Soy Phytochemicals Decrease Nonsmall Cell Lung Cancer Growth In Female Athymic Mice

Daniela Gallo, Gian Franco Zannoni, Ilaria De Stefano, Marco Mosca, Cristiano Ferlini, Elisabetta Mantuano, and Giovanni Scambia

Abstract
This study investigated the effects of a phytoestrogen-containing standardized soy extract (SSE) on the growth of nonsmall cell lung cancer (NSCLC; A549) xenografts in female athymic mice. Tumor-bearing mice received either sterile water or SSE [50 or 100 mg/(kg·d), per os], 5 d/wk, until the mean tumor weight in each group was at least 900 mg. Treatment with SSE reduced tumor growth in the high-dose group compared with control (P < 0.01); tumors in both treated groups had reduced proliferation and greater apoptosis compared with controls (P < 0.05). SSE treatment also induced diffuse central necrosis, reducing the viable tissue mass within the tumor. Whereas tumor levels of epidermal growth factor receptor were comparable in control and treated mice, the expression of phosphorylated protein kinase B (p-Akt) was lower in tumors of mice treated with 100 mg SSE/(kg·d) than in controls (P < 0.01). The protein level of phosphorylated mitogen-activated protein kinase also tended to be lower (P = 0.07) in this group than in controls. Estrogen receptor (ER)α and ERβ were present in tumors, but their expression levels did not differ among groups. Serum insulin-like growth factor-1 concentrations also were not affected by the treatments. In conclusion, we found that soy phytochemicals slow the in vivo growth of NSCLC xenografts; the modulation of the Akt-signaling pathway observed in tumors of SSE-treated mice may have a role in the activity observed. Our research provides further support for the concept that consumption of phytoestrogens may be effective in delaying lung cancer progression. J. Nutr. 138: 1360–1364, 2008.

Introduction
Lung cancer is the leading cause of death from cancer in U.S. men and women (1); in Europe, in 2004, it accounted for 13.3% of all cases and one-fifth of all cancer deaths (2). An interesting finding is that the incidence of lung cancer in U.S. women has increased by 600% in 50 y, surpassing breast cancer as the leading cause of cancer mortality by nearly 20,000 patients yearly; genetic, metabolic, and hormonal factors have been all implicated in the way women react to carcinogens and lung cancer (3). In particular, estrogen status has been recognized as a modulator of lung cancer in women, although the effects are not so clear-cut as that for the modulation of breast cancer. There is substantial evidence that estrogen receptors (ER)α are expressed in healthy lung tissue and in lung tumors and that lung cancer responds to estrogen by proliferation. Estrogen is able to induce cell proliferation in culture and in tumor xenografts by activating growth factor signaling pathways, and is also able to change gene expression in nonsmall cell lung cancer (NSCLC) cell lines from women and men (reviewed in 4). Other authors have presented evidence that exogenous and/or endogenous estrogens may play a role in the development of lung adenocarcinoma in women (5,6). In particular, Tàtolli and Wynder (5), using case-control data, showed that early age at menopause (40 y or younger) is associated with a reduced risk of adenocarcinoma of the lung, the use of estrogen therapy is associated with a higher risk of this cancer, and a positive interaction exists between estrogen therapy, smoking, and the development of adenocarcinoma of the lung. However, other studies have contradicted these findings, showing a decreased risk of lung cancer in women using hormone therapy (7,8).

Phytoestrogens are a broad group of nonsteroidal compounds of different structure that bind to ER. There are 3 main classes of phytoestrogens: isoflavones, coumestans, and lignans. Among the isoflavones, genistein and daidzein are the most investigated. Functionally, phytoestrogens can exert both estrogenic and antiestrogenic effects depending on many factors, including their concentration, the concentration of endogenous sex hormones, the relative levels of ERα and ERβ, and the nature of the response elements with which the receptors interact on the estrogen-related genes; phytoestrogens can also interact with pathways of cellular activity that do not involve ER.
Soy phytochemicals in lung cancer

