INTERACTIVE EFFECTS OF FLAXSEED OIL AND TRASTUZUMAB ON THE GROWTH OF BREAST TUMOURS OVEREXPRESSING HER2

by

Julie Mason

A thesis submitted in conformity with the requirements for the degree of Master of Science

Graduate Department of Nutritional Sciences
University of Toronto

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ABSTRACT

Flaxseed oil (FO), rich in α-linolenic acid, has been shown to inhibit breast cancer growth. One suggested mechanism is through modulation of HER2 expression and signalling. This study determined the effect of FO on the growth of established HER2-overexpressing breast tumours (BT-474) and its interaction with two doses of a primary anti-HER2 therapy, trastuzumab (TRAS), in athymic mice. FO alone had no effect on tumour size, cell proliferation and apoptosis. TRAS (2.5 and 5 mg/kg) reduced tumour size and cell proliferation but had no effect on apoptosis. TRAS (2.5 mg/kg) combined with FO reduced tumour size and cell proliferation and increased apoptosis compared to TRAS (2.5 mg/kg) alone and was just as effective as 5 mg/kg TRAS. TRAS (5 mg/kg) resulted in almost complete tumour regression with or without FO. In conclusion, FO has no effect on BT-474 tumour growth but can enhance the effectiveness of low dose TRAS.
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LIST OF ABBREVIATIONS

AA  arachidonic acid
ADCC antibody dependent cellular cytotoxicity
ALA alpha linolenic acid
ANOVA analysis of variance
BCIRG 006 breast cancer international research group trial 006
BD basal diet
DHA docosahexaenoic acid
DMBA 7,12-dimethylbenz[a]anthracene
E2 estrogen (17β-estradiol)
EGF epidermal growth factor
EGFR epidermal growth factor receptor
EPA eicosapentaenoic acid
ER estrogen receptor
ERK extracellular signal-regulated kinase
FAS fatty acid synthase
FO flaxseed oil
FS flaxseed
HER2 human epidermal growth factor receptor-2
HERA           herceptin adjuvant trial
IGF-IR         insulin-like growth factor-1 receptor
LA             linoleic acid
LVEF           left ventricular ejection fraction
MAPK           mitogen-activated protein kinase
MMTV           mouse mammary tumour virus
mTOR           mammalian target of rapamycin
NCCTG N9831    North Central Cancer Treatment Group N9831 trial
NF-κB          nuclear transcription factor -kappa B
NK             natural killer
NMU            N-methyl-N-nitrosurea
NSABP B31      National Surgical Adjuvant Breast and Bowel Project B-31
OVX            ovariectomized
PPAR           peroxisome proliferator-activated receptors
PI3K           phosphoinositide-3 kinase
PR             progesterone receptor
PRKDC          protein kinase, DNA activated catalytic polypeptide
PTEN           phosphatase and tensin homologue
PUFA           polyunsaturated fatty acid
SCID           severe combined immunodeficiency
<table>
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<th>Full Form</th>
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<tr>
<td>SDG</td>
<td>secoisolariciresinol diglucoside</td>
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<tr>
<td>SEM</td>
<td>standard error of mean</td>
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<tr>
<td>TAM</td>
<td>tamoxifen</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl-trasnferase-mediated nick end labeling</td>
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<td>TRAS</td>
<td>trastuzumab</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
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1.0 INTRODUCTION

Breast cancer is the type of cancer with the highest incidence and is the second leading cause of cancer-related death in North American women (Canadian Cancer Society 2009, Esteva and Hortobagyi, 2008; World Health Organization, 2008). The human epidermal growth factor receptor 2 (HER2/neu/erbB2) is overexpressed in 25-30% of breast cancer and its overexpression is associated with aggressive tumours, a high rate of metastasis and relapse, poor prognosis and limitations in treatment (Baselga et al., 2006; Ravdin and Chamness, 1995). HER2 overexpression is also common in tumours that have become resistant to endocrine therapy such as tamoxifen (TAM) (Dowsett, 2001). Trastuzumab (TRAS, Herceptin™), a recombinant humanized monoclonal antibody (rhumab4D5), is a first line therapy in HER2-overexpressing breast cancer (Hudis, 2007). It effectively regresses tumours in patients who respond, however, clinical studies have shown a response rate of only 12-26% when it is used as a single agent and responders develop resistance (i.e. tumours regrow) within one year (Baselga et al. 2005; 1996; Cobleigh et al. 1999; Vogel et al., 2002). Furthermore, 5% of patients suffer cardiotoxicity due to treatment (Baselga et al. 2005; 1996; Cobleigh et al. 1999; Vogel et al., 2002). Therefore, there is a need to enhance its effectiveness and alternative approaches are being sought.

Flaxseed (FS) is rich in phytoestrogens called lignans, namely secoisolariciresinol diglucoside (SDG), and contains up to 40% oil which is exceptionally rich in the n-3 polyunsaturated fatty acid (PUFA) alpha-linolenic acid (ALA) (Cunnane, 2003; Hall et al., 2006; Thompson et al., 2006). Animal models have shown that FS, SDG and flaxseed oil (FO) can reduce the growth of both estrogen receptor (ER) positive (Bergman Jungestrom et
al., 2007; Chen et al., 2007a; 2007b; 2004; Saggar et al., 2010b; Truan et al., 2010) and negative human breast tumours (Chen et al., 2002; Dabrosin et al., 2002; Wang et al., 2005b). Furthermore, 10% dietary FS has been shown to increase the tumour cell apoptosis and reduce cell proliferation and HER2 expression in ER+ MCF-7 tumours in athymic mice which have undergone prolonged treatment with TAM and may be progressing towards TAM resistance and regrowth (Chen et al., 2007a; 2007b). Importantly, in a randomized controlled trial, daily consumption of 25g of FS by post-menopausal patients was shown to reduce HER2 expression and to increase tumour cell apoptosis (Thompson et al., 2005).

The component most responsible for FS effect depends on the experimental conditions. In ER+ MCF-7 tumours, at high circulating levels of estrogen (E2), FO has a greater effect than SDG (Thompson et al., 2010) while at low circulating levels of E2, SDG has a greater effect than FO (Saggar et al., 2010b). However, at low circulating levels of E2 in combination with TAM treatment, FO is more effective than SDG at reducing MCF-7 tumour growth (Saggar et al., 2010a). Evidently the more effective component of FS depends on the treatment conditions; at high E2 level or in combination with TAM therapy, FO seems to be most effective.

Interestingly, some of the demonstrated actions of FS and ALA-rich FO are similar to those of TRAS. TRAS has been shown to downregulate the expression of HER2 in HER2-overexpressing breast cancer (BT-474) (Baselga et al., 2001). Reduction of HER2 expression has also been shown with ALA treatment of breast cancer cells (BT-474 and SkBr-3) in vitro (Menendez et al., 2006), with dietary FO in a nude mouse model with MCF-7 breast tumours (Truan et al., 2010) and with FS consumption by post-menopausal breast cancer patients (Thompson et al., 2005). TRAS treatment has also been noted to reduce
HER2 signaling through pathways including the mitogen-activated protein kinase (MAPK) and the phosphoinositide-3 kinase (PI3K) cascades, pathways which play a role in regulation of cell proliferation and apoptosis (Ripple et al., 2005); these effects have also been shown with FS and FO treatment in the nude mouse model (Chen et al., 2009; Saggar et al., 2010a; Truan et al., 2010). TRAS has been shown to reduce angiogenesis (Izumi et al., 2002), an effect also seen with dietary FS in nude mice (Bergman-Jungestrom et al., 2007) and long chain n-3 PUFAs (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) in vitro (Szymczak et al., 2008). Evidently there are common and complementary mechanisms that exist between FO and TRAS.

Hence, this study aims to determine the effect of FO, alone and in combination with TRAS, on the growth (i.e. tumour size, cell proliferation and apoptosis) of HER2-overexpressing breast tumours (BT-474). Since there are similar suggested mechanisms between TRAS and FO, it is hypothesized that FO will inhibit tumour growth and will enhance the anti-tumour effect of TRAS. If FO can increase the effectiveness of TRAS, it could lead to a simple, inexpensive, complementary treatment of breast cancer. About 80% of breast cancer patients take complementary/alternative medicine including dietary supplements to treat their breast cancer and improve health; FS is one of the most commonly used supplements (Boon et al., 2007; Greenlee et al., 2009; Hietala et al., 2010; Rausch et al., 2010). Therefore our research will also help understand any harmful or beneficial interactions of a supplement with a widely used breast cancer drug.
2.0 LITERATURE REVIEW

2.1 Breast Cancer and HER2

2.1.1. Statistics and Risk Factors

Breast cancer is the form of cancer with the highest incidence and is the second leading cause of cancer mortality in North American women (Canadian Cancer Society, 2009; Esteva and Hortobagyi, 2008; World Health Organization, 2008). While the incidence of breast cancer in Canadian women has shown a modest increase in the past twenty years, there has been a steady decrease in mortality that may be attributed to enhanced screening and improved adjuvant therapies (Canadian Cancer Society, 2009). The World Cancer Report shows that age-standardized incidence rates are higher in North American and European countries compared to Eastern countries (i.e. China, Korea, Japan). However, breast cancer incidence is on the rise in Eastern countries; for example, there has been a doubling or tripling in breast cancer incidence in Singapore, Japan and Korea and a greater than 20% increase in China in the past decade (World Health Organization, 2008). It is hypothesized that this may be due to the “westernization” of these countries which includes both positive (socioeconomic development) and negative (dietary changes, sedentary lifestyle) changes (Porter, 2008). This suggests that environmental factors play a role in breast cancer development.

While the exact cause of breast cancer development is not known, research suggests that there are several modifiable and non-modifiable risk factors. Modifiable risk factors include elevated body mass index, radiation exposure, diet and hormone replacement therapy
use and non-modifiable risk factors include early menstruation, late menopause and family history (World Health Organization, 2008).

2.1.2. Breast Cancer Development

The development of breast cancer is a multi-step process in which breast epithelial cells undergo a genetic mutation leading to uncontrolled proliferation. This complex process is distinguished into three stages: initiation, promotion and progression (Oliveira et al., 2007). The first stage, initiation, occurs when an epithelial cell is exposed to a carcinogen resulting in an irreversible change to its genetic make-up. Genetic mutations occur spontaneously in the cells of healthy individuals and many simply undergo apoptosis or remain in a dormant state and therefore not all initiated cells progress to the promotion stage of carcinogenesis (Pitot, 1993). The second stage, promotion, involves changes, driven by promoter agents, to the delicate balance of cell proliferation and apoptosis that occurs in all organisms. Mutated cells are driven to proliferate before DNA repair mechanisms are activated and thus mutations are passed to daughter cells. Unless apoptotic pathways are able to direct these damaged cells toward cell death, cells undergo the final stage of carcinogenesis: progression. These initiated and promoted cells undergo uncontrolled growth and develop into a tumour mass which will eventually lead to metastasis if left untreated.

2.1.3 Breast Cancer Subtypes

There are two main forms of malignant breast tumours based on their place of origin: the lobules or the ducts of the breast. Breast tumours are further classified based on their molecular profile and proliferation level into four main subtypes: luminal A, luminal B, HER2 (erbB2) positive and triple negative (Sorlie et al., 2003). Luminal A breast tumours
are ER+, progesterone (PR)+, HER2- and have a relatively low proliferation level. Luminal B breast tumours are ER+, PR+, HER2- and have a relatively high proliferation level. HER2+ breast tumours are either positive or negative for the hormone receptors but express high levels of HER2. Triple negative tumours or basal tumours do not express high levels of the hormone receptors or HER2. Prognosis and treatment differs based on tumour classification. Figure 2.1 shows the disease outcomes of these four subtypes. Evidently, the HER2+ and triple negative subtypes have the worst outcomes, with HER2+ subtype being the most metastatic (Figure 2.1 A) and having the second lowest overall survival (Figure 2.1 B).

