Dietary Flaxseed Interaction With Tamoxifen Induced Tumor Regression in Athymic Mice With MCF-7 Xenografts by Downregulating the Expression of Estrogen Related Gene Products and Signal Transduction Pathways

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Abstract: Our previous short-term study has shown that 10% flaxseed (FS) inhibits the growth of human estrogen dependent estrogen receptor positive breast tumors (MCF-7) xenografts in ovariectomized (OVX) athymic mice and enhances the tumor inhibitory effect of tamoxifen (TAM). This study determined the long-term effect of 5% and 10% FS, with or without TAM, on the growth of MCF-7 xenografts in athymic mice and the potential mechanisms of actions. OVX mice with established MCF-7 tumors were treated with basal diet (control), 5% FS (5FS), 10% FS (10FS), and TAM (5 mg/pellet, 60-day release), alone or in combination, for 16 wk without estrogen supplementation. Tumor growth was monitored weekly. At sacrifice, the tumors were analyzed by immunohistochemistry for cell proliferation, apoptosis, and expression of estrogen-related genes and signal transduction pathways. Both 5FS and 10FS regressed the pretreatment tumor size by over 90% similar to control. TAM initially regressed the tumors but then induced a regrowth; thus, only a final 6% reduction from pretreatment tumor size was achieved, which was attenuated by combining TAM with 10FS but not with 5FS. TAM combined with 10FS regressed tumors to 55% of pretreatment tumor size due to decreased cell proliferation and increased apoptosis. The expressions of cyclin D1, estrogen receptor α, human epidermal growth factor receptor 2, and insulin-like growth factor 1 receptor in the TAM group were significantly reduced when TAM was combined with 5FS or 10FS. In conclusion, after long-term treatment, FS did not stimulate tumor growth and combined with TAM, regressed tumor size in part due to downregulation of the expression of estrogen-related gene products and signal transduction pathways.

Introduction

Flaxseed (FS) is exceptionally rich in the phytoestrogen secoisolariciresinol diglycoside (SDG) (1,2), which is metabolized by intestinal microbiota to the mammalian lignans enterodiol (ED) and enterolactone (EL) (2). Both ED and EL have chemical structural similarity to the natural estrogen, 17-β estradiol (E2), suggesting that they may have weak estrogenic and/or antiestrogenic properties and hence potential anticancer effects. FS also contains 40% oil, about half of which is α-linolenic acid (ALA), an n-3 fatty acid (3). Flaxseed (FS) is exceptionally rich in the phytoestrogen secoisolariciresinol diglycoside (SDG) (1,2), which is metabolized by intestinal microbiota to the mammalian lignans enterodiol (ED) and enterolactone (EL) (2). Both ED and EL have chemical structural similarity to the natural estrogen, 17-β estradiol (E2), suggesting that they may have weak estrogenic and/or antiestrogenic properties and hence potential anticancer effects. FS also contains 40% oil, about half of which is α-linolenic acid (ALA), an n-3 fatty acid (3).

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tested, and the treatment time was short (8 wk). Thus, it is unclear whether the interactive action of FS and TAM is dose dependent and whether the dose of FS can be reduced without decreasing its efficacy. Because the acquired, not de novo, resistance to hormonal therapy usually occurs after prolonged treatment, it is also unknown if the interactive effect of FS and TAM is sustained for a longer treatment period, particularly under low E2 level.

The mechanism(s) by which FS interacts with TAM to regress tumor size is unclear. Mounting evidence indicates that breast cancer cells become resistant to TAM by developing hypersensitivity to the estrogenic properties of TAM after long-term treatment (17). TAM resistance is also associated with alteration of ER and signal transduction pathways such as overexpression of receptors of epidermal growth factor (EGF) family, e.g., EGF receptor (EGFR) and HER2, and insulin-like growth factor-I (IGF-I), e.g., IGF-I receptor (IGF-IR) (18). Besides its estrogen-related properties, FS has been shown to downregulate the expression of EGFR, HER2, and IGF-I in breast cancer cells (7,11). Thus, we hypothesize that dietary FS may attenuate TAM-induced tumor regrowth through the modulation of ER-related and/or signal transduction pathways.

