RESEARCH ARTICLE

Dietary flaxseed lignan or oil combined with tamoxifen treatment affects MCF-7 tumor growth through estrogen receptor- and growth factor-signaling pathways

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This study aimed to elucidate which component of flaxseed, *i.e.* secoisolariciresinol diglucoside (SDG) lignan or flaxseed oil (FO), makes tamoxifen (TAM) more effective in reducing growth of established estrogen receptor positive breast tumors (MCF-7) at low circulating estrogen levels, and potential mechanisms of action. In a 2×2 factorial design, ovariectomized athymic mice with established tumors were treated for 8 wk with TAM together with basal diet (control), or basal diet supplemented with SDG (1 g/kg diet), FO (38.5 g/kg diet), or combined SDG and FO. SDG and FO were at levels in 10% flaxseed diet. Palpable tumors were monitored and after animal sacrifice, analyzed for cell proliferation, apoptosis, ER-mediated (ER-α, ER-β, trefoil factor 1, cyclin D1, progesterone receptor, AIBI), growth factor-mediated (epidermal growth factor receptor, human epidermal growth factor receptor-2, insulin-like growth factor receptor-1, phosphorylated mitogen activated protein kinase, PAKT, BCL2) signaling pathways and angiogenesis (vascular endothelial growth factor). All treatments reduced the growth of TAM-treated tumors by reducing cell proliferation, expression of genes, and proteins involved in the ER- and growth factor-mediated signaling pathways with FO having the greatest effect in increasing apoptosis compared with TAM treatment alone. SDG and FO reduced the growth of TAM-treated tumors but FO was more effective. The mechanisms involve both the ER- and growth factor-signaling pathways.

Keywords:
Breast cancer / Flaxseed / Growth factor signaling / Lignan / Tamoxifen

1 Introduction

Tamoxifen (TAM), a selective estrogen receptor (ER) modulator, is the most commonly prescribed drug for estrogen receptor positive (ER+) breast cancer and is approved for usage as an adjuvant [1]. However, TAM has side-effects including the development of menopausal-like symptoms, an increased risk for endometrial cancer, and after prolonged treatment, TAM resistance, *i.e.* tumors re-grow [2].

The *in vivo* target of TAM is the ER. In classical ER signaling, estrogen binds to the ER, which results in the transcription of the estrogen-sensitive genes: trefoil factor 1 (PS2), cyclin D1 (CD1), and progesterone receptor (PGR) [3]. Membrane-bound ER can also interact with and activate the growth factor receptors such as insulin-like growth factor receptor-1 (IGF-1R), human epidermal growth factor receptor-2 (HER2), and epidermal growth factor receptor (EGFR) [4], leading to non-genomic effects and cellular

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Abbreviations: ALA, α-linolenic acid; **BD,** basal diet; **CD1,** cyclin D1; **E2,** estradiol; **EGFR,** epidermal growth factor receptor; **END,** enterodiol; **ENL,** enterolactone; **ER,** estrogen receptor; **ER−,** estrogen receptor negative; **ER+,** estrogen receptor positive; **FO,** flaxseed oil; **FS,** flaxseed; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **HER2,** human epidermal growth factor receptor-2; **IGF-1R,** insulin-like growth factor receptor-1; **MAPK,** mitogen activated protein kinase; **OVX,** ovariectomized; **PGR,** progesterone receptor; **PHER2,** phosphorylated human epidermal growth factor receptor-2; **PMAPK,** phosphorylated mitogen activated protein kinase; **PS2,** trefoil factor 1; **RT-PCR,** real-time PCR; **SDG,** secoisolariciresinol diglucoside; **TAM,** tamoxifen; **VEGF,** vascular endothelial growth factor

proliferation. It has been suggested that up-regulation in cross-talk between these pathways leads to TAM resistance [4]. Other suggested mechanisms of TAM resistance include loss of ER-α expression or function, altered expression of ER-β, and alterations in co-regulatory proteins and signal transduction (cellular kinase) pathways [2].

Flaxseed (FS) is an oilseed that contains high amounts of the phytoestrogens called lignans and oil rich in the n-3 fatty acid, α-linolenic acid (ALA) [5]. The predominant lignan in FS is secoisolariciresinol diglucoside (SDG), which is present in FS at levels 75–800 times higher than other plant foods [6]. SDG is converted to the active mammalian lignans enterolactone (ENL) and enterodiol (END) by bacteria in the human and animal colon [5]. END, ENL, and ALA have all been suggested to reduce cancer risk. The lignans may bind to the ER in a similar fashion as estradiol (E2) and TAM, and exert estrogenic as well as anti-estrogenic effects [7]. In addition, FS and the mammalian lignans can alter E2 metabolism and bioavailability, and action of the ER on ER-dependent gene transcription [8].

Previous studies in carcinogen-treated rats and in athymic mice with xenografts of ER+ or estrogen receptor negative (ER−) human breast tumors have shown that dietary FS can reduce the growth of established tumors [9–13]. Similarly, in a study of postmenopausal breast cancer patients, FS has been shown to reduce tumor-cell proliferation and HER2 protein expression and increase apoptosis [14]. In the ovariectomized (OVX) athymic mouse model, dietary 5 or 10% FS also did not interfere with but rather enhanced the tumor growth inhibitory effect of TAM at high circulating estrogen levels [9–10] and attenuated the tumor stimulatory/estrogenic effects of long-term exposure to TAM at low circulating estrogen levels [11]. However, it is unknown whether the beneficial effects of FS may be attributed to its SDG lignan, ALA-rich oil components or their combination, and the mechanisms whereby they act in combination with TAM are unclear. Thus, the objective of this study was to elucidate the component(s) of FS responsible for its anti-cancer effect and their potential mechanisms of action when combined with TAM treatment, with specific focus on the ER- and growth factor- mediated signaling pathways. The results may lead to a simple, inexpensive, complementary treatment whereby FS or its components make TAM more effective and possibly delay or prevent resistance and thus help prolong lives. The study has added significance considering that many breast cancer patients appear to supplement their medical treatments with FS [15].