(9). Epidemiological, human, and experimental studies have suggested an association between higher intake of phytoestrogens and reduced risk for cancer of the breast and prostate, thus supporting a preventive role for phytoestrogens (10); specific chemopreventive effects putatively associated with phytoestrogens include induction of apoptosis, cell cycle regulation, inhibition of angiogenesis, and inhibition of invasion and metastasis (11). Recently, Schabath et al. (12) reported results from a case-control study supporting evidence that phytoestrogens are also associated with a decreased risk of lung cancer. Particularly, the protective effect for the highest quartile of total isoflavone intake ($\geq 1$ mg/d) was significant for both women and men, with a 32% overall reduced risk (44% for men and 22% for women). The aim of this study was to evaluate the potential activity of standardized soy extract (SSE) in the A549 NSCLC xenograft model.

Materials and Methods

Cell line. A549 cells were purchased from the European Collection of Cell Cultures. Cells were grown in Kaighn’s nutrient mixture F12 and supplemented with 10% fetal bovine serum, 1% nonessential amino acids mixture, and 1% kanamycin. Cells, propagated as a monolayer culture, were trypsinized twice weekly and plated at a density of $1 \times 10^8$ cells/L. All cultures were incubated at 37°C with 5% CO$_2$ in a high humidity atmosphere. On the day of dosing, cells were trypsinized and a suspension of $8 \times 10^6$ cells was injected subcutaneously in the right flank of each mouse (0.2 mL/mouse).

Animals. Female athymic mice [Athymic Nude-nu], 5 wk old and within a weight range of 18–22 g, were obtained from Charles River and housed under controlled conditions. They were fed a phytoestrogen-free, purified diet (Harlan) (Table 1). Procedures and facilities followed the requirements of Commission Directive 86/609/EEC concerning the protection of animals used for experimental and other scientific purposes. Italian legislation is defined in the Decreto Legislativo No. 116 of 27 January 1992. In addition, the UK-Coordinating Committee on Cancer Research guidelines for the welfare of animals in experimental neoplasia were followed (13). The Animal Care and Use Committee of the Catholic University of the Sacred Heart (Rome, Italy) and the Italian Ministry of Health approved the project.

Human tumor xenograft growth. SSE (SOYSELECT, Indena spa) is a standardized extract from soy with a double standardization (13–17% of isoflavone glycosides genistin and daidzin and not <18% of B-group saponins, by HPLC). The product is prepared by extracting ripe whole soy beans or oil-free soy flour with aliphatic alcohols through an industrial manufacturing proprietary process (patents US 6,280,777 and US 6,607,757). One gram of extract also contains 0.058 g of protein, 0.035 g of fat, and 0.023 g of ash, with the remaining matter undefined. The batch used in the study contained 14.7% isoflavone glycosides and 21.2% B-group saponins. SSE was dissolved in sterile water at the concentrations required for dosing. There were 3 experimental groups in the study, each consisting of 10 mice. After A549 tumors grew to a limiting size of 30–75 mg, mice were assigned to 3 treatment groups: vehicle control, and 50 or 100 mg SSE/kg d, each having a similar mean tumor weight. Mice were treated by oral gavage 5 d/wk until the mean tumor weight in each group was at least 900 mg. During the study mice were checked daily for any adverse clinical reactions. Body weight and tumor dimensions were measured 2 times per week; food intake was determined randomly over several 24-h periods throughout the study. At the end of the study, mice were killed by CO$_2$ blood was collected and serum frozen at $-20^\circ$C for analysis; uteri were rapidly removed, cleaned of fat, and weighed. All tumors were also removed and subsequently cut into 2 fragments: one half of the tumor was snap-frozen in liquid nitrogen before storage at $-80^\circ$C for protein analysis and the other half was fixed in 10% formalin and subsequently dehydrated and blocked in paraffin. The paraffin block was cut into 3- or 5-μm sections and processed for light microscopy [hematoxylin/eosin staining (H&E)] or immunohistochemistry (see below). Tumor necrosis was assessed by light microscopy by an investigator who did not know the treatment group.