The current study was designed to determine, with E2 deprivation, 1) whether FS has dose-related tumor inhibitory effects on human ER+ breast cancer, 2) whether the inhibitory effect of FS on the growth of TAM-treated tumors can be sustained for a longer treatment period, and 3) the potential mechanisms by which FS exerts this action.

Materials and Methods

Cell Line and Cell Culture

MCF-7, human ER+ breast cancer cell line (The American Type Culture Collection, Manassas, VA), was routinely cultured in Dulbecco’s minimum essential medium/F12 with 10% fetal bovine serum and 1% antibiotics. For injection, the cells with 70–90% confluence were trypsinized and resuspended in serum-free medium with 1:1 Matrigel (BD Biosciences, Franklin Lakes, NJ) at a concentration of $1 \times 10^7$ cells/ml on ice. Cell viability, over 90%, was determined by trypan blue exclusion assay before and after cell injection.

Animal and Diets

OVX BALB/c nu/nu athymic mice (Charles River Canada, St-Constant, Province of Québec, Canada), 5–6 wk old, were maintained in microisolator cages (4/cage) within a pathogen-free isolation facility with 12 light/dark cycle at 22–24°C and 50% humidity. Animal care and use followed the Guide to the Care and Use of Experimental Animals (19), and the experimental protocol was approved by the University of Toronto Animal Care Committee.

The basal diet (BD) was based on the American Institute of Nutrition 93G formulation (20) modified to have 7% corn oil instead of soybean oil. The FS diets were the BD supplemented with 5% or 10% freshly ground FS (Linott Variety) corrected for the contribution of FS to fat, fiber, and protein components so that the energy values of the diets were the same (5,6,16). Diets were prepared by Dyets Inc. (Bethlehem, PA) and sterilized with Co$^{60}$ radiation by Isomedix Corp. (Whitby, Ontario, Canada).

Experimental Design

Mice were anesthetized i.p. with ketamine/zylazine mixture after 7-day acclimatization while being fed the BD. A 3 mm incision was made over the skin in the interscapular region, and a sterilized 17β-estradiol (E2) pellet (1.7 mg, 60-day release, produce 3–4 nM E2 blood level; Innovative Research of America, Sarasota, FL) was implanted subcutaneously followed by sealing of the incision with tissue adhesive Vetbond (3M Animal Care Products, St. Paul, MN). A 50 µl cell suspension containing 5 $\times 10^5$ cells was injected into each of the 4 mammary fat pads, thus producing 4 sites of mammary tumor growth per mouse. Tumors were palpated weekly. The tumor surface area was calculated using the formula $(length/2 \times width/2) \times \pi$. At Week 6, when tumor area reached ~40 mm$^2$, the mice were randomly divided into 7 groups such that their tumor size and body weight were similar.

To produce low circulating E2 level, the existing E2 pellet was removed except that the positive control (+E2) had a new E2 pellet (1.7 mg, 60-day release) implanted and fed BD. Thus, all 6 treatment groups (Groups 1–6) did not have the E2 pellet replaced, resulting in about 35 pM of circulating E2 in the ovariecotomized nude mice (21). Group 1(0FS) was fed the BD only to serve as negative control. Group 2 (5FS) was fed 5% FS diet, while Group 3 (10FS) was fed 10% FS diet. Group 4 (TAM/0FS) was fed the BD and subcutaneously implanted a TAM pellet (5 mg, 60-day release, produce 3–4 ng/ml blood level; Innovative Research of America). Group 5 (TAM/5FS) was implanted a TAM pellet and fed 5% FS diet. Group 6 (TAM/10FS) was implanted a TAM pellet and fed 10% FS diet. The mice in Groups 1–3, which had no TAM pellet implant, received a placebo pellet (Innovative Research of America) implanted subcutaneously. The food intake, body weight, and tumor size were monitored weekly. Primary tumor area was calculated based on the formula $(length/2 \times width/2) \times \pi$. Mice in the +E2 group were sacrificed at Week 14 (8 wk posttreatment) due to high tumor burden. All remaining mice had the TAM or placebo pellet replaced at Week 14 (8 wk posttreatment) and were sacrificed at week 22 (16 wk posttreatment) by CO$^2$ asphyxiation. At necropsy, body weight, primary tumor weight and volume, and weights of major organs including uterus were recorded. Primary tumor volume was calculated based on the formula $(length/2 \times width/2 \times thickness/2) \times \pi$. 
Assessment of Tumor Regression

For further analysis of tumor regression pattern, tumor areas at Week 21 were compared with their initial area at Week 6. Tumors that initially regressed in size in response to E2 pellet removal but grew back >30% bigger than the pretreatment surface area during the dietary treatment period were assessed as “tumor size increased.” The tumors that changed ±30% of pretreatment size were assessed as “not regressed.” Tumors that regressed to nonpalpable size were classified as “completely regressed” tumors. All the remaining tumors (i.e., tumor size reduced >30% of the pretreatment area but not completely regressed) were classified as “regressing tumors.”