2 Materials and methods

2.1 Cell line and cell culture

Human ER+ breast cancer cells (MCF-7; The American Type Culture Collection, Manassas, VA, USA), were maintained in Dulbecco's Minimum Essential Medium and supplemented with 10% fetal bovine serum and 1% antibiotics. Cells were grown to 70–90% confluence in T-150 flasks. One day before cell harvesting, cells were provided with fresh medium. To prepare for injection, cells were first trypsinized and then re-suspended in serum-free medium with 1:1 Matrigel at a concentration of 2×10^7 cells/mL on ice. Cell viability was determined by trypan blue exclusion assay and found to be greater than 95%.

2.2 Animals and diets

All animal care use and experimental protocols were approved by the University of Toronto Animal Care Ethics Committee (permission ♯ 20006109) and were in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993). BALB/c, nu/nu athymic mice (5–6 wk old, $n = 50$) were OVX (Charles River Canada, St-Constant, Que., Canada) and maintained in micro-isolator cages (four/cage) housed in a pathogen-free isolation facility with 12 h light/dark cycles at 22–24°C and 50% humidity.

FS was cold-pressed to obtain the flaxseed oil (FO, Pizzey's Milling, Angusville, Manitoba, Canada). SDG (99.5% purity) was isolated from FS as previously described [16]. The basal diet (BD) control was prepared based on the AIN-93G diet [17], modified to contain 20% fat from corn oil instead of soybean oil due to its low levels of phytosterols and n-3 fatty acids to minimize confounding factors. The anti-cancer effects of FS have been tested in both high [9] and low [10–11] fat diets and the results are similar. However, the high-fat diet established the tumors faster and therefore was used in the present study. The BD was supplemented with either FO (38.5 g/kg diet), SDG (1 g/kg in diet), or their combination with corrections for fat and carbohydrate content, respectively; hence all diets were isocaloric. The SDG and FO were at levels present in a 10% FS diet. The fatty acid composition of the test diets are presented in Table 1. Diets were prepared by Dyets (Bethlehem, PA, USA) and were sterilized by cobalt radiation by Isomedix (Whitby, Ont., Canada).

Table 1. Percent fatty acid composition of test diets

Fatty acids	BD	BD+SDG	BD+FO	BD+SDG+FO
Saturated	13.0	13.0	12.4	12.4
Monounsaturated	28.0	28.0	26.6	26.6
Polyunsaturated				
Linoleic acid	58.0	58.0	50.4	50.4
ALA	1.0	1.0	10.6	10.6

2.3 Experimental design

After 1 wk acclimatization while being fed the BD, OVX athymic mice were anesthetized with an isoflurane and oxygen mixture. A 50 μL cell suspension (1×10^6 cells) with Matrigel was injected subcutaneously into each of the four mammary fat pads (right and left thoracic and right and left abdominal), thus providing four tumor sites. A sterilized 17-β E2 pellet (0.36 mg, 60-day release; producing 3–4 nmol/L E2 blood level; Innovative Research of America, Sarasota, FL, USA) was implanted in the intrascapular region to help promote tumor growth. At 7 wk post-injection when tumors are established, the E2 pellet was then removed to simulate the low E2 in postmenopausal situation (27–38 pg/mL plasma E2 in mice; 10–40 pg/mL in women). All mice were then subcutaneously implanted with a TAM pellet (5 mg, 60-day release, producing 3–4 ng/mL blood level; Innovative Research of America). Mice were randomized into groups ($n = 12$/group) such that the mean tumor size and mice weight did not differ from one another. Group 1 was fed the BD (control) and Groups 2–4 were fed the BD supplemented with SDG, FO, or combined SDG and FO, respectively. Measurement of food intake was conducted three times a week while body weight and palpable tumor size were monitored weekly with the palpable tumor area calculated as length/2 × width/2 × π. After 8 wk of treatment, mice were sacrificed using CO_2 asphyxiation and cervical dislocation. Tumors to be used for real-time PCR (RT-PCR) were immediately frozen in liquid nitrogen and stored at −80°C for analysis of estrogen-sensitive genes: ER-α, ER-β, CD1, PS2, and PGR and growth factor-related genes: BCL2, HER2, IGF-1R, EGFR, and vascular endothelial growth factor (VEGF). For immunohistochemical analysis of tumor growth biomarkers (cell proliferation, apoptosis), phosphorylated mitogen activated protein kinase (PMAPK), PAKT, and AIBI, one to two tumors *per* mouse were taken and fixed in a 10% buffered formalin solution.

2.4 RNA preparation

RNA was extracted from frozen tumors using RNA tri reagent (Sigma-Aldrich, St. Louis, MO, USA). Tumors that were representative of the mean area for each treatment group were selected for analysis. Tumors frozen at −80°C (< 50 mg) were placed into RNA*later* solution (Applied Biosystems, Austin, TX, USA) and kept at −20°C for 16 h. Tumors were then removed from solution, placed into 1 mL of tri reagent solution, and homogenized with a polytron (Brinkmann, Switzerland). Isopropanol (0.5 mL) was added to the homogenate, mixed and allowed to stand for 10 min at room temperature, centrifuged at 12 000 *g* for 10 min at 4°C to form an RNA pellet. This was then washed by addition of 1 mL of 75% ethanol, vortexing and centrifuging at 7500 *g* for 5 min at 4°C. The RNA pellet was air-dried for 5–10 min, dissolved in RNase-free dH_2O, and stored at −80°C until needed. RNA concentration was measured at 260 and 280 nm to ensure a ratio of > 1.7, which indicate it is free of proteins and DNA.

