent across the level (e.g. presence or absence) of the other factor. An interaction effect indicates that the effect of one factor depends on the effect of the other factor. The significance level was set at P<.05. Tukey's pairwise comparison test was used to compare among groups if there was a significant interaction. Kaplan-Meier survival analysis with Log Rank comparison was used to determine treatment differences on survival.

5.3 Results

5.3.1 Body Weight, Food Intake and Survival

There were no differences in food intake, body weight or survival among treatment groups (data not provided).
5.3.2 Tumor Growth

By design, there was no significant difference in tumor size amongst treatment group at the beginning of treatment (Figure 5-1A). At the end of treatment (week 4), tumor area in all treatment groups significantly increased (P<0.0001) by 168, 167, 100, 74, 122 and 32% in the control, FSO, TRAS1, TRAS2.5, FSO+TRAS1 and FSO+TRAS2.5 groups, respectively. There was a significant FSOxTRAS interaction (P<0.001) on the extent of tumor growth. Multiple comparisons showed no significant difference between the control and FSO group, significant reductions in both the TRAS1 and TRAS2.5 groups compared to control (both P<0.001) and no difference between the TRAS1 and TRAS2.5 groups. Tumor growth was significantly lower by 33.6% in the FSO+TRAS2.5 group compared to TRAS2.5 alone (P<0.001) while there was no difference between the TRAS1 and FSO+TRAS1 groups. FSO+TRAS2.5 treatment resulted in significantly lower tumor area compared to FSO+TRAS1 (P<0.001).

5.3.3 Cell Proliferation and Apoptosis

With respect to cell proliferation (Figure 5-1B), there was a significant TRAS effect (P<0.001), a significant FSO effect (P=0.004) and a significant FSOxTRAS interaction (P<0.001). Multiple comparisons analysis revealed that compared to all other treatment groups, cell proliferation was significantly lower in the FSO+TRAS2.5. Cell proliferation was 63.8% lower in the FSO+TRAS2.5 group compared to TRAS2.5 group (P<0.001). There were no significant differences among the remaining treatment groups.

Regarding apoptosis (Figure 5-1C), there was a significant TRAS effect (P<0.001), a trend toward a FSO effect (P=0.061) and a significant FSOxTRAS interaction (P=0.035). FSO treatment did not result in a difference in apoptosis compared to control. TRAS2.5 (P=0.039), FSO+TRAS1 (P=0.045) and FSO+TRAS2.5 (P<0.001) treatment groups all had significantly
Figure 5-1. Effect of FSO and TRAS alone or in combination on (A) palpable tumor area (n=39–47 tumors), (B) cell proliferation (Ki-67 labeling index; n=8–10/group) and (C) apoptosis (n=8–10/group) of BT-474 breast tumors in athymic mice. Different letters (a, b, or c) indicate significant difference between groups at P<.05.
higher apoptosis compared to both control and FSO groups. Apoptosis was significantly higher in the FSO+TRAS2.5 group compared to TRAS2.5 alone (P=0.002) while there was no difference between the TRAS1 and FSO+TRAS1 groups. FSO+TRAS2.5 treatment resulted in significantly higher apoptosis compared to FSO+TRAS1 (P<0.001).

5.3.4 mRNA Biomarkers

Expression of HER2 (ERBB2), EGFR, IGFIR, MAPK3 (Erk1), MAPK1 (Erk2), Akt1, PTEN and FASN mRNA in BT-474 tumors were all measured. The results for mRNA biomarkers for which significant TRAS, FSO and FSOxTRAS effects were seen are presented in Figure 5-2. There was no effect of either FSO or TRAS on HER2, EGFR, IGFIR, Erk1, Erk2 or Akt1 mRNA expressions. There was a significant effect of TRAS in reducing FASN mRNA expression (P=0.027) and a significant effect of FSO in increasing PTEN mRNA expression (P=0.028). There was no effect of FSO on FASN mRNA and no effect of TRAS on PTEN mRNA. There were significant FSOxTRAS interactions for HER2 (P= 0.048), EGFR (P=0.038) and Erk2 (P=0.050) mRNA but no significant interaction observed for Akt1, FASN, PTEN, IGFIR or Erk2 (IGFIR and Erk2 data not shown). Multiple comparisons showed that the biomarker-reducing effects were driven largely by the large effect in the FSO+TRAS2.5 group: mRNA expressions of HER2 (61.8%), EGFR (66.3%) were significantly lower in FSO+TRAS2.5 treated tumors compared to TRAS2.5 alone (P<0.05). IGF1R mRNA expression was not affected by FSO or TRAS and there was no interaction (data not shown).

5.3.5 Protein Biomarkers

Protein expression of total and phosphorylated HER2, Akt1 and MAPK and total PTEN and FASN were measured. The results for protein biomarkers for which significant TRAS, FSO or FSOxTRAS effects were seen are presented in Figure 5-3. There were no significant effects of
Figure 5-2. Effect of FSO and TRAS alone or in combination on mRNA levels of various biomarkers in BT-474 tumors (n=10–12 tumors/group) from athymic mice. Different letters indicate significant difference between groups at P<.05.
Figure 5-3. Effect of FSO and TRAS alone or in combination on protein levels of various biomarkers in BT-474 tumors (n=8–10 tumors/group) from athymic mice. (A) pHER2 measured by IHC with representative images of slides. Western blot was used to measure the relative expressions of (B) pMAPK/MAPK, (C) pAkt/Akt and (D) PTEN. Different letters indicate significant difference between groups at P<.05. HER2 levels were also measured by western blot but no TRAS, FSO and FSO×TRAS effects were seen.
TRAS or FSO on HER2 or FASN proteins. There was a significant FSOxTRAS interaction (P=0.045) observed for pHER2 expression, which was driven by the 34-35% significantly lower levels in the FSO+TRAS2.5 group compared to both FSO alone and TRAS2.5 alone. Despite a lack of main effect of FSO or TRAS on pHER2 protein, pMAPK/MAPK protein expression was significantly lower in TRAS-treated (P<0.001) and FSO-treated (P=0.027) tumors and there was no FSOxTRAS interaction. Interestingly, there were significant FSO effects with higher PTEN protein (P=0.007) expression and lower pAkt1/Akt1 protein (P<0.01). TRAS treated tumors had lower pAkt1/Akt1 protein expression (P<0.01) while PTEN expression was unaffected. Although no multiple comparisons tests were conducted due to the lack of FSOxTRAS interactions, it is noted that pMAPK/MAPK was 49% lower, pAkt1/Akt1 was 49% lower and PTEN was 45% higher in the FSO+TRAS2.5 group compared to the TRAS2.5 group.

5.3.6 Tumor Fatty Acids

Tumor ALA, EPA and DHA concentrations were 49 nmol/g, 17 nmol/g and 572 nmol/g, respectively, in BD fed mice (i.e. control, TRAS1 and TRAS2.5) and 295 nmol/g, 80 nmol/g and 762 nmol/g, respectively, in FSO fed mice (i.e. FSO, FSO+TRAS1 and FSO+TRAS2.5). Figure 5-4 provides the major n-3 fatty acids as a percent of total fatty acids and the n-6:n-3 ratio. There were significant FSO effects (P<0.001) with higher levels of ALA, EPA and DHA and lower n-6:n-3 ratio. There was no significant TRAS effect on ALA, EPA, DHA or n-6:n-3 ratio, nor were there FSOxTRAS interactions.

5.4 Discussion

This study has shown that FSO can enhance the effectiveness of TRAS in reducing the growth of established HER2-overexpressing BT-474 tumors at high circulating levels of E2.
Figure 5-4. Effect of FSO and TRAS alone or in combination on tumor fatty acid composition and n-6:n-3 ratio in BT-474 tumors (n=8 tumors/group) from athymic mice.
Combining TRAS treatment (2.5 mg/kg) with a FSO-rich diet (4%) for 4 weeks led to a greater attenuation of tumor growth compared to treatment with this dose of TRAS alone. Tumor analysis showed that FSO+TRAS2.5 treatment caused a greater reduction in cell proliferation and a greater increase in apoptosis compared to TRAS2.5 treatment alone further confirming the reduction in tumor growth. These results not only support the findings from my Master’s thesis that showed that an 8% FSO diet combined with 2.5 mg/kg TRAS resulted in greater tumor reduction than TRAS alone (Mason et al. 2010), but also demonstrate that a lower level of FSO (4%) can be as effective. However, combining TRAS treatment at 1 mg/kg dose with dietary FSO showed no additional benefit. Interestingly, in the absence of TRAS treatment, FSO showed no significant effect on tumor size or biomarkers of tumor growth. These results suggest that combining TRAS treatment with ALA-rich dietary FSO may increase TRAS effectiveness, however, without TRAS treatment FSO shows no benefit, effects that will be further discussed later.

The current study furthers the understanding of the FSO-TRAS interaction through measurement of gene and protein biomarkers of the HER2 signaling pathway. HER2 status is an important prognostic marker in breast cancer and is used to predict TRAS response. HER2+ tumors show constitutive activation of the proliferation-inducing and anti-apoptotic PI3K/Akt and the MAPK signaling cascades, ultimately leading to tumor growth (Hudis 2007). The results showed that FSO enhanced TRAS effectiveness by increasing its anti-proliferative and pro-apoptotic effects perhaps through reduction in HER2 signaling (MacLennan et al. 2013). The effect was not modulated through reduced total HER2 protein expression which is likely beneficial for TRAS treatment as there is no change in the level of expression of the target to which TRAS binds. Rather the effect seems to be modulated at the level of HER2 activation since significantly lower pHER2 expression was observed when TRAS2.5 treatment is combined
with FSO. This enhanced reduction in pHER2 may have led to lower activation of downstream signaling pathways as approximately 50% lower levels of pAkt1/Akt1 and pMAPK/MAPK expression were observed in the FSO+TRAS2.5 group compared to TRAS2.5. At the mRNA level, enhanced reductions in HER2 and Erk2 were seen with FSO+TRAS2.5 compared to TRAS2.5. Neither of these reductions translated to differences in HER2 or MAPK protein. The lack of difference in MAPK protein is likely explained by the lack of difference in Erk1 mRNA, the second transcript that codes for the protein.

Further insight is gained when considering the independent effects of TRAS and FSO on protein signaling in this study. TRAS affects tumor growth by reducing the Akt and MAPK signaling pathways, indicated by significantly lower ratios of phosphorylated to total protein expression. Inhibition of Akt and MAPK signaling by TRAS is commonly seen in the literature (Wang et al. 2005a; Vu and Claret 2012; Mason et al. 2013a). In the current study, FSO showed independent effects in reducing MAPK and Akt activation. The FSO effect on pAkt protein may be attributed to the inhibitory effect of PTEN which was significantly higher at both the mRNA and protein level. These findings are supported by previous studies in MCF-7 xenografts where FSO reduced both pAkt and pMAPK expression (Saggar et al. 2010b; Truan et al. 2010). The effects of FSO+TRAS2.5 treatment on Akt1 and MAPK phosphorylation may be explained by the combined effect of the two independent treatments. For example, compared to control, FSO and TRAS2.5 alone caused 26% and 38% reductions in pAkt1/Akt expression, respectively, while FSO+TRAS2.5 caused a 69% reduction.

Few studies have looked at the effect of n-3 PUFA on the growth of HER2-overexpressing breast cancer in vivo (MacLennan et al. 2013; Mason et al. 2013a). In this study, BT-474 tumor growth was not affected by dietary FSO treatment alone. In study 1 (Chapter 4), it
was shown that while 10% dietary FS delayed tumor growth it did not affect the final tumor area compared to control (2.5 mg/kg) (Mason et al. 2013a). In contrast, long chain n-3 PUFA synthesized endogenously through the fat-1 gene or from dietary fish oil have been shown to reduce tumorigenesis in the MMTV-neu(ndl)-YD5 model which spontaneously develop HER2-overexpressing breast tumors (MacLennan et al. 2013). Differences in both the source of n-3 PUFA and the model of carcinogenesis (prevention vs. treatment) may explain the discrepancies in the findings between studies. Further research should explore the effects of FSO alone in a model of cancer prevention such as the MMTV-neu transgenic mouse model and the effect of long chain n-3 PUFA in the treatment of established HER2-overexpressing breast tumors.

FSO has been shown to reduce tumorigenesis in models of breast cancer with low HER2 expression (e.g. MCF-7) (Saggar et al. 2010b; Truan et al. 2010). In the present study, FSO alone showed no significant reduction in HER2-overexpressing tumor growth, yet when combined with TRAS significantly enhanced its tumor-reducing effect. It is hypothesized that the level of HER2 in BT-474 tumors is very high leading to constitutive activation and aggressive tumor cell growth. Therefore, while FSO may be causing effects similar to those demonstrated in past studies in MCF-7 xenograft models (Saggar et al. 2010a; Saggar et al. 2010b; Truan et al. 2010), the HER2-driven tumor growth signals may be so strong rendering the magnitude of FSO effect non-significant. In contrast, when TRAS is present, the HER2-driven tumor growth signals may be decreased to a point where FSO can elicit a significant effect as seen in MCF-7 tumors.