### 2.1.4. HER2-Overexpressing Breast Cancer

HER2/neu is a proto-oncogene that is amplified in 25-30% of breast tumours leading to the overexpression of the HER2 protein receptor, a member of the epidermal growth factor receptor (EGFR) family. Many properties of HER2 expression in breast cancer have rendered the receptor an effective therapeutic target (reviewed in Nahta and Esteva 2006). Firstly, high expression levels correlate strongly with cancer development and poor prognosis. Secondly, in HER2+ patients, HER2 is present in a high proportion in tumour cells and tumours 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 tumour sites as well as in metastatic sites, which suggests that targeting HER2 may be effective in all disease sites.
Figure 2.1 Disease outcomes in two patient cohorts by Kaplan-Meier analysis stratified by subtypes. A) Time to development of distant metastasis b) Overall survival. Modified from Sorlie et al., 2003
Figure 2.2 illustrates the HER2 signaling pathways. HER2 has no known direct ligand and becomes activated upon homodimerization or when it heterodimerizes with another member of the growth factor receptor family (i.e. EGFR, HER3 or HER4). Once activated, through its tyrosine kinase activity, it initiates signaling pathways including the proliferation-inducing MAPK and anti-apoptotic PI3K/Akt kinase cascades. These pathways activate transcription factors that play a role in the regulation of cell proliferation, differentiation, apoptosis and metastasis (Kennedy et al. 1997; Santen et al 2002; Vivanco and Sawyers, 2002; Yarden and Sliwkowski, 2001). In HER2-overexpressing breast tumours, HER2 is constitutively activated leading to tumour cell survival and growth. In addition to the proliferative activity and anti-apoptotic activities of the MAPK and PI3K/Akt pathways, these pathways have been implicated in the expression of the biosynthetic enzyme fatty acid synthase (FAS) (Menendez and Lupu, 2007). Glucose metabolism is increased in cancer cells beyond energy requirements and there is therefore a shift from oxidative to glycolytic metabolism (Menendez and Lupu, 2007). Pyruvate, the end product of glycolysis, is then directed to fatty acid synthesis where FAS catalyzes the de novo synthesis of fatty acids from acetyl-CoA and malonyl-CoA. These endogenously-derived fatty acids are used for membrane production (Menendez and Lupu, 2007). FAS is overexpressed in many cancers including those of the breast (Alo et al., 1999; Kusakabe et al., 2002; Menedez and Lupu, 2007; Rashid et al., 1997; Swinnen et al., 2002). A link has been shown between the expression of HER2 and FAS. Pharmacological blockade of FAS in HER2-overexpressing cells was shown to reduce HER2 mRNA and protein levels (Menendez et al., 2004a).
Figure 2.2: Signaling pathways activated by HER2 and their downstream effects. Once activated by homo- or heterodimerization, HER2 can stimulate proliferation, reduce apoptosis and increase the expression and activity of FAS by initiating phosphorylation cascades that activate the PI3K/Akt and MAPK pathways. EGFR = epidermal growth factor receptor; ERK = extracellular signal-regulated kinase; FAS = fatty acid synthase; HER2 = human epidermal growth factor receptor 2; MAPK = mitogen-activated protein kinase; mTOR = mammalian target of rapamycin; P = phosphorylation; PI3K = phosphoinositide-3 kinase
HER2 is overexpressed in both ER+ and ER- breast cancer. In ER+ HER2+ breast cancer the ER-mediated signaling pathway is also activated. In the classical mechanism of E2 action, E2 diffuses through the tumour 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 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 proliferation-inducing genes such as epidermal growth factor (EGF), cyclin D1 and pS2 (Bjornstrom and Sjoberg, 2005).

In HER2-overexpressing breast cancer, signaling through the growth factor receptor-mediated signaling pathway occurs to such a great extent that treatment strategies target this pathway as opposed to the ER-mediated signaling pathway. Transfection of ER+ MCF-7 cells with HER2 results in E2 insensitivity in vitro and TAM resistance in vitro and in vivo (Pietras et al., 1995). Interestingly, when HER2-transfected MCF-7 cells were treated with the monoclonal antibody against HER2 (rhuMAb 4D5), sensitivity to TAM was restored further supporting the role of HER2 in E2 sensitivity. Cross-talk has been demonstrated between the ER-mediated and growth factor receptor-mediated pathways. Pietras et al. went on to show that treatment of OVX mice with MCF-7 tumours with heregulin, a ligand for the EGFR family, resulted in tumour growth even in the absence of E2. It is suggested that this E2 insensitivity may be due to reduced E2 binding capacity and expression. Evidently there is a link between HER2 and E2 sensitivity suggesting that cross-talk occurs between pathways.
2.1.5. Ovariectomized Athymic Mouse Model of HER2-Overexpressing Breast Cancer

To study the biology of breast cancer and evaluate treatment effects, several useful animal models have been developed. A summary of rodent models including their advantages and disadvantages are outlined in Table 2.1. 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 level, diet and environment. The particular model of interest for this study is the ovariectomized (OVX) athymic nude mouse model. This 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). This immunodeficiency allows for human cancer cells to be injected without rejection (Kavanaugh et al., 2002). This mouse model also lacks E2-producing ovaries which reduces endogenous E2 levels. An E2 pellet can then be implanted to regulate E2 levels, an important factor to control in hormone-sensitive disease.

To study HER2-overexpressing breast cancer, a number of cell lines have been derived from human breast tumours. The BT-474 and Sk-Br3 cell lines are the most frequently used model system of HER2 overexpression (Zimonjic et al., 2000). BT-474 cells are ER+ while Sk-Br3 cells are ER- and both have been shown to induce tumour growth in nude mice, however, SK-Br3 tumours are poorly differentiated. TRAS lowers colony formation in both BT-474 and Sk-Br3 cultures in vitro (Baselga et al., 1998). The BT-474 cell line was used in some of the in vivo pre-clinical studies that led to the approval of TRAS (Baselga et al., 1998) and both BT-474 and Sk-Br3 cells have been used in the study of ALA
<table>
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<th>Examples</th>
<th>Characteristics</th>
<th>Advantages</th>
<th>Disadvantages</th>
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| Carcinogen-induced        | DMBA, NMU         | Carcinogen injection results in tumour development within days | Allows for investigation of early stages of carcinogenesis and malignant progression                                                              | Carcinogen activation may be affected by co-administered treatments  
Mutated ras not representative of human situation  
Tumours are not of human origin                                                                 |
| Spontaneously-developed   | MMTV-neu transgenic rodent | Genetic alterations result in spontaneous tumour development | Allows for investigation of early stages of carcinogenesis and malignant progression  
Can study HER2 overexpression                                                                 | Tumours are not of human origin                                                                                                                   |
| Xenograft                 | Athymic rat, mouse | Lacks functional thymus gland  
Lacks T cells  
B cells and NK cells present | Allows for injection of human tissue without rejection  
Capable of ADCC  
Can study HER2 overexpression                                                                 | Cells already transformed thus cannot see early stages of carcinogenesis  
Lacks ADCC                                                                                                      |
| SCID mouse                | Mutated Prkdc     | Mutated Prkdc  
Lacks T cells, B cells and NK cells                   | Allows for injection of human tissue without rejection  
Can study HER2 overexpression                                                                                                                | Cells already transformed thus cannot see early stages of carcinogenesis  
Lacks ADCC                                                                                                    |

ADCC= antibody dependent cellular cytotoxicity; DMBA= dimethylbenz(a)anthracene; MMTV= mouse mammary tumour virus; NMU= N-nitrosomethyl-urea; NK= natural killer; Prkdc= protein kinase, DNA activated catalytic polypeptide; SCID= severe combined immunodeficiency
effect (Menendez et al., 2006). Therefore, the OVX athymic mouse with BT-474 tumours is an effective model for the investigation of drug and diet treatments on human breast tumour development.

2.2. Trastuzumab

2.2.1. 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. Its efficacy and safety as a monotherapy have been tested in a number of clinical trials described in Table 2.2 and it has been confirmed as an effective therapy for HER2-overexpressing breast cancer patients. Four pivotal phase III clinical trials have been conducted and are outlined in Table 2.3. The Breast Cancer International Research Group trial (BCIRG 006) showed a significant benefit of incorporating TRAS into common adjuvant chemotherapy regimens (Slamon et al., 2009). The Herceptin Adjuvant (HERA) trial showed a significant benefit of TRAS treatment following the standard treatment regimen in North Americans (surgery, chemotherapy and radiation (if indicated)) (Piccart-Gebhart et al., 2005; Smith et al., 2007). 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 TRAS treatment following surgical removal of HER2+ breast tumours (Romond et al., 2005). The development and approval of TRAS was
Table 2.2: Summary of clinical studies of trastuzumab effectiveness and safety as a monotherapy

<table>
<thead>
<tr>
<th>REFERENCE</th>
<th>TREATMENT</th>
<th>SUBJECTS</th>
<th>RESULTS</th>
</tr>
</thead>
</table>
| Baselga et al., 1996 | LD: 250 mg MD: 100 mg weekly | 46 metastatic breast cancer patients with 25% of cells positive for HER2 by IHC | • Overall response rate= 11.6%  
  • 37% of patients had minimal responses or stable disease lasting a median of 5.1 months  
  • Minimal toxicity observed |
| Cobleigh et al., 1999 | LD: 4mg/kg MD: 2mg/kg weekly | 222 metastatic breast cancer patients with 2+ or 3+ HER2 score* by IHC | • Objective response rate= 15%  
  • Median duration of response= 9.1 months  
  • Median survival= 13 months |
| Vogel et al, 2002  | LD: 4mg/kg MD: 2mg/kg weekly or LD: 8mg/kg MD: 4mg/kg | 114 previously untreated metastatic breast cancer patients with 2+ or 3+ HER2 score* by IHC | • Overall response rate= 26%  
  • No difference in response between treatment regimes |

IHC= Immunohistochemistry, LD= Loading dose, MD=Maintenance dose  
*HER2 score of 2+ or 3+ = weak to strong complete membrane staining observed in 10% of the tumour cells
Table 2.3: Summary of pivotal phase III clinical trials of trastuzumab

<table>
<thead>
<tr>
<th>TRIAL</th>
<th>REFERENCES</th>
<th>DESIGN</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCIRG 006</td>
<td>Slamon et al., 2009</td>
<td></td>
<td>At third interim follow-up (median=65 months):</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Surgery</td>
<td>• Greater DFS and overall survival in both TCH and AC→TH vs. Control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AC→T</td>
<td>• Fewer deaths from all causes in both TCH and AC→TH vs. Control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(control)</td>
<td>• No differences between the TCH and AC→TH groups</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=1073</td>
<td>• Significantly lower LVEF in AC→TH compared to both AC→T and TCH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCH</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=1075</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AC→TH</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=1074</td>
<td></td>
</tr>
<tr>
<td>HERA</td>
<td>Piccart-Gebhart et al., 2005;</td>
<td>Surgery,</td>
<td>At first interim report (median= 1 year) (Piccart-Gebhart et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Smith et al., 2007</td>
<td>chemotherapy,</td>
<td>• Greater DFS in 1 year TRAS vs. observation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>radiotherapy</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Observation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=1693</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 year TRAS</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>n=1693</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 years TRAS</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=1694</td>
<td></td>
</tr>
<tr>
<td>NSABP B31 and NCCTG</td>
<td>Romond et al., 2005</td>
<td></td>
<td>At second interim report (median= 2 years) (Smith et al., 2007)</td>
</tr>
<tr>
<td>N9831 (jointly</td>
<td></td>
<td>Surgery</td>
<td>• Greater DFS and overall survival in 1 year TRAS vs. observation</td>
</tr>
<tr>
<td>analyzed)</td>
<td></td>
<td>AC→T</td>
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<tr>
<td></td>
<td></td>
<td>(control)</td>
<td></td>
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<td></td>
<td></td>
<td>n=1679</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AC→TH</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=1672</td>
<td></td>
</tr>
</tbody>
</table>

AC→T= Doxorubicin and Cyclophosphamide followed by Taxotere; AC→TH= Doxorubicin and Cyclophosphamide followed by Taxotere plus Herceptin; BCIRG= Breast Cancer International Research Group; DFS= Disease free survival; HERA= Herceptin Adjuvant; LVEF= Left ventricular ejection fraction; NCCTG= North Central Cancer Treatment Group; NSABP= National Surgical Adjuvant Breast and Bowel Project; TCH= Taxotere + Carboplatin plus Herceptin; TRAS= Trastuzumab
a breakthrough in the treatment of HER2-overexpressing breast cancer, however, limitations include a low response rate (12-26% for TRAS monotherapy), the development of resistance (i.e. tumours regrow) within one year of treatment and cardiotoxicity (i.e. reduced left ventricular ejection fraction (LVEF)).