2.5 RT-PCR and cDNA synthesis: ER-α, ER-β, CD1, PS2, PGR, BCL2, HER2, IGF-1R, EGFR, and VEGF

cDNA formation for RT-PCR analysis was performed with the one-step QIAGEN multiplex RT-PCR kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used for all analysis. The fluorescently labeled VIC/MGB dye housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified as control and all other genes were fluorescently labeled with a FAM/TAMRA dye obtained from Taqman® gene expression Assay-on-Demand kits (Applied Biosystems). cDNA production was performed by incubation of RNA (< 125 ng/25 uL reaction) for 20 min at 50°C, followed by 95°C for 15 min, and then 40 cycles of 45 s at 94°C and 45 s at 60°C. The fixed threshold (C_T) was used to quantify expression of target genes. C_T values were determined by triplicate sample runs in separate tubes for test and reference samples for each target and for GAPDH. C_T values of the target gene were averaged and the GAPDH C_T was subtracted to obtain ΔC_T. The relative expression level of target gene was determined as $2^{\Delta\Delta CT}$ where $\Delta\Delta C_T$ is the tumor sample ΔC_T minus the reference ΔC_T (control sample).

2.6 Immunohistochemistry

All primary antibodies used were rabbit antihuman polyclonal diluted accordingly: Ki-67 (H-300) at 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); PMAPK 1:100 (Cell Signaling, Pickering, Ont., Canada), AIBI 1:200 (Santa Cruz Biotechnology), PAKT 1:100 (Cell Signaling), HER2 1:400 (Dako Cytomation, Mississauga, Ont., Canada), phosphorylated human epidermal growth factor receptor-2 (PHER2) 1:200 (Cell Signaling), ER-α protein 1:800 (Santa Cruz Biotechnology). In brief, 5-μm sections of formalin-fixed paraffin-embedded tumor tissue, deparaffinized and rehydrated, were used for cell proliferation; 3% H_2O_2 was used to block endogenous peroxidase and antigen retrieved by heating in 0.01 mol/L citrate buffer (pH 6) for 20 min in a microwave oven. To block any nonspecific antigens present, Ki-67 antibody was diluted in diluent buffer and incubated overnight at 4°C; this was then followed by incubation with biotinylated swine antirabbit IgG. To show antigens, streptavidin-horseradish peroxidase and 3-amino-9-ethylcarbazole substrate were used and slides were read blindly with a light microscope at 400× magnification. A minimum of 1000 cells from different fields were counted. Cell proliferation (Ki-67 labeling index) was calculated as the

percentage of positive cells over total cells counted. The analysis of other tumor biomarkers (PMAPK, AIBI, PAKT) was conducted using a similar method; however, scoring was used based on the Allred method [18], which considered both a proportion score (range 0 = 0% positive to 5 = 100% positive), and a staining intensity score (negative = 0, weak = 1, intermediate = 2, or strong = 3) with a total maximum score of 8 (range 0–8).

Apoptosis was measured as the degree of DNA fragmentation present using *in situ* terminal deoxynucleotidyl transferase-mediated nick end labeling assay by ApopTag Detection kit (Intergen, Purchase, NY, USA) following the manufacturer's protocol. Briefly, deparaffinized and rehydrated sections were pre-treated with proteinase K (20 μg/mL) for 15 min followed by incubation at 37°C with terminal transferase and digoxigenin dUTP for 1 h. Sections were incubated with antidigoxigenin antibody coupled to horseradish peroxidase for 30 min at room temperature. Slides were then incubated with diaminobenzidine for 6 min, and counter-stained with methyl green. Positively stained breast carcinoma cells were counted and expressed as apoptotic cell number *per* millimeter square at 400× magnification. All assays were performed blindly.

2.7 Statistical analysis

All data showed no marked skewness. Data were presented as mean ± SEM and, in the case of the biomarkers, in both bar and line graphical forms. The arithmetic of the analysis of variance associated with a 2 × 2 factorial design was used to determine the main and interaction effects of SDG and FO. No multiple comparison adjustments were made for the three planned comparisons of the FO, SDG, and the FO + SDG interaction effects. When a modest interaction exists ($p < 0.10$), the remaining two degrees of freedom were spent comparing each factor, SDG or FO, within the levels of the other factor. One-way ANOVA followed by Tukey's multiple range test was used to test differences ($p < 0.05$) amongst groups in food intake and body weight with BD as the control. All statistical analyses were done using Statistical Analysis Software (SAS) (Cary, NC, USA).

3 Results

3.1 Food intake and body weight

There were no significant differences in food intake and body weight amongst treatment groups (data not provided).

3.2 Palpable tumor growth

The mean pretreatment tumor size was 29 mm^2 at week 0 (Fig. 1). Palpable tumors regressed in size in all groups after 7 wk because of E2 removal, but regressions were greater in all treatment groups (dietary SDG, 24%; FO, 44%; SDG + FO 42%; all diets combined with TAM treatment) than the control group (TAM treatment alone, 18%). However, only FO had an overall effect in reducing palpable tumor area ($p = 0.001$) and in tumor regression rate (slope) ($p = 0.087$).

3.3 Cell proliferation and apoptosis

SDG and FO caused significant reductions in TAM-induced cell proliferation (Ki-67 labeling index) (Fig. 2A). There was a significant interaction in the effects of FO and SDG on cell proliferation ($p = 0.0011$) but FO alone in the absence of SDG significantly lowered the cell proliferation by 35% ($p = 0.0001$) while SDG alone in the absence of FO reduced it by 29% ($p = 0.0001$) with combined SDG and FO reducing it slightly less (25%). There was also a significant interaction of the FO and SDG effects on apoptosis ($p = 0.001$) but FO alone increased apoptosis by 76 % ($p = 0.0001$) while SDG alone increased apoptosis by 29% ($p = 0.029$) with similar increase observed with SDG combined with FO (28%) (Fig. 2B).

