Review

Can the combination of flaxseed and its lignans with soy and its isoflavones reduce the growth stimulatory effect of soy and its isoflavones on established breast cancer?

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Consumption of phytoestrogen (PE)-rich foods (*i.e.*, soy and flaxseed (FS)) is increasing because of their suggested health benefits. However, recent studies raise concern over the safety of soy and its isoflavones, particularly genistein (GEN), for postmenopausal breast cancer (BC), due to their potential stimulatory effects on human breast tissue and on the growth of existing tumors in rodents. FS, rich in PE lignans, which is metabolized to the mammalian lignans enterolactone (ENL) and enterodiol (END), has consistently been shown to have tumor inhibitory effects in a human clinical trial as well as rodent BC models. Using the preclinical athymic mouse postmenopausal BC model, combining FS with soy protein or GEN with END and ENL, was found to negate the tumor stimulatory effects of soy protein or GEN alone. The mechanism may be related to the modulation of estrogen receptor and MAPK signaling pathways. If these studies can be confirmed in clinical trials, then consumption of combined soy and FS, or their PEs, may reduce the tumor growth stimulatory effect of soy or GEN. This may indicate that if soy is consumed with lignan-rich foods, it may continue to induce its other beneficial health effects, without inducing adverse effect on postmenopausal BC.

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1 Introduction

Dietary phytoestrogens (PEs) are compounds that can elicit both weak estrogenic and antiestrogenic activities and are thus being investigated for their role in modulating breast cancer (BC) growth [1]. The two main PEs are the lignans, found in most plant foods but is high in concentration in flaxseed (FS), and the isoflavones, which are rich in soy products [2–4]. The major plant lignan in FS is secoisolariciresinol diglycoside (SDG), which is metabolized into the mammalian lignans, enterodiol (END), and enterolactone (ENL), by colonic bacteria [5, 6]. Soy isoflavones, daidzin, glycitin, and genistin, are also converted in the colon to their more biologically active and estrogenic forms, daidzein, glycitein, and genistein (GEN), respectively [7]. Of the PEs, the soy isoflavones, particularly GEN, have been studied most extensively for their role in BC growth and development.

The intake of PE-rich foods, such as FS and soy are increasing, particularly by postmenopausal women to help reduce the risk of BC, osteoporosis, heart disease, and menopausal symptoms [8]. However, although soy products and isoflavones have generally been thought as having anticancer properties, recent controversy has emerged with respect to their use by postmenopausal BC patients [9–11]. This was in part due to observations that, in ovariectomized (OVX) animal models of BC treatment, under low estrogen levels simulating postmenopausal situation, soy protein isolate (SPI) [12, 13], GEN [13–16], and genistin (the glycoside form of GEN found in soy) [17, 18] have all been shown to stimulate the growth of existing estrogen receptor

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Abbreviations: ALA, α-linolenic acid; BC, breast cancer; **BD,** basal diet; **DMBA,** 7,12-dimethylbenz[*a*]anthracene; **E2,** estradiol; **END,** enterodiol; **ENL,** enterolactone; **EPA,** eicosapentaenoic acid; **ER,** estrogen receptor; **FA,** fatty acids; **FS,** flaxseed; **GEN,** genistein; **HER2,** human epidermal receptor 2; **MAPK,** mitogen-activated protein kinase; **NAF,** breast nipple aspirate fluid; **OVX,** ovariectomized; **PE,** phytoestrogen; **PR,** progesterone receptor; **SDG,** secoisolariciresinol diglycoside; **SPI,** soy protein isolate

positive (ER+) human breast tumors. On the other hand, FS and its lignans have consistently been shown to exhibit inhibitory effects on existing tumor growth in several models of BC [19] and in clinical trials [20]. Nevertheless, PE-rich foods contain many different types of PE (*i.e.*, lignans and isoflavones) [4, 21], which may interact adversely or beneficially on existing or established BC. However, very little is known regarding these interactions.

This paper will review some of the animal and clinical studies on the role of soy and FS and their PEs alone on existing or established BC. Studies supporting our hypothesis that combining FS and its lignans with soy and its isoflavones may be more beneficial in reducing established tumor growth, than soy or its isoflavones alone, will then be described. Some of the potential mechanisms of the interactive effects of soy and FS will also be discussed. This review may be of interest particularly to postmenopausal BC patients who may consume soy and FS, alone and in combination, as complementary treatments.

2 Soy, isoflavones, and BC treatment

2.1 Animal studies

Two animal models have generally been used to study the effects of soy and the isoflavones on the growth of established breast tumors: the OVX athymic nude mouse model and carcinogen-induced rat model. The athymic mouse model is a useful model to determine the effect of compounds on human tumors *in vivo* because, depending on the type of BC cells implanted in mice, different mechanisms of action can be elucidated for a specific treatment. The MCF-7 cell line contains both ER subtypes, ERα and ERβ [22–25], and thus is commonly used to represent human tumors that grow in response to estrogens and to establish the estrogenic or antiestrogenic potential of treatments. To study the effects of PE and PE-rich foods on the growth of established MCF-7 tumors, OVX athymic mice are often implanted with MCF-7 cells and an estradiol (E2) pellet to stimulate MCF-7 tumor growth. Once tumors are established the E2 pellet is either removed, to establish a low circulating E2 level typical of postmenopausal conditions, or a new E2 pellet is implanted, to establish a high E2 level typical of premenopausal conditions, before treatment initiation.