2.2.2. Proposed Mechanisms of Trastuzumab Action

The antitumour mechanisms of TRAS have yet to be fully elucidated; however, in vitro and in vivo models have revealed many molecular and cellular effects (Figure 2.3). (1) It has been shown to bind to the HER2 and thus reduce HER2 signaling through pathways including the MAPK and PI3K/Akt cascades. These pathways are known to play a role in tumour cell survival, activating several genes involved in increasing cell proliferation and decreasing apoptosis (Ripple et al 2005). The reduction of signaling through these pathways leads to the accumulation of p27kip1, a cyclin-dependent kinase inhibitor that promotes cell cycle arrest and apoptosis (Baselga et al 2001; Sliwkowski et al 1999). (2) It has been shown to inhibit 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) It may act via induction of an immune response via antibody-dependent cellular cytotoxicity (ADCC) (Lewis et al., 1993). (4) Studies have shown that TRAS may lead to HER2 internalization and degradation, resulting in downregulation of the receptor (Baselga et al., 2001; Sliwkowski et al., 1999).
Figure 2.3 Proposed mechanisms of trastuzumab (TRAS) action. ADCC = antibody dependent cellular cytotoxicity; VEGF = vascular endothelial growth factor; VEGFR = vascular endothelial growth factor receptor; for remaining abbreviations see figure 2.2
2.3. Diet and HER2

Dietary compounds, including FS and its components, have been shown to modulate many important cancer biomarkers and there is an increasing body of literature to support that this holds true for HER2. Examples of compounds that have been shown to reduce HER2 expression and signaling and HER2-overexpressing tumor growth include: curcumin (Hong et al., 1999), FS and FO (Saggar et al., 2010a; Thompson et al., 2005; Truan et al., 2010), isothiocyanates (Warin et al., 2009) and n-3 PUFAs (Menendez et al., 2006). Other foods such as soy and its components showed conflicting results. High intake of dietary soy during early life resulted in lower HER2 expression in malignant tissue sample from breast cancer patients (Maskarinec et al., 2009). On the other hand, dietary soy increased the HER2 mRNA expression in N-methyl-N-nitrosurea (NMU)-induced tumours, however, there was an overall reduction in tumour incidence and enhancement in tumour latency in soy-fed rats (Simmen et al., 2005). Furthermore, the major component of soy, genistein, promoted the growth of MCF-7/erbB-2 transfected cells in vitro (Yang et al., 2010). Interestingly, some compounds have different effects depending on tumour cell type. The trans10,cis12-conjugated linolenic acid isomer inhibits cell proliferation and increases apoptosis of MCF-7 human breast cancer cells (Chujo et al., 2003; Majumder et al., 2002; Miller et al., 2003), however stimulates the growth of established HER2-overexpressing breast tumours in transgenic mice (Ip et al., 2007; Meng et al., 2008). Diet may also increase HER2-overexpressing tumour growth as a high fat diet has been implicated in tumour promotion in mouse mammary tumour virus (MMTV)-HER2/neu transgenic mice (Khalid et al., 2010). Evidently, HER2-overexpressing breast cancer is influenced by dietary components and further research should determine mechanisms behind these effects.
2.4. Flaxseed

2.4.1. Flaxseed and its Components

FS is a dietary supplement or ingredient that has been increasingly used by consumers for its positive health benefits. Its incorporation into commonly consumed food products such as breads and spaghetti tripled from 2003 to 2006 (Datamonitor's Product Scan Online; Fitzpatrick et al., 2007). A recent study indicates that approximately 80% of breast cancer patients make diet and lifestyle modifications upon diagnosis to include some form of complementary or alternative medicine including dietary supplements (Boon et al., 2007). In a number of studies, FS has been shown to be one of the highest consumed supplements among breast cancer patients (Boon et al., 2007; Greenlee et al., 2009; Hietala et al., 2010; Rausch et al., 2010)

Of interest and the main reason for its use as a 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 phytoestrogen called lignan, in addition to its high quality protein. While the exact composition of FS varies by cultivar, growth location and environment, it is comprised of approximately 30% fiber, 20% protein, 40% oil and contains approximately 820 μmol lignan per 100g of FS (Daun et al., 2003; Thompson et al., 2006). The predominant lignan in FS is SDG, which can be metabolized by bacteria in the colon to the enterolignans enterodiol and enterolactone (Thompson et al., 2006; 1991). These phytoestrogens have a structural similarity to E2, thus their effect in hormone-related diseases, such as breast cancer, is of interest (Adlercreutz, 2007). Approximately 57% of FO consists of ALA which can be metabolized to a limited extent to the longer chain PUFAs,
EPA and DHA, through a series of elongation and desaturation steps (Figure 2.4) (Cunnane, 2003; Hall et al., 2006). Recent reviews on ALA conversion to longer chain n-3 PUFA consistently showed an increase in plasma EPA with ALA supplementation and no significant increase in DHA levels (Barcelo-Coblijn et al., 2009; Brenna et al., 2009). Studies have shown a <0.3-8% and up to 21% conversion of ALA to EPA in men and women, respectively. The conversion through to DHA is even lower at <4% but often undetectable in men and up to 9% in women (Arterbern et al., 2006). This variability may stem from the influence of factors such as energy balance, the presence of other dietary factors such as n-6 fatty acids and cofactors influencing desaturation-chain elongation. The physiological action of ALA is not yet fully established but since most is catabolized for energy (Arterbern et al., 2006), its action may be due to its direct effect or through its longer-chain metabolites.

### 2.4.2. Flaxseed Effects in Breast/Mammary Cancer

Several studies have been conducted using rodent models investigating the role of FS in relation to breast/mammary cancer as summarized in Tables 2.4 and 2.5. FS has been shown to modulate the various stages of breast cancer development. Feeding Sprague-Dawley rats a 5% dietary FS diet before (pre-initiation stage) or after (promotion stage) 7,12-dimethylbenz[α]anthracene (DMBA) carcinogen administration resulted in reduced tumour size (Serraino et al., 1992). Furthermore, 2.5 and 5% FS fed two days after NMU administration (early promotion stage) resulted in reduced tumour invasiveness and grade; however, there was no effect on final tumour weight, volume or incidence (Rickard et al., 1999). To determine the effect of FS on the progression and tumour development stages, Thompson et al (1996a) fed Sprague-Dawley 2.5 and 5% FS diets 13 weeks after DMBA.
Figure 2.4 Metabolism of n-3 PUFA. ALA is converted to longer chain n-3 PUFA through a series of desaturation and elongation steps and a final β-oxidation step.
Table 2.4. Summary of in vivo studies of the effect of flaxseed and its components on mammary/breast cancer at low circulating levels of estrogen (E2)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Model</th>
<th>Treatments</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saggar et al., 2010a</td>
<td>OVX athymic mouse model with MCF-7 (ER+, HER2-) xenografts</td>
<td>● FO (38.5g/kg), SDG (1g/kg) and FO+SDG</td>
<td>● Tumour Growth: greater ↓ in all treatment groups vs. control but FO had greatest effect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>● 20% fat diets</td>
<td>● Cell Proliferation: ↓ in all groups vs. control but FO had the greatest effect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>● 7 week treatment</td>
<td>● Apoptosis: ↑ in all groups vs. control but FO had the greatest effect; Bcl-2 mRNA ↓ in all groups</td>
</tr>
<tr>
<td></td>
<td></td>
<td>● All mice received TAM pellet implant</td>
<td>● ER-related biomarkers: ↓ PGR (mRNA) in FO group; ↓ CD1 (mRNA) in FO, SDG and combination; ↓ ERα in all treatments</td>
</tr>
<tr>
<td></td>
<td></td>
<td>● 2x2 factorial design</td>
<td>● Growth factor-related biomarkers: ↓ HER2, pHER2, pMAPK (protein) and IGF-IR (mRNA) in all groups; ↓ EGFR(mRNA) in SDG</td>
</tr>
<tr>
<td>Saggar et al., 2010b</td>
<td>OVX athymic mouse model with MCF-7 (ER+, HER2-) xenografts</td>
<td>● FO (38.5g /kg), SDG (1g/kg) and FO+SDG</td>
<td>● Tumour Growth: ↓ in all groups vs. control; SDG had greatest effect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>● 20% fat diets</td>
<td>● Cell Proliferation: ↓ in all groups vs. control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>● 7 week treatment</td>
<td>● ER-related biomarkers:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>● 2x2 factorial design</td>
<td>● ↓ PGR, ERβ (mRNA) in SDG and FO but not in FO+SDG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>● ↓ ERα (mRNA) in SDG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>● Growth factor-related biomarkers:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>● ↓ EGFR (mRNA) in SDG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>● ↓ IGF-IR (mRNA) in FO+SDG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>● ↓ Bcl-2 (mRNA) and pMAPK (protein) in SDG and FO but not in FO+SDG</td>
</tr>
</tbody>
</table>
| Chen et al., 2009 | OVX athymic mouse model with MCF-7 (ER+, HER2-) xenografts | • 10% FS, SDG and FH (at levels present in 10% FS)  
• 20% fat diets  
• 7 week treatment | • **Tumour growth:** greater ↓ in FS and SDG groups vs. control; FH did not differ from any other group  
• **Cell Proliferation:** ↓ in all groups vs. control; no difference between FS, SDG and FH groups  
• **Apoptosis:** ↑ in FS and SDG vs control  
• **Apoptotic mRNA biomarkers:** ↓ Bcl-2 in FS and SDG vs. control and FH; ↑ in FH vs. control  
• **ER-related mRNA biomarkers:** ↓ cyclin D1, pS2, ERα and ERβ in FS and SDG groups vs. control; FH did not differ from control  
• **Growth factor receptor-related mRNA biomarkers:**  
  • ↓ EGFR in FS and SDG groups vs. control  
  • ↓ HER2 in FS vs. control  
  • ↓ IGF-IR in FS and SDG groups vs. control  
  • ↓ pMAPK in SDG vs. control; no difference in other groups  
  • No difference in pAkt among treatment groups |
| Power et al., 2008; Saarinen et al., 2006 | OVX athymic mouse model with MCF-7 (ER+, HER2-) xenografts | • 10% FS diet, 20% soy protein isolate (SPI) diet and combined SPI+FS  
• 20% fat diets  
• Two treatment lengths: 2 or 25 weeks  
• Control= BD with E2 pellet removed | • **Tumour size:** Week 25: ↑ in SPI group vs. all other treatments; no difference between control, FS and SPI+FS  
• **Tumour weight:** Week 2: no effect; Week 25: ↑ in SPI vs. control; no difference among other treatment  
• **Cell Proliferation (Ki-67 and Cyclin D1):** Week 2: ↑ in SPI and FS+SPI vs. control; Week 25: ↑ in SPI and FS+SPI vs. control; ↑ in Ki-67 SPI vs. FS+SPI  
• **Apoptosis:** Week 25: ↑ SPI and SPI+FS vs. control  
• **ER-related protein biomarkers:** ERα: ↑ in SPI and FS+SPI vs. control and FS (Week 25); ERβ: ↓ in SPI vs. all other treatments (Week 25); PR: no effect  
• **Growth factor receptor-related protein biomarkers:** IGF-IR: ↑ in SPI vs. FS and control; HER2: ↓ in SPI vs. control and FS (Week 25); pMAPK: ↑ in SPI vs. control (Week 25) |
<table>
<thead>
<tr>
<th>Study</th>
<th>Model and Characteristics</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chen et al., 2007b</td>
<td>OVX athymic mouse model with MCF-7 (ER+, HER2-) xenografts</td>
<td>- Tumour Growth: TAM+10% FS ↓ tumour regrowth seen with TAM alone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Cell Proliferation: ↓ in TAM+10% FS vs. TAM alone</td>
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<td></td>
<td></td>
<td>- Apoptosis: ↑ in TAM+10% FS vs. TAM alone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- ER-related protein biomarkers: ↓ cyclin D1 and ERα in TAM+5% FS</td>
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<tr>
<td></td>
<td></td>
<td>and TAM+10% FS vs. TAM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Growth factor receptor-related protein biomarkers: ↓ HER2 and IGF-IR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>in TAM+5% FS and TAM+10% FS vs. TAM</td>
</tr>
<tr>
<td>Thompson et al., 2005</td>
<td>Human breast cancer patients</td>
<td>- Cell Proliferation: ↓ in FS group</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- HER2: ↓ in FS group</td>
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<tr>
<td></td>
<td></td>
<td>- Apoptosis: ↑ in FS group</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- ↑ urinary lignan excretion in FS group vs. control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- FS intake correlated with changes in HER2 score and apoptotic index</td>
</tr>
<tr>
<td>Chen et al., 2004</td>
<td>OVX athymic mouse model with MCF-7 (ER+, HER2-) xenografts</td>
<td>- Tumour growth: ↓ in FS and FS+TAM vs. TAM and control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Cell Proliferation: ↓ in FS and FS+TAM vs. TAM and control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Apoptosis: ↑ in FS and FS+TAM vs. TAM and control</td>
</tr>
</tbody>
</table>