Evaluation of antitumor activity. The tumor weight was calculated from 2-dimensional measurements (mm) (14): tumor weight = length $\times$ width$^2$/2. The ratio between the mean tumor weight of treated mice (T) and that of control mice (C) ($\times$ 100) (T:C%) was assessed on each day of measurement. Differences in efficacy between treatment groups were expressed as the percentage of tumor weight inhibition (TWI%), calculated as follows: TWI% = 100 – T:C%. The optimum value for TWI% obtained during the study was considered for each experimental group. In addition, the tumor growth delay (TGD) was calculated by the difference in the mean time required for the treated and control group tumors to reach a predetermined size (900 mg, size criteria). The tumor doubling time (Td) was estimated from the best fit straight line from the log linear plot of the control group tumors in exponential growth (100–900 mg range). The log$_{10}$ cell kill (LCK) achieved by drug treatment was calculated from the following formula: LCK = TGD value in d/3.32 $\times$ Td (14); Td = 7 d in control mice.

Histological analysis of apoptotic bodies. Apoptosis was evaluated on paraffin sections by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay, using TumorTACS In Situ Apoptosis Detection kits (Trevenig) according to the manufacturer’s recommendations; sections were analyzed with light microscopy. Apoptosis was evaluated by an investigator without knowledge of treatment groups who counted the positive cells (brown-stained cells), as well as the total number of cells, at 5 arbitrarily selected fields in nonnecrotic areas of each section. The apoptotic index was calculated as number of apoptotic cells $\times$ 100/total number of cells. Tumor sections were also stained with H&E.

Immunohistochemical analysis. Cells expressing Ki67 were identified as previously described (15), with the following modifications: briefly, after the antigen retrieval procedure and blocking, tumor sections were incubated with Unconjugated AfiniPure Fab Fragment goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Labs) for 1 h at room temperature (1:10 dilution) to avoid background staining. After incubation with the primary antibody (mouse anti-Ki67, M7240, DAKOCYTOMATION, 1:100 dilution, 30 min, room temperature), slides were incubated with Biotin-SP-conjugated AfiniPure Fab Fragment goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Labs, 1:500 dilution) for 20 min at room temperature. Detection was evaluated with horseradish peroxidase-streptavidin (1:500, Vector Labs) and we used diaminobenzidine as a chromogen (DAB substrate System, DAKO). Positive control corresponding

### TABLE 1 Diet composition

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Diet</th>
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<td>g/kg</td>
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<tr>
<td>Corn starch</td>
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<tr>
<td>Casein</td>
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<tr>
<td>Vitamin mix$^2$</td>
<td>10</td>
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</tbody>
</table>

$^1$ Supplied the following (g/kg diet): calcium carbonate, 6; potassium chloride, 3; magnesium oxide, 4; sodium chloride, 1.3; dicalcium phosphate, 3; copper sulfate - SH$_2$(O)$_2$, 0.046; manganese sulfate - H$_2$O, 0.166; iron sulfate - H$_2$O, 0.896; zinc sulfate - H$_2$O, 0.185; cobalt-basic-carbonate - H$_2$O, 0.006; calcium iodate, 0.003.

$^2$ Supplied the following (mg/kg diet): retinyl acetate, 4.96; cholecalciferol, 0.032; thiamin HCl, 13.5; riboflavin, 6.8; pyridoxine HCl, 5.85; cyancobalamin, 0.03; all-rac-a-tocopherol, 49.5; menadione nicotinamide bisulfite 2.79; niacin, 54.0; folic acid, 1.89; pantothenic acid, 14.4; biotin, 0.279; choline, 1000. Proximate analysis: protein 19%, fat 4%, fiber 6%, carbohydrate 59.5%. Metabolizable energy, 15.4 kJ/g.