The mechanisms by which FSO may influence tumorigenesis and HER2 signaling pathways are thought to be through altering the membrane fatty acid profile. We have shown that dietary FSO increases the level of ALA and its downstream metabolites (EPA and DHA) in tumor tissue. Changes in tumor cell membrane fatty acids, particularly within the lipid raft
microdomain, are suggested to affect signaling through alterations in the receptor localization within the membrane (Schley et al. 2007; Corsetto et al. 2012; Lee et al. 2014). For example, EPA and DHA supplementation to MDA-MB-231 cells in vitro resulted in a significant decrease in EGFR in the lipid raft domain with no significant difference in total cellular EGFR levels and concomitant alterations in MAPK signaling (Schley et al. 2007). Further research is needed to determine whether ALA-rich FSO has similar effects on growth factor receptor localization within the cell membrane.

Changes in other growth factor receptors were measured, including EGFR and IGFIR, that have been shown to be affected in MCF-7 xenografts by FSO (Saggar et al. 2010a; Saggar et al. 2010b; Truan et al. 2010). At the mRNA level, IGFIR expression was not affected by FSO, TRAS nor their combinations. EGFR mRNA expression was significantly lower in the FSO+TRAS2.5 group compared to all other groups while neither TRAS nor FSO alone altered its expression. Both western blot and IHC were used to measure EGFR protein expression; however, quantifiable results were not achieved. Up to 60 µg of protein was tested for western blot and no signal was detected despite strong signal in the positive control extract. Stained sections showed sparse positive regional staining isolated to the necrotic tumor core in all treatment groups; however, there were insufficient positively stained cells for comparison between groups. While some studies have shown that BT-474 cells express EGFR protein, others have shown very low or negative EGFR expression (deFazio et al. 2000). Taken together, these findings suggest that in this model, modulation of EGFR and IGFIR expressions do not contribute to the effects on tumor growth.

The doses of TRAS used in this study fall within the range (0.1-30 mg/kg) used in previous studies investigating the effect of TRAS in the nude mouse model (Baselga et al. 1998;
Wang et al. 2005a; Ritter et al. 2007) and are similar to the levels often used in the clinical setting (2 mg/kg) when using body weight to compare between mice and humans. Based on body surface area, which has been suggested to be a better estimator (Reagan-Shaw et al. 2008), the selected doses were 5-10 times lower than those used in humans. These suboptimal levels are important to allow for a FSO effect to be seen. However, if HER2 inhibition by TRAS is required in order for FSO to exert an antitumorigenic effect, then a very low dose of TRAS (i.e. 1 mg/kg) may be too low. This may explain the difference in effect of FSO between the two doses of TRAS studied.

The 4% FSO diet contained levels equivalent to that in 10% dietary FS. It is estimated that, depending on the amount of other foods consumed in the human diet, this level is approximately equivalent to a daily intake of about 5-10 g (1-2 tablespoons) FSO. FSO can be easily incorporated into the diet in a liquid form (i.e. in a salad dressing), pill form or can be used to enrich foods. This dietary modification can be made by patients being treated with TRAS, however, clinical trials still need to be conducted before any recommendations are made as it is not known how the observed effect will translate into a human population. However, the findings on the anticancer effect of FS using the athymic mouse model with MCF-7 xenografts was reproduced in a randomized controlled trial of postmenopausal women thus validating the athymic mouse model (Thompson et al. 2005). As shown in Study 1, in contrast to FSO, 10% dietary FS does not alter the effectiveness of TRAS in reducing the growth of BT-474 tumors in the athymic mouse model (Mason et al. 2013a). This suggests that other components in FS may alter the ability of FSO to enhance TRAS effectiveness and that the form of FSO consumption is an important consideration with pure FSO showing greater effect. However, more research is needed to understand the interaction between FS components.
This study provides support for FSO as a potential complementary agent to be used with TRAS in the treatment of HER2+ breast cancer; however, there are several limitations. The methods employed in this study were very similar to those used in study 1 (Chapter 4); therefore the limitations described in Chapter 4 related to the athymic mouse model, the use of only one cell line and the measurement of selected biomarkers of signaling pathways apply to this study as well.

In conclusion, this study has shown that while 4% FSO alone does not affect the growth of HER2+ BT-474 xenografts in athymic mice, it enhances the tumor-reducing effect of TRAS (2.5 mg/kg). The interactive effect is suggested to be modulated through a greater reduction of HER2 signaling as indicated by lower levels of pHER2, pMAPK and pAkt expression, leading to reduced cell proliferation and increased apoptosis. Alterations in the tumor fatty acid profile likely contributed to the observed alterations in HER2 signaling by FSO, however, the exact mechanism through which this occurs requires further elucidation. These findings are significant because they provide support for clinical trials to explore the use of FSO as a simple, inexpensive complementary agent to be used with TRAS in the treatment of HER2+ breast cancer.
CHAPTER 6
Study 3: Effects of $\alpha$-linolenic acid and docosahexaenoic acid, alone and combined with trastuzumab on BT-474 cell growth and biomarkers of HER2-signaling \textit{in vitro}
6.1 Introduction

Dietary approaches to improve breast cancer outcomes are of interest to the scientific, medical and patient communities (Boon et al. 2007; World Cancer Research Fund/American Institute for Cancer Research 2007; Eccles et al. 2013). n-3 PUFAs have anticancer effects in experimental models and are commonly consumed by breast cancer patients (Boon et al. 2007; Brasky et al. 2010; Boucher et al. 2012). ALA, found in plant sources such as nuts and seeds, particularly FS, cannot be synthesized in the body. ALA can be metabolized to the long chain n-3 PUFAs found in fish including EPA and DHA. Whether ALA has independent effects in breast cancer or whether its benefits are due to its long-chain n-3 PUFAs metabolites remains unclear (Anderson and Ma 2009). This is important to establish because it will lead to better dietary recommendations for the use of n-3 PUFAs as complementary treatments in breast cancer.

Animal studies using ALA-rich FS and FSO diets suggest that plant-based n-3 PUFA sources reduce breast cancer growth (Wang et al. 2005b; Chen et al. 2009b; Saggar et al. 2010b; Truan et al. 2010). This effect is subtype-dependent as FS and FSO alone do not affect the growth of HER2-overexpressing BT-474 tumors in athymic mice (Mason et al. 2013a; Mason et al. 2015). Interestingly, FSO diets at 4% (Mason et al. 2015) and 8% (Mason et al. 2010) levels enhance the effectiveness of the HER2-targeted drug TRAS in reducing tumor growth and cell proliferation and increasing apoptosis of BT-474 tumors. This beneficial interaction is at least partially due to reduced signaling through HER2 as indicated by reduced activation of HER2, Akt and MAPK (Mason et al. 2015). FSO has also been shown to reduce MAPK and Akt signaling in MCF-7 xenografts (Saggar et al. 2010b; Truan et al. 2010). Together, this suggests that ALA-rich FSO may be a beneficial complementary treatment approach in breast cancer.
Serum and tumor levels of ALA and long chain n-3 PUFAs EPA and DHA are significantly higher in mice fed 4% FSO or 10% FS diets compared to CO oil-based BD (Truan et al. 2010; Mason et al. 2013a; Mason et al. 2014; Mason et al. 2015). DHA and DHA-rich fish oil have demonstrated anticancer effects (Yee et al. 2005; Cao et al. 2012; Ewaschuk et al. 2012; Zou et al. 2013; Leslie et al. 2014; Xue et al. 2014) and have also been shown to affect HER2 signaling (Zou et al. 2013) in models of HER2-overexpressing breast cancer. It is therefore of interest to determine the independent effects of ALA and DHA at serum levels seen after FSO feeding as well as their interaction with TRAS.

It is suggested that breast cancer cell lines lack the ability to convert ALA to its downstream metabolites, importantly EPA and DHA, due to a lack of Δ6 desaturase enzyme. This has been demonstrated in MCF-7, MDA-MB-231 and T47D cells where downstream products of reactions catalyzed by of Δ6 desaturase including gamma linolenic acid, stearidonic acid and DHA were not produced following treatment with their precursors LA, ALA, and EPA, respectively (Grammatikos et al. 1994; Bardon et al. 1996; Corsetto et al. 2011). In the current study we have found for the first time that BT-474 cells are also unable to metabolize ALA to DHA, thus presenting a good model to study the independent effects of ALA and DHA.

The overall objective was to determine using cell culture methods whether ALA is the component of FSO responsible for the effects seen in vivo on HER2 signaling in BT-474 xenografts. Specifically, we aimed to determine the effect of ALA with and without TRAS on cell growth, phospholipid fatty acid profile and protein biomarkers of HER2 signaling in BT-474 cells. Subsequently, the effects of physiologically relevant doses of the ALA metabolite, DHA, alone and combined with TRAS, on cell growth and HER2 signaling biomarker expression were measured. Overall, the findings demonstrate that both ALA and its DHA metabolite reduce BT-
474 cell growth, however, show for the first time that only DHA reduces activation of proteins in HER2 signaling pathways. This finding is significant because it emphasizes the differences in mechanisms whereby n-3 PUFAs exert their effect in breast cancer.

### 6.2 Materials and Methods

#### 6.2.1 Cell Line

The BT-474 (HTB-20) cell line was obtained from ATCC. Cells were authenticated by short tandem repeat (STR) analysis at the Centre for Applied Genomics at the Sickkids Research Institute (Toronto, ON). Cells were maintained in RPMI medium (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS (Sigma-Aldrich) and 1% antibiotic-antimycotic solution containing penicillin, streptomycin and amphotericin B (Gibco).

#### 6.2.2 Fatty Acid and TRAS Treatment

ALA and DHA (>99% pure) were obtained from Sigma-Aldrich. Stock solutions were prepared in 100% ethanol, aliquoted, flushed with nitrogen gas and stored at -20°C. In preparation for treatment, ethanol was evaporated with nitrogen gas. 4 mM solutions were prepared in CS-FBS and incubated for 1 hour at 37°C to allow for the fatty acids to complex with albumin in the CS-FBS. TRAS was purchased from the Princess Margaret Cancer Centre Pharmacy (Toronto, ON). A 20 mg/ml stock solution of TRAS was prepared every two weeks using bacteriostatic water and stored at 4°C.

Cells in the log phase of growth were plated in tissue culture plates or flasks (Sarstedt, Numbrecht, Germany) in RPMI maintenance medium. After approximately 72 hours, the medium was removed and replaced with treatment medium. Treatment solutions were prepared using phenol red-free RPMI 1640 medium (Gibco) containing 1% antibiotics-antimycotics, 5% CS-FBS and 1 nM E2 dissolved in ethanol. The control treatment medium contained no fatty acids.
acids or TRAS. TRAS treatment was at a dose of 10 µg/ml. In experiment 1, cells were treated with ALA (50 and/or 100 µM) alone and combined with TRAS and cell growth, apoptosis, phospholipid fatty acids and HER2 signaling protein biomarkers were measured. In experiment 2, cells were treated with DHA (50 and 100 µM) alone and combined with TRAS and cell growth and HER2 signaling protein biomarkers were measured.

In both experiments, treatment medium was refreshed after 48 hours. To demonstrate that the effect was not simply due to the addition of fatty acids to the medium, an additional experiment was conducted to confirm the effect of ALA on cell growth using a background of other fatty acids (40 µM oleic acid (OA) and 40 µM LA) in both the control and treatment groups.

6.2.3 Cell Growth

Cell growth was analyzed using the trypan blue exclusion assay as described in Section 4.2.12.

6.2.4 Apoptosis

After 96 hour treatment, cells grown in 6 well plates (Sigma) were collected and washed in PBS. Cells were then incubated with 5µL Annexin V-APC and 7-AAD stain (BD Biosciences, Mississauga, ON) for 15 minutes at room temperature in the dark. Controls were prepared by adding no stains, Annexin V only and 7-AAD. 400 µl of binding buffer (BD Biosciences) was added to the samples. Samples were immediately analyzed by flow cytometry using a BD LSR Fortessa. Viable (Annexin V-/7-AAD-) and apoptotic cells (Annexin V+/7-AAD- and Annexin V+/7-AAD+) were quantified as a percent of total cells.
6.2.5 Protein Signaling Biomarkers

Cells were treated for 48 and/or 96 hours and then protein was extracted using RIPA lysis buffer (Cell Signaling Technology, Beverley, MA, USA) containing protease inhibitors (Complete Mini, EDTA-free Protease Inhibitor Cocktail Tablets, Roche) and phosphatase inhibitors (PhosSTOP Phosphatase Inhibitor Cocktail Tablets, Roche). Samples were prepared and analyzed as described in Section 4.2.9. Primary antibodies included HER2 (CST 2165), pHER2 (CST 4290), Akt1 (CST 2938), pAkt1 (CST 9018), MAPK (MAPK; CST 4695), pMAPK (CST 4377), caspase-3 (CST 9665) and β-actin (CST 3700). As in Study 3, LumiGLO® chemiluminescent reagent was used for protein detection and densitometric analysis was performed using ImageJ software. For this study, signal was detected on X-ray film (Clonex Corporation, Markham, ON) using a Konica Minolta™ SRX-101A Film Processor.