BD= basal diet; E2= estrogen; EGFR= epidermal growth factor receptor; ER= estrogen receptor; FH= flaxseed hull; FO= flaxseed oil; FS= flaxseed; HER2= human epidermal growth factor receptor 2; IGF-IR= insulin-like growth factor 1 receptor; MAPK= mitogen-activated protein kinase; OVX= ovariectomized; pMAPK= phosphorylated mitogen-activated protein kinase; PR= progesterone receptor; SDG= secoisolariciresinol diglycoside; SPI= soy protein isolate; TAM= tamoxifen
Table 2.5. Summary of in vivo studies of the effect of flaxseed and its components on mammary/breast cancer at high circulating levels of estrogen (E2)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Model</th>
<th>Treatment</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Truan et al., 2010</td>
<td>OVX athymic mouse model with MCF-7 (ER+, HER2-) xenografts</td>
<td>• 4% FO&lt;br&gt;• 20% fat diets&lt;br&gt;• 8 week treatment</td>
<td>• Tumour growth: ↓ in FO vs. control&lt;br&gt;• Cell Proliferation: ↓ in FO vs. control&lt;br&gt;• Apoptosis: ↑ in FO vs. control&lt;br&gt;• Protein Biomarkers: ↓ HER2, EGFR in SDG vs. control; no effect on IGF-IR, VEGFR, Akt, p-Akt, MAPK, p-MAPK</td>
</tr>
<tr>
<td>Thompson et al., 2010</td>
<td>OVX athymic mouse model with MCF-7 (ER+, HER2-) xenografts</td>
<td>• 0.1% SDG&lt;br&gt;• 20% fat diets&lt;br&gt;• 8 week treatment</td>
<td>• Tumour growth: no effect&lt;br&gt;• Cell Proliferation: ↓ in SDG vs. control&lt;br&gt;• Apoptosis: no effect&lt;br&gt;• Protein Biomarkers: ↓ HER2, EGFR, Akt, p-MAPK in FO vs. control; no effect on IGF-IR, VEGFR, p-Akt, MAPK</td>
</tr>
<tr>
<td>Chen et al., 2007a</td>
<td>OVX athymic mouse model with MCF-7 (ER+, HER2-) xenografts</td>
<td>• 0%, 5% and 10% FS diets alone and in combination with TAM&lt;br&gt;• 7% fat diets&lt;br&gt;• 8 week treatment</td>
<td>• Tumour growth: ↓ in all groups vs. control; 10% FS as effective as TAM alone; TAM+5% FS more effective than TAM or 5% alone&lt;br&gt;• Cell Proliferation: ↓ in all groups vs. control; ↓ in TAM+5% FS vs. 5% FS&lt;br&gt;• Apoptosis: ↑ in all groups except 5% FS vs. control; ↑ in TAM+5% FS vs. 5% FS&lt;br&gt;• ERα: ↑ in TAM+5% FS vs. control&lt;br&gt;• PgR: ↓ in all groups vs. control; ↓ in TAM+10% FS vs. TAM alone&lt;br&gt;• IGF-I: ↓ in 10% FS, TAM+5% FS and TAM+10% FS vs. control&lt;br&gt;• HER2: ↓ in 5% FS and 10% FS vs. TAM alone</td>
</tr>
<tr>
<td>Bergman Jungestrom et al., 2007</td>
<td>OVX athymic mouse model with MCF-7 (ER+, HER2-) xenografts</td>
<td>• ED (15mg/kg), EL (15mg/kg) or 10% FS&lt;br&gt;• 3 week (control) or 5 week treatment (all other groups)&lt;br&gt;• 20% fat diet</td>
<td>• Tumour Growth: ↓ in all treatments vs. control&lt;br&gt;• Tumour Extracellular VEGF: ↓ in all treatments vs. control&lt;br&gt;• Tumour Angiogenesis: ↓ in all treatments vs. control</td>
</tr>
<tr>
<td>Reference</td>
<td>Model</td>
<td>Treatment</td>
<td>Results</td>
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<tr>
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</tbody>
</table>
| Wang et al., 2005b | OVX athymic mouse model with MDA-MB-435 (ER-,HER2-) xenografts | • 10% FS, SDG and FO at levels present in 10% FS or SDG+FO  
• 20% fat diets  
• 6 week treatment | • Tumour growth: ↓ in all treatments except SDG vs. control  
• Cell Proliferation: ↓ in all treatments except SDG vs. control  
• Apoptosis: ↑ in all treatments except SDG vs. control  
• Metastasis: Lung: ↓ FS and SDG+FO vs. control; Lymph node: ↓ in FO vs. control; Total: ↓ in SDG+FO vs. control  
• Lipid Peroxidation: No effect |
| Chen et al., 2004 | OVX athymic mouse model with MCF-7 (ER+, HER2-) xenografts | • TAM alone or combined with 10% FS, 10% FS alone  
• 20% fat diets | • Tumour growth: ↓ in all treatments vs. control; ↓ in FS+TAM vs. FS  
• Cell Proliferation: ↓ in all treatments vs. control; ↓ FS+TAM vs. both FS and TAM alone  
• Apoptosis: ↑ in all treatments vs. control; no differences between treatments |
| Chen et al., 2002 | Athymic mouse model with MDA-MB-435 xenografts | • 10% FS  
• 20% fat diet  
• 7 week treatment | • Tumour Growth: ↓ in FS vs. control  
• Metastasis: ↓ lymph node metastasis in FS vs. control  
• Cell Proliferation: ↓ in FS vs. control  
• Growth Factor and Receptor Biomarkers: ↓ IGF-I and EGFR in FS vs. control; no effect on VEGF |
| Dabrosin et al., 2002 | Athymic mouse model with MDA-MB-435 xenografts | • 10% FS diet  
• 20% fat diets  
• 6 week treatment | • Tumour Growth: ↓ in FS vs. control  
• Metastasis: ↓ in FS vs. control  
• Angiogenesis: ↓ extracellular VEGF in FS vs. control |
| Rickard et al., 1999 | Sprague-Dawley rats with NMU-induced tumours (Early promotion stage) | • 2.5% or 5% FS diets  
• 20% fat diets  
• Diet treatment started 2 days after carcinogen administration  
• 22 week diet treatment | • Tumour Invasiveness and Grade: ↓ in 2.5% and 5% FS vs. control  
• No effects on final tumour weight, volume, multiplicity and incidence |
<table>
<thead>
<tr>
<th>Study</th>
<th>Animal Model</th>
<th>Diet and Treatment</th>
<th>Findings</th>
</tr>
</thead>
</table>
| Thompson et al., 1996a               | Sprague-Dawley rats with DMBA-induced tumours     | • 2.5% or 5% FS diet or control diet containing FO or SDG gavage at levels present in 5% FS  
• Diet treatment started 13 weeks after and continued for 7 weeks  
• 20% fat diets | • Established Tumour Growth: ↓ in 2.5% and 5% FS and FO vs. control; no effect of SDG  
• New Tumour Volume: ↓ in SDG vs. control; no effect of 2.5% or 5% FS or FO  
• Established + New Tumour Volume: ↓ in 2.5% and 5% FS and SDG groups; no effect of FO  
• Tumour Incidence and Number: No difference in incidence between groups; ↓ new tumour incidence in 2.5% FS and SDG  
• Tumour Number: No difference in established tumour number; ↓ new tumour number in SDG  
• Negative correlation between established tumour volume and urinary mammalian lignan excretion in control, 2.5% and 5% FS and SDG groups |
| Serraino et al., 1992                | Sprague-Dawley rats with DMBA-induced tumours     | • 5% FS flour (FF) diet  
• 20% fat diets  
• FF fed for 4 weeks pre DMBA then switched to BD (initiation) or starting 1 week post DMBA (early promotion) | • Tumour Size: ↓ in rats fed FF at promotional stage; no effect of FF fed at initiation |
| Serraino et al., 1991                | Sprague-Dawley rats with DMBA-induced tumours     | • 5% or 10% FS flour (FF) or defatted FS meal (FM)  
• 20% fat diets  
• Diets fed for 4 weeks before DMBA exposure and rats sacrificed 24h post DMBA | • Mitotic index: ↓ in terminal end buds of 5 and 10% FF groups  
• Cell proliferation: ↓ in terminal end buds of 5% FF groups  
• Nuclear Aberrations: ↓ in terminal end buds of 5% FF; ↓ in terminal duct of 5 and 10% FM; ↓ in alveolar buds of 10% FF and 10% FM; ↓ in total structure of mammary gland in 10% FM  
• Urinary lignans: ↑ in all treatments vs. control |

For abbreviations see table 2.2. In addition: DMBA= 7,12-dimethylbenz[a]anthracene; ED= enterodiol; EL= enterolactone; FF= flaxseed flour; FM= flaxseed meal; VEGF= vascular endothelial growth factor
administration resulting in significant attenuation of tumour growth. Together, these suggest that FS can modulate breast tumour development.

A number of xenograft studies have shown that dietary FS can attenuate the growth of both established ER+ (MCF-7) (Chen et al., 2007a, 2007b, 2004; Saarinen et al., 2006) and ER- (MDA-MB-435) human breast tumours (Chen et al., 2002; Dabrosin et al., 2002; Wang et al., 2005b). The effect on ER+ tumours has been demonstrated under both high and low circulating levels of E2. Importantly, the anti-tumour effect of FS is seen in postmenopausal breast cancer patients (Thompson et al., 2005). Doses used in these studies range from 2.5%-10% FS. Studies are conflicting on whether 5% FS is as effective as 10% FS (Chen et al., 2007a, 2007b, Serraino et al., 1991).

Research has also investigated the potential interaction of FS with TAM, a primary adjuvant therapy for the treatment of ER+ breast cancer. It was hypothesized that FS could enhance the effect of TAM and potentially reduce adverse effects and prevent the development of resistance. At high circulating levels of E2, FS enhanced the tumor-suppressing effect of TAM in the nude mouse model with MCF-7 xenografts (Chen et al., 2007a; 2004). Additionally, at low circulating levels of E2, 10% FS prevented the tumour regrowth seen with TAM treatment alone through a decrease in cell proliferation and an increase in apoptosis (Chen et al., 2007b; 2004).

Investigations into the mechanisms of FS effect suggest that this may be due to reduction in angiogenesis (reduced VEGF secretion) (Bergman-Jungestrom et al, 2007; Dabrosin et al., 2002), metastasis (Chen et al., 2002; Dabrosin et al., 2002; Wang et al., 2005b), E2 signaling (Chen et al., 2007a; Saggar et al., 2010a; 2010b) and/or growth factor
receptor signaling (Chen et al., 2002; 2007a; Power et al., 2008; Saggar et al., 2010a, 2010b; Thompson et al., 2005) including the reduction in HER2 expression (Chen et al., 2009; 2007a; 2007b; Saggar et al., 2010a; Thompson et al., 2005).

2.4.3 Effects of Flaxseed Lignans on Breast/Mammary Cancer

Because of their structural similarity to E2, the effects of lignans on hormone-sensitive cancers, including breast cancer, have been investigated. A recent meta-analysis that included 6 cohort, 6 nested case control and 10 case control studies showed no association between plant lignan intake and breast cancer risk in all women. However, when subjects were classified based on menopausal status, a significant reduction in risk was observed in postmenopausal but not in premenopausal women (Velentzis et al., 2009). This suggests that the lignans may be more effective only at low E2 level but the reason for this is still not very clear.

Exposure to SDG, the predominant lignan in FS, results in protective effects in various stages of tumour development. A daily gavage of SDG at equivalent levels to those found in 2.5 or 5% dietary FS to rats two days after treatment with NMU (early promotion), resulted in lower tumour invasiveness, grade and multiplicity in the high dose treatment (Rickard et al., 1999). In addition, Thompson et al. (1996b) gavaged rats with 1.5mg/day SDG (approximately equivalent to level in 5% FS) starting one week after exposure to DMBA resulting in lower tumour multiplicity and fewer tumours in the SDG group. Furthermore, 13 weeks after DMBA administration (progression and tumour development stage) a daily gavage of SDG at levels present in 5% FS in rats reduced new tumour formation and tumour volume of both new and established tumours (Thompson et al.,
Together these suggest that the lignan component of FS contributes to the anti-tumourigenic effect of FS.