3.4 Cellular biomarkers

The expression of estrogen-sensitive genes (PS2, PGR, ER-α, ER-β, CD1) involved in ER signaling pathway (Fig. 3), of genes (EGFR, IGF-1R, BCL2, HER2) involved in growth factor- signaling pathways (Fig. 4), of proteins (ER-α, HER2, PHER2, PMAPK, A1B1, PAKT) (Fig. 5) involved in ER- and growth factor-signaling pathways, and of gene involved in angiogenesis (VEGF; Fig. 4) are provided.

Significant interactions in the effects of FO and SDG on mRNA expressions of PGR ($p = 0.049$), CD1 ($p = 0.029$), IGF-1R ($p = 0.059$), BCL2 ($p = 0.087$) and protein expressions of PMAPK ($p = 0.056$), HER2 ($p < 0.001$), PHER2 ($p = 0.040$), and ER-α ($p = 0.022$) were observed. FO alone lowered the mRNA expressions of PGR by 83% ($p = 0.024$), CD1 by 32% ($p = 0.027$), IGF-1R by 64% ($p = 0.006$), BCL2 by 56% ($p = 0.017$), and protein expressions of PMAPK by 29% ($p = 0.025$), HER2 by 32% ($p = 0.0004$), PHER2 by 19% ($p = 0.017$), and ER-α by 29% ($p = 0.0002$). FO also tend to have overall (main) effects in reducing HER2 mRNA expression ($p = 0.067$). In contrast, SDG alone lowered the mRNA expressions of CD1 by 31% ($p = 0.024$), IGF-1R by 42% ($p = 0.045$), and BCL2 by 47% ($p = 0.042$) and protein expressions of PMAPK by 31% ($p = 0.015$), HER2 by 22% ($p = 0.012$), PHER2 by 17% ($p = 0.031$), and ER-α by 16% ($p = 0.022$). In addition, SDG has an overall effect in reducing the expressions of EGFR mRNA ($p = 0.047$) and AIB1 protein ($p = 0.001$).

When SDG and FO are combined, the patterns of their interactive effect are either slightly increased from that caused by FO or SDG alone (cell proliferation, PGR, CD1, PMAPK, HER2 protein, PHER2), slightly lowered than that caused by FO or SDG alone (apoptosis), intermediate

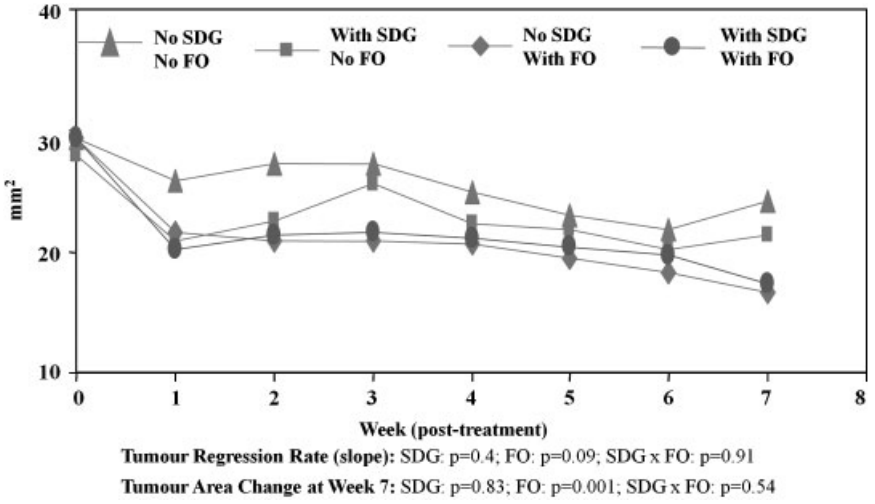

Figure 1. Effect of SDG, FO, and their combination, in the presence of TAM, on palpable MCF-7 breast tumor area over treatment time in OVX athymic mice.

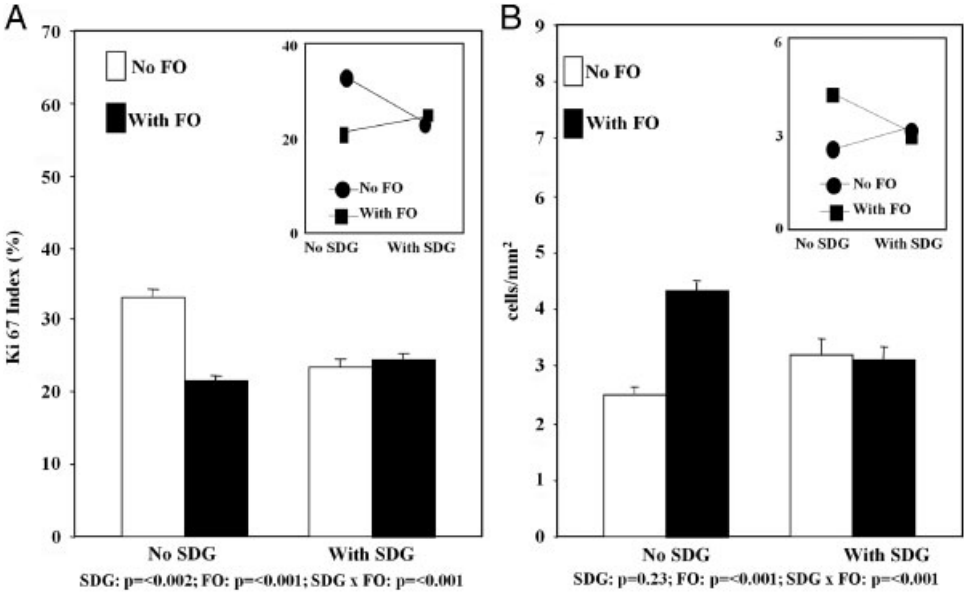

Figure 2. Effect of SDG, FO, and their combination, in the presence of TAM on (A) cell proliferation (Ki-67 labeling index) and (B) apoptotic index of MCF-7 breast tumors in OVX athymic mice.

between FO and SDG (IGF-1R, ER protein), or the same as SDG but higher than FO (BCL2). However, with the exception of HER2 protein, the effects of combined SDG and FO did not differ significantly from that of SDG or FO alone. No significant associations of SDG, FO, or combined SDG and FO with expressions of PS2, ER-α, ER-β, and VEGF mRNA and PAKT protein were observed.