Using this mouse model under low E2 conditions, mice with established MCF-7 tumors were treated with dietary GEN (750 ppm) or E2 to test the estrogenic potential of GEN *in vivo* [26]. After 12 wks, GEN treatment stimulated MCF-7 tumor growth to a similar level as 2 wks of E2 treatment. This study was the first to demonstrate that, although weaker than E2, GEN induced tumor growth under low E2 conditions and thus raised concern for postmenopausal BC patients. Subsequently, it was found that SPI and GEN dose-dependently stimulated MCF-7 tumor growth in OVX athymic mice [13, 14]. In addition, diets containing the glycoside form of GEN, genistin (primary form found in human diet), also stimulated MCF-7 tumor growth [17]. Genistin was converted to GEN, which was responsible for the tumor stimulatory effect of genistin [17]. These studies also showed that GEN was acting through the ER since the ER activation marker, pS2, was increased by soy and GEN [13].

Postmenopausal women have low circulating E2 levels as a result of a cessation of E2 production from the ovaries. In OVX athymic mice, circulating E2 is in the lower range (~25 pM) [15] than that found in postmenopausal women (20–100 pM) [27] and thus compounds that are weak estrogens may stimulate growth. This low level of circulating E2 in OVX mice has been an issue with regards to the clinical relevance of this model in representing postmenopausal BC. However, a study in which OVX athymic mice with established MCF-7 were treated with a physiologically achievable dose of GEN (3 μM) in the presence of postmenopausal plasma levels of E2 (~40 pM), showed that GEN alone stimulated tumor growth as did the low dose E2 [15]. In addition, when GEN and E2 were combined, tumor growth was stimulated beyond that of either compound alone [15]. Thus, the above-described studies continuously demonstrate that GEN may stimulate postmenopausal BC and therefore may induce adverse effects in BC patients.

Since soy is a complex food containing several bioactive components (*i.e.*, lignans, coumestans, saponins, plant sterols, phytates, and protease inhibitors (Bowman–Birk inhibitor and Kunitz–Trypsin inhibitor)), it has been suggested that interactions between these components may influence the estrogenic effect of GEN on tumor growth [28]. This is of particular interest because whole soy foods are generally consumed in Asian populations where soy consumption has been related to low BC development [2]. However, in North American and European diets, soy is commonly consumed after being highly processed or in a supplement form, in which many bioactive components are removed [29]. With this in mind, Allred *et al.* [18] conducted a study to determine the effect of soy, at different degrees of processing, on MCF-7 tumor growth. The diet treatment consisted of soy flour (ground defatted roasted soybeans), two soy extracts (molasses and Novasoy), a mixture of purified isoflavones, and GEN [18]. Although each diet contained soy at different levels of processing, they were all standardized to contain the same level of GEN. The authors found that as the level of soy processing increased, so did the growth of established MCF-7 tumors, with GEN and soy concentrate (Novasoy) inducing the greatest proliferative effects [18]. This study suggests that other components in whole soy may interfere with proliferative effects of GEN. In addition, this study suggests that North American and European use of soy products may be more detrimental to BC patients as they are highly processed; however, ingestion of whole soy foods may minimize the adverse effects of GEN.

Recently, a study was carried out in OVX mice with established MCF-7 tumors, in which mice were treated with

a soy extract (SOYSELECT, containing approximately 13% GEN, 17% daidzein, and 18%, saponins) [30]. Unlike GEN and SPI in previous studies [13, 14], the soy extract did not stimulate growth of MCF-7 tumors. However, the treatment duration was only 5 wks and as seen in previous studies, GEN- and SPI-induced tumor growth stimulation did not occur until later treatment time points [13, 14]. This could indicate that the treatment period in this study was not long enough to observe an estrogenic effect of the soy extract. In fact, although tumor growth and proliferation were not altered by the soy extract, estrogen-sensitive genes (pS2 and progesterone receptor (PR)) were increased [30]. This may indicate an estrogenic potential of the soy extract that may have resulted in tumor growth if the treatment period was prolonged. However, it cannot be ruled out that other components of the extract, *e.g.*, higher level of daidzein than GEN, and saponins and other unidentified compounds, had interactive effects with GEN and inhibited its tumor stimulatory effects.

Although the majority of studies analyze GEN as the active component of soy, daidzein is the second highest isoflavone in many soy foods and can be found as high or in higher concentrations than GEN in some isoflavone supplements [31, 32]. The effects of daidzein and its metabolite equol on established BC growth are unclear. However, Ju *et al.* [33] recently showed in OVX mice with established MCF-7 tumors that daidzein treatment (125–1000 ppm) resulted in tumors that were larger than controls, while equol treatment did not differ from control. Although daidzein showed an estrogenic effect, its tumor growth stimulatory effects were less than that observed with similar doses of GEN in a previous study [13]. This suggests that, of the soy isoflavones, GEN exerts the greatest estrogenic effect on established estrogen responsive tumors compared to daidzein and equol, with the latter inducing no estrogenic effect.

The effect of GEN combined with the BC drug tamoxifen is also of interest since many BC patients using tamoxifen supplement their therapy with soy to help alleviate tamoxifen-induced side effects, decrease osteoporosis risk, and alleviate menopausal symptoms [8]. However, it was not known if soy or GEN interferes with the tumor inhibitory effects of tamoxifen. A study was conducted using a similar experimental design as used above, except once MCF-7 cells were implanted, E2, tamoxifen, and GEN+ tamoxifen treatments were initiated, instead of treatment initiation after MCF-7 tumors were established [34]. While tamoxifen alone did not increase tumor growth, when combined with GEN, tumor growth was stimulated. An adverse interactive effect was also shown in Wild-type erbB-2/neu transgenic mice in which a low dose isoflavone diet (211 μg/g diet) inhibited tamoxifen-induced mammary tumor prevention [35]. These findings raise concern regarding the use of soy or isoflavones by BC patients who are taking tamoxifen.