6.2.6 Phospholipid Fatty Acid Analysis

After 96 hours of treatment, cells were trypsinized, washed twice in PBS and resuspended in fatty acid free medium. Total lipids were extracted as described by Bligh and Dyer (Bligh and Dyer 1959) using NaCl, methanol and choloform (Sigma-Aldrich) in a 1:2:2 ratio. Thin-layer chromatography was used to separate lipid classes using silica G-plates (EMD Chemical, Gibbstown, NJ, USA). Total phospholipid fatty acids were collected into test tubes and a known amount of 17:0 standard (Avanti, Alabaster, AL, USA) was added. Fatty acids were converted to FAME by incubating for 1 hour at 100°C in hexane and boron trifluoride-methanol (Sigma-Aldrich). FAME were transferred to GC vials and samples were analyzed by GC-flame ionization detection (GC-FID) using a Varian-430 GC (Varian, Lake Forest, CA, USA) equipped with a Varian FactorFour capillary column (VF-23ms; 30 m · 0.25 mm i.d. · 0.25 lm film thickness) and a FID. Samples were injected in splitless mode as previously described (Chen et al. 2011a). Fatty acids were identified by comparison to a reference standard consisting of GLC-
68 and GLC-455 supplemented with 8:0, 10:0, and 12:0 methyl esters (Nu-Chek Prep, Elysian, MN) and quantified by comparing the area of the peaks to the 17:0 peak. Results are presented as mole percentages of total fatty acids.

### 6.2.7 Statistical Analysis

Statistical analyses were performed using SigmaPlot (version 12.0; Systat Software Inc., San Jose, CA). Data are expressed as mean ± SEM. Unpaired t-test was used to determine differences in phospholipid fatty acids between control and ALA treated cells. For all other outcomes, two-way ANOVA was used to evaluate main effects of fatty acids (ALA or DHA), TRAS and their interactions. If significant interactions were observed (P < 0.1), Tukey’s post-hoc test was used to compare each treatment group with significance set at P<0.05.

### 6.3 Results

#### 6.3.1 Experiment 1. Effect of ALA with and without TRAS

##### 6.3.1.1 Cell Growth and Apoptosis

TRAS had main effects in reducing cell growth (P<0.001) (Figure 6-1a) and increasing the number of apoptotic (annexin-V positive) cells (P=0.042) (Figure 6-1b) while ALA had main effect in reducing cell growth (P<0.001). The same growth reduction results as those shown in Figure 6-1 were observed when ALA was tested on a background of other fatty acids (data not shown) indicating that it is not simply the presence of fatty acids that is affecting cell growth. ALA did not significantly affect the number of apoptotic cells based on annexin-V staining (Figure 6-1b). Since there was a small but non-significant increase in apoptotic cells in the ALA treated cells compared to control, caspase 3 protein expression was measured by western blot but no difference in total or cleaved protein expression was detected (data not shown).
Figure 6-1. Effect of ALA and TRAS, alone and in combination, on the (A) growth and (B) apoptosis of BT-474 cells. n=3-5
6.3.1.2 Protein Biomarkers of HER2 Signaling Pathways

Figure 6-2 shows that pHER2/HER2 expression was not affected by ALA and TRAS alone and in combination at 48 and 96 hours. TRAS significantly reduced pAkt/Akt at 48 (P<0.001) and 96 hours (P<0.01) and pMAPK/MAPK at 96 hours (P<0.001). ALA treatment resulted in significantly higher pAkt/Akt (P<0.001) and pMAPK/MAPK (P=0.042) at 48 hours but had no significant effect at 96 hours. ALA×TRAS interactions were not seen for any biomarkers at any time point.

6.3.1.3 Phospholipid Fatty Acid Profile

TRAS had no effect on the fatty acid composition of the cells. Therefore, the results were pooled for cells treated with 0 uM ALA (control and TRAS) and with 100 µM ALA (ALA and ALA+TRAS). As seen in Table 6-1, ALA treatment caused a dramatic increase in phospholipid ALA (18:3n-3; 4926%, P<0.01) but no formation of ALA metabolites; in fact, levels of EPA (P<0.01) and DHA (P<0.001) were significantly lower in ALA-treated cells.

6.3.2 Experiment 2. Effect of DHA with and without TRAS

6.3.2.1 Cell Growth

Both DHA and TRAS showed main effect in reducing the growth of BT-474 cells (Figure 6-3; P<0.001). There was a significant DHA×TRAS interaction (P= 0.053). Multiple comparisons showed a significant dose-dependent reduction with DHA alone (P<0.05). 50 and 100 µM DHA combined with TRAS significantly reduced cell growth compared to TRAS alone (P<0.01) and there was no significant difference between the two DHA doses.

6.3.2.2 Protein Biomarkers of HER2 Signaling Pathways

Neither DHA nor TRAS had main effects on pHER2/HER2 expression nor did they show any interaction (Figure 6-4). Although not statistically significant, there was 45% lower
Figure 6-2. Effect of 100 µM ALA and 10 µg/ml TRAS, alone and in combination, on the expression of total and phosphorylated HER2, Akt and MAPK after 48 and 96 hours of treatment of BT-474 cells. (n=5-6). Representative blots are displayed.
Table 6-1. Phospholipid fatty acid profile of control and ALA-treated BT-474 cells. Results are presented as molar % of total fatty acids. N=6

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>100 µM ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>2.03 ± 0.62</td>
<td>1.27 ± 1.08</td>
</tr>
<tr>
<td>16:0</td>
<td>28.95 ± 4.94</td>
<td>27.16 ± 4.68</td>
</tr>
<tr>
<td>18:0</td>
<td>18.11 ± 3.14</td>
<td>17.95 ± 2.62</td>
</tr>
<tr>
<td>20:0</td>
<td>0.94 ± 0.21</td>
<td>0.56 ± 0.13</td>
</tr>
<tr>
<td>22:0</td>
<td>0.10 ± 0.03(^a)</td>
<td>1.14 ± 0.10(^b)</td>
</tr>
<tr>
<td>16:1 n-7</td>
<td>6.97 ± 2.86</td>
<td>6.24 ± 3.55</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>16.45 ± 1.03</td>
<td>11.74 ± 1.62</td>
</tr>
<tr>
<td>18:1n-7</td>
<td>4.36 ± 0.18(^a)</td>
<td>2.68 ± 0.34(^b)</td>
</tr>
<tr>
<td>20:1n-9</td>
<td>0.32 ± 0.08</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>22:1n-9</td>
<td>1.35 ± 0.70(^a)</td>
<td>0.53 ± 0.20(^b)</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>4.68 ± 0.86</td>
<td>3.11 ± 0.42</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>1.95 ± 0.92</td>
<td>1.15 ± 0.29</td>
</tr>
<tr>
<td>20:2n-6</td>
<td>0.35 ± 0.05(^a)</td>
<td>0.18 ± 0.03(^b)</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>6.34 ± 0.63(^a)</td>
<td>2.83 ± 0.79(^b)</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>0.38 ± 0.05(^a)</td>
<td>0.14 ± 0.02(^b)</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>0.08 ± 0.04</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.39 ± 0.15(^a)</td>
<td>19.60 ± 3.60(^b)</td>
</tr>
<tr>
<td>20:3n-3</td>
<td>2.64 ± 0.22(^a)</td>
<td>1.09 ± 0.10(^b)</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>1.29 ± 0.81</td>
<td>1.14 ± 0.19</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>1.18 ± 0.06</td>
<td>0.84 ± 0.17</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>1.15 ± 0.12(^a)</td>
<td>0.55 ± 0.14(^b)</td>
</tr>
<tr>
<td>n-6:n-3</td>
<td>2.09 ± 0.08(^a)</td>
<td>0.34 ± 0.07(^b)</td>
</tr>
</tbody>
</table>

*values with different superscripts in the same row are significantly different by unpaired T-test.
Figure 6-3. Effect of DHA (50 and 100 µM) and TRAS (10 µg/ml), alone and in combination, on the growth of BT-474 cells. n=3
**Figure 6-4.** Effect of DHA (50 and 100 µM) and TRAS, alone and in combination, on the expression of total and phosphorylated HER2, Akt and MAPK after 96 hours of treatment of BT-474 cells. (n=5-6). Representative blots are displayed. For HER2 protein biomarkers, blot images were cropped and rearranged for consistency of presentation of treatment groups. Bands for each biomarkers are from the same blot.
pHER2/HER2 expression in the DHA100+TRAS treated cells compared to TRAS treated cells. There were significant main effects of TRAS in reducing the expression of pMAPK/MAPK and pAkt/Akt (P<0.01). DHA had main effects in reducing pMAPK/MAPK and pAkt/Akt. DHA+TRAS100 resulted in 27% lower pMAPK/MAPK and 56% lower pAkt/Akt compared to TRAS alone.

6.4 Discussion

This study has shown that both ALA and DHA reduce the growth of BT-474 cells and enhance the effect of TRAS in reducing cell growth. The first experiment showed that BT-474 cells were shown to lack the ability to convert ALA to downstream metabolites thereby presenting a good system to study the independent effects of ALA and its metabolite DHA. The results strongly suggest that DHA reduces growth factor receptor signaling as indicated by reductions in phosphorylation of Akt and MAPK while the opposite effect is seen for the plant-based n-3 PUFA ALA. Because in vitro ALA treatment reduced phospholipid DHA level and increased phosphorylation of Akt and MAPK, it is possible that the in vitro effect of ALA on Akt and MAPK activation is driven by the decrease in phospholipid DHA. It is important to note that feeding an ALA-rich diet does not decrease but rather increases tumor DHA level in mice as shown in studies 1 and 2 (Chapters 4 and 5) (Mason et al. 2013a; Mason et al. 2015) suggesting that this loss of DHA is isolated to in vitro models.

The mechanism driving the growth reducing effect of ALA in this HER2 overexpressing cell line requires further elucidation. Based on the current study, ALA is not acting through an induction of apoptosis or reduction of HER2 signaling pathways. Although the effect of ALA on breast cancer cell growth has been previously explored (Grammatikos et al. 1994; Chajes et al. 1995; Horia and Watkins 2005; Kim et al. 2009; Tran et al. 2010; Truan et al. 2010), only two
studies have looked at effects on HER2-overexpressing BT-474 cells (Menendez et al. 2006; Wiggins et al. 2015). Similar to the current findings, ALA alone (Menendez et al. 2006; Wiggins et al. 2015) and combined with TRAS (5 µg/ml) (Menendez et al. 2006) reduced BT-474 cell growth; however, Menendez et al. (2006) saw significant reductions in HER2 mRNA and protein following treatment with 20 µM ALA in that study. Several, but not all, studies looking at the effect of ALA in other breast cancer cell lines have shown significant growth reductions (Grammatikos et al. 1994; Chajes et al. 1995; Horia and Watkins 2005; Kim et al. 2009; Tran et al. 2010; Truan et al. 2010). Though apoptosis has been suggested as a mechanism of ALA effect in MCF-7 cells (Kim et al. 2009), no such effect of ALA on apoptosis was seen in BT-474 cells. It is possible that ALA modulates cell growth through ER-related mechanisms. ALA has been shown to bind to the α and β isoforms of the ER and exert mixed agonistic/agonistic effects at different doses (Tran et al. 2010). Dietary FSO reduces the expression of E2-sensitive genes including PGR and cyclin D1 in MCF-7 xenografts (Saggar et al. 2010a; Saggar et al. 2010b). Further research is needed to determine the mechanism for ALA effect in breast cancer.

More studies have investigated the effect of DHA on cell growth and apoptosis (Chajes et al. 1995; Chamras et al. 2002; Schley et al. 2005; Schley et al. 2007; Ghosh-Choudhury et al. 2009; Rogers et al. 2010; Corsetto et al. 2011; Cao et al. 2012; Ewaschuk et al. 2012; Ravacci et al. 2013). Others have similarly found that DHA reduces the growth of HER2-overexpressing breast cancer cells (BT-474 and SkBr-3) (Ewaschuk et al. 2012; Zou et al. 2013). Ewaschuk et al (2012) studied the effect of DHA with and without TRAS on SkBr-3 cells. They found a 27% reduction with TRAS treatment alone yet when 100 µM DHA was combined with TRAS a much greater reduction was observed (63%). Similar to the findings presented in this chapter, there was no significant difference between the cells treated with 100 µM DHA in the absence or presence of TRAS (Ewaschuk et al. 2012). Others have suggested that DHA affects cell signaling
pathways through incorporation into membrane rafts thereby affecting the distribution and activity of associated receptors (Schley et al. 2007; Corsetto et al. 2011; Ravacci et al. 2013). DHA has been fairly consistently shown to induce apoptosis or affect the expression of markers involved in apoptosis pathways in breast cancer cells (Schley et al. 2005; Blanckaert et al. 2010; Kang et al. 2010; Corsetto et al. 2011; Xiong et al. 2012; Lee et al. 2014). Furthermore, it is well established that treatment with DHA increases phospholipid DHA in breast cancer cells (Grammatikos et al. 1994; Blanckaert et al. 2010; Corsetto et al. 2011). Apoptosis and fatty acid analysis following DHA treatment was therefore not conducted in the current study.