4 Discussion

This study demonstrated in athymic mice with low circulating levels of estrogen mimicking the post-menopausal situation, that TAM, alone or in combination with SDG, FO, or combined SDG and FO, can reduce the growth of established ER+ human breast tumors (MCF-7). However, FO gave the strongest effect in reducing the palpable tumor size of TAM-treated tumors. FO and SDG combined with TAM also reduced the cell proliferation and increased the apoptosis but TAM+FO had a stronger effect in increasing apoptosis; this may explain why FO had a greater tumor growth inhibitory effect than SDG when combined with TAM. The results agree with our earlier observations that FS has components that can increase the effectiveness of TAM [9–11] and demonstrate that FO and SDG are two components contributing to this effect.

In our unpublished study conducted at the same time, with the same experimental design and mice and BD from the same batch as those used in this study, the palpable tumor in mice treated for 8 wk with the BD alone (*i.e.* without TAM treatment) regressed by 43±16%. In this study, mice treated with TAM and fed the same BD had palpable tumor reduction of 18±20%. In addition, TAM-

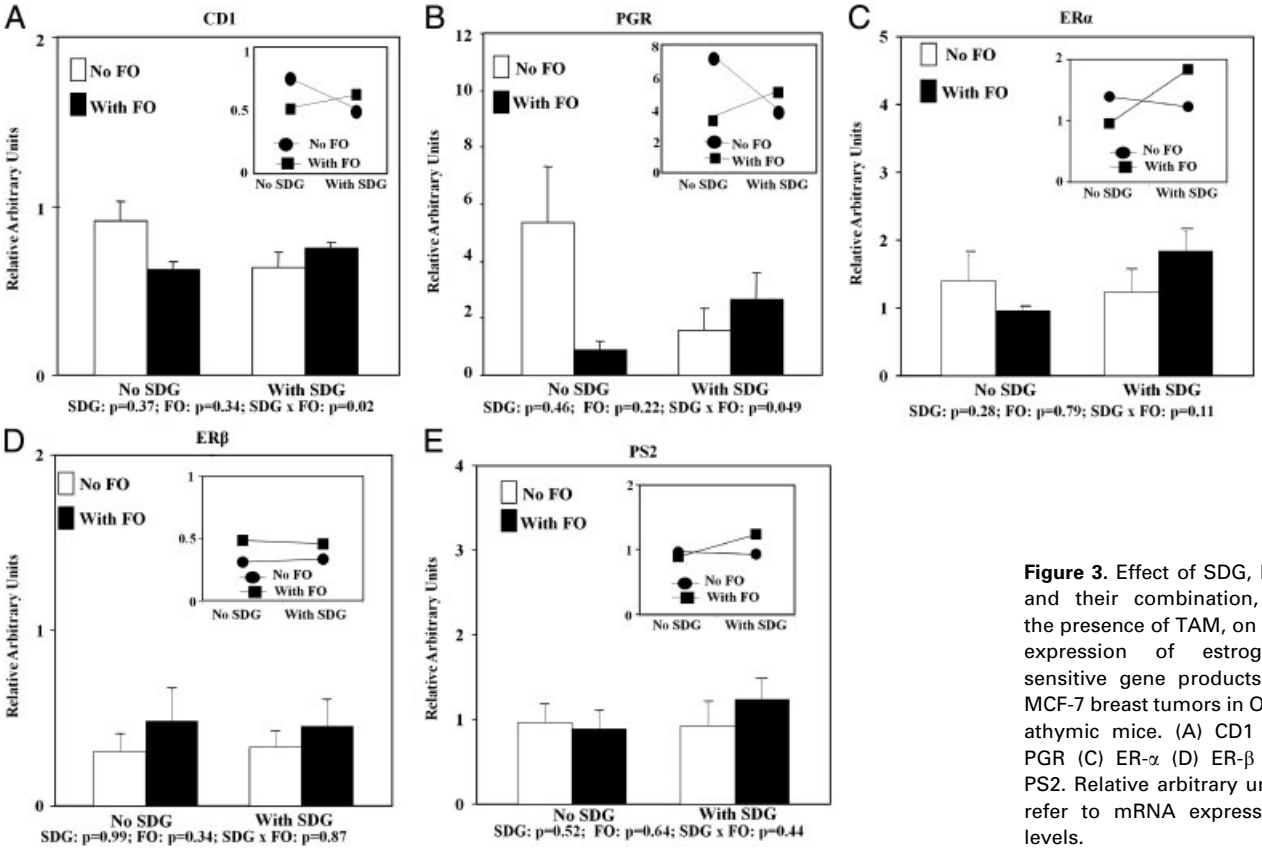

Figure 3. Effect of SDG, FO, and their combination, in the presence of TAM, on the expression of estrogen-sensitive gene products of MCF-7 breast tumors in OVX athymic mice. (A) CD1 (B) PGR (C) ER-α (D) ER-β (E) PS2. Relative arbitrary units refer to mRNA expression levels.