Mineral mix

1:100 dilution, 30 min, room temperature), slides were incubated with TdT (1:10 dilution) to avoid background staining. After incubation with the primary antibody (mouse anti-Ki67, M7240, DAKOCYTOMATION, 1:100 dilution, 30 min, room temperature), slides were incubated with Biotin-SP-conjugated AfiniPure Fab Fragment goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Labs, 1:500 dilution) for 20 min at room temperature. Detection was evaluated with horseradish peroxidase-streptavidin (1:500, Vector Labs) and we used diaminobenzidine as a chromogen (DAB substrate System, DAKO). Positive control corresponding
to human breast cancer and negative control obtained by omission of the primary antibody were conducted in the assay. Immunohistochemical scoring was determined without knowledge of the treatment groups. The number of positive (brown-stained) cells was determined as a percentage of the total number of cells counted in 5 separate fields of 100 cells in nonnecrotic areas of each section.

Western blotting of tumor tissues. Tumor tissues were homogenized in lysis buffer (T-PER, Pierce) containing protease plus phosphatase inhibitors. Samples were incubated on ice for 30 min, centrifuged, and the supernatant protein quantified by the Bradford assay (Bio-Rad Laboratories). Equal amounts of protein (30–60 μg) were separated by 8–10% SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes HybondP+ (Amersham Biosciences). The membranes were then blocked 1 h at room temperature with 5% skim milk in Tris-buffered saline with Tween 20 and incubated (for 16 h at 4°C) with the following antibodies: rabbit anti-phospho-protein kinase B (p-Akt) (Ser-473), and anti-total-Akt (1:1000); rabbit anti-phospho-p44/42 mitogen-activated protein kinase (p-p44/42 MAPK) (Thr-202/Tyr-204), and anti-total-p44/42 MAP kinase (1:1000) (all from Cell Signaling Technology); rabbit anti-ERα (Santa Cruz Biotechnology, 1:500); rabbit anti-ERβ (Upstate, 1:500); rabbit anti-epidermal growth factor receptor (EGFR) (1005, Santa Cruz Biotechnology, 1:250); mouse anti-β-actin (1:4000) (Sigma-Aldrich). After incubation with secondary antibodies (horseradish peroxidase-conjugated) (Santa Cruz Biotechnology), proteins were detected by chemiluminescence detection (SuperSignal West Pico Chemiluminescent Substrate, Pierce). The level of β-actin expression was used as a loading control. For p-Akt/Akt and p-p44/42 MAPK/ p44/42 MAPK, the following procedure was applied: the membranes were chemically stripped of antibodies using stripping buffer and rebloked before reprobing. p-Akt (or p-p44/42 MAPK) was probed first, followed by Akt (or p44/42 MAPK) and then β-actin. Protein levels were quantified by densitometric analysis using the Scion Image Beta 4.02 software package.

Serum insulin-like growth factor-1. Circulating levels of insulin-like growth factor-1 (IGF-I) were determined by radioimmunoassay following the instructions provided by the manufacturer (product code IGF-R22, Medignost).

Statistical methods. Tumor weight data were analyzed by repeated-measures ANOVA followed by the Bonferroni method as post-test. The remaining data were analyzed by 1-way ANOVA followed by Dunnett’s multiple comparison test. Statistical analysis was carried out with GraphPad Prism5 software. Values in the text are means ± SD, P < 0.05 was considered significant.

Results

A549 tumors in athymic female mice. Tumors in the control group grew rapidly (Fig. 1). Treatment with 100 mg SSE/(kg·d) suppressed the growth of A549 xenografts in athymic mice beginning at d 41 (P < 0.01) (Fig. 1). The efficacy of the treatment was also confirmed by the achievement of a LCK value of 1.2 (Table 2). Treatment with 50 mg SSE/(kg·d) did not affect tumor growth (Fig. 1; Table 2). Body weight and food consumption did not differ among treatment groups (data not shown).

Tumor necrosis in the untreated tumors was ~10%. Diffuse and more extensive necrosis was present in tumors of mice treated with 50 or 100 mg SSE/(kg·d); these showed ~30 and 50% necrosis, respectively. In the high-dose group, the majority of mice showed only a thin rim of viable tumor tissue at the periphery (Supplemental Fig. 1).