This study has some limitations. One limitation is that only one ALA metabolite was studied. EPA is another n-3 ALA metabolite but we focused specifically on DHA for a number of reasons. EPA is a minor constituent of tumor and serum fatty acids in the athymic mouse model (Truan et al. 2010; Mason et al. 2013a; Mason et al. 2014; Mason et al. 2015). Feeding of 10% FS or 4% FSO resulted in serum levels of only 15-22 µM EPA (Truan et al. 2010; Mason et al. 2014) while studies looking at EPA effect on cell growth in vitro have seen growth reduction at or above approximately 100 µM (Chamras et al. 2002; Corsetto et al. 2011; Ewaschuk et al. 2012). On the other hand, DHA concentration in the serum of 10% FS and 4% FSO-fed mice was shown to be 244 and 193 µM (Truan et al. 2010; Mason et al. 2014), respectively, while DHA has been shown to reduce breast cancer growth at doses as low as 25 µM in HER2-overexpressing SkBr-3 cells (Ewaschuk et al. 2012). Furthermore, there was support for the hypothesis that DHA affects HER2 signaling pathways (Cao et al. 2012; Zou et al. 2013). An additional limitation is that only one cell line was used in the current study. However, literature suggests that the DHA and ALA effects observed in this study are likely not cell-line specific since DHA alone has been shown to reduce pAkt in both BT-474 and SkBr-3 cells (Zou et al. 2013) and DHA has been shown to enhance TRAS effect in SkBr-3 cells (Ewaschuk et al. 2012).
Furthermore, work from our research group has shown that ALA treatment reduces the growth of four breast cancer cell lines with varying receptor expression (Wiggins et al. 2015).

This study was conducted to clarify whether ALA or its metabolite, DHA, caused the effects seen in Chapter 5 where a diet with ALA-rich FSO significantly enhanced the effectiveness of TRAS in athymic mice (Mason et al. 2015). Therefore, n-3 fatty acid concentrations used were derived from studies in the athymic mouse model with feeding of 4% FS oil or 10% FS diets. Serum ALA levels of mice fed these diets ranged from 33 to 108 µM (Truan et al. 2010; Mason et al. 2014). Hence, in the current study, cells were treated with 50 and 100 µM ALA, levels within this range. In mice fed a 10% FS or 4% FSO diet, approximately 100-150 µM higher serum DHA was detected compared to mice fed the BD. However, the cells were not treated with 150 µM DHA as preliminary results suggested that DHA treatment higher than 100 µM was cytotoxic. Thus, cells were treated with 50 and 100 µM. Overall, these findings suggest that treating BT-474 cells with serum levels of ALA seen in animal model reduces cell growth with and without TRAS but does not match the effects on HER2 signaling pathway markers seen in vivo. On the other hand, treating BT-474 cells with the concentration of DHA seen following FSO feeding reduces cell growth and biomarkers of the HER2 signaling pathway in a similar manner to study 2 (Chapter 5).

Humans are known to be poor converters of ALA to DHA and it is suggested that the best way to increase serum levels of DHA is through dietary intake (Brenna et al. 2009). Several factors are suggested to affect this conversion including background fatty acids in the diet and sex (Brenna et al. 2009; Kitson et al. 2010). Daily consumption of approximately 6 g of ALA from FSO for 12 weeks has been shown to increase serum ALA by approximately 154 µM but increase DHA only by 15 µM (Austria et al. 2008). The findings from the current study suggest
that interventions that significantly increase serum DHA are required for modulation of HER2 signaling pathway. Interestingly, a randomized controlled trial showed that consumption of 25 g of FS per day, providing approximately 6 g of ALA, by breast cancer patients significantly reduced cell proliferation and HER2 expression (Thompson et al. 2005). This suggests that despite the low conversion to DHA in humans, ALA-rich diets may significantly reduce breast tumor growth in breast cancer patients.

The overall objective of this study was to determine whether ALA is the component of FSO responsible for the effects seen in vivo on HER2 signaling in BT-474 xenografts. Because ALA alone did not cause significant downregulation of HER2 signaling while DHA did, the presented findings suggest that the effects of FS and FSO seen in animal studies on growth factor signaling pathways is likely due to DHA produced from hepatic conversion of ALA to DHA and not due to ALA itself. Despite this lack of effect on growth factor receptor signaling, ALA significantly reduced cell growth perhaps by different mechanisms including through ER-related signaling, which merits further exploration. Findings suggest that there are differences in the mechanisms of ALA and DHA growth effects in HER2 overexpressing cells. These significant findings contribute to the understanding of the role of n-3 PUFAs in breast cancer and may help in the development of nutritional approaches for breast cancer treatment.
CHAPTER 7
Study 4: Effect of α-linolenic acid on MCF-7 cell growth \textit{in vitro}
and exploration of potential mechanisms
7.1 Introduction

ALA is an essential fatty acid that is the metabolic precursor to the long chain n-3 PUFA EPA and DHA. EPA, DHA and their major dietary source, fish oil, are suggested to have anticancer effects and reduce the growth of various cancer types (Cao et al. 2012; Ewaschuk et al. 2012; Leslie et al. 2014; Xue et al. 2014). ALA-rich diets from FS and FSO reduce tumor growth and cell proliferation and increase apoptosis of ER+ MCF-7 breast tumors in athymic mice (Chen et al. 2007b; Saggar et al. 2010b; Truan et al. 2010). Furthermore, canola oil and walnut-based diets which have high ALA have been shown to reduce ER negative MDA-MB-231 tumor growth and cell proliferation (Hardman 2007; Hardman and Ion 2008) in mouse models. Tumor levels of ALA and its n-3 metabolites EPA and DHA are all significantly elevated following consumption of ALA-rich diets in mouse models (Hardman 2007; Mason et al. 2013a; Mason et al. 2015). The in vivo effects of ALA-rich diets may be due to either ALA itself, the long-chain n-3 PUFAs, EPA and DHA, or their combined effects, but this has not been delineated.

In vitro studies allow for the determination of the independent effect of ALA since several breast cancer cell lines are unable to convert ALA to EPA and DHA (Grammatikos et al. 1994; Bardon et al. 1996; Wiggins et al. 2013). Despite the lack of conversion to EPA and DHA, ALA has been shown to reduce the growth of several breast cancer cell lines in vitro (Grammatikos et al. 1994; Chajes et al. 1995; Menendez et al. 2006; Kim et al. 2009; Tran et al. 2010; Truan et al. 2010; Vanden Heuvel et al. 2012; Wiggins et al. 2015). Some inconsistencies have been observed, however, between and within cell lines. For example, in the three studies that have compared the effect of ALA at concentrations between 50-100 µM in ER+ MCF-7 cells and ER- MDA-MD-231 cells, one only saw a significant growth reduction in MCF-7 cells
(Tran et al. 2010), one only saw significant growth reduction in MDA-MB-231 cells (Chajes et al. 1995) and one saw a growth reduction in both cell lines (Wiggins et al. 2015). Several additional studies have looked at the effect of ALA in ER+ MCF-7 cells (Gore et al. 1994; Grammatikos et al. 1994; Kim et al. 2009; Truan et al. 2010; Vanden Heuvel et al. 2012) and all have shown significant growth reduction. More variability has been seen in the response of the ER- MDA-MB-231 cells to ALA with some studies showing a growth reduction (Chajes et al. 1995; Horia and Watkins 2005; Wiggins et al. 2015) and others showing no effect (Tran et al. 2010; Yu et al. 2011). Taken together, these findings suggest that ALA reduces the growth of both ER+ and ER- breast cancer growth, although the effect is more consistent in ER+ MCF-7 cells.

The mechanisms for the effect of ALA in reducing breast cancer growth are unclear. Induction of apoptosis has been suggested to play a role (Kim et al. 2009), however, this has not been consistently shown across all cell lines (Wiggins et al. 2013). ALA-rich diets in vivo have been shown to reduce growth factor receptor expression and signaling in MCF-7 and BT-474 xenografts (Saggar et al. 2010a; Saggar et al. 2010b; Truan et al. 2010; Mason et al. 2015); however, study 3 (Chapter 6) suggests that in HER2-overexpressing BT-474 cells, the effect on growth factor receptor signaling is due to the DHA metabolite rather than ALA itself. Whether this is specific to BT-474 cells is unknown. ALA has been shown to bind to both ERα and ERβ (Tran et al. 2010), thus, it is possible that ALA may affect the growth of ER+ breast cancer cells through antiestrogenic mechanisms but this has not yet been explored.

The overall objective of this study was to explore potential mechanisms of ALA effect in ER+ MCF-7 cells. Effects on biomarkers of growth factor receptor and ER-related signaling were of particular interest. The results suggest that modulation of ER-related signaling more so
than growth factor receptor signaling contributes to the mechanism of ALA effect as evidenced by reduced expression of gene and/or gene products of biomarkers of ER-related signaling and the lack of ALA effect when combined with the selective estrogen receptor downregulator (SERD) ICI 182,780 (ICI, fulvestrant). These findings, shown for the first time, are significant as they provide a plausible, specific biological mechanism of ALA in ER+ breast cancer that is independent of long-chain n-3 PUFA metabolites. This contributes to the understanding of the beneficial effect of ALA in breast cancer risk reductions seen in some observational studies (Saadatian-Elahi et al. 2004) and of ALA-rich FS in a clinical trial of postmenopausal breast cancer patients (Thompson et al. 2005).

7.2 Materials and Methods

7.2.1 Cell Line

The MCF-7 (HTB-22) cell line was obtained from ATCC. Cells were authenticated by STR analysis using the Cell Line Authentication Service through ATCC and Promega as previously described in (Capes-Davis et al. 2013) and found to be 100% match to known database profiles. Cells were maintained in DMEM medium (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS and 1% antibiotic-antimycotic solution containing penicillin, streptomycin and amphotericin B.

7.2.2 Experimental Treatments

ALA (>99% pure), OA (18:1n-9, >99% pure), LA (18:2n-6, 99% pure), E2 and the ER antagonist ICI 182,780 (ICI, fulvestrant, >98% pure) were obtained from Sigma-Aldrich. Stock solutions of fatty acids, E2 and ICI were prepared in 100% ethanol, aliquoted and stored at -20°C. TRAS was obtained and prepared as described in Section 6.2.2. Fatty acid solutions were prepared as described in section 6.2.2. Treatment solutions were prepared using phenol red-free
medium (Gibco) containing 1% antibiotics-antimycotics, 5% CS FBS. Experimental agents were added to the treatment medium at the following doses: E2: 0 or 1 nM, ICI: 0 or 100 nM, ALA: 0, 50 or 100 µM, OA: 40 µM, LA 40 µM and TRAS: 10 µg/ml. ALA was tested alone or combined with a background of fatty acids (OA and LA) to ensure that the effect on cell growth was not simply due to the presence of any fatty acid. It was also tested (a) alone or combined with ICI, an ER antagonist, to determine whether its effect on cell growth relates to ER function and (b) alone or combined with TRAS, a HER2 inhibitor, to determine its role in HER2 signaling. Cells in the log phase of growth were plated in tissue culture plates or flasks (Sarstedt, Numbrecht, Germany) in DMEM maintenance medium at the following densities: 9.6 x 10^4 cells/well in 6 well plates, 3.6 x 10^5 cells/well in 24 well plates and 1.0 x 10^6 cells/flask in T75 flasks. After 72 hours of adherence, the medium was removed and replaced with treatment medium. For experiments with 96 hour duration, treatment medium was refreshed after 48 hours.

7.2.3 Cell Growth, Apoptosis and Phospholipid Fatty Acid Composition

Cell growth, apoptosis and phospholipid fatty acids were measured as described in Sections 6.2.3, 6.2.4 and 6.2.6 respectively. Cell growth was measured following 96 hours of treatment with 1 nM E2 alone (control) or combined with ALA (50 and 100 µM) both in the absence and presence of background fatty acids (40 µM OA and 40 µM LA). Apoptosis and phospholipid fatty acids were measured following 96 hours of treatment with 1 nM E2 alone (control) or combined with 100 µM ALA.

7.2.4 qRT-PCR Array

Cells were treated for 24 hours with 1 nM E2 alone (control) or combined with 100 µM ALA (ALA) in 6 well plates. Cells were then collected and cell pellets were immediately stored at -80°C. RNA was extracted using the RNeasy mini kit with on-column DNase digestion kit
according to the manufacturer’s protocol (Qiagen, Frederick, MD, USA). RNA concentration and purity were measured using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA; Mean OD260/280= 2.1 ± 0.0; Mean OD 260/230= 1.8 ± 0.1 ). The Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) was used to measure RNA integrity (Mean RNA Integrity Number = 9.9 ± 0.1). cDNA was synthesized from 0.5 µg of RNA using the RT2 First Strand kit (Qiagen) using the procedure outlined by the manufacturer. Gene expression analysis was conducted using a customized RT2 Profiler Breast Cancer PCR array (Qiagen) which uses SYBR Green technology. This array measures the expression of 88 genes of interest in one sample per plate. Three biological replicates were run per treatment. Gene expression was measured using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Three housekeeping genes were used: B2M (β-2 microglobulin), HPRT1 (hypoxanthine-guanine phosphoribosyltransferase), and RPLP0 (ribosomal protein P0) as these had the lowest variation (standard deviation) in ΔCt across all plates. Data was analyzed using the downloadable excel spreadsheets available from Qiagen/SABiosciences which performs a student’s t-test to compare expression levels using the ΔΔCt method.

7.2.5 Protein Biomarkers

MCF-7 cells were treated in 6 well plates with 1 nM E2 alone (control) or combined with ALA. Protein biomarker expression was performed by western blot as described in Section 6.2.5. Primary antibodies were targeted to (i) growth factor receptor signaling pathways biomarkers: HER2 (CST 2165), pHER2 (CST 4290), Akt1 (CST 2938), pAkt1 (CST 9018), p44/42 MAPK (CST 4695), pMAPK (CST 4377), (ii) ER-related biomarkers: ERα (CST13258), cyclin D1 (CST2978), progesterone receptor A/B (CST8757), survivin (CST2808) and TFF-1/pS2
(CST12419), (iii) caveolin-1 (CST3267) which is suggested to regulate growth factor receptors and ER; and (iv) β-actin (CST 3700) as a housekeeping protein.