Immunohistochemistry

The 5 μm sections of formalin-fixed paraffin-embedded tumor tissue, n = 8–12/group, were deparaffinized and rehydrated. Endogenous peroxidase was blocked with aqueous 3% H2O2. The antigen was retrieved by heating in 0.01 M citrate buffer at pH 6.0 for 20 min in a microwave oven. The primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, except otherwise indicated) used were rabbit antihuman polyclonal and diluted as follows: Ki-67 (H-300), at 2 μg/ml; cyclin D1 (H-295), 1 μg/ml; ERα (HC-20), 0.5 μg/ml; IGF-IRα (sc-712), 1 μg/ml; HER2, 0.25 μg/ml (DakoCytomation, Mississauga, Ontario, Canada); ERβ (PA1-311), 4 μg/ml (Affinity BioReagents, Golden, CO). The antibodies were diluted with the Diluent Buffer (DakoCytomation) that blocks nonspecific antigens, incubated at 4°C overnight followed by incubation with biotinylated swine antirabbit IgG (DakoCytomation). Streptavidin-HRP and AEC substrate chromogen incubation with biotinylated swine antirabbit IgG (DakoCytomation). Streptavidin-HRP and AEC substrate chromogen were used to demonstrate the antigens. A positive and a negative, without primary antibody, control slides were run at the same batch staining. All slides were read blindly under a light microscope at ×400 magnification.

Apoptosis

In situ TUNEL assay was utilized to demonstrate DNA fragmentation by ApopTag Detection Kit (Chemicon, Temecula, CA) and ran based on the manufacturer’s protocol as reported previously (11,16). Briefly, following pretreatment with proteinase kinase (20 μg/ml), the sections were incubated with terminal transferase and digoxigenin dUTP at 37°C for 1 h. After washing, the sections were incubated with antidigoxigenin antibody coupled to horseradish peroxidase for 30 min at room temperature. The slides were then incubated with diaminobenzidine for 6 min and counterstained with methyl green. The number of breast carcinoma cells showing positive nuclear immunoreactivity was counted and expressed as apoptotic cell number/mm2 at ×400 magnification. All assays were done blind to the treatment groups.

Statistical Analysis

Data are presented as means ± SE of the mean (SEM). Analysis of variance (ANOVA) with general linear model repeated measures procedure was used to determine palpable tumor growth difference among treatment groups over treatment time followed by post hoc Tukey test. The differences in tumor area between the pretreatment, i.e., at Week 6, and posttreatments in the same group were assessed by paired t-test. One-way ANOVA followed by post hoc Tukey test was used to determine the differences among groups in food intake, relative organ weights, final tumor volume and weight, and various biomarkers, whereas the body weight gain was assessed by Kruskal-Wallis 1-way ANOVA on ranks followed by Dunn test due to non-normality. χ2 test was used to assess the proportions (frequencies) of tumor with different growth (regression) patterns. The relationship of several tumor biomarkers to cell proliferation and apoptosis was analyzed by linear regression. Because of the shorter treatment time and high E2 supplementation, the +E2 group just served as positive control for the model system; results from this group were not compared with the other treatment groups except for tumor data. All statistical analyses were done by SPSS version 10 (SPSS Inc., Chicago, IL), and the significant level was set at P < 0.05.

Results

Food Intake, Body, and Organ Weights

Food intake in the SFS and 10FS groups did not differ from each other and the BD group (0FS) (Table 1). The TAM group (TAM/0FS) had significantly lower food intake than the control and the FS groups. However, food intake increased significantly when TAM was combined with 10FS (TAM/10FS; Table 1). Body weight gain followed a similar pattern as the food intake, where TAM alone caused the lowest final body weight and body weight gain, which were improved by combining it with dietary FS, although not significantly (Table 1). Except for the uterus, there was no significant difference among groups in the weight of major organs such as liver, lungs, kidneys, and brain (data not shown). The TAM group had a significantly higher uterine weight compared to the FS groups, which was decreased when dietary FS was combined with TAM treatment, although not significantly (Table 1).

