Dietary intake of a plant phospholipid/lipid conjugate reduces lung cancer growth and tumor angiogenesis

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It is well recognized that early detection and cancer prevention are significant armaments in the ‘war against cancer’. Changes in lifestyle and diet have significant impact on the global incidence of cancer. For over 30 years, many investigators have studied the concept of chemoprevention. More recently, with the demonstration that antiangiogenic activity reduces tumor growth, the concept of angio-prevention has emerged as a novel strategy in the deference of cancer development (carcinogenesis). In this study, we utilized a fast growing, highly aggressive murine Lewis lung cancer model to examine the in vivo antitumor effects of a novel, dietary supplement, known as plant phospholipid/lipid conjugate (pPLC). Our goal was to determine if pPLC possessed direct antitumor activity with relatively little toxicity that could be developed as a chemoprevention therapy. We used pPLC directly in this in vivo model due to the lack of aqueous solubility of this novel formulation, which precludes in vitro experimentation. pPLC contains known antioxidants, ferulic acid and lipoic acid, as well as soy sterols, formulated in a unique aqueous-insoluble matrix. The pPLC dietary supplement was shown to suppress in vivo growth of this tumor model by 30%. We also demonstrated a significant decrease in tumor angiogenesis accompanied by increased apoptosis and present preliminary evidence of enhanced expression of the hypoxia-related genes pentraxin-3 and metallothionein-3, by 24.9-fold and 10.9-fold, respectively, compared with vehicle control. These findings lead us to propose using this plant phospholipid/lipid conjugate as a dietary supplement that may be useful in cancer prevention.

Introduction

The National Cancer Institute recognizes that prevention is an essential component to the strategic plan to eliminate the suffering and death due to cancer. It is estimated that 30–40% of cancers can be directly linked to dietary habits. People who eat a diet high in plant foods have a much lower risk of developing cancer. Despite a growing body of evidence supporting an association between diets rich in fruits and vegetables and reduced risk of cancer, human clinical trials are now underway to examine the cancer prevention effectiveness of select, purified phytochemicals. Accordingly, the challenging nature of these studies will require a significant amount of time before the effectiveness of potential preventive therapies can be definitively established.

Plant sterols (phytosterols) are a specific group of phytochemicals similar in structure to cholesterol, but found exclusively in plants. There are known to exist (4,5). Plant sterols are known to interfere with cholesterol transport from the gastrointestinal tract and to reduce serum cholesterol (6,7). An inverse correlation between plant sterol consumption and serum cholesterol has also been observed in epidemiologic studies (8). Evidence from case–control studies conducted in Uruguay also supports an anticancer role of plant sterols in lung, stomach, estrogen-dependent breast and ovarian cancer (9-12). Although multiple mechanisms for the anticancer effects of plant sterols have been proposed, the detailed mechanisms associated with individual plant sterols from a specific plant source have not yet been studied in detail (13). To our knowledge, the simultaneous administration of specific plant sterols and other purified phytochemicals on in vivo tumor growth has not been examined. Such studies would advance the creation of dietary databases and thereby facilitate future investigations and clinical trials.

Plant polyphenols are a structurally diverse group of phytochemicals that act on multiple targets and mechanisms involved in carcinogenesis, cell proliferation, apoptosis, inflammation and angiogenesis. The polyphenols are known to have strong antioxidant activities that result in cytoprotective effects that are mediated in part through hormetic mechanisms (14,15). Although the antioxidant activity of these compounds has long been considered a principal mechanism for reduced tumor growth, interest in the antiangiogenic and anti-inflammatory activity of polyphenol compounds has increased significantly in recent years. The concept of angioprevention, first proposed in 2002, seems to converge with the effects of polyphenols on the redox imbalance of the tumor microenvironment resulting in an overall antitumor response (1,16). Indeed, several purified compounds from this group such as the epigallocatechin gallate from green tea and resveratrol from grapes and red wine are now in clinical trials for prevention of cancer. However, epigallocatechin gallate has been shown to have a complex role in carcinogenesis through alteration of enzymes involved in both modification (Phase-I) and conjugation (Phase-II) of a variety of pharmaceutical agents (17). These effects range from enhancing the therapeutic potential of chemotherapeutic agents observed by doxorubicin’s ability to augment cytotoxicity to strongly reducing the therapeutic potential of chemotherapeutic agents demonstrated by reduced antiblastic activity of bortezomib (reviewed in ref. 17). The in vivo effects of other potent plant polyphenols, such as ferulic acid (abundant in eggplants and artichokes), are less well studied (18). However, ferulic acid esters demonstrate potent antioxidant activity with significant inhibition of tumor cell proliferation (breast, lung, colon, central nervous system and gastric carcinoma cell lines), as well as cyclooxygenase (COX-1 and 2) and lipid peroxidation inhibitory activity (19). The antioxidant activity of ferulic acid has also been shown to contribute to the cytoprotective effects in normal, human dermal fibroblasts in vitro, and neuroprotective effects in vivo (20,21).