7.2.6 Statistical Analysis

Student’s t-test was used to compare the control and ALA treatments. For experiments with multiple treatment groups, one-way ANOVA with Tukey’s post-hoc test was used. Significance was set at $P < 0.05$. All analyses were conducted using SigmaPlot version 12.0.

7.3 Results

7.3.1 Cell Growth

Irrespective of background fatty acids (40 µM OA and 40 µM LA), 100 µM ALA combined with 1 nM E2 resulted in a significantly lower final live cell count compared to the E2-containing control ($P<0.05$) (Figure 7-1A and B). With background fatty acids, 50 µM ALA treatment resulted in significantly fewer live cells compared to control ($P<0.05$). No reduction in cell growth was seen with 50 µM ALA alone. All subsequent experiments were thus conducted with 100 µM ALA and 1 nM E2 without the background fatty acids.

7.3.2 Apoptosis

The majority of cells (>70%) in the control (+E2) and ALA (100 µM, +E2) groups were healthy and showed no staining for Annexin-V or 7-AAD. There was no significant difference in early apoptotic cells (Annexin-V +/7-AAD-) between control (24.9%) and ALA (25.8%) treated cells (Figure 7-1C). There were significantly more late apoptotic cells (Annexin-V +/7-AAD+) in the ALA treatment group (2.1%) compared to control (1.3%) ($P= 0.03$).
Figure 7-1. Effect of ALA on (A and B) growth and (C) apoptosis in MCF-7 cells. Cells were treated for 96 hours and all treatments (ALA and control) contained 1 nM E2. Bars with different letters are significantly different by one-way ANOVA with Tukey’s post-hoc test. * indicates significant difference by unpaired t-test.
7.3.3 Phospholipid Fatty Acid Profile

Treatment with 100 µM ALA caused a dramatic 78 fold increase in phospholipid ALA (P<0.001) but no formation of ALA metabolites; in fact, the level of DHA was significantly lower in ALA-treated cells (P<0.01) (Table 7-1). Several other fatty acids were significantly lower (16:1n-7, OA 18:1n-9, 18:1n-7, 20:1n-9, LA 18:2n-6, ARA 20:4n-6, 22:4n-6, 22:5n-6 and 22:5n-3) or higher (18:0, 22:0 and 22:5n-6) in ALA-treated cells (P<0.05).

7.3.4 mRNA Expression

A complete dataset from the RT-PCR array which was conducted after a 24 hour treatment period comparing MCF-7 cells treated with E2 alone (control) and combined with 100 µM ALA is presented in Appendix 1. Table 7-2 shows the genes that were significantly up- or down-regulated following treatment with 100 µM ALA + E2. Caveolin-1 and ABCG2 both showed greater than 2 fold lower expressions in ALA+E2-treated cells compared to +E2 control while other genes showed more modest effects. Expressions of genes involved in growth factor receptor pathways including those that code for Akt1, EGFR, HER2, Grb7, IGFIR, p44/42 MAPK and PTEN were not significantly different between +E2 control and ALA+E2 treated cells. The expression of genes that have been shown to be upregulated by E2 treatment in MCF-7 cells (Lam et al. 2011) including PGR and Survivin (BIRC5) was significantly downregulated by ALA treatment.

7.3.5 Growth Factor Receptor Signaling

Both total and phosphorylated levels of HER2 were significantly higher in E2-stimulated MCF-7 cells treated with 100 µM ALA (P< 0.01) while pHER2/HER2, an indicator of HER2 activation, was not significantly different (Figure 7-2). Total and phosphorylated levels of MAPK were not affected by ALA. Total Akt1 expression was not affected; there was a
<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Control</th>
<th>ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>3.43 ± 0.24</td>
<td>2.84 ± 0.09</td>
</tr>
<tr>
<td>16:0</td>
<td>26.05 ± 1.01</td>
<td>28.50 ± 0.97</td>
</tr>
<tr>
<td>18:0</td>
<td>13.58 ± 0.41</td>
<td>17.12 ± 0.70</td>
</tr>
<tr>
<td>20:0</td>
<td>0.16 ± 0.01</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>22:0</td>
<td>0.12 ± 0.02</td>
<td>2.06 ± 0.09</td>
</tr>
<tr>
<td>16:1 n-7</td>
<td>6.11 ± 1.00</td>
<td>1.77 ± 0.12</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>25.99 ± 0.57</td>
<td>9.84 ± 0.81</td>
</tr>
<tr>
<td>18:1n-7</td>
<td>8.94 ± 0.53</td>
<td>2.39 ± 0.16</td>
</tr>
<tr>
<td>20:1n-9</td>
<td>0.57 ± 0.03</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>22:1n-9</td>
<td>0.28 ± 0.07</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>6.55 ± 0.34</td>
<td>3.14 ± 0.27</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>0.08 ± 0.01</td>
<td>0.19 ± 0.10</td>
</tr>
<tr>
<td>20:2n-6</td>
<td>0.33 ± 0.04</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>5.03 ± 0.29</td>
<td>1.39 ± 0.19</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>0.22 ± 0.03</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>0.04 ± 0.01</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.37 ± 0.04</td>
<td>28.91 ± 1.62</td>
</tr>
<tr>
<td>20:3n-3</td>
<td>0.41 ± 0.06</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.21 ± 0.06</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.68 ± 0.04</td>
<td>0.36 ± 0.06</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>0.46 ± 0.04</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>n-6:n-3</td>
<td>5.79 ± 0.24</td>
<td>0.18 ± 0.02</td>
</tr>
</tbody>
</table>

*indicates significant difference by unpaired t-test.
Table 7-2. Genes significantly up- or down-regulated by 100 µM ALA in E2-treated MCF-7 cells.

<table>
<thead>
<tr>
<th>Gene</th>
<th>RefSeq #</th>
<th>Fold change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCG2</td>
<td>NM_004827</td>
<td>-2.5</td>
<td>0.01</td>
</tr>
<tr>
<td>BIRC5</td>
<td>NM_001168</td>
<td>-1.4</td>
<td>0.02</td>
</tr>
<tr>
<td>CAV1</td>
<td>NM_001753</td>
<td>-2.2</td>
<td>0.00</td>
</tr>
<tr>
<td>CCNA1</td>
<td>NM_003914</td>
<td>-1.5</td>
<td>0.01</td>
</tr>
<tr>
<td>CCNE1</td>
<td>NM_001238</td>
<td>-1.2</td>
<td>0.03</td>
</tr>
<tr>
<td>CDK2</td>
<td>NM_001798</td>
<td>-1.3</td>
<td>0.02</td>
</tr>
<tr>
<td>CDKN1C</td>
<td>NM_000076</td>
<td>-1.5</td>
<td>0.03</td>
</tr>
<tr>
<td>GSTP1</td>
<td>NM_000852</td>
<td>1.7</td>
<td>0.01</td>
</tr>
<tr>
<td>KRT18</td>
<td>NM_000224</td>
<td>-1.3</td>
<td>0.02</td>
</tr>
<tr>
<td>MAPK8</td>
<td>NM_002750</td>
<td>1.1</td>
<td>0.04</td>
</tr>
<tr>
<td>PGR</td>
<td>NM_000926</td>
<td>-1.3</td>
<td>0.04</td>
</tr>
<tr>
<td>XBP1</td>
<td>NM_005080</td>
<td>1.2</td>
<td>0.04</td>
</tr>
</tbody>
</table>
Figure 7-2. Effect of 96 hour treatment with 1 nM E2 alone (control) or combined with 100 µM ALA on protein biomarkers of growth factor receptor signaling. *indicates significant difference by unpaired t-test. n=3. Representative blots are shown.
significant increase in pAkt (P= 0.04) but no significant difference was seen in the ratio of pAkt/Akt.

MCF-7 cells have low expression of HER2; therefore, a follow up experiment was conducted to explore the biological significance of the upregulation of HER2 expression seen with ALA treatment. TRAS, which is known to inhibit HER2 (Hudis 2007), had no effect on MCF-7 cell growth when given alone or in combination with ALA (Figure 7-3). ALA reduced cell growth to a similar extent when provided alone or combined with TRAS (P< 0.05) (Figure 7-3).

7.3.6 Biomarkers of ER signaling

The protein expression level of various markers related to ER signaling in breast cancer were compared between cells treated for 96 hours with 1 nM E2 alone (control) and combined with 100 µM ALA. No effect was seen on PS2/TFF1 or survivin expression (data not shown). Caveolin-1, PGR and cyclin D1 protein levels were all significantly lower in ALA treated cells compared to control by 83% (P= 0.05), 79% (P<0.01) and 30% (P<0.01), respectively (Figure 7-4). Compared to control, ERα protein expression was significantly higher by 29% (P=0.03) in ALA treated cells (Figure 7-4). To further explore the role of ER in the mechanism of ALA effect, the effect of ALA alone and combined with the ER antagonist ICI was examined (Figure 7-5). As expected, culturing MCF-7 cells in the absence of E2 or inhibiting the ER with ICI significantly reduced growth (P<0.05). Consistent with the previous experiments, 100 µM ALA (+E2) significantly reduced growth compared to control (+E2) (P<0.05), however, there was no significant benefit of combining ICI+ALA compared to ICI alone.
Figure 7-3. Effect of 96 hour treatment with 100 μM ALA and 10 μg/ml TRAS alone and in combination on the growth of MCF-7 cells. All treatments included 1 nM E2. Bars with different letters are significantly different by one-way ANOVA with Tukey’s post-hoc test. n=5.
Figure 7-4. Effect of 96 hour treatment with 100 μM ALA on protein biomarkers of ER-related signaling. Both control and ALA treatments contain 1 nM E2. *indicates significant difference by unpaired t-test. n=3. Representative blots are shown.
Figure 7-5. Effects of 96 hour treatment with E2 (1nM), ICI (100nM) and ALA (100µM) alone and in combinations on the growth of MCF-7 cells. Bars with different letters are significantly different by one-way ANOVA with Tukey’s post-hoc test. (n=4). ICI, ALA and ALA+ICI treatments were given in combination with 1 nM E2.
7.4 Discussion

This study agrees with previous findings that ALA reduces the growth of MCF-7 cells (Grammatikos et al. 1994; Kim et al. 2009; Tran et al. 2010; Truan et al. 2010; Wiggins et al. 2015). The reduction in growth is not explained by an induction in apoptosis. Although there was a statistically significant increase in late apoptotic cells, very few cells stained positive for both 7-AAD and Annexin V in control cells, as seen by others (Kang et al. 2010), as well as ALA-treated cells so it is unlikely that the increase seen would have any biological significance. MCF-7 cells did not convert ALA to DHA as previously reported (Grammatikos et al. 1994). Therefore the observed effects of ALA on cell growth are directly due to ALA and not its metabolites. The main objective of this study was to explore potential mechanisms of ALA effect and results from the RT-PCR array and protein biomarker analyses suggest that ALA may reduce cell growth in part through interfering with ER-related signaling pathways. ALA was shown to reduce the expression of E2-sensitive genes and/or gene products including caveolin-1, PGR and cyclin D1 despite an increase in ERα protein. Furthermore, there was no additional growth reduction seen when ALA treatment was given in combination with ICI compared to ICI alone. Assuming that reductions beyond that seen with ICI are possible, these results highlight the importance of ERα in ALA effect. Previous work conducted in our laboratory using similar methods have demonstrated growth reductions of greater than 90% in MCF-7 cells with ALA doses of 125 µM and higher suggesting that large reductions in cell growth can be achieved.

This hypothesis-generating study explored the independent effect of ALA in E2-stimulated MCF-7 cells. Previous research from our laboratory using the same methods as the current study looked at the independent and combined effects of ALA and E2 and showed that ALA reduced the MCF-7 cell growth in the presence and absence of E2 and that the stimulatory effect of E2 is
completely negated by the addition of 50 µM ALA (Wiggins et al. In press) suggesting that ALA effect is at least in part due to antiestrogenic effects. Future studies should directly compare the independent and combined effects of ALA, E2 and ER modulators such as TAM on ER-related signaling biomarkers in order to confirm the hypothesis that ALA interferes with ER signaling. However, comparing the mRNA and protein biomarker results for the independent effect of ALA in E2-stimulated MCF-7 cells from the current study to previously observed effects of E2 and the selective estrogen receptor modulator (SERM) TAM, supports the suggestion that ALA has SERM-like effects. E2 is known to reduce ERα and increase survivin (BIRC5), cyclin D1 and PGR mRNA and protein expression (Altucci et al. 1996; Martin et al. 2000; Frasor et al. 2003; Stoica et al. 2003). Similar to the observations with ALA treatment, treating MCF-7 cells with TAM or 4-OH-TAM increased ERα expression post-transcriptionally (Wijayaratne and McDonnell 2001; Thomas et al. 2010), decreased PGR mRNA and protein (Horwitz et al. 1978; Murphy et al. 2005; Lebedeva et al. 2012), and decreased cyclin D1 mRNA and protein (Johnson et al. 2010; Anbalagan et al. 2012; Zhao and Wang 2013) (note a non-significant reduction in CCND1 mRNA was observed with ALA+E2 treatment). Cyclin D1 is an important regulator of the cell cycle. That ALA reduces cyclin D1 protein expression suggests that it may reduce cell growth by reducing cell proliferation as has been shown in previous studies using BrdU incorporation assays (Truan et al. 2010; Vanden Heuvel et al. 2012). An important consideration is that in some of these cited studies the TAM effects were demonstrated in the absence of E2 with comparison to a –E2 control (Wijayaratne and McDonnell 2001; Thomas et al. 2010) whereas in others TAM treatment was combined with E2 and compared to a +E2 control (Horwitz et al. 1978; Lebedeva et al. 2012).