Palpable Tumor Growth

The average tumor size at the beginning of treatments at Week 6 did not significantly differ among groups (Table 2). The palpable tumor area of the positive control (+E2) increased consistently (Fig. 1A). At Week 14, 8 wk posttreatment, the tumor area in the positive control group
increased by 250% (P < 0.01) from the pretreatment value and was higher than that in the treatment groups without E2 supplementation (P < 0.01; Fig. 1A), indicating that the model system was working, and the tumor growth was not affected by the surgical procedures. This group was sacrificed at Week 14 because of the large tumor burden. After withdrawal of E2 pellet at Week 6, the tumors in all treatment groups initially regressed (Fig. 1A). The primary tumors in the 0FS, 5FS, and 10FS groups consistently regressed to the lowest level at Week 14 (8 treatment wk); this was retained to the end of the experiment at Week 21, resulting in significant >90% reduction of the pretreatment value (P < 0.01; Fig. 1A, Table 2). TAM alone (TAM/0FS) initially regressed the tumor size by 31% at Week 14 (P < 0.05) but then induced a regrowth so that at the end of the experiment, the tumor size was 36% higher (P < 0.05) than that at Week 14 and only 6% lower (not significant) than the pretreatment size (Fig. 1A, Table 2). The final tumor size in the TAM/5FS group did not differ significantly from the pretreatment size or the TAM group (TAM/0FS). However, the tumors in the TAM/10FS group significantly regressed by 55% of the pretreatment size and were 54% smaller than that in the TAM/0FS group (P < 0.05; Fig. 1A, Table 2). At sacrifice, both the final tumor weight and volume followed a similar pattern as the palpable tumor area (Fig. 1B and 1C).

At endpoint, all tumors were categorized as growing or regressing to further clarify the response of individual tumor to treatments (Table 2). Tumors in the 0FS, 5FS, and 10FS groups either completely or partly regressed. However, the TAM/0FS group had the least tumor regression, with none completely regressed,<25% partly regressed, most not regressed, and some increased in size. Similarly, no significant influence on the tumor growth pattern was seen when 5FS was combined with TAM. However, on increasing FS to 10% (TAM/10FS group), 67% of the tumors regressed, of which 30% (11 out of 36) completely regressed.

**Cell Proliferation and Apoptosis**

The 5FS and 10FS groups did not differ significantly from each other, and both did not differ from the control (0FS) in cell proliferation measured as Ki67 LI (Fig. 2A). However, the TAM alone (TAM/0FS) group had a significant >50% higher LI than the control and FS groups (Fig. 2A). The 10FS combined with TAM (TAM/10FS) significantly reduced the cell proliferation to a similar level as the control, whereas TAM/5FS was not significantly different from TAM alone.

There was no significant difference in tumor cell apoptosis among 0FS, 5FS, and 10FS groups (Fig. 2B); however, they were all significantly greater than TAM group. Similar to the Ki-67 index, combining 5FS with TAM did not affect apoptosis. However, apoptosis was significantly increased when 10FS was combined with TAM, although it was still significantly lower than that of 0FS, 5FS, and 10FS (Fig. 2B).

### Table 1. Effect of 5% (5FS) and 10% Flaxseed (10FS), and Tamoxifen (TAM), Alone or in Combination, on Food Intake, and Body and Uterine Weight in Ovariectomized Nude Mice With Established MCF-7 Xenografts