In addition to using MCF-7 xenografts, ER negative (–) human cell lines have also been used to study the effects of GEN on established BC growth. Santell *et al.* [36] treated athymic mice with established MDA-MB-231 tumors with GEN (750 μg/g diet) for 5 wks. While GEN (>20 μM) reduced MDA-MB-231 cell proliferation *in vitro*, GEN had no tumor inhibitory effects *in vivo* [36]. In another study, mice with established MDA-MB-231 tumors were given daily subcutaneous injections of GEN (100–500 μg/kg BW) for 2 wks resulting in a significant reduction in tumor volume in the 500 μg/kg GEN group [37]. Thus, depending on the method of GEN administration, dietary or injection, different effects on tumor growth may result.

Using the 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced rat model, Ueda *et al.* [38] demonstrated that in rats with established tumors, dietary treatment with 25 ppm GEN induced a weak tumor stimulatory effect, while 250 ppm GEN induced an inhibitory effect [38]. This result may be due to the known biphasic effect of GEN, *i.e.*, inducing BC cell proliferation at low concentrations but decreasing proliferation at high concentrations [26]. While the authors suggest no adverse effects of GEN [38], it cannot be excluded that an adverse effect could occur at the low GEN dose. In contrast, using the MNU-induced breast tumor model, 750 ppm GEN for 3 months stimulated estrogen-dependent tumor growth in OVX rats [39]. This GEN dose resulted in plasma levels that were physiologically achievable in humans (3.4 μM) [40–42]; thus, it was suggested that GEN may stimulate tumor growth in postmenopausal BC patients.

The above animal studies suggest that the effect of soy on established tumor growth depends on the experimental design, concentration of GEN used, level of processing of soy products, and duration of the treatment period. Although it appears that GEN can induce estrogenic effects and increase growth of tumors in a postmenopausal preclinical model of BC, this effect may be modulated by the presence of other bioactive components found in whole soy foods. Therefore, studies analyzing the effects of soy food component interactions should be conducted to determine the potential safe use of soy foods for BC patients.

2.2 Clinical studies

Several human intervention studies were also conducted to determine the effects of soy or isoflavone supplementation on various markers of BC risk (Table 1). While some studies looked at alterations in hormone levels [46] as indicators of BC risk, others looked directly at the breast tissue or breast nipple aspirate fluid (NAF) to observe soy-induced effects [43–45, 47, 48]. Of particular interest is the observation in three studies in which soy acts estrogenically to induce hyperplastic epithelial cells [43], and increase markers of breast cell proliferation [44, 45]. These studies further raise concern for use of soy in BC patients since it may indicate the ability to induce tumor growth.

Table 1. Clinical studies on soy, isoflavones, and BC

Study design and biomarker	Subjects	Source of isoflavones	Isoflavone levels	Outcome	Reference
Intervention-12 months (NAF)	Healthy premenopausal and postmenopausal subjects (n = 24)	18.7 g SPI containing 37 mg GEN per day	Mean urinary excretion of GEN was 3.12 mg (7.4% available GEN from soy protein); daidzein (6.01 mg) and its metabolites (42.1% available daidzein from soy protein)	Only premenopausal NAF volume increased with soy intervention. 30% subjects (pre- and postmenopausal) had an increase in hyperplastic epithelial cells suggesting adverse effects	[43]
Intervention-2 wks-epithelial cell proliferation	Premenopausal BC patients (n = 19 intervention; n = 29 controls)	60 g soy supplement containing 45 mg isoflavones	Mean serum concentrations after supplement (GEN = 500 nmol/L; daidzein = 300 nmol/L)	Significant increase in proliferation and PR levels suggesting an estrogenic adverse effect	[44]
Intervention-2 wks-NAF	Premenopausal BC patients (n = 84 intervention; n = 23 controls)	Four bread rolls per day containing 60 g soy (45 mg isoflavones)	Mean serum total isoflavones was ~200 ng/mL intervention group and NAF contained 27.3 ng/g daidzein compared to 5.2 ng/g in control (p = 0.028).	Markers of proliferation (pS2) in NAF were enhanced by soy supplementation suggesting an estrogenic effect, while no changes in breast epithelial cell markers	[45]
Intervention -2 years – serum hormone status	Healthy premenopausal (n = 97 intervention; n = 92 control)	2 servings/day of soy products (soymilk, tofu, soy protein bar, soy protein powder, or roasted soy nuts) resulting in ~50 mg of isoflavones/day	Mean urinary isoflavone excretion was 64.1 nmol/mg creatinine intervention group and 9.9 nmol/mg creatinine control	No significant intervention effect was observed for serum estrone, E2, SHBG, progesterone, and adione suggesting no effect of soy on hormonal status of premenopausal women	[46]
Intervention -2 years breast density	Healthy premenopausal subjects (n = 109 intervention; n = 111 control)	2 servings of soy foods/day (soymilk, tofu, soy protein bar, soy protein powder, or roasted soy nuts) resulting in 58 mg of isoflavones/day and estimated lifetime soy exposure (FFQ)	Mean urinary isoflavone excretions was 32.2 ± 34.9 and 64.1 ± 67.8 nmol/mg creatinine in the intervention group after y 1 and 2 and 7.2 ± 19.4 and 10.0 ± 18.7 nmol/mg in the control group	No intervention effect was observed, however estimated soy intake throughout life found that soy exposure during childhood resulted in an increase in breast density suggesting adverse effects	[47]
Intervention-2 wks BC biopsies	Pre- and postmenopausal BC patients (n = 17 intervention; n = 26 controls)	Daily 50 mg isoflavone tablet	Mean urinary daidzein after supplement = 59.6 μmol/g creatinine; GEN = 22.7 μmol/g creatinine	No changes in ER, PR, HER2, p53, or apoptosis between soy tablet group and controls	[48]