Lipoic acid was originally considered a vitamin; however, subsequent studies demonstrated that it is synthesized in plants and animals. Humans can synthesize lipoic acid from fatty acids and cysteine, but only in very small amounts (22). Lipoic acid contains two oxidized or reduced thiol groups. The oxidized form, referred to as lipoic acid, is principally responsible for inactivation of free radicals and metal atom

Abbreviations: FGF2, fibroblast growth factor-2; IL, interleukin; LL2-LUC, luciferase-labeled Lewis lung carcinoma; MAP, mouse antibody production; pPLC, plant phospholipid/lipid conjugate; RT-PCR, real-time PCR.
chelation. The reduced form known as dihydrolipoic acid has potent antioxidant activity and inactivates reactive oxidative species (23). Given that food intake may reduce the absorption of lipoic acid, it is therefore recommended that lipoic acid be taken for therapeutic purposes from exogenous sources such as dietary supplements between meals. Dietary supplements containing up to 600 mg are available providing 1000 times greater dose than that available from diet alone (22). Lipoic acid supplements in dosages as high as 1800 mg/day are associated with significant weight loss, and doses of 2400 mg/day have been shown to be useful in clinical trials for the prevention of cardiovascular disease with little or no adverse effects (24-26). Lipoic acid acts as an important coenzyme for several mitochondrial enzymes and protects these organelles from oxidative stress. Lipoic acid also inhibits growth of a variety of cancer cells in vitro including human colon cancer (HT-29) cells, Jurkat (acute T-cell leukemia) and FaDu (pharyngeal squamous carcinoma) cells, as well as murine B16F10 melanoma cells. The principal mechanisms involved in these in vitro effects were antioxidant activity and induction of apoptosis (8,23,27,28).

We identified a commercially available dietary supplement plant phospholipid/lipid conjugate (pPLC) available from Conjugated Functional Foods (Hackensack, NJ), which combines soy sterols, ferulic acid and lipoic acid through a proprietary process with a variety of other ingredients that are crucial to the formulation and conjugation process. As discussed above, previous studies have shown that soy sterols, ferulic acid and lipoic acid each possess low bioavailability but overlapping biological antioxidant and antiangiogenic activities. Given the long time course involved in true ‘chemoprevention and angioprevention’ studies, we instead selected a highly aggressive syngeneic mouse lung cancer model (Lewis lung carcinoma) in order to more rapidly (24-28 days) evaluate the in vivo antitumorogenic and antiangiogenic potential of pPLC. In addition, we utilized histologic staining and messenger RNA expression profiling to examine changes in gene expression possibly associated with antimutator mechanisms.

Materials and methods

Feed composition and preparation

The composition of pPLC and vehicle control conjugates are shown as weight percent in Table I. Purina mouse chow is the standard diet given to all mice housed in the Clinical Cancer Research Mouse Vivarium at National Institutes of Health. Accordingly, we used this feed to deliver both the pPLC-containing and control-conjugated formulations since they are insoluble in aqueous solution. In these experiments, pPLC or the vehicle control conjugates were physically milled into the Purina mouse chow, resulting in powdered chow containing different weight percent concentrations of the vehicle or pPLC conjugates. Powdered chow was fed ad libitum using Follower feeders.

Preliminary experiments demonstrated that mice fed chow containing concentrations of pPLC greater than 5% failed to ingest sufficient feed to demonstrate the normal gain in body weight observed in control mice.