Xenograft studies in athymic nude mice support a protective effect of lignans; however these effects differ depending on circulating E2 level and ER status. At low circulating levels of E2, SDG treatment in established ER+ MCF-7 tumours resulted in a significant tumour regression compared to control (Saggar et al., 2010b). Similar results were observed when SDG treatment was combined with TAM treatment (Saggar et al., 2010a). At low circulating levels of E2, treatment with the SDG metabolites enterolactone and enterodiol results in increased MCF-7 tumour cell apoptosis compared to the negative control treatment (E2 pellet removed) suggesting an anti-tumour effect although this model does not allow for tumour growth effects to be observed as MCF-7 tumours deprived of E2 rapidly regress (Power et al., 2006). Under high circulating E2, SDG had no effect on the growth of established ER- MDA-MB-435 tumours (Wang et al., 2005) nor did it affect the tumour growth rate of established ER+ MCF-7 tumours (Thompson et al., 2010). There was, however, a significant reduction in cell proliferation, HER2 and EGFR protein expression in MCF-7 tumours from mice treated with SDG at high circulating levels of E2 (Thompson et al., 2010). On the other hand, at high levels of E2, enterolactone and enterodiol treatment of MCF-7 tumours resulted in significantly lower tumour growth compared to control (Bergman-Junestrom et al., 2007). The effect of enterolactone on MCF-7 tumour growth at high levels of E2 was confirmed in a study by Saarinen et al. (2010) where enterolactone treatment alone and in combination with genistein significantly reduced tumour growth rate which has been attributed to reduced angiogenesis.
Suggestions for the potential mechanisms of lignan action include: reductions in ER signaling (Saggar et al., 2010b), growth factor receptor signaling (Saggar et al., 2010b, Thompson et al., 2010) and angiogenesis (Bergman-Jungestrom et al., 2007) pathways. Other proposed mechanisms include the modulation of E2 metabolism through competition for metabolic enzymes such as sulfokinase and sulfatase enzymes (Mousavi et al., 1992) and/or 17-hydroxysteroid dehydrogenase type I and aromatase enzymes (Brooks et al., 2005).

2.4.4 Effects of Omega-3 Fatty Acids on Breast/Mammary Cancer

The role of dietary fat in breast cancer is complicated as different fat classes have different effects. Most animal fat is rich in n-6 PUFA and saturated fat but a specific animal fat, fish oil, is rich in n-3 PUFA. Highly saturated animal fat was positively correlated while fish oil was negatively correlated with breast cancer mortality in a study of 30 countries (Sasaki et al., 1993). However, this pattern has not been consistently shown (Stampfer et al., 1987; Willett, 1997) and therefore it is suggested that the ratio of n-6:n-3 PUFA maybe a better measure of effect on breast cancer.

Fish oil is rich in the longer chain n-3 PUFAs, EPA and DHA while FO is rich in their precursor PUFA ALA (Hall et al., 2006; Larsson et al., 2004). As described in section 2.4.1, ALA can be converted to a limited extent to EPA and DHA. While the majority of n-3 PUFA effects have been attributed to EPA and DHA (Larsson et al., 2004), there is evidence that ALA also has effects in breast cancer. First, low levels of ALA in human breast tissue have been linked epidemiologically to both the development of breast cancer and metastasis (Bougnoux et al 1994; Klein et al 2000). Second, when diets rich in FO, fish oil or corn oil were fed to mice prior to mammary cancer cell injection (410.1), there was a reduction in
tumour growth 4 weeks post injection in FO-fed mice compared to mice fed either the corn oil diet or a fish oil diet (Fritshe et al., 1990). At sacrifice (29-45 days post injection), FO fed mice had lower tumour diameter and pulmonary metastasis compared to corn oil-fed mice, while fish oil had an intermediate effect. Third, ALA-rich dietary FO at levels present in 5% FS reduced tumour progression and development in DMBA-treated rats (Thompson et al., 1996a). Fourth, dietary FO reduced the growth of established breast cancer xenografts in the OVX athymic mouse model. At high circulating levels of E2, FO reduced the growth of ER+ MCF-7 tumours (Truan et al., 2010) and the growth and metastasis of ER- MDA-MB-435 tumours (Wang et al., 2005b). At low circulating levels of E2, FO reduced MCF-7 tumour growth both in the presence (Saggar et al., 2010a) and absence (Saggar et al., 2010b) of TAM treatment. Mechanisms suggested for the FO effect include: reduction of ER- (Saggar et al., 2010a; 2010b) and growth factor receptor- signaling (Saggar et al., 2010a; 2010b; Truan et al., 2010). HER2 expression was downregulated by dietary FO at high circulating levels of E2 (Truan et al., 2010) and at low circulating levels of E2 (Saggar et al., 2010a). These results are also supported by in vitro work which showed that incubation with ALA resulted in a significant downregulation of HER2 in HER2-overexpressing BT-474 and Sk-BR3 cells (Menendez et al., 2006).

Other suggested mechanisms of n-3 PUFA action include:

1. Competitive inhibition of n-6 fatty acid metabolism and reduction in n-6-derived pro-inflammatory cytokines.

ALA is metabolized to the EPA and DHA by the elongation-desaturation pathway (Larsson et al 2004). Linoleic acid (LA), the parent n-6 PUFA, utilizes the same desaturase
and elongase enzymes (Figure 2.4), for metabolism into arachidonic acid (AA, 20:4n-6). ALA and LA, therefore, compete for the same enzymes. However n-3 fatty acids have greater affinities for the enzymes than do n-6 fatty acids and thus ALA reduces the conversion of LA to AA (Larsson et al. 2004). Eicosanoids generated from AA, including the 4-series leukotrienes, the 2-series prostaglandins and the 2-series thromboxanes, have been positively linked to carcinogenesis whereas those produced from EPA and DHA, including the D- and E-series resolvins, neuroprotectin D1 and the 3-series prostaglandins, have been shown to be protective in carcinogenesis (Rose and Connolly 1999).

1. Alterations of tumour cell membrane lipid composition.

Dietary intake of n-3 PUFA has been shown to result in changes to the lipid composition of tissue cell membranes, including tumour cells (Ma et al., 2004; Rose and Connolley, 1999; Schley et al., 2007; Thompson et al., 1996a). This leads to alterations in membrane fluidity and growth factor expression and signal transduction that influence tumor growth.

2. Effects on signaling pathways.

N-3 PUFA may also exert their effects by downregulating the transcription and expression of nuclear transcription factor-kappa B (NF-κB), which regulates many important cell effects including cell cycle activation, apoptosis and carcinogenesis (Novak et al., 2003). In addition, n-3 PUFA effects on cell signaling may be due to a direct binding of n-3 PUFA to nuclear receptors including peroxisome proliferator-activated receptors (PPARs) which regulate gene expression (Edwards et al., 2008).

3. Increased lipid peroxidation.
As n-3 PUFA are highly unsaturated they are susceptible to oxidation resulting in the production of free radicals and reactive oxygen species (Larsson et al., 2004). Mammary tumour growth was decreased in vitro and in vivo with n-3 PUFA treatment and this effect related to the extent of lipid peroxidation in the tumours (Chajès et al., 1995; Cognault et al., 2000; Gonzalez et al., 1991). The effect was decreased with the addition of anti-oxidants and was enhanced with the addition of pro-oxidants (Cognault et al., 2000).

4. Effects on estrogen metabolism.

Prostaglandin E₂, a derivative of AA, stimulates aromatase activity which converts androgens to E2 (Noble et al., 1997). As indicated above, n-6 metabolism to AA is inhibited by n-3 PUFA, suggesting that n-3 PUFA can reduce the prostaglandin E₂-driven increase in tumourigenic-E2.

2.4.5 Effectiveness of Flaxseed Oil Versus Lignan

In an effort to determine which component of FS was most responsible for FS effect in our model of breast cancer, a number of xenograft studies have been conducted comparing the effectiveness of FO and SDG. At high circulating E2, FO reduced the growth of ER+ MCF-7 tumours while SDG showed no effect (Thompson et al., 2010). At low circulating E2, both FO and SDG reduced MCF-7 tumour growth, however, SDG showed a greater effect (Saggar et al., 2010b). Interestingly, when TAM treatment was combined with dietary SDG and FO at low circulating E2 situation, FO showed a greater effect than SDG. These results suggest that the component most responsible for FS effect depends on the conditions. With respect to HER2 expression, both SDG and FO reduced HER2 expression by 58% and 79%, respectively, at high circulating levels of E2 (Thompson et al., 2010) and had no effect
at low circulating levels of E2 (Saggar et al., 2010b). However, at low circulating levels of E2 when dietary treatment was combined with TAM treatment, SDG and FO both reduced HER2 expression by 22% and 32% respectively (Saggar et al., 2010a). Therefore, studies indicate that the component of FS responsible for reduction in tumour growth and HER2 expression depends on the serum levels of E2 and the presence of breast cancer drugs.

2.5 Summary and Questions

HER2 is overexpressed in 25-30% of breast cancer and its overexpression is associated with aggressive tumours, worse prognosis and earlier death. TRAS is used to treat HER2-overexpressing breast cancer but is limited by intrinsic and acquired resistance, thus there is a need to increase its effectiveness. FS and FO have been shown to reduce tumourigenesis in a number of breast cancer models. Some of their mechanisms of action include the reduction in the expression and signaling of growth factor receptors, such as HER2. This suggests that FS and FO can modulate breast tumour growth through the HER2 pathway. The studies of effects of FS and its components have been conducted in carcinogen-induced (DMBA, NMU) and xenograft (MCF-7, MDA-MB-435) rodent models and in a postmenopausal patient population, all with normal HER2 levels. This raises the question of how FS and its components act in breast cancer with HER2 overexpression. Furthermore, since breast cancer patients change their diet upon diagnosis to include dietary supplements, such as FO, it is imperative that research determines how and if these supplements interact, either positively or negatively, with common breast cancer therapies. No studies have yet looked at how FO interacts with TRAS in vivo. TRAS and FO have some similarities in mechanisms of action including reduced HER2 signaling resulting in reduced tumour cell proliferation and increased apoptosis. This raises the question of
whether FO, not only does not interfere with TRAS action, but rather can enhance its anti-tumour effect.

3.0 OBJECTIVES AND HYPOTHESES

3.1 Objectives

To determine:

1) the effect of dietary FO and 2 dosing regimens of TRAS (2.5 and 5 mg/kg body weight maintenance doses; 5 and 10 mg/kg loading doses), alone and in combination, on the growth (i.e. tumour size, cell proliferation and apoptosis) of established human breast tumours overexpressing HER2 (BT474)

2) the potential adverse effects of the treatments by analyzing body weight gain, and organ gross pathology and weights

3.2 Hypotheses

FO alone will reduce the growth (i.e. decrease tumour size and cell proliferation and increase apoptosis) of established BT-474 tumours under high circulating levels of E2. TRAS will dose dependently reduce BT-474 tumour growth and FO will not interfere with its action, but, will enhance its tumour-reducing effect. Neither TRAS nor FO will result in changes in body weight, and organ gross pathological appearance or weight.
4.0 MATERIALS AND METHODS

4.1 Cell Line and Cell Culture

The BT-474, ER+ human breast cancer cell line which exhibits amplified HER2, was used as it is one of the most common cell lines used to test the effect of compounds on tumours with amplified HER2. It was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotics including penicillin, streptomycin and amphotericin (Sigma, St. Louis, Missouri, USA). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air with periodic screening for Mycoplasma contamination. Once cells reached 70% confluence in T-150 flasks, they were subcultured by trypsinization. Medium was removed from the flask, cells were washed with 5 ml of phosphate-buffered saline then incubated with 5 ml of 0.25% trypsin-EDTA solution for 5 minutes to remove cells from the bottom of the flask and 5 ml of medium was added to stop trypsinization. The cell suspension was then centrifuged for 10 minutes at 1500 rpm to produce a cell pellet. The supernatant was removed and the cell pellet was reconstituted in complete medium with dimethyl sulfoxide and stored at -80°C.

A BT-474 cell suspension was prepared fresh for injection. Cells were subcultured, counted and then resuspended in serum-free medium with 1:1 Matrigel (BD Biosciences, Mississauga, ON) at 1x10^8 cells/ml and kept on ice. Cell viability was >95% as determined by trypan blue exclusion assay before and after each injection each day.