There are a number of potential mechanisms by which ALA may interfere with ER-related signaling in MCF-7 cells. First, we saw a significant 2.45 fold downregulation of ABCG2
mRNA expression in ALA-treated MCF-7 cells. These findings agree with previous findings from our research group using a lower dose of ALA (75 µM) (Wiggins et al. 2015). This gene codes for the ATP-binding cassette sub-family G member 2, a transporter that moves molecules across cell membrane including drugs, lipids and steroids including E2 (Hardwick et al. 2007). Thus, ALA may reduce ER-related signaling by reducing intracellular levels of E2. Secondly, we saw significant reductions of caveolin-1 mRNA (2.2 fold reduction) and protein (83% reduction). Caveolin-1 is a structural protein localized in caveolae within the lipid raft domain. Some suggest that MCF-7 cells do not express caveolin-1 (Schlegel et al. 1999; Agelaki et al. 2009), yet, we like several others (Razandi et al. 2002; Thomas et al. 2010) have detected caveolin-1 in these cells. The biological role of caveolin-1 is not yet fully clear and it is suggested to play both tumor suppressor and oncogenic roles depending on the context. Caveolin-1 has been shown by others to induce ligand-independent activation of ERα (Schlegel et al. 1999; Schlegel et al. 2001); therefore, the effect of ALA in reducing the expression of E2-sensitive genes may be related to its downregulatory effect on caveolin-1 expression. Finally, Tran et al. (2010) have shown that ALA can bind both ERα and ERβ, thus, ALA may directly regulate ER activity.

We observed elevated total and phosphorylated HER2 protein expressions in ALA-treated cells which was unexpected given previous work from our group showing a reduction in HER2 protein in MCF-7 xenografts in mice fed an ALA-rich FSO diet (Truan et al. 2010). We have found that tumor levels of ALA, EPA and DHA in these MCF-7 tumors were all significantly higher in FSO-fed mice compared to mice fed the BD (unpublished data). In the current study, we found that phospholipid levels of ALA were significantly higher, EPA levels were unchanged and DHA levels were significantly lower in ALA-treated cells. It is possible that the reduction in HER2 seen in vivo was due to the elevated levels of DHA thereby explaining the discrepancy in results. Caveolin-1 has also been shown to negatively regulate a number of proteins including
HER2 (Patani et al. 2012); therefore, the observed upregulation of HER2 may be related to the reduction in caveolin-1. It is unlikely that the upregulation in HER2 expression has significant biological consequences in MCF-7 cells. First, ALA did not significantly affect the MAPK pathway an important downstream pathway of HER2. ALA caused a modest but significant increase in pAkt expression but no significant effect on pAkt/Akt thereby suggesting minimal effect of the elevated HER2 on the Akt pathway another downstream pathway of HER2. Secondly, HER2 inhibition by TRAS did not affect MCF-7 cells growth when given alone and combined with ALA. Taken together, growth factor receptor modulation does not appear to explain the effect of ALA in reducing the growth of E2-stimulated MCF-7 cells.

ALA-rich diets have been consistently shown to reduce the growth of MCF-7 xenografts in vivo in the athymic mouse model (Chen et al. 2007b; Saggar et al. 2010a; Saggar et al. 2010b; Truan et al. 2010). Analysis of tumor biomarkers from these in vivo studies supports the hypothesis that modulation of ER signaling pathways may play a role as PGR protein and mRNA expressions and cyclin D1 mRNA were reduced by ALA-rich diets from FS and FSO (Chen et al. 2007b; Saggar et al. 2010a; Saggar et al. 2010b), although other pathways may have affected these biomarkers. Serum levels of ALA, EPA and DHA are all elevated in athymic mice fed FS and FSO diets (Truan et al. 2010; Mason et al. 2014). As MCF-7 cells lack the ability to convert ALA to EPA and DHA, the use of in vitro methods in the current study shows that ALA specifically exerts anticancer effects independent of EPA and DHA. The level of ALA used in this study (100 µM) is physiological and achievable through diet based on findings in animal and human studies. Consumption of FS and FSO diets results in serum ALA concentrations with ranges upwards of 100 µM in athymic mice (Truan et al. 2010; Mason et al. 2014). Plasma levels of ALA in a multiethnic cohort of healthy young Canadians ranged from 12.0-186.9 µM (mean= 54.4 µM) (Abdelmagid et al. 2015). Daily consumption of approximately 6 g of ALA from FSO
for 12 weeks has been shown to increase serum ALA by approximately 154 µM (Austria et al. 2008).

The aim of this study was to explore potential mechanisms of ALA effect in reducing the growth of E2-stimulated ER+ MCF-7 cells. One of the study’s strengths is the use of a hypothesis-generating comprehensive breast cancer PCR array to screen a broad assortment of genes commonly involved in breast cancer processes. As shown in Appendix 1, a few of the 88 measured target genes were significantly affected by ALA treatment; however, those that were affected suggest that the growth effects may be a result of reduced ER-related signaling. Following up this hypothesis by measuring protein products of these genes and looking at results from published studies further supported these findings. We acknowledge some limitations in the current study. As mentioned previously, a limitation is the lack of measurement of the independent effect of E2 by including both – and + E2 controls and looking at the effect of ALA with and without E2. Only one ER+ cell line was used in this study and therefore it is of interest to explore the effects of ALA on other ER+ breast cancer cells. Furthermore, only one time point was used for each experiment while it has been shown that, particularly for gene expression, results may vary over time (Wiggins et al. 2015). Follow up studies employing reporter gene assays to study whether ALA transactivates genes containing an ERE are recommended to clarify the mechanism by which ALA modulates the expression of ER-related signaling biomarkers.

The novel finding that ALA alone reduces ER+ breast cancer cell growth an effect that may involve the disruption of ER-related signaling pathways improves our understanding of ALA in breast cancer and suggest that ALA has an effect independent of its long chain n-3 PUFA metabolites EPA and DHA. While there are a vast number of mechanisms which likely
contribute to the reduction in growth by ALA (Mason et al., 2013b), this exploratory study suggests a novel mechanism and new direction to pursue in subsequent research. Observational studies suggest that ALA may play a role in breast cancer prevention (Saadatian-Elahi et al. 2004) and ALA-rich FS has been shown to reduce biomarkers of tumor growth in postmenopausal breast cancer patients (Thompson et al. 2005). The results of the current study are significant as they offer one plausible biological mechanism for these beneficial effects of ALA. In conclusion, ALA reduces the growth of ER+ MCF-7 breast cancer cells in vitro and the disruption of ER-related signaling is suggested to contribute to this growth reduction.
CHAPTER 8

Study 5: The role of α-linolenic acid in the prevention of trastuzumab resistance and growth of trastuzumab-resistant cells
8.1 Introduction

TRAS, a first-line therapy for HER2+ breast cancer, was a major advancement in the treatment of this aggressive breast cancer subtype (Hudis 2007). TRAS, alone and combined with chemotherapy, has been shown to reduce tumor growth; however, the response rate is low and responders develop resistance within one year. Alternative and complementary approaches are being explored to overcome these barriers (Nahta and Esteva 2006; Murphy and Modi 2009; Pohlmann et al. 2009; Hurvitz et al. 2012).

Research is being done to better understand the molecular mechanisms driving TRAS resistance. A number of hypothesized mechanisms of action have been suggested including the reactivation of pathways downstream of HER2 either through other growth factor receptors or due to loss of PTEN (Pohlmann et al. 2009). While shown to have a greater impact in resistance to the anti-HER2 therapy lapatinib, increased ER signaling may also contribute to TRAS resistance (Xia et al. 2006; Wang et al. 2011). Cocktails of drugs that differentially target these pathways have been proposed to circumvent TRAS resistance.

Dietary FS and FSO, rich in the n-3 PUFA ALA, have been shown to reduce the expression of growth factor receptors including EGFR (Chen et al. 2002; Chen et al. 2007a; Truan et al. 2010). As shown in study 2 (Chapter 5) FSO increases tumor levels of PTEN in athymic mice with BT-474 xenografts. (Mason et al. 2015). Furthermore, the long chain n-3 PUFA-rich fish oil has been shown to increase PTEN expression (Ghosh-Choudhury et al. 2009). Results from study 4 suggest that ALA may reduce MCF-7 cell growth in vitro by interfering with ER-related signaling. Taken together ALA may be a beneficial complementary treatment to circumvent TRAS resistance.
Therefore, the current study used *in vitro* methods to test the hypotheses that treatment with ALA will prevent the development of TRAS resistance, reduce the growth of TRAS resistant cells and sensitize resistant cells to TRAS treatment. Previous *in vitro* studies have shown that exposing BT-474 cells to TRAS over a prolonged period results in TRAS resistance; however, the period of exposure used in those studies varied from 2 weeks to 6 months (Kute et al. 2009; Huang et al. 2010; Wang et al. 2011). The results of this study after 5 months of TRAS exposure suggest that ALA does not prevent the development of TRAS resistance but reduces the growth of TRAS resistant cells. These important findings suggest that the n-3 PUFA ALA, which has been previously shown to reduce the growth of TRAS-sensitive BT-474 cells, may be a beneficial complementary agent at any point in the treatment of HER2+ breast cancer.

### 8.2 Materials and Methods

#### 8.2.1 Cell Lines

Two HER2-overexpressing human breast cancer cell lines, BT-474 (ER+, PGR+) and TRAS-resistant UACC-732 (ER-, PGR+), were obtained from ATCC. Both cell lines were maintained in RPMI medium supplemented with 10% FBS and 1% antibiotic-antimycotic solution as described in Section 4.2.3.

#### 8.2.2 Fatty Acid and TRAS Treatment

ALA and TRAS were obtained and prepared for use in cell culture as described in Section 6.2.2.

#### 8.2.3 Development of BT-474-TR and BT-474-ALA+TR Cell Lines

BT-474 cells were cultured under three experimental conditions for a period of 5 months to derive 3 sub-cell lines. BT-474 Parental cells were maintained in standard RPMI maintenance medium. BT-474-TR cells were cultured in RPMI maintenance medium with 10 µg/ml TRAS.
BT-474-ALA+TR cells were cultured the same as BT-474-TR cells with 25 µM ALA added to the medium. Cells were passaged into new culture flasks when they reached 80% confluence. Passages were conducted weekly for the BT-474 Parental cells. Throughout the 5 month treatment period, the growth rates for the TRAS treated cell lines varied, thus the intervals between passages varied. Throughout the experimental treatment period, cells were monitored for phenotypic changes and signs of contamination. Cells were tested by STR analysis and all sub-lines were shown to match known database profiles for BT-474 cells.

8.2.4 TRAS Sensitivity

At monthly intervals, starting at month 2, cells were tested for sensitivity to TRAS using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI, USA). Cells were plated into 96 well plates (6000 cells/well) and allowed to adhere for 72 hours in each cell line’s respective maintenance medium. The medium was then replaced with phenol red-free RPMI medium containing 5% CS-FBS, 1% antibiotics, 1 nM E2 and TRAS (0, 0.1, 1, 10, 50 or 100 µg/ml) in a volume of 100 µl/well. The treatment medium was refreshed after 48 hours. After a total treatment period of 96 hours, 20 µl of the MTS reagent was pipetted into each well using a multichannel pipette. Plates were incubated for 2 hours at 37°C in a humidified, 5% CO₂ atmosphere and absorbance was read at 490 nm using the iMark™ Microplate Absorbance Reader (Bio-Rad). Treatments were run in quadruplicate wells and the experiments were repeated 3 times.

8.2.5 Effect of ALA on the Growth of TRAS-resistant Cells

UACC-732, BT-474-TR and BT-474-ALA+TR cells were plated into 24 well plates at a density of 3.6 x 10⁴ cells/well in their respective maintenance media. After 72 hours, existing media were removed and replaced with treatment solutions (3 wells per treatment). Treatment
solutions were prepared in phenol red-free RPMI medium with 5% CS-FBS, 1% antibiotics, 1 nM E2, TRAS (0 or 10 µg/ml) and/or ALA (0 or 100 µM). Treatment solutions were refreshed after 48 hours and after a total of 96 hours, cell growth was measured by trypan blue exclusion assay as described in Section 4.3.12.

8.2.6 Statistical Analysis

Statistical analysis was conducted using SigmaPlot version 12.0. Two way ANOVA was used to determine main effects of TRAS and cell line or TRAS and ALA as well as their interaction on TRAS sensitivity and cell growth, respectively. Tukey’s multiple comparisons testing was used when significant interactions (P<0.1) were observed. Significance was determined at P<0.05.