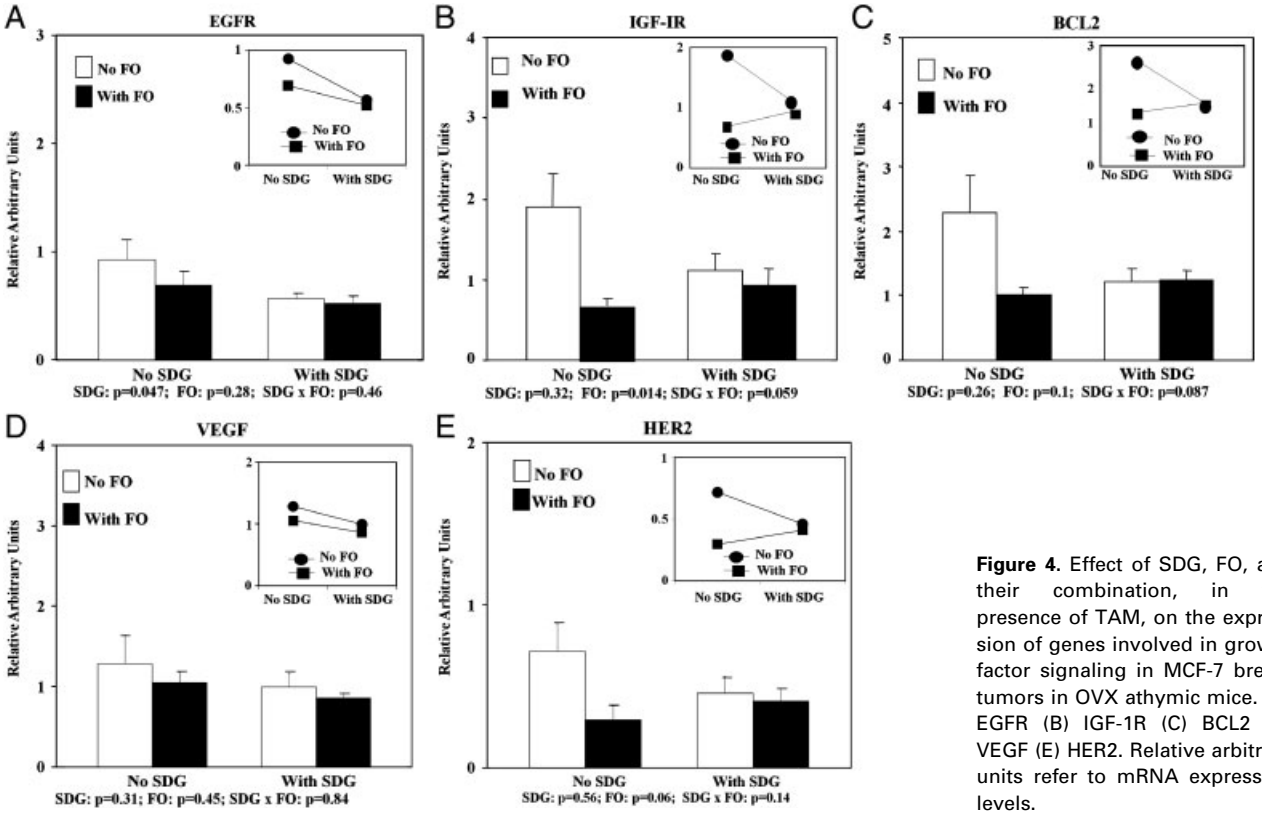

Figure 4. Effect of SDG, FO, and their combination, in the presence of TAM, on the expression of genes involved in growth factor signaling in MCF-7 breast tumors in OVX athymic mice. (A) EGFR (B) IGF-1R (C) BCL2 (D) VEGF (E) HER2. Relative arbitrary units refer to mRNA expression levels.