4.2 Trastuzumab

TRAS (Genentech Inc., San Francisco, CA) was purchased from the University Health Network Pharmacy (Toronto, ON). A stock solution (20mg/ml) was prepared every 3 weeks using bacteriostatic water for infusion containing 1.1% benzyl alcohol as a preservative (Genentec Inc.). The doses were prepared fresh in phosphate buffered saline for each injection. Two dosing regimens were used: a single 5 mg/kg loading dose followed by twice weekly 2.5 mg/kg maintenance doses (referred to as TRAS2.5) and a single loading dose of 10 mg/kg loading dose followed by twice weekly 5 mg/kg maintenance doses (referred to as TRAS5).

The doses of TRAS used in this study were selected as they fall within the range used in previous studies (0.1-30mg/kg body weight) looking at TRAS effect in the nude mouse model (Baselga et al., 1998; Wang et al., 2005a; Ritter et al., 2007). Our pilot study using a low dose of 0.375mg/kg body weight showed no effect on tumour growth (unpublished data). In the clinical setting and this study, an initial high loading dose of TRAS is administered followed by regular maintenance doses at half the level of the loading dose. Since TRAS has a relatively long half-life (6 days), a loading dose helps to achieve optimal blood levels quicker which can be maintained with lower regular maintenance doses (Genentech Inc, 2009). Based on bodyweight in the formula to convert human dose to animal dose, the levels used in the clinical setting (4 mg/kg loading dose, 2 mg/kg weekly maintenance dose) appear to be lower than those used in the current study (5 and 10 mg/kg loading dose, 2.5 and 5 mg/kg twice weekly maintenance dose), but based on body surface area, which has been suggested to be a better estimator (Reagan-Shaw et al., 2008), our selected doses were 2.5-5 times lower than those used in humans. The latter conversion
formula is human equivalent dose (mg/kg) = animal dose (mg/kg) x [animal \( K_m \)/human \( K_m \)]

where \( K_m \) is a conversion factor based on body weight and body surface area; mice \( K_m = 3 \) and human \( K_m = 37 \). These suboptimal levels are important to allow for a FO effect to be seen.

### 4.3 Experimental Diets

The basal diet (BD; control) composition is presented in Table 4.1. It was formulated based on the AIN-93G diet (Reeves et al. 1993) modified such that 20% fat was provided by corn oil in place of soybean oil to minimize complications brought about by ALA or other components such as phytosterols or phytoestrogens in the soybean oil. The effects of FS and its components in breast cancer have been tested in both a low (7%) (Chen et al. 2007a; 2007b) and high (20%) (Chen et al. 2004) fat diet and the results are similar at both low and high circulating levels of estrogen. Tumours were more quickly established in mice fed the high fat diet and therefore a 20% fat diet was used in the present study.

Pure FO was prepared from FS by cold pressing and was provided by Pizzey Nutritionals (Angusville, MB, Canada). Its fatty acid composition, was analyzed by Medallion Labs (Minneapolis, MN, USA) by gas chromatography (Mossoba et al. 2003), and is presented in Table 4.2. BD supplementation with FO (80g/kg diet), in place of corn oil, was at levels equivalent to that found in a 20% FS diet. Corrections for fat and carbohydrate contents were conducted to ensure diets were isocaloric. Diets were prepared by Dyets Inc. (Bethlehem, PA) and sterilized by gamma irradiation by Isomedix Corp. (Whitby, ON) and stored at 4°C until used. Mice were fed respective diets and sterilized water ad libitum and diets were replaced every 2-3 days. The peroxide values (meq/kg) of the prepared BD and FO diets, a marker of oxidative stability, were 1.0 and 1.8, respectively, and did not increase
Table 4.1 Diet composition (g/kg)*

<table>
<thead>
<tr>
<th>Ingredients (g/kg)</th>
<th>Basal Diet</th>
<th>FO Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200.0</td>
<td>200.0</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>305.5</td>
<td>305.5</td>
</tr>
<tr>
<td>Dyetrose</td>
<td>86.3</td>
<td>86.3</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Corn oil (no antioxidant)</td>
<td>200.0</td>
<td>120.0</td>
</tr>
<tr>
<td>AIN-93G minerals</td>
<td>40.3</td>
<td>40.3</td>
</tr>
<tr>
<td>AIN-93VX vitamins</td>
<td>11.5</td>
<td>11.5</td>
</tr>
<tr>
<td>L-cystine</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.9</td>
<td>2.9</td>
</tr>
<tr>
<td>Flaxseed oil</td>
<td>0.0</td>
<td>80.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1000</strong></td>
<td><strong>1000</strong></td>
</tr>
</tbody>
</table>

*Modified AIN-93G purified rodent diet (Reeves et al., 1993)
Table 4.2 Fatty acid profile of flaxseed oil

<table>
<thead>
<tr>
<th>Component Name</th>
<th>% of Total Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated Fatty Acids</strong></td>
<td>9.02</td>
</tr>
<tr>
<td>10:0 Capric</td>
<td>0.05</td>
</tr>
<tr>
<td>13:0 Tridecanoic</td>
<td>0.03</td>
</tr>
<tr>
<td>14:0 Myristic</td>
<td>0.05</td>
</tr>
<tr>
<td>16:0 Palmitic</td>
<td>5.59</td>
</tr>
<tr>
<td>17:0 Margaric</td>
<td>0.06</td>
</tr>
<tr>
<td>18:0 Stearic</td>
<td>2.78</td>
</tr>
<tr>
<td>20:0 Arachidic</td>
<td>0.10</td>
</tr>
<tr>
<td>22:0 Behenic</td>
<td>0.13</td>
</tr>
<tr>
<td>24:0 Lignoceric</td>
<td>0.23</td>
</tr>
<tr>
<td><strong>Monounsaturated Fatty Acids</strong></td>
<td>19.01</td>
</tr>
<tr>
<td>16:1 Palmitoleic</td>
<td>0.05</td>
</tr>
<tr>
<td>17:1 Margaroleic</td>
<td>0.04</td>
</tr>
<tr>
<td>18:1 Oleic</td>
<td>18.59</td>
</tr>
<tr>
<td>20:1 Gadoleic</td>
<td>0.33</td>
</tr>
<tr>
<td><strong>cis-Polyunsaturated Fatty Acids</strong></td>
<td>71.92</td>
</tr>
<tr>
<td>18:2 Linoleic</td>
<td>14.00</td>
</tr>
<tr>
<td>18:3 α-Linolenic</td>
<td>57.81</td>
</tr>
<tr>
<td>20:2 Eicodienoic</td>
<td>0.04</td>
</tr>
<tr>
<td>20:3Eicosatrienoic</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Trans Fatty Acids</strong></td>
<td>0.05</td>
</tr>
<tr>
<td>18:2 t-Octadecadienoic</td>
<td>0.05</td>
</tr>
</tbody>
</table>
after 2 days at room temperature (usual diet replenishment period) because of antioxidants in
the diet, indicating stability.

4.4 Animals and Housing Conditions

Animal care and use conformed to the Guide to the Care and Use of Experimental
Animals (Canadian Council on Animal Care, 1984) and experimental protocol was approved
by the University of Toronto Animal Care Committee. OVX athymic mice (BALB/c nu/nu,
4-5 weeks old; n= 6-7 per group) were purchased from Charles River (Senneville, QC,
Canada) and maintained in micro-isolator cages (4 per cage) within a pathogen-free isolation
facility with a 12:12-hr light-dark cycle at 22-24°C and 50% humidity. Mice were given ad
libitum access to sterile water and diets.

4.5 Experimental Design

The experimental design is depicted in Figure 4.1 After one week of acclimatization
while being fed the BD, OVX athymic mice were anaesthetized with a mixture of isoflurane
and oxygen. They were subcutaneously injected with 1x10^7 BT-474 cells in 100µl of culture
medium with 1:1 Matrigel medium into four sites of mammary fat pad (right and left thoracic
and right and left abdominal). Mice were 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) subcutaneously into the intrascapular region to promote tumour growth and
produce E2 levels that fall within the range of the E2 level seen in premenopausal women
during the menstrual cycle.
**OVX athymic mice (n=6-7 per group)**

- **Week -3**  
  Acclimatization; fed BD

- **Week -2**  
  Inject 1x10^7 BT-474 cells; implant E2 pellet
  - *Weekly tumour palpation and body weight measurement*

- **Week 0**  
  Group separation; start diet and TRAS treatment
  - Treatments= control (BD), flaxseed oil (FO diet), TRAS2.5, TRAS5, FO+TRAS2.5, FO+TRAS5
  - *Weekly tumour palpation and body weight measurement*

- **Week 4**  
  Stop TRAS treatment; continue diet treatment
  - *Weekly tumour palpation and body weight measurement*

- **Week 6**  
  Sacrifice; tumour and organ excision

*Figure 4.1 Experimental Design.* BD= basal diet; E2= 17β-estradiol; FO= flaxseed oil; OVX= ovariectomized; TRAS= trastuzumab
Tumours were palpated weekly after injection and surface area was calculated as length/2 x width/2 x π. All mice were fed with the BD for 2 weeks. When tumours had reached an average area of 16.5mm², the mice were separated into 6 treatment groups (n=6-7/group) such that tumour size and body weight were similar among treatment groups: 1) Control group was fed the BD, 2) FO group was fed the FO diet, 3) TRAS2.5 group received a single 5mg/kg body weight loading dose TRAS injection and twice weekly 2.5mg/kg body weight maintenance dose TRAS injections and fed the BD, 4) TRAS5 group received a single 10mg/kg body weight loading dose TRAS injection and twice weekly 5mg/kg body weight maintenance dose TRAS injections and fed the BD, treatment as TRAS2.5 group and was fed the FO diet, and 6) FO+TRAS5 group received the same treatment as TRAS5 group and was fed the FO diet.

Food intake, body weight and palpable tumour area were measured weekly. Diet and TRAS treatment were continued for 4 weeks and TRAS treatment was stopped as mice in the high dose TRAS group were showing adverse reactions (i.e. respiratory distress) to the treatment. Diet treatment was continued for 2 additional weeks and palpable tumour continued to be measured weekly. Combination groups are referred to as FO+TRAS2.5 and FO+TRAS5 for all analyses, however, it should be noted that these mice were not receiving TRAS and were only receiving diet treatment, for the final two weeks of treatment.

Mice were sacrificed at week 6 by CO₂ asphyxiation. Tumours were excised, weighed and volume was measured. Uterus, kidney, heart, liver, lung and brain weights were weighed and examined for gross (macroscopic) pathological changes as other indicators of toxicity. A
portion of the tumours were preserved in 10% buffered formalin for immunohistochemical analysis of tumour growth biomarkers (cell proliferation and apoptosis).

4.6 Immunohistochemistry: Cell Proliferation and Apoptosis

Cell proliferation was measured using 5μm sections of formalin-fixed paraffin-embedded tumour tissue which was deparaffinised and rehydrated. Endogenous peroxidase was blocked with 3% H₂O₂ and antigen was retrieved by heating in Tris-EDTA buffer (at pH=9) for 22 minutes in a microwave oven in a pressurized bath. The Ki-67 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit anti-human polyclonal antibody, was diluted at 1:200 in diluent buffer (Dako Cytomation, Mississauga, ON) in order to block any nonspecific antigens. Sections were incubated overnight at 4ºC followed by incubation with biotinylated swine antirabbit IgG against the primary antibody (Dako Cytomation, Mississauga, ON). Streptavidin-horseradish peroxidase and 3-amino-9-ethylcarbazole substrate chromogen (both Dako) were used to show the antigens. Mayer’s hematoxylin (Sigma) was used to counterstain the slides. Positive and negative (diluent buffer) controls were used in each staining batch, which contained 3 samples per treatment group at the same time. One tumour per mouse, therefore 6-7 tumours per group, was analyzed. Slides were read blindly with a light microscope at 400x magnification and a minimum of 1000 cells from 5-10 randomly selected fields were counted. Cell proliferation as Ki-67 labelling index was calculated as percentage of positive cells over total cells counted.