GEN, genistein; NAF, breast nipple aspirate fluid; BC, breast cancer; PR, progesterone receptor; SHBG, sex hormone binding globulin; FFQ, food frequency questionnaire; HER2, human epidermal growth factor receptor 2.

3 FS, lignans, and BC treatment

3.1 Animal studies

As in the soy and isoflavones studies, the athymic mouse and carcinogen-induced rat models have been utilized to study the effects of FS and the lignans on established BC. Using the OVX athymic mouse model with established ER+ MCF-7 tumors, our group has demonstrated the effect of a 10% FS diet alone or in combination with tamoxifen on tumor growth [49]. In one study, under low E2 conditions, mice with established MCF-7 tumors were given a basal diet (BD; control), 10% FS diet, tamoxifen (5 mg pellet; 60 day release), or tamoxifen and 10% FS treatment for 7 wks [49]. FS treatment continuously regressed tumors to the level of the control, while tamoxifen stimulated tumor

growth after 4 wks of treatment, suggesting tamoxifen resistance. When FS was combined with tamoxifen, the tumor stimulatory effect of tamoxifen was negated. This study demonstrates that FS does not act estrogenically on MCF-7 tumors and also it decreases tamoxifen-stimulated tumor regrowth. When the effect of 5 and 10% FS diet was tested alone or in combination with tamoxifen for 16 wks, similar effects were observed [50]. However, the 10% FS diet was more effective than 5% FS in negating the tumor-stimulating effects of tamoxifen [50].

To determine if 10% FS, alone or in combination with tamoxifen, can induce antiestrogenic effects, athymic mice with established MCF-7 tumors were implanted with E2 pellet to increase circulating E2 level to simulate premenopausal situation [49]. After 6 wks of treatment, final tumor volume and weight were smallest in the tamoxifen + FS group, followed by the tamoxifen, and then the FS group, compared to the control group. Although final tumor volume and weight of the tamoxifen + FS group were not significantly different from the tamoxifen alone, further analysis of the tumor cell proliferation (measured by Ki67) showed that the tamoxifen + FS group reduced tumor cell proliferation greater than the tamoxifen or FS groups alone [49]. This suggests that FS in combination with tamoxifen is more effective in reducing tumor growth, than either one alone, when circulating E2 level was high. Although under both the pre- and postmenopausal conditions, FS appears to induce inhibitory effects on tumor growth when combined with tamoxifen, clinical studies still need to be conducted to confirm these effects seen in animal studies.

Xenografts of ER negative (–) cell lines, such as the MDA-MB-435 human BC cell line, have also been used to study the effects of FS and lignans on BC growth. MDA-MB-435 cells do not require estrogen for growth, does not contain ERs, and are highly metastatic, thus, they can be used to determine the effect of FS and lignans on metastasis and other non-ER mediated mechanisms. In one study, athymic mice with established MDA-MB-435 tumors were fed either a BD control or a 10% FS diet for 7 wks [51]. The FS group not only had smaller final tumors but also had a 45% less incidence of total metastasis (including lung and lymph node) compared to control.

Apart from the lignans, FS oil is another component of FS that may be responsible for its tumor inhibitory effects [52]. FS contains approximately 40% oil of which over 50% is the n-3 fatty acids (FA), α-linolenic acid (ALA), and diets rich in n-3 FA have been shown to induce tumor inhibitory effects [53–55]. Thus, to determine the bioactive component(s) of FS that was responsible for the inhibitory effects on tumor growth and metastasis, mice with established MDA-MB-435 tumors were fed either BD, 10% FS, SDG, FS oil, or SDG + FS oil for 6 wks [56]. The levels of SDG and FS oil were equivalent to that found in the 10% FS diet. Palpable tumor area was significantly reduced in the FS, FS oil, and SDG + FS oil groups, which was accompanied by a significant increase in tumor cell apoptosis [56]. Furthermore, the FS oil and SDG + FS oil treatment groups significantly reduced tumor cell proliferation, indicating that the SDG and FS oil may be responsible for the tumor inhibitory effects of FS on ER– tumor growth. Tumor malondialdehyde levels were significantly increased in the FS, FS oil and SDG + FS oil tumors suggesting that lipid peroxidation may be in part responsible for their primary tumor inhibitory effects [56]. Lung metastasis incidence was significantly reduced in the mice treated with 10% FS and SDG + FS oil groups, suggesting that both the lignan and the ALA-rich oil components may interactively contribute to the antimetastatic effect of FS [56].