Proliferation was lower in tumors from mice administered 50 or 100 mg SSE/(kg·d) than in controls (P < 0.05), as shown by the reduction in the percentage of positive-stained cells (Fig. 2A; Supplemental Fig. 2A).

Apoptosis was higher in tumors from mice receiving 50 or 100 mg SSE/(kg·d) than in control tumors (P < 0.05) as shown by the increase in the percentage of brown apoptotic bodies (Fig. 2B; Supplemental Fig. 2B). When stained with H&E, tumors from treated mice had very dark and dense nuclei and smaller nucleoli compared with control tumors, indicative of degenerative cells undergoing to apoptosis (Supplemental Fig. 2C).

Tumor EGFR, p-Akt/Akt, p-p44/42 MAPK/p44/42 MAPK, ERα, and ERβ concentrations and ratios. EGFR levels in A549 tumors did not differ among treatment groups (overall mean, 0.74 ± 0.25 arbitrary units; n = 30).

The p-Akt:Akt ratio was decreased in A549 tumors from mice treated with 100 mg SSE/(kg·d) compared with controls (P < 0.01), whereas no changes occurred in the 50 mg SSE/(kg·d) group (Fig. 3). Tumors from mice receiving 100 mg SSE/(kg·d) also tended to have a lower p-p44/42 MAPK:p44/42 MAPK ratio compared with controls (i.e. 0.57 ± 0.27 vs. 0.80 ± 0.17; P = 0.07).

Using an antibody to the COOH terminus, we observed in A549 tumors both the full-length ERα 66 kDa and also a smaller band at 54 kDa; SSE treatment did not change protein levels compared with control tumors (Supplemental Table 1). Immunoblots of the same tumors using an ERβ polyclonal antibody showed that A549 expressed the ERβ1 isofrom (i.e. the short isoform of ERβ) as 33-kDa size; no differences in the receptor protein levels were observed among treatment groups (Supplemental Table 1).

Serum IGF-I and uterus weight. The treated groups did not differ from controls in serum IGF concentrations (overall,

![FIGURE 1 A549 growth in female athymic mice receiving 0 (control), 50, or 100 mg SSE/(kg·d). Values are means ± SD, n = 10. *Different from control at a time, P < 0.01.](https://academic.oup.com/jn/article-abstract/138/7/1360/4670212/Downloaded-from-hps://academic.oup.com/jn/article-abstract/138/7/1360/4670212 by guest on 23 February 2019)

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<th>Treatment schedule</th>
<th>TWI%</th>
<th>TGD</th>
<th>LCK</th>
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<td>SSE 50</td>
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<td>33 (23)</td>
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<td>Per os, 5 d/wk</td>
<td>50 (23)</td>
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1 Mice were not treated on weekend days.
2 TWI% in treated vs. control mice. The optimum value for TWI% obtained during the study is reported; in parentheses the day on which it was evaluated.
3 Difference in the mean time for treated (T) and control (C) group tumors to reach a predetermined size (900 mg); the time for the mean control tumors to reach 900 mg was 38 d.
4 LCK = TGD value in days/3.32 × Td; Td was 7 d in control mice.