<table>
<thead>
<tr>
<th>Group</th>
<th>Food intake (g/mouse)</th>
<th>Body weight (g)</th>
<th>Uterine weight (mg/g bodyweight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 6</td>
<td>Week 22</td>
<td>Gain</td>
</tr>
<tr>
<td>0FS</td>
<td>315.6 ± 2.2*</td>
<td>19.49 ± 0.37</td>
<td>1.36 ± 0.22*</td>
</tr>
<tr>
<td>5FS</td>
<td>319.8 ± 7.8*</td>
<td>18.89 ± 0.43</td>
<td>1.57 ± 0.19*</td>
</tr>
<tr>
<td>10FS</td>
<td>318.3 ± 4.6*</td>
<td>18.97 ± 0.32</td>
<td>1.41 ± 0.18*</td>
</tr>
<tr>
<td>TAM/0FS</td>
<td>287.3 ± 5.4†</td>
<td>18.34 ± 0.33</td>
<td>2.45 ± 0.21†</td>
</tr>
<tr>
<td>TAM/5FS</td>
<td>304.7 ± 2.2†</td>
<td>19.3 ± 0.46</td>
<td>2.14 ± 0.19†</td>
</tr>
<tr>
<td>TAM/10FS</td>
<td>311.2 ± 5.5†</td>
<td>19.37 ± 0.45</td>
<td>1.88 ± 0.21†</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Different symbols (*) indicate significant difference among treatment groups [1-way analysis of variance (ANOVA), followed by post hoc Tukey’s test for food intake and uterine weight and Kruskal-Wallis 1-Way ANOVA on ranks, followed by Dunn’s test for body weight gain].

### Table 2. Effect of 5% Flaxseed (5FS) and 10% FS (10FS) and Tamoxifen (TAM), Alone or in Combination, on Tumor Area and Regression Pattern in Ovariectomized Nude Mice With Established MCF-7 Xenografts

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of regressed tumor (%)</th>
<th>Tumor area/mouse (mm²)</th>
<th>n</th>
<th>Week 6</th>
<th>Week 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>0FS</td>
<td>12</td>
<td>40.7 ± 3.6</td>
<td>3.3 ± 1.6†,**</td>
<td>48</td>
<td>36 (75.0)</td>
</tr>
<tr>
<td>5FS</td>
<td>10</td>
<td>41.2 ± 2.8</td>
<td>3.2 ± 1.7†,**</td>
<td>39</td>
<td>25 (64.1)</td>
</tr>
<tr>
<td>10FS</td>
<td>12</td>
<td>41.6 ± 4.0</td>
<td>2.7 ± 1.4†,**</td>
<td>48</td>
<td>36 (75.0)</td>
</tr>
<tr>
<td>TAM/0FS</td>
<td>10</td>
<td>41.3 ± 3.4</td>
<td>38.8 ± 4.6†</td>
<td>36</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>TAM/5FS</td>
<td>10</td>
<td>42.0 ± 3.6</td>
<td>34.6 ± 4.0†</td>
<td>36</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>TAM/10FS</td>
<td>9</td>
<td>39.6 ± 1.4</td>
<td>17.7 ± 5.2†,**</td>
<td>36</td>
<td>11 (30.6)</td>
</tr>
</tbody>
</table>

Abbreviation is as follows: MCF-7, estrogen receptor positive human breast cancer. Data are means ± SEM. Different symbols (†, §, ‡) indicate significant difference among groups (1-way analysis of variance followed by Tukey test); ***, P < 0.01 vs. Week 6 within the same group (paired t-test).

χ² test for trend; different symbols (†, §, ‡) indicate significant difference among groups (P < 0.05).
Figure 1. Effect of 5% flaxseed (5FS) and 10% dietary FS (10FS) and tamoxifen (TAM), alone or in combination, on palpable tumor growth over treatment time (A); final tumor volume (B); and final tumor weight at sacrifice (C) of MCF-7 breast cancer in ovariectomized nude mice. Data are means ± SEM. Treatment groups with different letters (a–d) indicate significant difference at $P<0.05$, using general linear model, 1-way analysis of variance (ANOVA) repeated measures followed by Tukey test (A), or 1-way ANOVA followed by Tukey test (B and C).

Cellular Biomarkers

No significant difference in tumor cyclin D1 (Fig. 3A) expression was observed among 0FS, 5FS, and 10FS groups. However, TAM alone (TAM/0FS) significantly enhanced the expression of cyclin D1 compared to 0FS, 5FS, and 10FS groups. This enhanced cyclin D1 expression was significantly reduced when 5FS or 10FS was combined with TAM treatment.

FS showed a dose-related tendency to decrease ERα expression, whereas TAM induced a slight increase in its expression compared to the control (Fig. 3B). The combination of 5FS or 10FS with TAM significantly reduced ERβ expression.