Table I. Weight percent compositions of vehicle control and pPLC conjugates feed administered to mice

<table>
<thead>
<tr>
<th>Additive</th>
<th>Vehicle (weight %)</th>
<th>Vehicle g/kg/day</th>
<th>pPLC (weight %)</th>
<th>pPLC g/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy lecithin</td>
<td>30</td>
<td>2.01</td>
<td>27</td>
<td>1.81</td>
</tr>
<tr>
<td>Soy oil</td>
<td>30</td>
<td>2.01</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Xantham gum</td>
<td>25</td>
<td>1.68</td>
<td>25</td>
<td>1.68</td>
</tr>
<tr>
<td>Aloe vera</td>
<td>10</td>
<td>0.67</td>
<td>7.55</td>
<td>0.506</td>
</tr>
<tr>
<td>Calcium sulfate</td>
<td>3.75</td>
<td>0.251</td>
<td>7.10</td>
<td>0.476</td>
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<tr>
<td>Magnesium sulfate</td>
<td>0.35</td>
<td>0.023</td>
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<td>0.245</td>
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<tr>
<td>Glycerin</td>
<td>0.53</td>
<td>0.036</td>
<td>7.0</td>
<td>0.469</td>
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<tr>
<td>Oil of lemon</td>
<td>0.19</td>
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<td>0.101</td>
</tr>
<tr>
<td>Citric acid</td>
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<td>0.012</td>
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<tr>
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<tr>
<td>Lipoic acid</td>
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<tr>
<td>H2O</td>
<td>100</td>
<td>6.7</td>
<td>100</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Mice and tumorigenicity studies

Five- to 8-week-old female C57BL/6j mice (Jackson Laboratories, Bar Harbor, ME) were housed 5 mice/cage, under a 12:0 h light/dark cycle with access to Purina chow containing 5% vehicle control or 5% pPLC conjugate and water ad libitum in accordance with an approved National Institutes of Health Institutional Animal Care and Use Committee protocol LP-003. Luciferase-labeled Lewis lung carcinoma (LL2-LUC) cells (Caliper Life Sciences, Hopkinton, MA) were grown in Dulbecco’s Modified Eagle Medium/F-12 media 1:1 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma–Aldrich, St. Louis, MO) in a humidified incubator containing 5% CO2, at 37°C. Prior to subcutaneous injection, LL2-LUC cells were mouse antibody production tested (NCI/FCRF, Frederick, MD), grown to ~80% confluence and counted using the Z1 Coulter Particle Counter (Beckman Coulter, Brea, CA). The viability of LL2-LUC cell cultures were determined by Trypan Blue staining (Invitrogen, Carlsbad, CA). Each mouse was injected subcutaneously in the right flank with 0.25 × 10^6 LL2-LUC cells resuspended in 100 µl phosphate-buffered saline. Five or 15 mice per experimental group were used in three replicate experiments. Mice were switched to chow containing 5% weight volume pPLC or vehicle control conjugate 3 days posttumor cell inoculation to allow time for tumor take. Feed was changed every 48 h. At the indicated number of days posttumor cell injection, mice were weighed, tumor growth was monitored by luminescence using the IVIS Spectrum Imaging System (Perkin Elmer) and the Living Image Software v3.1 (Caliper Life Sciences, Hopkinton, MA), and tumor volumes were calculated by manual caliper measurements using the following formula: Volume = Length × Width × Height/2. For luminescence measurements, mice were injected intraperitoneally with 100 µl of 37.5 mg/ml D-luciferin diluted in sterile phosphate-buffered saline 15 min prior to imaging. The experiment was repeated at least 3 times using either 5 or 15 mice per group in each experiment.

Immunohistochemistry

Immunohistochemistry was performed to examine CD-31 expression in paraffin-embedded formalin-fixed tumor tissue using the avidin–biotin peroxidase method with the Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Prior to staining, slides were deparaffinized in a series of xylene and graded alcohols, followed by antigen retrieval in 0.01M Citrate buffer (pH 6.0) and incubation in 3% hydrogen peroxide to quench endogenous peroxidase. Intratumoral microvascular density, a measure of tumor angiogenesis, was assessed using the rat anti-mouse CD-31 antibody (PECAM-1, Dianova, Hamburg, Germany) at a dilution of 1:40. We investigated the mechanisms associated with the antitumorigenic effect of pPLC by immunohistochemistry using antibodies to Pentraxin-3 (Lifespan BioSciences, Seattle, WA), Macrophage Inhibitory Protein-3 (9829), CD11b (EPR13434), and F4/80 (C57-1-1) (Abcam, Cambridge, MA). Formalin-fixed, paraffin-embedded tumor tissues were subject to heat-induced antigen retrieval in 0.01M Citrate buffer (pH 6.0) prior to incubation with the primary antibody overnight at 4°C. Color development was achieved using 3,3’-diaminobenzidine (Sigma Chemical, St. Louis, MO). Quantification of staining was assessed by calculating the mean number of positive cells within 10 high-power (×200) fields per animal in each group (vehicle control n = 5 and pPLC n = 6). Individual comparisons were performed using unpaired t-test (GraphPad Prism). Mean values for all parameters were used to determine statistical significance (P < 0.05).
Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay

Apoptotic cell death was investigated using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay (ApopTag Plus Peroxidase In Situ Detection Kit, Chemicon, Temecula, CA) according to the vendor’s instructions. Quantification of staining was assessed by calculating the mean number of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling positive cell nuclei within 10 high-power (×200) fields per animal in each group (vehicle control n = 5 and pPLC n = 6) and comparisons between groups were performed as previously described.

SA Biosciences real-time PCR array on tumor tissue

At the termination of the experiment, tumors were resected and a portion was submerged in RNAlater® Solution (Ambion®, Life Technologies, Grand Island, NY) and stored at 4°C until RNA isolation. Total RNA was isolated from ~30 mg of tumor tissue using the RNeasy™ Kit (Qiagen, Valencia, CA). Tumor tissue was homogenized using the gentleMACS™ Dissociator (Miltenyi Biotec, Cambridge, MA) and RNA purity and concentration was determined using the Nanodrop 1000 Spectrophotometer v3.7 (Thermo Fisher Scientific, Waltham, MA). The complementary DNA was generated for each tumor from 2 μg RNA using the RT² First Strand™ Kit (Qiagen, 330401) according to the manufacturer’s protocol. All real-time (RT)-PCR controls contained 2X SA Biosciences RT² SYBR Green/ROX Master Mix (Qiagen, 330521) and one reverse transcription reaction containing 2 μg RNA for every 96 wells of the Mouse Hypoxia Signaling Pathway RT²Profiler™ PCR Array (SA Biosciences, A Qiagen Company, PAMM-032E-4) or Mouse Inflammatory Cytokines and Receptors RT²Profiler™ PCR array (SA Biosciences, A Qiagen Company, PAMM-011A). Each array contained five housekeeping genes, one genomic DNA control, three reverse transcription controls, and three positive PCR controls in order to control for template concentration and consistency between arrays, as well as to detect any sample contamination or impurities. RT-PCR was performed on 10 tumor samples (five per group) and normalized to glyceraldehyde 3-phosphate dehydrogenase by the ΔΔCt method.

Pentraxin-3 (Ptx3) enzyme-linked immunosorbent assay and RT-PCR

Marine dermal fibroblasts isolated from C57BL/6 pups and Lewis lung carcinoma cells were plated at 1 × 10⁴ cells per well in a 12-well plate and grown until 80% confluent. Cells were serum starved for 4 h prior to treatment with 0.1 ng/ml interleukin (IL)-1β, 10 ng/ml IL-1β, or 1 ng/ml transforming growth factor-β. Supernatants and cells were collected 24 h later for mPtx3 enzyme-linked immunosorbent assay and RT-PCR analysis. Ptx3 levels were measured in tissue culture supernatants according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN—MPTX30). Sensitivity was 0.23 ng/ml. The complementary DNA was generated from 2 μg RNA using the RT² First Strand™ Kit (Qiagen, 330401) according to the manufacturer’s protocol. RT-PCR reactions were run using the Applied Biosystems 7900HT Fast Real-Time PCR System. SYBR Green master mix (Applied Biosystems) and the following primer sequences were added to each complementary DNA sample: forward, 5′-GCCTGTGAGCAACAGATT-3′ and reverse, 5′-CCAGAAGGCAAGGAGGAT-3′. Mean Ct values for Ptx3 were normalized to the Ct values of the endogenous control, glyceraldehyde 3-phosphate dehydrogenase, and the ΔΔCt method was used to analyze fold change in Ptx3 expression.

Statistical analyses

Tumor sizes and weights were tested for statistical significance by using a 1-tailed t-test with F-tests to evaluate variance (GraphPad Software) and P values ≤ 0.05 were considered statistically significant.