<table>
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<th>TABLE 2 Effects of SSE on the growth of A549 xenografts in female athymic mice</th>
<th>Treatment group</th>
<th>n</th>
<th>Treatment schedule</th>
<th>TWI%</th>
<th>TGD</th>
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In this study, we have shown that a phytoestrogen-containing soy extract is able to delay A549 lung tumor xenograft growth in intact athymic female mice; tumors from SSE-treated groups had reduced proliferation and greater apoptosis compared with controls. The SSE-mediated inhibition of tumor growth was associated with inactivation of Akt, a finding consistent with previous literature data showing that the phytoestrogen genistein is able to downregulate total and phosphorylated Akt proteins in A549 cells (17). Akt and MAPK are 2 major intracellular downstream signaling pathways that are directly or indirectly activated in response to ligand-dependent EGFR functional activation; specifically, Akt, a member of the Ser/Thr kinases, regulates cell survival and apoptosis in its activated state, whereas p44/42 MAPK regulate cell growth and proliferation (18). In lung carcinogenesis, Akt is regarded as a crucial mediator of tumor survival and therapeutic resistance and it has been shown to be constitutively active in most NSCLC cells, including the A549 (19). It has also been reported that Akt plays an important role in tumor angiogenesis, with studies showing that overexpression of phosphoinositide-3 kinase or Akt is associated with increased vascular endothelial growth factor levels (a fundamental regulator of tumor angiogenesis) (20–22). Results from the present study demonstrated that SSE treatment induced extensive central necrosis and reduced viable tissue mass within A549 tumor, a finding likely arising from a reduced vascularization of the tumor. Although we did not specifically evaluate the effect of treatment on tumor angiogenesis in the present experiment, we previously reported that SSE was able to modulate angiogenesis in a well-established preclinical in vivo model; the data showed a striking antiangiogenic activity in mice receiving 100 mg SSE/(kg·d) (23). Collectively, results from the present study suggest that the modulation of the Akt signaling pathway may possibly represent the underlying molecular mechanism by which SSE significantly induces apoptosis, reduces proliferation of cancer cells, and induces tumor necrosis in our experimental conditions.

It is noteworthy that our findings occurred at physiological plasma levels of phytoestrogens. In fact, a previous pilot study carried out in our laboratory showed that, following repeated administration of 50 or 100 mg SSE/(kg·d) to healthy athymic mice, plasma concentrations of total daidzein and genistein were as follows: total daidzein, 1.1 ± 0.3 and 1.6 ± 0.7 μmol/L; total genistein, 0.8 ± 0.1 and 0.9 ± 0.2 μmol/L, for the low and the high-dose group, respectively (our unpublished data). In agreement with previous results (24), formation of equol in athymic mice was highly variable, with only 1 of 10 mice in each group forming the metabolite (our unpublished data). These isoflavone blood levels are in the range of those in Japanese women consuming a traditional soy diet [i.e., daidzein mean concentrations 246.8 nmol/L (range 0–2407); genistein mean concentrations 501.9 nmol/L (range 0–4192)] (25), and to concentrations detected in a previous clinical trial in menopausal women receiving the tested extract (26). At these doses, effective to slowing down tumor growth, SSE did not cause stimulation in uterus.

Even if no treatment-related effects were observed in the tumor protein levels of ERα and ERβ, the possibility that the findings reported in this study arise from an anitioestrogenic activity of SSE cannot be ruled out. Actually, the precise molecular events governing ER activities in lung carcinogenesis are poorly defined, although 2 types of interactions between EGFR/ER pathways have been identified. First, there is a nongenomic, rapid signaling that is dependent on estrogen results in release of ligands for EGFR and activation of EGFR pathway. A second type of ER-EGFR interaction has been...
identified in the nucleus and is independent of estrogen; the proposed model involves activation of nuclear ER or ER myb-binding protein 1A coregulator proteins, via signaling through an EGFR-dependent MAPK cascade (4). Notably, Fulvestrant, an ER-antagonist with no agonistic effects, was effective in inhibiting lung tumor xenograft growth in mice by 40% (27). Likewise, it is possible that using SSE in intact (nonovariectomized) female mice with background circulating levels of estrogen may induce antiestrogenic activity, thus perturbing the functional interaction between the ER and the EGFR pathways in NSCLC.

Finally, results obtained in this study did not show any modulation of IGF-1 serum levels upon administration of SSE, a finding in keeping with previous results by our and other groups in preclinical models of estrogen-dependent breast cancer (28,29) but inconsistent with other literature data showing a reduction in circulating levels of this growth factor in mice bearing human prostate or bladder tumors following consumption of dietary soy products (30,31). The reason why effects of soy isoflavones on IGF-1 signaling are seen in some experimental systems but not in others is not understood, possibly involving differences in both cell lines and/or experimental conditions.

In conclusion, our research provides further support for the concept that consumption of phytoestrogens may be effective in delaying lung cancer progression, suggesting that direct or indirect modulation of Akt signaling pathways may have a role in the activity observed.

**Literature Cited**