Rats with established DMBA-induced tumors were fed a BD control, or BD supplemented with 2.5% FS, 5% FS, FS oil, or SDG (FS oil and SDG equivalent to the level found in 5% FS diet) for 7 wks [52]. All treatment groups reduced established tumor size compared to control [52]. In addition, the SDG group had significantly fewer new tumors that developed after the start of treatment [52]. This study demonstrated that both the SDG and oil components of FS were responsible for the tumor inhibitory effects of FS. Furthermore, mammalian lignan excretion negatively correlated with final established tumor size, suggesting that the metabolism of the FS lignans to mammalian lignans may be necessary for the tumor inhibitory effects of FS [52]. In another study, rats with established DMBA-induced tumors given a daily gavage of ENL (10 mg/kg BW) for 7 wks had smaller established tumors and less new tumor development compared to control [57]. These studies suggest that treatment with FS and the lignans can reduce the growth of established breast tumors in the carcinogen-treated rat model.

3.2 Clinical study

In a randomized double-blind placebo-controlled clinical trial, women with newly diagnosed BC consumed either a 25 g FS or placebo muffin for a mean duration of 32 and 39 days, respectively [20]. The objective of the study was to determine if FS could alter tumor growth biomarkers on biopsies taken at diagnosis and at time of surgery (end of treatment period). While the placebo did not cause significant changes in tumor biomarkers, treatment with FS significantly reduced tumor cell proliferation (Ki67 labeling index) and human epidermal receptor 2 (HER2)/c-erbB2 by 34 and 71%, respectively, and increased apoptosis by 30.7% [20]. In addition, urinary lignans increased by 14-fold in the FS-treated patients and the intake of FS was significantly correlated with changes in HER2 score and apoptotic index [20]. These results suggest that the lignans, perhaps in combination with other components such as FS oil, may in part be responsible for the changes in tumor growth biomarkers.

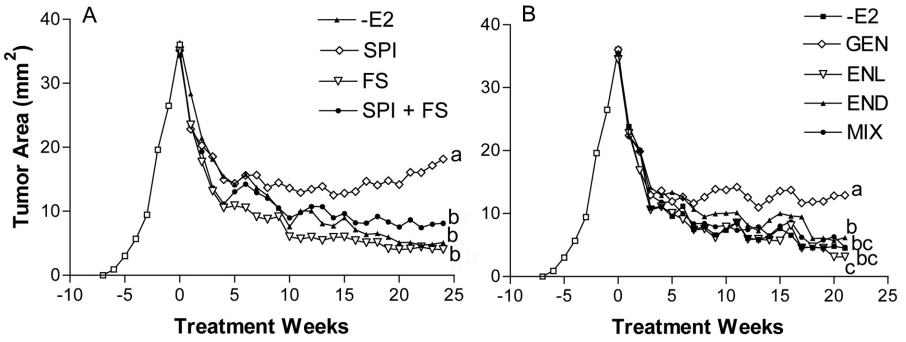

Figure 1. Effect of FS and SPI diet (A), and lignans ENL, and END and isoflavone GEN (B), alone and in combination (FS + SPI or MIX), on palpable tumor growth of MCF-7 xenografts in OVX athymic mice. Lines with different letters are significantly different ($p < 0.05$). Modified from [12] and [16].

4 Combination of FS and soy on BC treatment

Both FS and soy have the potential to induce biological effects on BC growth. While soy and FS differ in the majority of their bioactive components, they both contain high levels of PEs. However, the two main classes of PEs differ, with soy containing high isoflavone levels and FS containing high lignan levels [4]. When administered as single PE-rich sources alone (SPI or FS), it is apparent from the above reviewed studies that they do not induce the same effects on BC growth. For example, SPI stimulates MCF-7 tumor growth in OVX athymic mice [13], while FS does not [12, 49]. However, although experimental models test these food components in isolation, lignans are found ubiquitously throughout the plant kingdom and in soy [4] and soy-based health supplements [31]; thus, these two PE classes are generally not consumed by humans in isolation.

While the effect of FS and soy, or lignans and isoflavones, in combination has not been analyzed in clinical studies, an epidemiological study showed that the greatest BC risk reduction was found in women who had the highest estimated intake of both lignans and isoflavones, compared to those who had low intake levels of both lignans and isoflavones [58]. This BC risk reduction was not observed in individuals with intake of high lignans and low isoflavones, or high isoflavones and low lignans [58], suggesting a potential advantage of combining foods that are rich in both isoflavones and lignans.

To test the hypothesis that soy and FS will have beneficial interactive effects on BC compared to soy alone, OVX athymic mouse mice with established MCF-7 tumors were treated with BD (control), 10% FS, 20% SPI, or combined 20% SPI and 10% FS (SPI + FS), for 25 wks [12]. While SPI alone initially regressed MCF-7 tumor size, prolonged treatment (>16 wks) stimulated tumor growth resulting in significantly larger final tumor size (Fig. 1A) and higher cell proliferation than control (Fig. 2A) [12]. Further, this study showed that long-term FS treatment did not stimulate tumor growth, and when combined with SPI, it significantly attenuated the stimulatory effects of SPI on tumor growth and cell proliferation (Figs. 1A and 2A) and enhanced tumor cell apoptosis (Fig. 2C) [12]. FS treatment also resulted in greater tumor regression in the first 7 wks of treatment compared to control, indicating an early tumor inhibitory effect of FS [12].