8.3 Results

8.3.1 Effect of Prolonged Culture of TRAS Alone and Combined with ALA on TRAS Sensitivity

After 2 months of culture in the presence of TRAS, TRAS sensitivity analysis showed significant main effects of both TRAS (P< 0.001) and cell line (P< 0.001) and a significant TRAS x cell line interaction (P< 0.001) (Figure 8-1A). Similar patterns of results were seen at 3, 4 and 5 months (data not shown). Compared to control (0 µg/ml TRAS), BT-474 Parental cells showed reduced growth starting at 0.1 µg/ml (P<0.01), BT-474 TR cells showed no significant response to TRAS and BT-474 ALA+TR cells saw a significant reduction with 10 µg/ml TRAS (P=0.04) but no significant effect at any other doses. BT-474-Parental cells showed a significantly greater response to TRAS at all doses tested compared to BT-474-TR and BT-474-ALA+TR cells which did not differ in their response to TRAS. TRAS treatment (10 µg/ml) caused a 17% reduction in growth in the BT-474-TR cells at 2 months. Experimental treatments
Figure 8-1. Growth response of BT-474 Parental, BT-474-TR and BT-474-ALA+TR cells to (A) increasing doses of TRAS after 2 months of treatment and (B) 10 µg/ml TRAS from 2-5 months.
were continued up to 5 months. There was no difference in the response of the different cell lines to 10 µg/ml TRAS over time (Figure 8-1B).

8.3.2 Effect of ALA on the Growth of TRAS Resistant Breast Cancer Cells

The effects of 100 µM ALA, 10 µg/ml TRAS and their combination in the presence of 1 nM E2 on the growth of TRAS-resistant breast cancer cells (BT-474-TR, BT-474-ALA+TR and UACC-732) are shown in Figure 8-2. There were no TRAS main effects or ALA×TRAS interactions in any of the cell lines. ALA reduced the growth of all TRAS resistant cell lines (P<0.05).

8.4 Discussion

This study has shown that ALA has limited effect in preventing the development of TRAS resistance and in increasing the sensitivity of TRAS resistant cells to TRAS; however, ALA can reduce the growth of TRAS-resistant breast cancer cells. These important findings suggest that the n-3 PUFA ALA, which was shown to reduce the growth of TRAS-sensitive BT-474 cells in study 3 (Chapter 6), may be a beneficial complementary agent if added at any point in the treatment of HER2+ breast cancer.

The response of BT-474 cells to TRAS was reduced after exposure to TRAS for 2 months and this resistance did not change over an additional 3 months. BT-474 cells cultured with TRAS alone or combined with 25 µM ALA for 2 months did not differ in their response to TRAS over the doses tested (0-100 µg/ml). An exception is at 10 µg/ml TRAS where compared to their respective untreated controls, BT-474-ALA+TR showed a small but significant reduction in growth at 2 months measured while BT-474-TR cells showed no response.
**Figure 8-2.** Effects of 100 µM ALA, 10 µg/ml TRAS and their combination on the growth of BT-474-TR, BT-474-ALA+TR and UACC-732 cells.
Previously measured biomarkers expressions in BT-474 Parental and TRAS-resistant derivatives suggest that reactivation of pathways downstream of HER2 including Akt and MAPK contribute to resistance development (Kute et al. 2009; Wang et al. 2011). However, since no significant differences were observed between the BT-474-TR and BT-474-ALA+TR cells, no further biomarkers analyses were conducted in this study. Taken together, these findings suggest no significant benefit of combining a low dose of ALA with TRAS treatment in the prevention of TRAS resistance.

Treatment with 100 µM ALA reduced the growth of all TRAS-resistant cell lines tested including, UACC-732, BT-474-TR and BT-474-ALA+TR cells. Furthermore, 100 µM ALA has been shown to reduce the growth of BT-474 Parental cells (Study 3). These findings suggest that the introduction of a higher level of ALA (100 µM) is beneficial both at the outset of TRAS treatment or after long-term TRAS treatment when cells have become resistant. If these preclinical findings are confirmed in a clinical setting, it would have important implications as it supports the intervention with ALA at any time point in the course of TRAS treatment.

The mechanism of effect of ALA in reducing the growth of TRAS resistant cells is not yet known but may in part be related to modulation of ER-related signaling. The relationship between resistance to anti-HER2 therapies, including TRAS and lapatinib, and ER pathways has been previously explored in BT-474 cells (Wang et al. 2011). The upregulation of ER pathways was suggested to strongly contribute to lapatinib resistance in BT-474 cells but played a less significant role in the development of TRAS resistance. Similar growth reductions were caused by the antiestrogen fulvestrant (ICI) in BT-474 parental and TRAS-resistant BT-474 derivative. This suggests that TRAS-resistant BT-474 cells remain sensitive to ER inhibition. ALA has been shown to have antiestrogenic effects in ER+ MCF-7 cells in vitro (Study 4) which may explain
the reduction in cell growth seen with ALA treatment in BT-474 parental cells (Study 3), BT-474-TR and BT-474-ALA+TR cells in the current study. That UACC-732 cells which are ER- were also reduced by ALA suggests that other potential mechanisms such as lipid peroxidation (Gonzalez et al. 1991; Cognault et al. 2000) may play a role in this cell line. BT-474 and UACC-732 cell lines are both PGR+. It is possible that ALA effect of reducing PGR as seen in study 4 (Chapter 7) may contribute to growth reduction.

Different concentrations of ALA were used in experiments 1 and 2. The use of a lower concentration of ALA (25 µM) in experiment 1 was necessary as preliminary work demonstrated that culture with higher concentrations of ALA (50-100 µM) combined with TRAS resulted in a complete loss of cells after 2 weeks of culture. However, 25 µM ALA may have been too low to exert any biological effect. The effects of ALA-rich FS and FSO have been shown in studies with serum ALA levels greater than 60 µM and the effects of ALA in reducing ER-related signaling biomarkers (PGR, cyclin D1, survivin) seen in MCF-7 cells was with 100 µM ALA treatment. Thus, intervention with higher dose of ALA may be required to see any benefit. Importantly, a clinical study has shown increases of more than 150 µM ALA following consumption of approximately 6 g of ALA from FSO for 12 weeks demonstrating that the level used in this study is achievable through diet.

The current study tested two hypotheses. The hypothesis that ALA can prevent the development of TRAS resistance in BT-474 exposed to TRAS is rejected based on the data presented. In contrast, the hypothesis that ALA can reduce the growth of TRAS-resistant cells is confirmed. The findings that ALA, alone and combined with TRAS, can reduce the growth of two genetically different TRAS-resistant cells combined with previous work (Study 3) showing
the same effect in TRAS-sensitive BT-474 cells supports the use of ALA as an anticancer agent at any stage in the TRAS treatment period.
CHAPTER 9
General Discussion and Conclusions
The above experimental chapters are presented based on the published (Chapters 4-5), submitted (Chapters 6-7) manuscripts from which they were adapted. More detailed discussion of each experiment is, therefore, found within each chapter. The following general discussion will integrate the findings in the context of my overall thesis hypotheses and objectives.

9.1 Overall Findings

A major aim of my Doctoral thesis was to build on the findings from my Master’s that showed that diet with high level of FSO (8%) enhanced the effect of TRAS (2.5 mg/kg) in reducing the growth of ER+, HER2-overexpressing BT-474 tumors in the athymic mouse model. Several questions stemmed from my Master’s work and have been addressed in my doctoral thesis as discussed below.

Question 1: Does dietary FS show similar results to dietary FSO?

Synthesizing the findings from the two in vivo studies presented in this thesis and from my Master’s thesis (Mason et al. 2010) suggest that the form of consumption of ALA-rich diets may play an important role in whether an enhancement of TRAS effect is observed. In study 1, dietary FS did not interfere with TRAS action; however, a beneficial effect of FS was only seen after 2 weeks of treatment and no FS effect, alone or combined with TRAS, was observed for final tumor growth, cell proliferation or apoptosis after 5 weeks. On the other hand, in study 2, dietary FSO at a level similar to that found in the FS diet used in study 1, significantly enhanced the effect of TRAS in reducing final tumor growth and cell proliferation and in increasing apoptosis. A necessary consideration when synthesizing the results of these studies is the unexpected difference in body weight between the BD- and FS-fed groups in study 1 which was likely explained by differences in food intake between groups. This was not seen in study 2 or in my Master’s thesis project. Because caloric restriction has been shown to influence HER2+
breast tumorigenesis in mice (Mizuno et al. 2013), it is possible that the tumors in mice fed the BD were growing at the slower rate than if they had been consuming similar amount as their FS-fed counterparts and this effect is not fully accounted for by statistically controlling for body weight. It is also possible that other components in FS may interfere with the TRAS-reducing effect of FSO; however, the in vitro study presented in study 1, looking specifically at the effects of ALA and the lignans ED and EL, suggests that FS lignans do not interfere with the growth reducing effect of ALA. Perhaps components of FS other than lignans may interfere. A study directly comparing the effects of FS and FSO combined with TRAS in parallel would be required to specifically address the question of whether the food matrix in which the high ALA diet is provided alters the interaction with TRAS. The aim of my thesis was to build upon the findings from my Master’s and further characterize the interaction of FSO with TRAS. As such, subsequent experiments focused on determining the component responsible for the beneficial interaction between FSO and TRAS rather than further investigating the component(s) in FS responsible for interfering with the beneficial interaction between FSO and TRAS.

**Question 2: Does the lower level of FSO (4%) typically used in studies in the Thompson lab show a similar TRAS-enhancing effect to the 8% diet used in my MSc work?**

Yes, in agreement with the results seen with an 8% FSO diet, study 2 showed that combining a 4% FSO diet with 2.5 mg/kg TRAS treatment resulted in significantly lower tumor size and tumor cell proliferation and higher apoptosis compared to TRAS alone.

**Question 3: Is a beneficial interaction observed with an even lower dose of TRAS (1 mg/kg) and dietary FSO?**
Combining TRAS treatment at a 1 mg/kg dose with dietary FSO showed no additional benefit compared to the 1 mg/kg dose alone. There was no dose-response effect seen in the groups where TRAS treatment was given alone. The extent of tumor growth was similar between the mice fed the BD treated with 1 and 2.5 mg/kg TRAS and mice fed the FSO diet and treated with 1 mg/kg TRAS.

**Question 4:** Does the modulation of HER2 and biomarkers of its signaling pathways (Akt and MAPK) contribute to the effect of FSO as shown in models of breast cancer with low HER2?

Findings from study 2 suggest that a modulation of HER2 activation contributes to the FSO effect since we observed significantly lower pHER2 expression when TRAS2.5 treatment is combined with FSO. This enhanced reduction in pHER2 may have led to lower activation of downstream signaling pathways as we observed approximately 50% lower levels of pAkt1/Akt1 and pMAPK/MAPK expression in the FSO+TRAS2.5 group compared to TRAS2.5. The effect of FSO on Akt activation is likely attributed to the increase in PTEN which was seen for both mRNA and protein. These findings are supported by previous work in MCF-7 xenografts (Saggar et al. 2010a; Truan et al. 2010). Interestingly, despite the lack of effect on tumor growth, cell proliferation or apoptosis, there was a main effect of FS in reducing pAkt expression seen in study 1. Together these findings suggest that the in vivo effect of FSO involves the modulation of HER2 signaling as indicated by reductions in activation of HER2 and biomarkers of two HER2 signaling pathways Akt and MAPK.

**Question 5:** Is ALA the component responsible for FSO effect observed in vivo?
As shown in studies 1 and 2 tumor levels of ALA and long chain n-3 PUFAs EPA and DHA are significantly higher in mice fed 4% FSO or 10% FS diets compared to CO-based BD. Truan et al. (2010) showed this same elevation of ALA, EPA and DHA in the serum of athymic mice fed a 4% FSO diet and in a side project during my doctoral studies I showed this to also be true in the serum of mice fed a 10% FS diet (Mason et al. 2014). Study 3 showed that BT-474 cells do not convert ALA to DHA \textit{in vitro} suggesting that the elevation in tumor DHA \textit{in vivo} is due to hepatic conversion. Using \textit{in vitro} methods allowed for the isolation of ALA effect. ALA with and without TRAS reduced BT-474 cell growth suggesting that ALA contributes to the growth reducing effect of FSO. However, protein biomarker analysis showed that paradoxically ALA treatment increased the activation of Akt and MAPK. Evidently, ALA was not the component responsible for the effect of ALA on these biomarkers of HER2 signaling. On the other hand, treatment with concentrations of DHA derived from the \textit{in vivo} studies, decreased cell growth with and without TRAS and also reduced Akt and MAPK activation. These results strongly suggest that \textit{in vivo} the n-3 PUFA ALA metabolite contributes to the effect of FSO on biomarkers of HER2 signaling.

**Question 6: Can ALA prevent the development of TRAS resistance and affect the growth of TRAS resistant cells?**

Study 6 showed that prolonged culture of BT-474 cells with TRAS alone or combined with ALA has similar effect on TRAS sensitivity suggesting that ALA does not prevent the development of TRAS resistance. Treating TRAS-resistant BT-474-TR, BT-474-ALA+TR and UACC-732 cells with ALA alone and combined with TRAS significantly reduced cell growth. TRAS showed no effect on the growth of any of these cell lines alone and combined with ALA.
This suggests that ALA can reduce the growth of TRAS-resistant cells but does not sensitize these cells to TRAS.