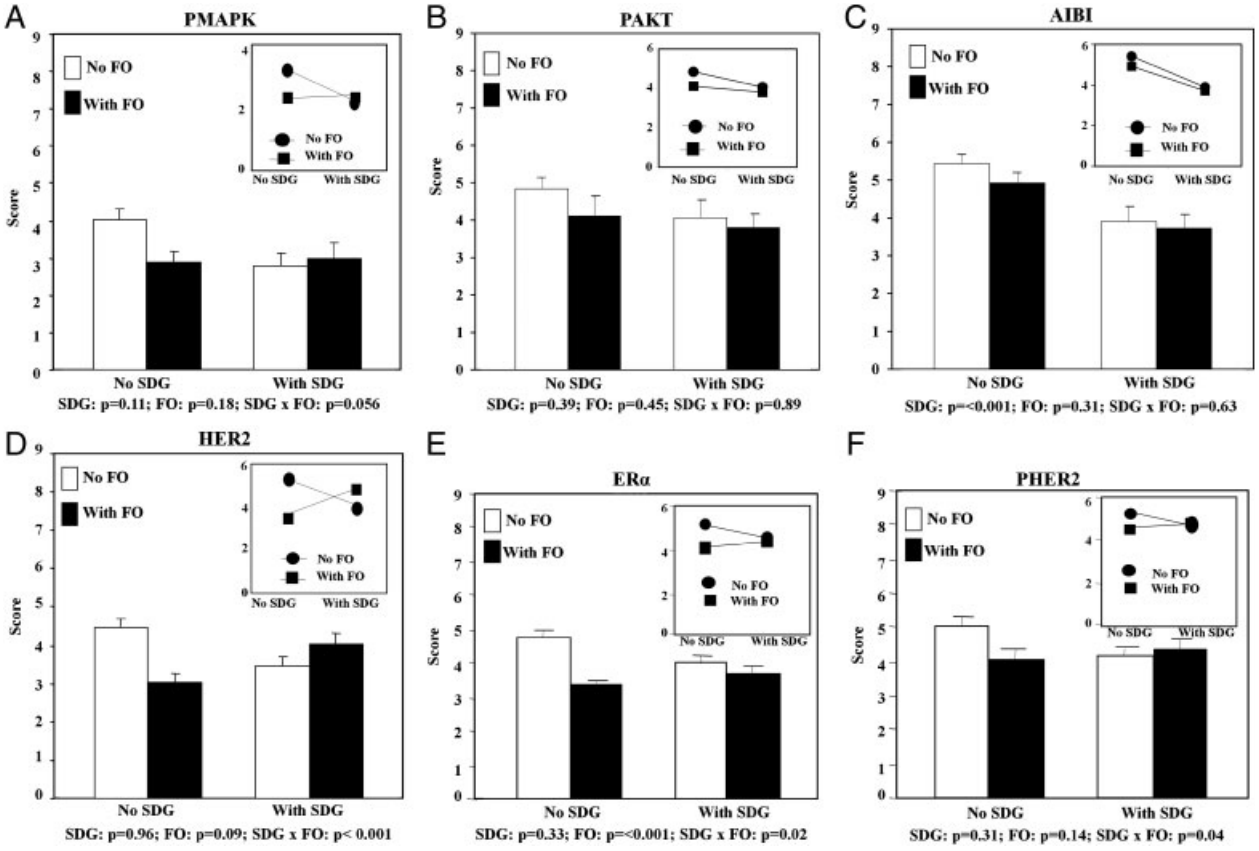

Figure 5. Effect of SDG, FO, and their combination, in the presence of TAM, on the expression of proteins involved in the growth factor signaling pathway in MCF-7 breast tumors in OVX athymic mice. (A) PMAPK (B) PAKT (C) AIBI (D) HER2 (E) ER-α (F) PHER2. Score refers to protein expression measured by immunohistochemistry.

treated tumors had higher cell proliferation ($33.15 \pm 3.2\%$ *versus* $27.47 \pm 5.5\%$) and lower apoptosis (2.48 ± 0.5 *versus* 3.20 ± 0.6 cells/mm^2) than tumors treated only with BD. This indicates that TAM may have exerted some weak estrogenic effect. This agrees with our previous studies that observed a greater tumor reducing effect of the BD than TAM+BD [9, 10] and thus also indicates that our model system is working.

In classical ER signaling, estrogen binds to the ER causing it to undergo a conformational change, dimerization, and phosphorylation, thereby activating the complex [19]. The activated estrogen–ER complex then binds to DNA, which then promotes recruitment of ER activators (*e.g.* AIBI) and transcription factors that activate the transcription of estrogen-sensitive genes such as PS2, CD1, and PGR [4], which promote cell proliferation. TAM works in part by competing with E2 for binding to the ER and causing the dimerized ER to bind to DNA and recruit co-repressor proteins, which inhibit the transcriptional activation of such genes, thereby decreasing cell proliferation [20]. At low circulating E2 levels or low tumor E2 synthesis, however, TAM binding to ER may also induce a weak estrogenic/tumor promoting effect after prolonged exposure [20], which was observed in this study.

Both SDG and FO induced a lowering of the expressions of PGR and CD1 mRNA and ER-α protein but had no effect on mRNA expressions of PS2, ER-α, and ER-β in TAM-treated tumors. However, only SDG, without or with FO, induced lower expression of AIB1 protein, a co-activator of ER, indicating that SDG may exert a stronger modulating effect than FO on the ER signaling pathway. Reduction of AIB1 expression is particularly important as high expression of A1B1 has been associated with shorter disease-free survival in patients treated with TAM as an adjuvant [20]. The SDG metabolites END and ENL have previously been shown to possess weak estrogenic and antiestrogenic properties *in vitro* [7, 21] but in agreement with other animal studies (reviewed in [8]), our results showed that they do not have estrogenic, tumor-promoting effect on ER+ tumors under low circulating estrogen concentration. Of greater significance in this study is that compared with the TAM control, the treatments did not induce significantly higher expression of the estrogen target biomarkers, suggesting that there is no synergistic action of these treatments with TAM to induce estrogenic effect; this is desirable for breast cancer patients currently having TAM treatment and consuming these compounds.

Growth factor signaling occurs through the growth factor receptors such as EGFR, HER2, and IGF-1R, which are present at the cell membrane and are known to interact. Activation through binding with ligands such as EGF and IGF-1 leads to increased AKT and MAPK activities [22, 23]. These can increase the expression of the anti-apoptotic gene BCL2 [23] or change the activity of cell cycle regulators such as CD1 [23], which then result in tumor growth and survival. The activation of MAPK, *i.e.* PMAPK, through increased growth factor activity, can also lead to phosphorylation and activation of the nuclear ER and increased transcription of the estrogen-sensitive genes in the absence of E2 ligand, which then leads to tumor growth [23, 24]. It has been postulated that TAM-induced tumor re-growth or resistance is associated with an up-regulation of the growth factor signaling pathway and increased cross-talk amongst the receptors with membrane-bound ER [24, 25]. Therefore, to improve the effectiveness of TAM, a treatment strategy is to inhibit the expression and activation of growth factor signaling pathway.

In this study, compared with TAM treatment alone (control), TAM combined with SDG or FO significantly reduced the mRNA expressions of IGF-1R and BCl2 and the protein expressions of HER2, PHER2, and PMAPK. In addition, SDG has significant overall effect in reducing EGFR mRNA while FO tends to have an overall effect in reducing HER2 mRNA expression. PAKT was reduced although not significantly. All these changes in gene and protein expressions indicate that SDG and particularly FO can strengthen the TAM effectiveness in part through down-regulation of the signaling of growth factors IGF-1R, HER2, and EGFR expressions, which are known to interact [22, 24, 26] and possibly also through decreased cross-talk between ER signaling and growth factor signaling, which then led to reduced downstream activation, particularly of MAPK, and decreased cell proliferation and increased apoptosis. Reduced IGF-1R may be due in part to a reduction in the IGF1 expression as FS and its SDG have been shown to lower the IGF1 level in plasma of carcinogen and non-carcinogen-treated rats [27] and in established ER− tumors [28] in athymic mice. Previous studies have shown that FS can increase the apoptosis and reduce the cell proliferation and protein expressions of HER2, IGF-1R, and CD1 in TAM-treated tumors [11] and this study indicates that FO and SDG likely contributed to that FS effect.