Results

Dietary intake of pPLC conjugate decreases Lewis lung carcinoma tumor growth

In order to test if milled chew containing the pPLC and vehicle control conjugates were consumed by non-tumor bearing C57BL/6 mice animal body weights were monitored on a daily basis. We first tested chew containing 25% of total weight as vehicle or pPLC milled with chew to determine if this high concentration was palatable and to deliver maximal concentrations of the pPLC constituents. Accordingly, at this concentration the mice lost an average of 14.4% total body weight compared with controls fed chew containing no additive, which gained an average of 4% total body weight over the 6-day test period (data not shown). We concluded that the 25% weight concentration of the vehicle or pPLC was too high and that the mice did not consume this mix sufficiently to increase body weight. Alternatively, the observed weight loss could be associated with the antiobesity effects of high-dose lipoic acid, as previously reported in rodents and human clinical trials (26,29).

To ensure milled chew consumption and avoid the weight loss associated with high-dose lipoic acid consumption, we tested chew with 5% of total weight concentrations of the pPLC or vehicle control conjugates. Using an in vivo, highly aggressive tumor model of short duration, the 5% weight composition was calculated to deliver adequate levels of bioavailable pPLC in the limited dosing period of the experiment. The groups of animals on this percent weight composition gained weight at exactly the same rate as the mice fed Purina chew containing no added conjugate. These results suggest that at 5% weight composition, the mice consumed the feed containing the conjugates at approximately the same rate as control feed to maintain a normal increase in body weight throughout the duration of the study and avoid the antiobesity (weight loss) associated with high-dose lipoic acid consumption (data not shown). The mean daily feed intake for C57BL/6 mice in these experiments was 4.0 g/30 g mouse/day, very close to the overall average daily intake of 4.4 g/mouse determined in 26 different mouse strains (30). We used a mean daily feed intake of 4 g/mouse to calculate the daily intake of components in the 5% pPLC and vehicle control feed using the percent compositions shown in Table I (total converted intake of pPLC or vehicle 6.7 g/kg/day).

To study the in vivo impact of pPLC conjugate on primary tumor growth, we injected C57BL/6 mice subcutaneously with luciferase-labeled Lewis lung carcinoma cells. Three days after tumor cell inoculation to allow tumor take, mice were switched from conventional diet to a milled diet containing 5% pPLC or vehicle control. At the indicated number of days posttumor cell inoculation, primary tumor growth was measured by luminescence (Figure 1A and B). Luminescence could be detected as early as 7 days posttumor cell inoculation in all mice, but manual caliper measurements could not be accurately determined until 14 days posttumor cell inoculation (data not shown), but demonstrated a rough correlation with the increase in luminescence measurements from day 14–24. In contrast, luminescence values became unreliable after 24 days, although tumor volume could still be calculated from manual caliper measurements at day 27 (when the experiments were terminated according to the animal study protocol with tumor volumes in excess of 2000 mm³). These findings are consistent with the greater sensitivity of the luminescence imaging compared with conventional caliper measurement.

Luminescence values revealed a statistically significant (P ≤ 0.05) decrease in tumor cell growth in mice on the 5% pPLC diet compared with vehicle control diet on days 19 (42.23 ± 6.56 × 10⁵ photon flux versus 46.20 ± 5.77 × 10⁵ photon flux, respectively, P ≤ 0.01) and 24 (95.92 ± 20.03 × 10⁵ photon flux versus 153.67 ± 312.0 photon flux, respectively, P ≤ 0.04) posttumor cell inoculation (Figure 1A, upper and lower panels). A statistically significant decrease in tumor volume was also recorded by manual caliper measurements 27 days posttumor cell inoculation in mice receiving 5% pPLC (2418.5 ± 312.0 mm³ versus 1654.1 ± 195.1 mm³, P ≤ 0.05; data not shown). These results suggest that dietary intake of pPLC conjugate suppresses tumor growth of a highly aggressive syngeneic mouse lung cancer model.

To confirm the tumor inhibitory effect of pPLC conjugate in vivo, we compared the average tumor weights between the two treatment groups at the conclusion of the experiment (27 days posttumor cell inoculation). Primary tumors isolated from C57BL/6 mice fed the pPLC conjugate diet weighed significantly less than tumors from mice fed the vehicle control diet (1.57 ± 0.20 g compared with 2.20 ± 0.21 g, respectively, P ≤ 0.05; Figure 1B, right panel).