Figure 2. Effect of 5% flaxseed (5FS) and 10% dietary FS (10FS) and tamoxifen (TAM), alone or in combination, on Ki-67 labeling (A) and apoptotic indices (B) of MCF-7 breast tumors in ovariectomized nude mice at low estradiol levels. Different letters (a–c) indicate significant difference at $P<0.05$ among groups by 1-way analysis of variance followed by post hoc Tukey test. Data are means ± SEM.
Figure 3. Effect of 5% flaxseed (5FS) and 10% dietary FS (10FS) and tamoxifen (TAM), alone or in combination, on the expression (score) of estrogen sensitive genes of MCF-7 breast tumors in ovariectomized nude mice at low estradiol levels. A: Cyclin D1. B: Estrogen receptor α (ERα). C: Estrogen receptor β (ERβ). Different letters (a–b) indicate significant difference at \( P < 0.05 \) among groups by 1-way analysis of variance followed by post hoc Tukey test. Data are means ± SEM.

There was no significant difference in ERβ expression among groups (Fig. 3C).

There was no significant difference in HER2 expression among FS and control groups (Fig. 4A). However, a significantly higher HER2 expression was observed with TAM treatment, compared to the control, which was then significantly reduced when 5FS or 10FS was combined with TAM. IGF-IR expression did not differ among FS and control groups (Fig. 4B). However, the high IGF-IR expression in the TAM/0FS group was significantly reduced when TAM treatment was combined with 5FS or 10 FS.

There was a significant inverse association between Ki-67 index and apoptosis (Table 3). The cell proliferation was significantly positively associated with cyclin D1, HER2, and IGF-IR, while apoptosis was negatively associated with cyclin D1 and HER2 expressions (Table 3).

| Table 3. Relationship of Tumor Biomarkers to Cell Proliferation and Apoptosis in Ovariectomized Nude Mice With Established MCF-7 Xenografts\(^a\) |
|-----------------|-----------------|-----------------|
|                 | \( r \) Value   | \( P \) Value   |
| Ki-67 vs.       | −0.62           | <0.001          |
| Apoptosis       | 0.33            | 0.046           |
| Cyclin D1       | 0.32            | 0.060           |
| ERα             | 0.22            | 0.192           |
| ERβ             | 0.479           | 0.003           |
| HER2            | 0.459           | 0.005           |
| IGF-IRα         | −0.23           | 0.148           |

| Apoptosis vs.   | −0.37           | 0.014           |
| Cyclin D1       | −0.19           | 0.196           |
| ERα             | −0.12           | 0.430           |
| ERβ             | −0.39           | 0.040           |
| HER2            | −0.23           | 0.148           |

\( ^a \)Abbreviations are as follows: MCF-7, estrogen receptor positive human breast cancer; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IGF-IR, insulin-like growth factor-I receptor.

Discussion

This study showed that dietary 5FS and 10FS can regress established MCF-7 tumors to the same extent as the negative control and that 10FS but not 5FS can attenuate the late-stage tumor stimulating effect of TAM. The inhibitory effect of 10FS on TAM-induced tumor regrowth, which previously has been shown for 8 wk (16), was sustained for 16 wk. Unlike TAM, FS does not cause tumor regrowth. Dietary FS combined with TAM treatment significantly downregulates the expressions of estrogen sensitive gene products such as ERα and cyclin D1 and receptors of tyrosine kinase growth factors such as HER2 and IGF-IR.

The anticancer effects of FS have been suggested to be related to the ability of its lignans to compete with E2 for ER (23,24) or inhibit E2 synthesis and metabolism in cancer cells (25,26). With normal circulating estrogen level, FS, SDG, and EL have been shown to reduce the growth of the dimethylbenzanthracene-induced hormone responsive mammary tumors in intact rats (4–6,27), suggesting an antiestrogenic role in breast cancer. Conversely, lignans may act as weak estrogens when endogenous estrogen levels are low such as during postmenopausal years (28) as seen in an in vitro study where lignans promoted MCF-7 tumor cell growth with E2 deprivation (29). However, in our OVX mouse model system with the E2 pellet removed, the 5FS and 10FS treatments did not elicit any estrogenic response in the tumors or uterus, which is in agreement with our previous long-term study in which 10FS was fed to athymic mice for 25 wk (30). A similar result was also observed when pure ED and EL were injected into tumor-bearing OVX mice (31), suggesting that lignans in FS do not exert an estrogenic effect on established breast cancer cells.