Without TAM, FS and the END and ENL metabolites of SDG have been shown to reduce the VEGF expression and angiogenesis in established ER+ tumors at high levels of estrogen [29] and in ER− tumors [30]. However, in this study, no similar effect of SDG or FO on VEGF expression was observed in the ER+TAM-treated tumors at low level of estrogen. This indicates that the reduction in the growth of TAM-treated tumors was not primarily due to reduction in angiogenesis.

The effect of FO may be related to its high content of the n-3 fatty acid, ALA [31]. ALA has previously been shown to have anti-cancer effects with multiple mechanisms of action [32, 33]. ALA can be metabolized to a small extent to eicosapentaenoic acid and docosahexaenoic acid, competing for the same elongase/desaturase enzymes that the n-6 fatty acids use for metabolism. This eventually leads to reduced production of inflammatory eicosanoids, which are found in higher concentrations in cancer cells and are known to promote cell proliferation [34]. Eicosapentaenoic acid has been shown to restore by 35% the TAM sensitivity in ER+ breast cancer cells with high kinase activity through a reduction in kinase activity [35]. In cancer cells that naturally overexpress HER2 (BT-474 and SKBr-3), ALA has been shown to suppress HER2 mRNA expression and diminish the proteolytic cleavage site of the growth factor, thereby reducing the probability of activation that leads to tumor growth [36]. Docosahexaenoic acid has been shown to reduce the growth of colon cancer cells in part through an accumulation of cells in the G1 phase of the cell cycle by decreasing CD1 levels and increasing cyclin-dependent kinase inhibitors [37]. Since both FO and SDG lowered the CD1 expression, it would be of interest to analyze the levels of cyclin-dependent kinase inhibitors in the future to further verify the effect of FO, ALA, and SDG and its metabolites END and ENL.

Interaction was observed between SDG and FO on several biomarkers of tumor growth. However, the effect of combined SDG and FO on biomarkers of growth of TAM-treated tumors did not differ significantly from those caused by SDG or FO alone except for the increase in expression of HER2 protein. It is surmised that any antagonist interaction may in part be related to the ability of ALA in FO to reduce tumor growth through increased lipid peroxidation in the tumors [38], with SDG counteracting it by acting as an anti-oxidant [39, 40]. The generation of reactive oxygen species, a by-product of lipid metabolism, is one of the suggested mechanisms whereby apoptosis may take place [41] but reactive oxygen species may be scavenged by antioxidants, such as SDG, which can then inhibit apoptosis [42, 43]. The mechanisms of any antagonistic interaction between SDG and ALA remain to be determined.

Although there was a significant interaction between SDG and FO, the biomarker data indicate that the combined SDG and FO still resulted in a better tumor size reducing effect than TAM treatment alone. FS, which is rich in both SDG and FO, has not been shown to have a tumor-stimulatory or estrogenic effect when combined with TAM; in fact, FS has been shown to increase TAM's effectiveness [9–11]. It is possible, however, that other components in FS including dietary fiber and other phytochemicals also play a role in the anti-cancer properties of FS.

This study has several limitations, which may be considered in future research.

The study was not conducted long enough to demonstrate a definitive TAM resistance. Therefore, it would be of interest to test the effects of FO and SDG for a longer period of time or to test them using established TAM-resistant

MCF-7 cells. An OVX athymic mouse model was used because it allows the injection of human tumor tissues without rejection. However, although widely used, this model is different from humans including in immunology (the mice are immunodeficient), pharmacology, psychological characteristics, and endocrine factors that may all influence the tumor growth. Therefore, results may not always translate to humans and need to be confirmed in clinical trials in the future. MCF-7 cell line was used because it is ER+ and has been used extensively to study the effects of the drug TAM. However, one limitation of this cell line is its low expression of the aromatase gene, whose function is to convert circulating androgens into estrogens. Hence, tumors will not grow unless estrogenic compounds are provided. In contrast, post-menopausal breast cancer expresses aromatase and therefore can grow despite low levels of circulating estrogen. It would be of particular interest to test the effects of FS and its components on MCF-7 that is transfected with an aromatase gene, thus having the ability to produce estrogens from androgens. This, in combination with an androgen supplement, would allow for a more effective simulation of the post-menopausal breast cancer, which has aromatase gene and is able to synthesize estrogen. SDG and FO were the only FS components tested, and at levels equivalent to that found in a 10% FS diet, which was previously found to be effective. It would be of interest to do a dose response study to determine the optimum concentration for protective effect. Only selected receptors of the growth factor signaling and gene products of the estrogen-sensitive signaling pathway were tested to observe the effects of SDG and FO on tumor growth. It would be useful to conduct a pathway-related study in which all of the biomarkers including the genes, proteins, and phosphorylated proteins in certain signaling pathways (*e.g.* ER, growth factor, angiogenesis) are measured so that a more complete mechanistic picture may be drawn.

In conclusion, both SDG and FO can reduce the growth of TAM-treated tumors, with FO being more effective. The mechanisms include decreasing cell proliferation and increasing apoptosis, particularly with FO, in part through a modulation of the ER- and growth factor-mediated signaling pathways. A significant interaction between SDG and FO did not result in greater tumor reduction compared with the effect of SDG or FO alone, but also did not result in greater palpable tumor growth than TAM treatment alone. This might explain why FS, which contains both of these components, still has been shown to increase the effectiveness of TAM and to have no tumor promoting effect [9–11].

In this study, the FS components were administered at a level found in a 10% FS diet; this is equivalent to the human consumption of 25–50 g (2.5–5 tablespoons) of FS *per* day depending on the person's food consumption [11]. Although this dose of FS is feasible for human consumption, some individuals may not be able to tolerate the large bulk of FS. However, with the findings that either SDG or FO has protective effect with TAM, such individuals may opt to consume these isolated components instead of FS. Manufacturers have started to produce pills that contain large amounts of lignans, and FO is readily available in stores. Nevertheless, although the results are promising, definitive recommendations on the use of FS, SDG, or FO as complementary treatment with TAM will depend on the results of future clinical trials testing their effects on breast cancer patients.

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