A second study determined the effect on tumor growth of the PE components of SPI and FS, *i.e.*, GEN and lignans, respectively [16]. Although the mammalian lignans (ENL and END) are not technically PEs, they are the biologically active metabolites of the plant lignans found in FS that after ingestion are formed by colonic bacteria. Using the same animal model as described above, mice with established MCF-7 tumors were given daily subcutaneous injections of 10 mg/kg BW of GEN, ENL, END, GEN + ENL + END (3.33 mg/kg BW of each compound in the mixture), or vehicle control for 22 wks [16]. GEN treatment initially caused tumor regression, however, after prolonged treatment, regression ceased, resulting in a final tumor size that was greater than control (Fig. 1B) [16]. GEN treatment also resulted in an increase in tumor cell proliferation (Fig. 2B), indicating a tumor stimulatory effect [16]. Treatment with ENL and END, alone or in combination with GEN, continuously regressed tumor size to the level of the control (Fig. 1B) indicating that at the same concentration as GEN alone, there was no tumor stimulatory effects [16]. In addition, the lignans increased final tumor cell apoptosis suggesting a tumor inhibitory effect (Fig. 2D) [16]. Since the concentration of ENL, END, or GEN injected in the combination group was 1/3 (each 3.33 mg/kg BW) the concentration of that injected of each compound alone (10 mg/kg BW), it cannot be concluded that lignans inhibited the tumor stimulatory effect of GEN. However, it can be concluded that at the same total concentration (10 mg/kg BW), the combination group has a better tumor growth inhibitory effect on established MCF-7 tumor than GEN alone.

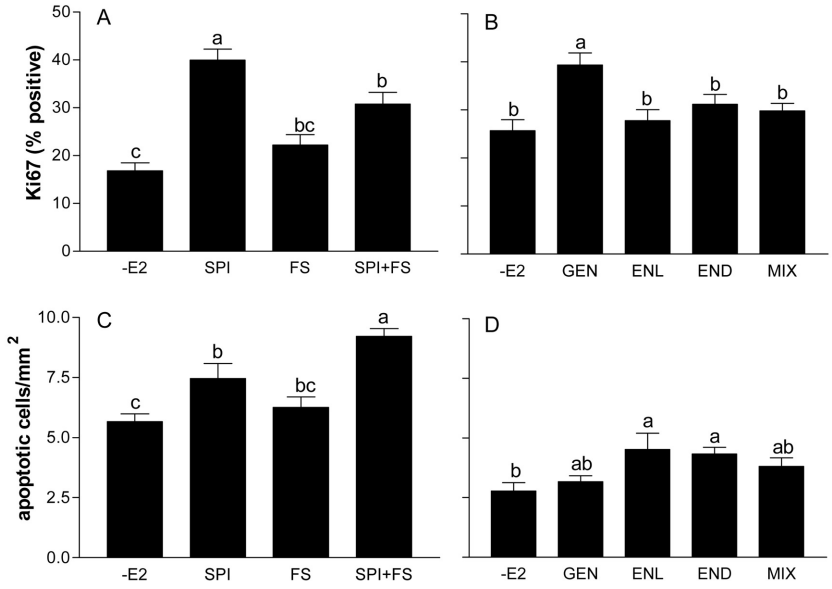

Figure 2. Effect of FS and SPI diet (A and C), and lignans ENL, and END and GEN (B and D), alone and in combination (FS + SPI or MIX), on cell proliferation (A and B) and apoptosis (C and D) in MCF-7 xenografts in OVX mice. Bars with different letters are significantly different ($p < 0.05$). Modified from [12] A and C and from [16] B and D.

Collectively, these studies suggest that combined FS and SPI, or the mammalian lignans and GEN, is more effective in reducing the growth of established MCF-7 tumors than SPI or GEN alone. However, the mechanisms of their action are still unclear.

5 Potential mechanisms

Since the tumor stimulatory effect of soy and GEN has been shown to be ER mediated [13], FS, and potentially the lignans, may negate the effects of soy and GEN through modulation of ER signaling. The mammalian lignan, ENL, and GEN have been shown to bind to both ERα and ERβ; however, ENL binds with a higher affinity for ERα [59], while GEN binds more to ERβ [60]. Thus, the lignans and isoflavones may induce different effects on tumor ER signaling, when present in combination compared to when present alone.

The ER is a member of the steroid nuclear receptor super family of ligand-activated transcription factors [61]. Estrogenic compounds bind to ERα or ERβ, initiating nuclear translocation of the ER, ER conformational changes, coactivator/corepressor binding, and ER dimerization [62, 63]. The ER complex then binds to specific DNA response elements and initiates transcription of estrogen sensitive genes including transforming growth factor alpha (TGF-α), epidermal growth factor (EGF), insulin-like growth factor (IGF-I/II), ras (oncogene), c-fos (protooncogene), c-jun (protooncogene), progesterone receptor, and cyclin D1 [64 – 66]. The protein products of these genes can act in an autocrine/paracrine fashion to increase proliferation of BC cells.

Enhanced growth factor signaling pathways have also been shown to activate the ER through phosphorylation at specific ER amino acid residues and coregulatory proteins [61]. Growth factor and ER signaling crosstalk in tumors is thought to result in hormone resistance or hypersensitivity to estrogens [63, 67, 68]. Mitogen-activated protein kinase (MAPK) is a signal transducing protein, which can be phosphorylated and thus activated (pMAPK) *via* transmembrane growth factor receptors such as epidermal growth factor receptor (EGFR), HER2, and insulin-like growth factor receptor (IGF-1R) [68]. Thus, with increased growth factor signaling pathways, along with estrogenic compounds (*e.g.*, GEN) acting through the ER, it may be possible for the ER and MAPK signaling pathways to be responsible for late-stage SPI-induced tumor growth.