ALA in FS may partly contribute to the above effect of FS and in reducing the tumor regrowth induced by TAM after prolonged treatment. ALA in FS can be metabolized into...
Tukey test. Data are means ± SEM.

**Figure 4.** Effect of 5% flaxseed (5FS) and 10% dietary FS (10FS) and tamoxifen (TAM), alone or in combination, on the expression (score) of human epidermal growth factor receptor 2 HER2 (A) and insulin-like growth factor–I receptor α (IGF-IRα) (B) of MCF-7 breast tumors in ovariectomized nude mice at low estradiol levels. Different letters (a–b) indicate significant difference at *P* < 0.05 among groups by 1-way analysis of variance followed by post hoc Tukey test. Data are means ± SEM.

docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which in turn can lead to reduced production of eicosanoids through the action of lipoxygenase and cyclooxygenase and inhibition of breast cancer growth and metastasis (3,32). The anticancer effect of ALA and its metabolites has also been attributed to increased lipid peroxidation (33), antiangiogenesis (34), modulation of signal transduction pathways (32), and inhibition of mevalonate pathway (35). All of these may complement the lignan effect in inhibiting TAM-induced tumor regrowth. A recent study has shown that cotreatment of TAM with EPA restored TAM sensitivity and induced breast cancer cells to be more responsive to the growth-inhibitory effects of TAM (36). However, because the dietary ALA level in this study is only about 2%, and the conversion of ALA to EPA and DHA is low (3), ALA may not be the major contributor to the inhibitory effect of FS on the TAM-induced tumor growth in this study.

This study mimicked the low circulating E2 level in postmenopausal condition. However, unlike breast tumors in postmenopausal women, which have aromatase activity and some available androgens for conversion to E2, MCF-7 cells have very low aromatase activity and insufficient androgens for conversion to E2 (37). Hence, after E2 withdrawal, the MCF-7 tumors received a much lower endogenous E2 supply in which any mild stimulation by estrogen-like compounds, such as TAM, would induce tumor cell proliferation. TAM treatment initially regressed tumors but to a lesser extent than the control and FS treatments; only 31% reduction was achieved at Week 14. By Week 21, tumors regrew to a level that did not differ from the pretreatment size. Hence, our results are supportive of the hypothesis that breast cancer cells become resistant to TAM by adapting or developing hypersensitivity to the estrogenic properties of TAM (17). Accordingly, some markers of estrogen sensitive genes, particularly cyclin D1, were upregulated by TAM in this and another study (21). The increase in cell proliferation occurred with an increase in cyclin D1, suggesting that an ER-dependent pathway, stimulated by TAM, may be responsible for the tumor regrowth. Several in vitro studies have shown that cyclin D1 can bind directly to the ER and increase its transcriptional activity (38) and induce the expression of HER2 (39). Both 5FS and 10FS might have acted as antagonist of TAM by downregulating the expression of ERα and particularly cyclin D1, thus resulting in a decreased cell proliferation.

The role of ERβ in ligand (E2 or TAM) induced cell proliferation and cell cycle modulation is not clear. Liu et al. (40) reported that in ERβ transfected HeLa cells, ERβ inhibits the E2-mediated but enhances the TAM-mediated activation of cyclin D1 promoter. Strom et al. (41), however, reported that when the ratio of ERβ to ERα is 4:1, ERβ induces E2-mediated cyclin D1 expression but inhibits cyclin E, cyclin A, and Cdc25A in T47D breast cancer cells. These findings indicate that ERβ action may be dependent on the ratio of ERα to ERβ. In mouse bone cells, ERβ inhibits ERα-mediated gene transcription in the presence of ERα, whereas in the absence of ERα, it can partially replace ERα (42). Nevertheless, ERβ plays an important role in estrogen-mediated cell proliferation by modulation of cyclin D1. In this study, we detected ERβ expression in the tumors, but it did not significantly differ among groups. On the other hand, significantly higher tumor ERα expression was observed in the TAM/0FS group compared with that in the combined FS and TAM groups, indicating a small change in the tumor ERα/ERβ expression. However, whether the change in the ratio of ERα to ERβ expressions was responsible for the reduced cyclin D1 expression in the interaction of FS and TAM requires further study.