Apoptosis was measured by in situ terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay which demonstrates DNA fragmentation, using the ApopTag Detection kit (Millipore, Temecula, CA, USA) and was performed according to
manufacturer’s protocol. Briefly, deparaffinised and rehydrated sections were pretreated with proteinase K (20μg/ml) for 15 minutes. The sections were incubated with terminal transferase and digoxigenin dUTP at 37ºC for 1 hour. Sections were then incubated with antidigoxigenin antibody coupled to horseradish peroxidase for 30 minutes at room temperature. The slides were incubated with diaminobenzidine for 6 minutes and counterstained with methyl green (Invitrogen, Carlsbad, CA, USA). One tumour per mouse, therefore 6-7 tumours per group, was analyzed. Slides were read blindly with a light microscope at 400x magnification and a minimum of 1000 cells from 5-10 randomly selected fields were counted. The total number of cells showing positive nuclear immunoreactivity was counted per field and expressed as apoptotic cell number/mm². All assays were performed blindly.

4.7 Statistical Analysis

Data are presented as means ± standard error of the mean (SEM). Palpable tumour area data was subjected to logarithmic transformation for statistical analysis. Differences amongst treatment groups in food intake, body weight, palpable tumour area at week 4 and week 6 and immunohistochemistry results were analyzed by one-way analysis of variance (ANOVA) with post-hoc Student Newman Keuls (SNK) test. Tumour area at week 6 vs. week 4 was analyzed using Student’s t-test. Regression analysis was performed on final tumor area vs Ki-67 labelling index or apoptosis and Ki-67 labelling index vs. apoptosis. (Graphpad Prism statistical package, version 3.0, San Diego, CA, USA). Significance was set at P<0.05 for all analyses.
5.0 RESULTS

5.1 Food Intake and Body Weight

There were no differences in total food intake over the 6 week treatment period nor were there differences in body weight at the start (week 0) or at the end (week 6) of the study (Table 5.1). At week 4 (end of TRAS treatment), body weight was significantly lower in the TRAS5 group compared to all other treatment groups. This was the time at which TRAS-treated mice, particularly those in the TRAS5 group, were showing adverse reactions (respiratory distress) to the treatment. Once the TRAS treatment was stopped they regained their body weight.

5.2 Organ Appearance and Weights

There were no differences between groups in gross pathology noted at necropsy nor were there differences in the weight of the uterus, right and left kidneys, liver, lungs, brain or heart (Table 5.2).

5.3 Palpable Tumour Area, Tumour Weight and Volume

At the start of treatment there was an average of 3.0 tumours/mouse in each group. At week 4 the average number of tumours/mouse was 3.5 in control, 3.5 in FO, 2.2 in TRAS2.5, 1.0 in TRAS5, 1.3 in FO+TRAS2.5 and 1.0 in FO+TRAS5. At week 6 there were no further changes in average number of tumours/mouse in any group. At the end of the study, the total number of tumours per group was 21 in control, 21 in FO, 13 in TRAS2.5, 7 in TRAS5, 9 in FO+TRAS2.5 and 7 in FO+TRAS5.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Food Intake (6 weeks, g/mouse)</th>
<th>Body Weight (g)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Week 0</td>
<td>Week 4</td>
<td>Week 6</td>
<td></td>
</tr>
<tr>
<td>CONTROL</td>
<td>114.9 ± 4.3</td>
<td>17.6 ± 0.7</td>
<td>18.5 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.0 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>TRAS2.5</td>
<td>110.7 ± 5.2</td>
<td>19.6 ± 0.3</td>
<td>19.0 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>TRAS5</td>
<td>114.8 ± 9.2</td>
<td>18.3 ± 0.6</td>
<td>16.6 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.8 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>FO</td>
<td>114.6 ± 4.7</td>
<td>18.0 ± 0.9</td>
<td>18.8 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.5 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>FO+TRAS2.5</td>
<td>118.4 ± 5.6</td>
<td>19.2 ± 0.4</td>
<td>18.7 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.4 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>FO+TRAS5</td>
<td>116.1 ± 3.4</td>
<td>18.1 ± 1.2</td>
<td>19.1 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.1 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

FO = Flaxseed Oil; TRAS = Trastuzumab. Different letters (a-b) within the same column indicate a significant difference at P<0.05 by one-way ANOVA followed by SNK test. Data are means ± SEM. N=6-7 mice/group.
Table 5.2. Effect of various treatments on the final relative weight (g/kg body weight) of various organs in ovariectomized athymic mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Uterus</th>
<th>Left Kidney</th>
<th>Right Kidney</th>
<th>Liver</th>
<th>Lungs</th>
<th>Brain</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>5.07 ± 0.53</td>
<td>7.90 ± 0.16</td>
<td>8.49 ± 0.20</td>
<td>57.09 ± 1.39</td>
<td>7.03 ± 0.27</td>
<td>20.50 ± 0.34</td>
<td>5.31 ± 0.24</td>
</tr>
<tr>
<td>TRAS2.5</td>
<td>6.63 ± 0.39</td>
<td>8.82 ± 0.53</td>
<td>8.91 ± 0.29</td>
<td>60.84 ± 1.69</td>
<td>7.41 ± 0.46</td>
<td>19.75 ± 0.30</td>
<td>5.32 ± 0.18</td>
</tr>
<tr>
<td>TRAS5</td>
<td>5.39 ± 0.98</td>
<td>9.52 ± 0.91</td>
<td>8.81 ± 0.42</td>
<td>62.86 ± 1.40</td>
<td>8.20 ± 0.88</td>
<td>21.14 ± 0.93</td>
<td>6.06 ± 0.62</td>
</tr>
<tr>
<td>FO</td>
<td>5.37 ± 0.75</td>
<td>7.53 ± 0.29</td>
<td>7.66 ± 0.24</td>
<td>59.99 ± 2.45</td>
<td>6.80 ± 0.30</td>
<td>19.77 ± 0.51</td>
<td>5.16 ± 0.08</td>
</tr>
<tr>
<td>FO+TRAS2.5</td>
<td>6.05 ± 0.89</td>
<td>8.26 ± 0.33</td>
<td>7.55 ± 0.64</td>
<td>57.97 ± 2.02</td>
<td>7.16 ± 0.54</td>
<td>19.89 ± 0.57</td>
<td>5.42 ± 0.36</td>
</tr>
<tr>
<td>FO+TRAS5</td>
<td>5.86 ± 0.83</td>
<td>9.65 ± 0.71</td>
<td>8.45 ± 0.52</td>
<td>58.22 ± 1.86</td>
<td>7.50 ± 0.50</td>
<td>20.10 ± 0.54</td>
<td>5.80 ± 0.32</td>
</tr>
</tbody>
</table>

FO= flaxseed oil; TRAS= Trastuzumab. Data are means ± SEM. N=6-7 mice/group
At week 4 (end of TRAS treatment), tumours from the control and FO groups significantly increased in size by 167% (P<0.0001) and 176% (P=0.02), respectively, compared to week 0 (Figure 5.1) suggesting that FO does not attenuate nor stimulate BT-474 tumour growth. Tumours in the TRAS2.5 group were not significantly different in size at week 4 compared to week 0 while tumours in the FO+TRAS2.5, TRAS5 and FO+TRAS5 groups significantly regressed by 89% (P=<0.001), 75% (P<0.01) and 84% (P<0.01), respectively. Palpable tumour area at week 4 was significantly lower in the FO+TRAS2.5 group compared to the TRAS2.5 (P<0.05) group and was not different from both TRAS5 treated groups, with and without FO. TRAS5 treatment caused a rapid and almost complete regression in tumour size and therefore at week 4 no further effect of combining with FO was seen. Importantly, combining FO with low dose TRAS (2.5mg/kg) was just as effective at reducing tumour size as higher dose TRAS (5mg/kg). There were no differences in tumour area between weeks 4 and 6 (two weeks after stopping TRAS treatment while continuing on the same diet) in any treatment. At week 6, tumour size in FO+TRAS2.5 group was 87% lower (P<0.05) than in TRAS2.5 group and was not different from TRAS5 group with or without FO (Figure 5.1). At sacrifice, there were no differences in final tumour volume or weight between the control and FO groups (Figure 5.2). All other treatments (TRAS2.5, TRAS5, FO+TRAS2.5, FO+TRAS5) had significantly lower tumour volume and weight compared to control and did not differ from each other.
Figure 5.1 Effect of various treatments on palpable BT-474 tumour area in ovariectomized athymic mice. Points with different letters indicate significantly different palpable tumour area at week 4 (end of TRAS treatment) or week 6 (end of study) (P<0.05) by one-way ANOVA followed by SNK test. N=17-23 tumours/group from 6-7 mice. FO= flaxseed oil; TRAS= trastuzumab. Modified from Mason et al., 2010
Figure 5.2 Effect of various treatments on the final tumour weight (A) and volume (B) of BT-474 tumours at necropsy in ovariectomized athymic mice. Bars with different letters are significantly different (P<0.05) by one-way ANOVA followed by SNK test. N=7-21 tumours/group from 6-7 mice. FO= flaxseed oil; TRAS= trastuzumab
5.4 Cell Proliferation and Apoptosis

There was no difference in cell proliferation (Ki-67 Labelling Index) between the control and FO groups. All other treatments significantly reduced cell proliferation compared to control (Fig. 5.3). In agreement with palpable tumour area, there was no difference in cell proliferation between the TRAS5 and FO+TRAS5 treatments. TRAS2.5 treatment caused a significant 48% (P<0.05) reduction in cell proliferation compared to control. Combining TRAS2.5 with dietary FO caused a significant 37% reduction in cell proliferation compared to TRAS2.5 alone (P<0.05) to a total of 85% reduction compared to control. Combining low dose TRAS with FO has a similar effect to high dose TRAS. There was no difference in apoptosis between the control and FO groups. TRAS2.5 treatment caused a 44% (P<0.05) increase in apoptosis compared to control (Fig. 5.3). Combining TRAS2.5 treatment with dietary FO further significantly increased the apoptosis by 135% (P<0.05) compared to TRAS2.5 treatment alone to a total of 179% increase compared to control. Apoptosis was similar for FO+TRAS2.5 and TRAS5 treatments with or without dietary FO (Fig 5.3). Again, in agreement with palpable tumour area data, there was no difference in apoptosis between TRAS5 and FO+TRAS5 treatment groups (Fig 5.3).

5.5 Regression Analysis

There was a significant positive relationship observed between final palpable tumor area and cell proliferation (Ki-67 labelling index) (r=0.804, p<0.001). Significant negative relationships were seen between final palpable tumor area and apoptosis (r=0.474, p=0.013) and cell proliferation and apoptosis (r=0.676, p<0.001) (Fig 5.4).
Figure 5.3 Effect of various treatments on BT-474 tumour cell proliferation (A) and apoptosis (B) in ovariectomized athymic mice. Bars with different letters are significantly different (P<0.05). N=6-7 tumours per group. FO= flaxseed oil; TRAS= trastuzumab Modified from Mason et al., 2010
Figure 5.4 Regression analysis of final palpable tumour area and Ki-67 labelling index (A), final palpable tumour area and apoptosis (B) and Ki-67 labelling index and apoptosis (C) of tumours from ovariectomized athymic mice with BT-474 xenografts. N= 36 tumours from all treatment groups included in analysis.
6.0 DISCUSSION

This study has shown for the first time, that combining dietary FO with TRAS treatment does not interfere with its tumour-reducing effect but rather enhances the effectiveness of low dose TRAS in reducing the growth of established HER2-overexpressing BT-474 tumours. The combination of low dose (2.5mg/kg) TRAS treatment with dietary FO for 4 weeks led to a greater reduction in tumour size compared to treatment with this dose of TRAS alone. Cell proliferation and apoptosis are both significantly related to tumor area and FO+TRAS2.5 treatment caused a greater reduction in tumour cell proliferation and a greater increase in apoptosis compared to TRAS2.5 treatment alone. TRAS5 treatment with or without FO caused a rapid and almost complete tumour regression and therefore did not allow for a further effect of FO to be seen. Importantly, combining low dose TRAS with dietary FO was just as effective as high dose TRAS indicating that with FO consumption, only lower doses of the drug may be required to observe the same effect. This may help relieve some of the side effects of TRAS treatment although more research is needed in this area. Furthermore, ALA and FO have been shown to have many potential health benefits beyond breast cancer (Thompson and Cunnane, 2003).

No significant change in tumour size was seen in FO+TRAS groups in the two weeks after cessation of TRAS treatment suggesting that dietary FO did not stimulate tumour growth in the absence of TRAS treatment. FO treatment alone resulted in no significant differences in palpable tumour area, cell proliferation or apoptosis compared to control. This suggests that FO alone may have no effect on HER2-overexpressing tumour growth but has effects that are able to enhance the effectiveness of TRAS. Our results showed that FO
increased TRAS effectiveness by increasing its anti-proliferative and pro-apoptotic effects perhaps through reduction in HER2 signaling although other mechanisms may also be involved.