To determine if the ER and MAPK pathways played a role in the tumor growth effects induced by FS and SPI, alone and in combination, tumor growth biomarkers (ERα, cyclin D1, HER2, and pMAPK) were analyzed after short- (2 wks) and long-term (25 wks) treatment periods ([69]; Power *et al.*, unpublished data]. SPI-treated tumors had increased cyclin D1 after short-term treatment (a time point when tumor size did not differ between treatment groups) and increased ERα, cyclin D1, and pMAPK, and decreased HER2 after long-term treatment. This may indicate that SPI stimulates tumor growth by modulating the ER and MAPK signaling pathways. After short-term FS treatment, alone and in combination with SPI, tumor pMAPK was significantly reduced compared to control, indicating an early tumor inhibitory effect (Power *et al.*, unpublished data). Long-term FS treatment did not significantly affect tumor biomarkers compared to control, however, when combined with SPI, FS attenuated the effects of SPI on HER2, pMAPK, and cyclin D1 expression ([69]; Power *et al.*, unpublished data). These results suggest that SPI-stimulated tumor growth may be due to enhanced activation of both the ER and MAPK pathways, which FS can attenuate.

Similarly, tumors from the mice treated with subcutaneous injections of GEN, ENL, END, and GEN + ENL + END were analyzed for biomarkers of the ER and MAPK signaling pathways [69]. After short-term treatment (2 wks), there were no differences between treatment groups in biomarker expression. However, after long-term treatment (22 wks), ERα and cyclin D1 levels were significantly higher in the GEN-treated tumors compared to control, which may have contributed to the increase in cell proliferation [69]. The lignans, either alone or in combination with GEN, did not alter tumor growth biomarkers compared to control after the long-term treatment period [69]. This suggests that the tumor stimulatory effect of GEN may be related to increased activation of the ER signaling pathway.

Another possible mechanism relates to the interaction between the long chain polyunsaturated n-3 FA, eicosapentaenoic acid (EPA), and GEN. A study in ER+ and ER– cells showed that when GEN and EPA were combined, inhibition of BC cell growth was reduced to a greater extent than with GEN alone [70]. EPA is converted from ALA, which is found in high concentrations in FS. Therefore, combining FS and soy could result in a GEN–EPA interaction. However, although the dose of GEN used in the above study [70] was higher than that physiologically achievable (>90 μM), the authors suggest the potential for GEN to be concentrated in tissues at levels that exceed plasma levels. This has been shown in the breast tissue of women given isoflavone supplements, however, only for the isoflavone equol and not GEN or daidzein, which were more concentrated in the plasma than breast tissue [71]. Therefore, further studies should be conducted to determine the interactive effects of soy and n-3 FA on breast tumor growth.

Alterations in soy isoflavone absorption and metabolism when given in a more complex food matrix, such as when consumed in combination with FS, is another potential mechanism of the interactive effects. Since both isoflavones and plant lignans require colonic bacteria for conversion to their aglycone forms and for further metabolism [72], they may compete for bacterial action when consumed in combination. This may result in a reduction of PE absorption and metabolism and thus biological activity in target tissues. In fact, Allred *et al.* [73] demonstrated that in OVX mice fed diets containing the same amount of GEN but which varied in the degree of soy processing, the less processed soy diet resulted in the lowest amount of circulating free GEN aglycone. This could indicate that other components in soy or ingesting soy with other food components, as in FS, could reduce the estrogenic potential of soy.

6 Soy *vs.* GEN and FS *vs.* lignans

Are the mammalian lignans and GEN responsible for the effects induced by FS and SPI, respectively? In our two studies described above, long-term treatment with GEN [16] and SPI [12] resulted in MCF-7 tumors that were larger than controls (Fig. 1). In addition, long-term treatment of GEN and SPI-treated tumors had enhanced cell proliferation, ERα, and cyclin D1 expression, indicating that GEN may be responsible for the effects induced by SPI (Fig. 2 and Power *et al.*, unpublished data). However, there were some differences in tumor growth pattern and biomarker expression observed between the two treatment groups. For example, palpable tumor area over time indicates that GEN-treated tumors initially regressed, however, after 4 wks of treatment, regression ceased and palpable tumor size remained stable until the end of the treatment period (Fig. 1B) [16]. On the other hand, SPI-treated tumors also initially regressed, however, after 16 wks of treatment tumor growth was stimulated (Fig. 1A) [12]. Furthermore, GEN did not modulate HER2 or pMAPK expression, while SPI increased pMAPK and decreased HER2 (Power *et al.*, unpublished data). Since enhanced activation of the MAPK pathway leading to enhanced ER signaling is thought to result in hormone resistance or hypersensitivity to estrogens [63], the lack of effect of GEN on this pathway may explain why GEN did not stimulate MCF-7 tumor growth while SPI treatment did [12].

The differences observed between GEN and SPI may be due to the lower dose of GEN subcutaneously injected (10 mg/kg BW) [16] compared to the level of GEN present in the 20% SPI diet [12]. In the GEN study, with the 10 mg/kg BW dose, mice were injected approximately 0.2 mg GEN/day [16]. In the SPI study, mice received GEN through a 20% SPI diet containing 1.6 mg GEN/g protein or 320 μg GEN/g diet [12]. Since mice ate ~3 g SPI diet/day, they ingested approximately 0.96 mg GEN/day. However, since plasma GEN levels were not measured in these studies, the actual differences in GEN exposure cannot be accurately estimated due to the potential differences in GEN absorption and metabolism as a result of the different methods of GEN administration (injection *vs.* diet). In addition, other components or combination of components in SPI (*i.e.*, daidzein, coumestans, saponins, plant sterols, phytates, and protease inhibitors) may have caused the additional effects induced by SPI compared to those induced by GEN.