An important finding in the present study is that both 5FS and 10FS significantly prevented the increase in the expression of receptors in the signal transduction pathways enhanced by TAM. Our previous studies have shown that dietary FS reduces the expression of IGF-I in carcinogen treated rats (43), IGF-I and EGFR in ER negative human breast cancer in athymic mice (7), and HER2 in postmenopausal breast cancer patients (11). Thus, one of the possible mechanisms by which FS interacts with TAM may be through modulation of signal transduction pathways. Compared to the negative control under low E2 condition, both doses of FS did not significantly change the expression of signaling transduction pathways. However, TAM increased the expression of...
HER2 and IGF-IR concurrent with tumor regrowth. Increasing evidence indicates that increased expression, or over-expression, of HER2 and IGF-IR and activation of related signal-transduction pathways is highly associated with TAM resistance (18, 44–47). Signaling via HER2 and IGF-IR can activate the ER and the important ER coactivators. In turn, ER located in the cell membrane can activate the growth factor receptor pathways (47–50), resulting in an increase of cell proliferation and inhibition of apoptosis to promote tumor growth. This study demonstrated a high correlation between the expression of HER2 and IGF-IR and cell proliferation as well as apoptosis. Therefore, we speculate that both of these receptors related to signal transduction pathways are major therapeutic targets for the nonhormonal action of FS to inhibit cell proliferation, induce apoptosis, and then retard TAM-induced tumor growth.

The 10FS, but not the 5FS, reduced the tumor regrowth induced by TAM. Although 5FS downregulated some cellular biomarkers when combined with TAM, it may not be strong enough to elicit inhibitory effect on TAM-induced tumor growth. The intake of 10FS diet is equivalent to an intake of about 25–50 g of FS per day depending on the amount of other foods consumed (9, 11). This FS dose, which is tolerated by breast cancer patients, has also been shown to reduce their tumor cell proliferation and HER2 expression and increase apoptosis (11). Because either dose of FS in this study did not exert any estrogenic effect on the tumor parameters and uterine weight, or other side effects such as food intake and body weight gain, it may be safe for postmenopausal breast cancer. However, the effect of higher amounts of FS is still unknown.

The 5 mg TAM pellet producing 3–4 ng/ml TAM is relevant to clinical treatment. The primary active metabolite of TAM for breast cancer treatment is 4-hydroxytamoxifen (4-OHTAM), which is 100 times as potent as TAM in inhibiting growth of human breast cancer (12). In patients, daily consumption of 40 mg TAM produces 6.7 (2.8–11.4) ng/ml of 4-OHTAM in the serum (51), and the ratio of 4-OHTAM to TAM is 0.02 to 0.06 (12, 51–53). However, the levels of 4-OHTAM in nude mice are about 1.5–2 times those of TAM (53, 54). Therefore, the blood levels of 4-OHTAM in mice with 3–4 ng/ml TAM in serum released by 5 mg pellet would likely reach 4.5–8 ng/ml, which is in the range of human level of 4-OHTAM for breast cancer treatment.

It should be noted that the model system in this study was used to illustrate the interactive effect of FS and TAM on tumor growth at extremely low E2 level. A tumor regrowth pattern was observed in the TAM group, but it is unclear whether it is due to acquired TAM resistance or just adaptive hypersensitivity to TAM. Nevertheless, the biomarkers detected in the tumors treated with TAM are early features of TAM resistance. This study detected only the expression of cyclin D1, ER, HER2, and IGF-IR. Investigation on the functional activities of these signal pathways, such as phosphorylation of ERα, HER2, IGF-IR, and their downstream MAPK and Akt pathways will further clarify the mechanism of the FS-TAM interactive effects on tumor growth.

In conclusion, FS has no tumor-inducing effect even in the longer term and can dose-dependently attenuate the tumor regrowth induced by TAM, with FS at 10% level having a greater effect. This beneficial effect did not cause adverse side effect on food intake and body, uterus, and other organ weights. The inhibitory effect of FS on TAM-induced tumor regrowth may be in part due to downregulation of the expression of receptors in estrogen-related and signal transduction pathways. FS has the potential to increase the effectiveness of TAM and merits further testing in future clinical trials.

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