None of the groups differed in their total food intake, nor were there differences in initial (week 0) and final (week 6) body weight. However, at week 4 (end of TRAS treatment) mice in the TRAS5 group had significantly lower body weight compared to all other treatment groups. This was the time point at which TRAS treatment was stopped as mice in the high dose TRAS groups were showing adverse reactions (respiratory distress) to the treatment. Once TRAS treatment was stopped, these mice quickly regained their lost body weight.

The adverse reactions to TRAS post injection were not unusual as similar post-infusion reactions have been seen in clinical settings (Baselga, 2000). The mice that demonstrated these reactions recovered within 12 hours of treatment. The cardiotoxicity in TRAS treated patients is a result of TRAS inhibition of HER2 in the heart tissue (Crone et al., 2002). Although FO may act similarly to TRAS in inhibiting HER2 action in the heart tissue no gross cardiotoxicity so far has been noted for FO in any of our past studies (Chen et al., 2002; 2004; Wang et al., 2005b). No cardiotoxicity was expected to be caused by TRAS in this study because TRAS binds specifically to the human form of HER2 and does not bind to the rodent form and therefore would not affect cardiac tissue and possibly other mouse tissues as well (Crone et al., 2002). On the other hand, the hypothesized mechanisms of action of FO do not involve the specific binding of FO to HER2 and therefore it would be expected that if FO had cardiotoxic effects it would be seen in both humans and animal models. Nevertheless, FO and FS have been fed in many of our past studies in the nude mouse model and no difference was consistently seen in gross (macroscopic) appearance as
well as the weight of the heart and any other major organs (Chen et al. 2002, 2004; Wang et al. 2005b). Therefore, since the main focus of this study is the effect on the tumours, we did not do further histological examination of the apparently normal looking heart and other major organs.

FO was shown to have no effect on the growth of BT-474 tumours (i.e. tumour area, cell proliferation, apoptosis), however, when combined with TRAS treatment (2.5mg/kg) it significantly enhanced the tumour reducing effect of TRAS. The mechanism behind this synergistic effect merits further investigation. However, a number of hypotheses exist. First, the level of HER2 in BT-474 is very high leading to constitutive activation and aggressive tumour cell growth. Therefore, while FO may be causing effects similar to those demonstrated in past studies in MCF-7 xenograft models (Saggar et al., 2010a; 2010b; Truan et al., 2010), the HER2-driven tumour growth signals may be so strong rendering the FO effect non significant. In contrast, when TRAS is present the HER2-driven tumour growth signals may be decreased to a point where FO can elicit a significant effect. Second, FO may be changing the tumour membrane rendering TRAS more accessible to HER2. Caveolae are flask-like invaginations of the plasma membrane measuring about 70nm in size (Parton et al., 2006). Perhaps the membrane invagination acts as a physical barrier to TRAS and reduces its binding to HER2. N-3 PUFA supplementation to cancer cells has been shown to decrease the n-6:n-3 ratio and increase total n-3 in the caveolae (Ma et al., 2004). This n-3 PUFA enrichment of the caveolae may result in a translocation of HER2 from the caveolae/lipid raft domains of the membrane to the non-caveolae/raft domain. EPA and DHA supplementation to MDA-MB-231 cells in vitro resulted in a significant decrease in EGFR, a close relative to HER2, in the lipid raft domain with no significant difference in total EGFR levels (Schley et
al., 2007). It remains to be determined whether similar effects are seen with ALA supplementation rather than EPA and DHA and in relation to HER2 translocation rather than EGFR. Alternatively, FO may be increasing TRAS accessibility to HER2 by rendering the tumour cell membrane more permeable to the drug so that TRAS can penetrate more deeply into the tumours and act not only on peripheral tumour cells but also at cells at the tumour interior.

The exact mechanism whereby ALA-rich FO modulates HER2 signalling is not fully understood but there are many possibilities (Figure 6.1). 1) FO consumption may lead to ALA incorporation into tumour cell membrane thus modulating the expression and signaling of growth factor receptors, an effect seen with the longer chain n-3 fatty acids (Ma et al., 2004; Schley et al., 2007). HER2 has no known ligand, thus redistribution or reduced expression of growth factors receptors may lead to a decrease in HER2 activation through reduced dimerization. 2) FO may be modulating the expression and activity of FAS, an important lipogenic enzyme, which is often upregulated in tumours including those of the breast (Kuhajda et al., 2008; Menendez et al., 2004a). A link has been demonstrated between the expression of FAS and HER2 (Menendez et al., 2004a). Incubating HER2-overexpressing breast cancer cells with ALA reduced both FAS expression (Menendez et al., 2004b) and HER2 expression (Menendez et al., 2006). 3) ALA-rich FS diets have been shown to reduce tumour VEGF indicating reduced angiogenesis (Bergman Jungestrom et al., 2007). TRAS has been shown to decrease pro-angiogenic factors including VEGF (Izumi et al., 2002). Hence, TRAS treatment combined with dietary ALA-rich FO may lead to a greater anti-angiogenic effect. 4) Fish oil, rich in long chain n-3 fatty acids, has been shown to increase phosphatase and tensin homolog (PTEN) expression (Ghosh-Choudhury et al.,
Figure 6.1: Proposed mechanisms of FO modulation of HER2-overexpressing breast cancer. See figures 2.3 and 2.4 for abbreviations. In addition: IGF-IR= insulin-like growth factor-1 receptor; PTEN= phosphatase and tensin homologue.
PTEN dephosphorylates and inactivates PI3K thus inhibiting the anti-apoptotic Akt pathway (Vivanco and Sawyers, 2002). ALA-rich FO may have a similar effect. Evidently, there are common and complementary mechanisms whereby TRAS and FO may interact in vivo to better reduce the growth of cancer cells overexpressing HER2. Overall, this study demonstrated that FO had no effect on the growth of HER2-overexpressing BT-474 tumours; however, when combined with TRAS (2.5mg/kg), it significantly enhanced TRAS effectiveness. It is still unclear how FO is enhancing TRAS effect but many hypotheses exist. Therefore, it is of interest to explore the mechanisms driving the FO-TRAS interaction in HER2-overexpressing breast cancer.

7.0 CONCLUSIONS

At high circulating E2 in OVX mice with HER2-overexpressing BT-474 tumours:

1) FO alone has no effect on HER2-overexpressing breast tumour growth (tumour size, cell proliferation, apoptosis). TRAS (2.5 and 5mg/kg) regresses tumour growth. FO does not interfere with TRAS but rather enhances its effectiveness in reducing the growth of established HER2-overexpressing BT-474 tumours. This effect may be modulated through a greater reduction of HER2 signalling leading to reduced cell proliferation and increased apoptosis. Low dose TRAS (2.5mg/kg body weight) combined with dietary FO is just as effective as high dose TRAS (5.0 mg/kg body weight).

2) Neither dietary FO or TRAS exposure have effects on organ gross pathology or weight suggesting that these treatments have no adverse effects on major body
organs. However, body weight loss was observed in the TRAS5 treatment but not in the FO+TRAS5 treatment.

8.0 STUDY LIMITATIONS AND FUTURE DIRECTIONS

This study supports the use of FO as a potential complementary treatment in breast cancer patients receiving TRAS therapy, however, several limitations exist that must be considered in future studies.

Firstly, although the OVX athymic mouse model used in this study is useful as it permits the study of human cancer cell growth in vivo and was in fact used in the development of TRAS (Fendly et al., 1990; Hudziak et al., 1989), the disadvantage is that these immune-compromised mice may not be reflective of human biology due to the lack of T-cell function (Rygaard et al., 1976). It is, therefore, unknown how our findings will translate into humans. However, our studies on the effect of FS on ER+ human breast tumours (MCF-7) in athymic mice (Chen et al., 2007a; 2007b; 2004; 2002; Saggar et al., 2010a; 2010b; Truan et al., 2010; Wang et al., 2005b) have been reproduced in post-menopausal breast cancer patients, thus validating our mouse model (Thompson et al., 2005).

Secondly, this study was conducted using only the BT-474 cell line, an ER positive breast cancer cell line overexpressing HER2 which has been used in several studies of both TRAS and ALA action. Because HER2 is overexpressed in both ER positive and negative tumours it would be of interest to verify the FO-TRAS interaction in ER negative breast cancer cells overexpressing HER2 such as Sk-BR3.
Thirdly, although useful, fatty acid analysis was not conducted due to limited amount of serum samples. However, serum fatty acids analyzed in a similar athymic mouse model fed with FO showed significant increase in serum ALA, EPA and DHA (Truan et al., 2010). Previous studies have shown that dietary profiles reflect serum and mammary tumour fatty acid profiles (Cohen et al. 1986). Furthermore, tumour ALA and EPA were increased in rats fed FS with DMBA-induced tumours (Thompson et al 1996a). Tumour fatty acid analysis would have provided information regarding fatty acid levels that may have caused tumour effect in this study and should be considered in future work.

Fourthly, the FO diet contained FO levels equivalent to that in 20% FS, equivalent to a daily intake of about 10-20 g (1-2 tablespoons) FO in human diet depending on the amount of other foods eaten. This level is higher than used in previous studies (based on 10% FS diet), which showed effectiveness in reducing growth of ER+ human tumours (MCF-7). Therefore future studies should determine whether even lower levels of FO can be effective in enhancing TRAS effects.

Fifthly, the TRAS5 dose caused a rapid and almost complete regression of the tumours and therefore no effect of FO could be seen. Furthermore, mice receiving this dose showed adverse reactions to the treatment. Therefore, future studies should use lower doses of TRAS to allow for an interactive effect to be seen and to avoid adverse reactions. Also it would be of interest to determine whether even lower TRAS dose than 2.5mg/kg (e.g. 1mg/kg) in combination with dietary FO can achieve a similar anti-tumour effect as high dose TRAS alone. In an effort to avoid adverse reactions and rapid tumour regression the loading dose may be omitted in future studies. In nude mouse xenograft studies a TRAS
effect is seen both in studies where the loading dose is included (Fujimoto-Ouchi et al., 2002; Ritter et al., 2007) and omitted (Baselga et al., 1998; Khalili et al., 2005; Wang et al., 2005a).

Finally, there are numerous molecules involved in carcinogenesis that were not measured in this study. Two of the major endpoints of the growth factor receptor signalling pathways, cell proliferation and apoptosis, were measured however it remains to be determined whether the differences seen in these markers are related to changes in signalling protein and mRNA biomarkers. Other mechanisms illustrated in figure 6.1 and related to HER2 accessibility should also be explored.

Other considerations in the design of future studies would be to begin with a larger established tumour size which may help to avoid too rapid of a regression of the tumours. Dose response studies should be conducted before recommendations can be made regarding combined FO and TRAS with further emphasis on the safety and pharmacokinetic and pharmacodynamic parameters of the combined treatment.

Future studies should investigate whether FS can be used instead of FO to exert the same interactive effect. Furthermore, since FS previously has been shown to be protective in tumour initiation and promotion stages (Serraino et al., 1992a; Thompson et al., 1996) it would be of interest to determine whether FO and FS play a preventative role in the development of HER2-overexpressing tumour development using the MMTV-neu mouse model (Jolicoeur et al., 1998) or the more aggressive strain MMTV-neu-YD5 (Dankort et al., 2001). Also, since it has been suggested that FS and FO have potential benefits to cardiovascular health (Hall et al., 2006), research should be done to determine whether these dietary compounds may reduce the cardiotoxic side effect of TRAS.
9.0 IMPLICATIONS

This research is significant as it provides evidence for a potential use of FO as a complementary treatment for premenopausal women with HER2-overexpressing breast cancer undergoing TRAS treatment. Furthermore, the combination treatment may reduce the TRAS dose required for treatment, therefore leading to lower health care cost, fewer side effects and potentially increased survival.

As dietary supplements including FS are highly used by breast cancer patients (Boon et al., 2007), it is important to know that the oil component of FS has the potential to be protective against HER2-overexpressing breast cancer when combined with common therapy such as TRAS. However, further studies are required to fully understand the mechanisms of the FO-TRAS interaction, to verify that FO does not stimulate BT-474 tumour growth, and to confirm the effectiveness of FO-TRAS combination in a breast cancer patient population before a more definitive recommendation can be made regarding FO use.
10.0 REFERENCES


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