Treatment of tumors with mammalian lignans or a 10% FS diet also resulted in some differences in tumor growth and biomarker expression. FS induced a greater tumor regression compared to control in the first 7 wks of treatment and reduced pMAPK levels after 2-wks of treatment, while the lignans did not [12, 16]. However, the lignans significantly increased tumor cell apoptosis [16] while FS did not [12]. The differences in results on tumor growth and pMAPK expression between the lignans and FS may indicate that other components (*e.g.*, FS oil) or combination of FS components (FS oil + lignans) may be responsible for the early tumor inhibitory effects. As discussed previously,

FS oil inhibits the growth of established mammary tumors in rats and human tumors in athymic mice, which may be related to its high ALA content [52, 56]. Furthermore, FS oil, in combination with SDG, reduced the growth and metastasis of established ER– tumors in athymic mice to a greater extent than SDG alone indicating a potential tumor inhibitory effect of the combined treatments [56].

The lignan dose (10 mg/kg BW) used in the injection study [16] was estimated to be similar to the amount of SDG present in a 10% FS diet. However, depending on the analytical method used to determine the level of SDG in FS, there may be large variations in SDG values [74]. Thus, the 10 mg/kg BW lignan dose used in our study [16] may be less than the amount of lignan produced after the ingestion of the 10% FS diet [12]. In addition, differences in lignan absorption, metabolism, and duration of treatments (22 wks in the injection study and 25 wks in the diet study), may be responsible for the differences in tumor effects observed between FS and the mammalian lignans.

7 Conclusion and future studies

It is evident that, under low circulating E2 conditions, SPI and its isoflavone GEN can induce the growth of established breast tumors after long-time exposure, while FS and the lignans do not. Also, combining FS and SPI or the lignans and GEN is more effective in reducing established breast tumor growth than SPI or GEN alone. While the effects of SPI were in part due to GEN, the effects of FS were not solely due to the mammalian lignans at the concentration used. The mechanisms of action were related to the modulation of ER and MAPK signaling pathways.

However, several future studies may still need to be conducted to help further establish the beneficial interactive effect between SPI and FS and their PE. The preclinical animal model of postmenopausal BC used in our studies has been used for decades in the development of tamoxifen as a BC drug, and has shown that MCF-7 xenografts eventually develop resistance to tamoxifen, as it does in many BC patients [75]; thus this model has clinical relevance. Nevertheless, testing the interaction of FS and SPI or their PE in other animal models of postmenopausal BC will further verify the effectiveness of FS and SPI and their potential mechanisms of action. Our studies have used only MCF-7 tumor cells and thus testing these compounds also in other BC cell lines will help confirm our findings. One example is the use of MCF-7 xenografts that have been transfected with the human aromatase gene (MCF-7Ca) in OVX mice with an androgen supplement [76]. This model enables MCF-7 tumors to synthesize estrogen from androgen precursors, which then may more accurately represent breast tumors in postmenopausal women. Although postmenopausal women have low levels of circulating E2 (100–200 pM), they can have enough androgens and high breast tumor aromatase expression, which enables tumor exposure to estrogen [77, 78].

It is still unclear if the interactive effects of FS and SPI are due to their PE. As mentioned above, the concentration of each PE in the MIX (combination) group (3.33 mg/kg BW) was 1/3 the concentration of each PE used alone (10 mg/kg BW) [16]. Therefore, the effects of ENL, END, and GEN in combination may be due to lower concentrations of each PEs and not a synergistic effect [16]. This can be further studied by testing the PEs in combination at the same concentration as each PE alone. In addition, using similar levels of GEN and lignans that would be found in the 20% SPI diet and converted from a 10% FS diet, respectively, would help determine if PE are responsible for the effects induced by SPI and FS. Measuring lignan and isoflavone plasma levels after the consumption of a 20% SPI diet, or a 10% FS diet, respectively, may help more accurately determine the lignan and isoflavone doses that should be analyzed in future studies.

To confirm the potential benefits of combining FS and soy in reducing human BC growth, future clinical trials should ultimately be conducted in postmenopausal BC patients with ER+ breast tumors. Before and following dietary treatments, tumor biopsies can be analyzed for biomarkers of tumor growth, *e.g.*, cell proliferation, apoptosis, ER, cyclin D1, MAPK; this will also help assess the potential mechanisms of the treatment effects.

If it can be further confirmed that the combined soy and FS or their PE is more antitumorigenic than soy or its isoflavones (*i.e.*, GEN) alone, then it may be one way of counteracting any potential adverse effect of soy or GEN intake by postmenopausal BC patients while maintaining their other health beneficial effects, *e.g.*, cholesterol lowering and maintaining bone health. No adverse effect on bone health was observed with combined GEN and lignan than GEN treatment alone under low circulating E2 conditions [79]. The lignans and isoflavones are naturally present in combination in PE-rich diets and soy is often consumed in a background diet with plant foods such as grains, fruits and vegetables, which also contain lignans. Hence, consumption of soy in a human diet rich in lignan-containing plant foods may not normally pose an adverse effect on postmenopausal women with BC.

8 References

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