Answering the questions that stemmed from my Master’s thesis, led to a new question: despite an increase in activation of Akt and MAPK, how and why does ALA treatment \textit{in vitro} reduce breast cancer cell growth? This was addressed in Study 4 using the MCF-7 cell line which shares some characteristics with BT-474 cells in that they are both ER+ and PGR+; however, MCF-7 cells have low HER2 expression. Switching to this cell line was thought to allow for more clear demonstration of non-HER2 related effect of ALA. Similar to BT-474 cells, MCF-7 cells were shown to lack the ability to convert ALA to downstream metabolites as shown by others (Grammatikos et al. 1994). In agreement with previous studies, ALA was shown to reduce the growth of MCF-7 cells (Kim et al. 2009; Tran et al. 2010; Truan et al. 2010; Vanden Heuvel et al. 2012; Wiggins et al. 2015). As was the case in BT-474 cells, this reduction in growth was not due to an induction of apoptosis. Results of a breast cancer-focused qRT-PCR array combined with protein biomarker analysis suggest that disruption of ER-related signaling may contribute to the mechanism of ALA effect. ALA reduced the expression of E2-sensitive genes and/or gene products including caveolin-1, PGR and cyclin D1. Furthermore, combining treatment with ALA and the ER downregulator ICI did not reduce cell growth compared to ICI alone indicating that intact ER expression may be required for ALA effect. Potential mechanisms by which ALA modulates the ER include (i) reducing intracellular E2 levels through a reduction in ABCG (Hardwick et al. 2007), (ii) reducing caveolin-1 expression, a protein which has been shown to activate ERα (Schlegel et al. 1999; Schlegel et al. 2001), and/or (iii) directly binding the ER as has been shown by Tran et al. (2010).

Figure 9-1 depicts a proposed mechanism for the observed effects of FSO derived from
Figure 9-1. Novel hypothesized mechanism for *in vivo* effect of dietary FSO in breast cancer. ALA= α-linolenic acid; DHA= docosahexaenoic acid; E2= 17β-estradiol; ER= estrogen receptor; HER2= human epidermal growth factor receptor 2; MAPK= mitogen-activated protein kinase; PGR= progesterone receptor. Red arrows represent effects for which DHA is primarily responsible. Yellow arrows represent effects for which ALA is primarily responsible.
the studies presented in this thesis, supported by published literature. The findings from a side project conducted during my Doctoral work suggest that mice metabolize dietary ALA to EPA and DHA (Mason et al. 2014). This results in elevated tumor levels of ALA, EPA and DHA. The extent of increase in serum DHA is sufficient to reduce activation of the MAPK and Akt signaling pathways contributing to a reduction in tumor/cell growth. Several studies suggest that DHA induces apoptosis and/or affects the expression of markers involved in apoptosis pathways in breast cancer cells (Schley et al. 2005; Blanckaert et al. 2010; Kang et al. 2010; Corsetto et al. 2011; Xiong et al. 2012; Lee et al. 2014). Thus, DHA likely contributes to the induction in apoptosis seen with FSO+TRAS treatment in vivo. ALA specifically reduces breast cancer cell growth (BT-474, MCF-7, UACC-732) and results from Study 4 and previous studies (Tran et al. 2010) suggest that disruption of ER-related signaling may also contribute to this growth reduction.

9.2 Strengths and Limitations

An important strength of the research presented in this thesis is that TRAS treatment serves as a positive control throughout the majority of the experiments. I have consistently seen the expected results with TRAS treatment (reductions in tumor and cell growth, induction of apoptosis, reductions in HER2 signaling biomarkers) (Fendly et al. 1990; Lewis et al. 1993; Sliwkowski et al. 1999; Baselga et al. 2001; Hudis 2007). The only inconsistency was in study 1 where no effect of TRAS was observed on cell proliferation, an effect that was seen in study 2 and is consistently shown in the literature. We suggest that this was related to the fact that the measurements were done only on tumors at the end of the study when the rate of change in tumor size in the control tumors was already low suggesting that the tumors were no longer growing. The assumption is that if cell proliferation was also measured at an earlier time point (i.e. week
4), TRAS would have caused a reduction. An additional strength of the work presented is that the effect of dietary FSO in enhancing TRAS (2.5 mg/kg) effect seen in my Master’s thesis was replicated in study 2. This provides greater confidence that it is a true effect.

The use of a comprehensive, breast cancer-focused qRT-PCR array allowed for examination of ALA effects on a broad spectrum of potential pathways. The use of validated assays for RNA extraction, cDNA synthesis and qRT-PCR ensured that all quality control requirements were met. The findings of this array generated a novel hypothesis for how ALA acts. Analysis of mRNA biomarkers in BT-474 cells following ALA treatment would have provided a greater understanding of whether the SERM-like effects seen in MCF-7 cells explain the reduction in growth in BT-474 cells. The RT-PCR array was used, however, due to equipment issues the data was not interpretable as none of the known effects of TRAS, which serves as a positive control in this study, were seen. Previous work from our research group has shown that treatment of BT-474 cells with 75 µM ALA for 24 hours results in non-significant downregulations of ER-related genes including PGR (Fold regulation= -1.8), ABCG1 (Fold regulation= -1.8), CAV1 (Fold regulation= -1.2) and BIRC5 (Fold regulation= -1.3) (Wiggins et al. 2015). It is possible that with the higher level of ALA used in Study 3 greater and significant reductions would be observed.

The studies presented in this thesis use animal and cell culture models. In the words of Dr. Howard Skipper, a pioneer in using animal models in cancer research, “A model is a lie that helps you see the truth.” (Mukherjee 2010). Cell and animal models have been invaluable in strengthening the understanding of cancer biology, screening potential anticancer agents and investigating their safety and mechanisms of action. How accurately these models mimic the complex physiology of humans and translate from one model to another is contentious and
imperfect. As described in Sections 2.1.7.1., 4.4 and 5.4, athymic mice are immunodeficient and thus do not accurately reflect human physiology as they lack B and T cells. Nonetheless, several lines of research suggest that the findings from OVX athymic mice are valuable and have been translated to a clinical setting. Interventions with FS diets at comparable levels were shown to reduce proliferation and increase apoptosis in both athymic mice under low circulating levels of E2 (Chen et al. 2007a; Chen et al. 2007b; Chen et al. 2009b) and in postmenopausal breast cancer patients (Thompson et al. 2005). Furthermore, athymic mice were used in the development of TRAS (Fendly et al. 1990; Baselga et al. 1998; Sliwkowski et al. 1999). The use of cell culture models was an asset for the isolation of specific effects of ALA and allowed for the exploration of an array of potential mechanisms. A common pitfall of in vitro studies is the use of non-physiological levels of experimental agents. As discussed within the experimental chapters, the doses of ALA, DHA and TRAS used were derived from or reflect levels seen in vivo in animal, observational or clinical studies. However, discrepancies were observed between the in vivo and in vitro studies as ALA-rich FS and FSO alone showed no significant effect on the growth of tumor. The beneficial effect of FSO was only observed when combined with TRAS (2.5 mg/kg), whereas in vitro ALA reduced cell growth alone and when combined with TRAS. Beyond the differences in fatty acid metabolism between these two systems, the mouse model is a much more complex system than cell culture as it is exposed to many additional factors such as hormones, growth factors and other nutrients. Even so, these two model systems contributed to the understanding of FS and FSO effect in HER2+ breast cancer.

We limited the scope of the research to focus on specific questions; however, the findings left a large number of questions for future directions. These are described in greater detail within chapter-specific discussions. An example is that in study 3 we only looked at only one ALA metabolite, DHA, while others including EPA may also exert biological effects that contribute to
the effects of FSO in vivo. In addition, only select biomarkers of the signaling pathways discussed in this study were measured and a greater understanding could be achieved by analyzing the full signaling pathways. Furthermore, tumor fatty acid analyses were limited to total lipids and cellular fatty acid analyses were limited to total phospholipids. Greater insight could be gained by looking at specific fatty acid and phospholipid classes.

Finally, though novel, the results of Study 5 are very preliminary. The study achieves the objectives for which it was designed to address in that it shows that ALA does not prevent the development of TRAS resistance but does reduce the growth of TRAS-resistant cells. It would be of interest to explore in greater detail the mechanisms of ALA in TRAS-resistant cells.

9.3 Future Research

Studies to address the limitations outlined above are required to more clearly understand the benefit of ALA-rich FSO in breast cancer and specifically in combination with TRAS. Before any specific recommendations can be made, studies in humans are needed to confirm that the results from the athymic mouse model apply to humans. Given the low conversion of ALA to DHA seen in humans (Brenna et al. 2009), the finding that DHA and not ALA reduces the activation of biomarkers of HER2-signaling pathways combined with the growth reducing effects of both ALA and DHA in HER2-overexpressing BT-474 cells, it would be of interest to compare the effects of ALA and DHA-rich diets combined with TRAS. The use of a prospective cohort design would provide a convenient method to explore these issues as estimated intakes of these fatty acids could be related to treatment outcomes in TRAS-treated patients. Finally, confirmation of these findings in a randomized controlled trial, the gold-standard of evidence, is required before recommendations can be made.
The findings that ALA impacts the expression of ER-related biomarkers and depends on intact expression of the ER are quite novel. The qRT-PCR array results provide some possible explanations for how this occurs including reduced activation by caveolin-1 and reduced transport of E2 into the intracellular environment. Tran et al. (2010) present another possible explanation with their finding that ALA binds both ERα and ERβ. Additional studies are recommended to further elucidate these findings. Furthermore, greater clarification is needed to understand why some but not all studies show a benefit of ALA in ER negative breast cancer cell lines.

Finally, the assumption was made that the findings from MCF-7 cells apply to BT-474 cells and that the SERM-like effect contributes to the growth reducing effect in both of these ER+ cell lines. This is the likely explanation attributed to the growth reduction seen in the TRAS-resistant cell lines tested. Future studies should confirm whether these assumptions are correct by measuring similar ER-related biomarkers in BT-474 cell line following ALA treatment.

9.4 Implications

The findings presented in this thesis have important implications for nutrition scientists in designing future clinical studies and understanding the mechanisms of ALA. Study 2 results provide additional support that ALA-rich FSO has biological effects that reduce tumor growth which should be followed up in clinical studies. Studies 1 and 2 results suggest that the form of consumption of ALA-rich diets may have an important influence which, if confirmed, has important implications for dietary recommendations. Study 3 results highlight that both ALA and its long-chain n-3 PUFA metabolite DHA reduce cancer growth but do so through distinct mechanisms. Study 4 results present a plausible biological mechanism of independent effect of
ALA in reducing the growth of ER+ breast cancer. Finally, study 5 results suggest that the beneficial effect of ALA during TRAS treatment of HER2-overexpressing breast cancer is not limited to TRAS-sensitive cells but may also apply to TRAS-resistant cells.

9.5 Conclusions

The following conclusions can be made from this research:

- 10% FS, alone and in combination with TRAS (2.5 mg/kg), does not affect the growth of breast tumors with amplified HER2 expression in the athymic mouse model.

- 4% FSO alone does not affect the growth of established breast tumors with amplified HER2 expression in athymic mice. Combining TRAS treatment at 2.5 mg/kg with dietary FSO enhances TRAS effect in reducing the growth of HER2-overexpressing breast tumors in athymic mice. Combining TRAS treatment at 1.0 mg/kg with dietary FSO does not enhance its effect.

- The in vivo effect of FSO but not FS involves a reduction in activation of pathways downstream of HER2 including the MAPK and Akt pathways.

- ALA and DHA likely both contribute to the growth-reducing effect of FSO; however, DHA, not ALA, contributes to the effect of FSO on the regulation of MAPK and Akt pathway activation.

- Other potential mechanism of ALA effect is that it exerts SERM-like effects and reduces the expression of selected biomarkers of ER-related signaling.

- ALA does not prevent the development of TRAS resistance.
• ALA reduces the growth of TRAS-resistant cells.

Therefore:
• The hypothesis that FSO (4%) enhances TRAS effectiveness at reducing the growth of HER2 overexpressing tumors (BT-474) through a combination of mechanisms including altered membrane fatty acid profile and reduced signaling through HER2 is accepted.
• The hypothesis that FS (10%) enhances TRAS effectiveness is rejected.
• The hypothesis that ALA will prevent the development of TRAS resistance is rejected.
• The hypothesis that ALA will enhance TRAS effectiveness in resistant cells is accepted.

Overall, these findings provide a better understanding of the effects and mechanisms of FS and its components, particularly ALA in breast cancer and supports further testing of ALA-rich FSO in clinical settings. This may ultimately lead to recommendations for the use of FSO as a cost-effective complementary treatment for women with breast cancer being treated with TRAS.
References


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Truan, J. S., Chen, J. M. and Thompson, L. U. 2010. Flaxseed oil is more effective than lignan in reducing the growth of estrogen sensitive human breast cancer (MCF-7) at high circulating estrogen level. 63rd Meeting of the Flax Institute of the United States, Fargo, ND, Flax Institute of the United States.


Appendices

**Appendix 1.** Relative gene expression ($\Delta C_T$) and fold regulation in MCF-7 cells treated with and without 100 $\mu$M ALA in the presence of 1 nM E2 for 24 hours. Target gene expression is expressed relative to 3 housekeeping genes (B2M, HPRT1 and RPLP0).

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