Dietary pPLC decreases lung tumor vascularity and increases tumor cell apoptosis in vivo

The previously reported in vitro antiangiogenic and/or antiapoptotic effects of soy sterols, lipoic acid and ferulic acid led us to examine whether similar mechanisms contributed to decreased tumor growth in vivo. Formalin-fixed, paraffin-embedded tumor sections were analyzed for microvascular density (CD-31 staining, Figure 1C upper panels) and tumor cell apoptosis (terminal deoxynucleotidyl
Fig. 1. Lewis lung carcinoma tumor growth, tumor angiogenesis and apoptosis in vivo. (A, upper and lower panels) C57BL/6J mice were injected (subcutaneous) with luciferase-labeled Lewis lung carcinoma cells and at day three started on a diet with 5% weight percent pPLC (upper panel) or vehicle control (lower panel). At the indicated number of days posttumor cell inoculation, primary tumor growth was measured using an IVIS spectrum imaging system to determine photon flux within the areas outlined in red using the Living Image Software v3.1. (B, left panel) Tumor growth over time measured by luminescence is shown in units of photon flux (photons/s/cm²/sr). (B, right panel) Tumor weights were obtained at the termination of the experiment on day 27 posttumor cell inoculation. Experiment was repeated three times and each time the pPLC diet showed a 22–30% reduction in tumor volume by luminescence and manual caliper measurements (data not shown). Shown is a representative experiment with n = 15 mice per condition. * indicates P ≤ 0.05. (C, upper panel, red arrows) Tumors were harvested 27 days posttumor cell inoculation and stained for CD-31 expression to measure blood vessel formation. (D, upper panel) Expression was
Dietary consumption of pPLC increases metallothionein-3 and pentraxin-3 gene expression within LL2 tumors

The growth of primary tumors can occur more quickly than the infiltration of new blood vessels, thereby creating hypoxic microregions within the tumor mass. In areas of these hypoxic conditions, tumor cells generate excess production of reactive oxygen species. Since, soy sterols, ferulic acid and lipoic acid conjugate, the active ingredients in pPLC, are all known to have potent antioxidant activity, we analyzed differential gene expression in tumors from the two experimental groups using a mouse hypoxic signaling pathway RNA microarray (RT²Profiler™). Results of these analyses on tumors from mice receiving pPLC conjugate feed showed a significant upregulation of at least five genes and downregulation of four genes compared with mice fed vehicle control conjugate, Table II. The greatest increases were found in metallothionein-3 (Mt3) and pentraxin-3 (Ptx3) expression with a 10.9 and 24.1-fold increase, respectively. Further investigation of the cells expressing Mt3 were hampered by the lack of antibodies that worked well in immunohistochemical staining or western blot.

In contrast, pentraxin 3 (Ptx3) is a member of the long pentraxin superfamily that is rapidly produced and released by several cell types including mononuclear phagocytes, dendritic cells, fibroblasts and endothelial cells in response to primary inflammatory signals (including IL-1β) and has been reported to be a significant biomarker for human non-small lung cancer through its elevation in the inflammatory tumor microenvironment and adipose tissue (31–33).

We tested the contribution of the LL2-LUC cells to observed elevated levels both in vivo and in vitro. Immunohistochemical analysis of LL2-LUC tumor-bearing mice treated with pPLC showed a significant increase in highly positive cell staining in non-tumor cells at both the periphery and the center of the tumors (Figure 2B and D–F). Whereas, in control tumors, only slight non-specific staining for Ptx3 was observed at the periphery of the tumor mass. These findings are consistent with the increased cell expression of Ptx3 not in the LL2-LUC tumor cells, but in other, probably inflammatory, cell types in a statistically significant fashion, both in the centers and periphery of the tumors grown in vivo, (Figures 2E and F). These findings are highly consistent with the evolving concept of the tumor microenvironment in the regulation of tumor progression, and that cellular suppression of tumor growth by host cells may be exploited therapeutically.

We also examined whether LL2-LUC cells contributed to the large (24.1-fold) increase in Ptx3 expression within the tumor samples, using an in vitro-stimulated cell expression model. LL2-LUC cells were treated with 0.1 or 10 ng/ml of IL-1β, a known inducer of Ptx3 expression (33), and expression levels were determined by RT-PCR and enzyme-linked immunosorbent assay (Figure 3). LL2-LUC cells had undetectable (<0.2 ng/ml) levels of secreted Ptx3 as determined by a murine Ptx3 enzyme-linked immunosorbent assay (R&D Systems, data not shown). Although basal levels of Ptx3 expression were detectable in LL2-LUC cells by quantitative RT-PCR, treatment with 10 ng/ml IL-1β did not increase Ptx3 expression. Conversely, treatment with 10 ng/ml treatment of IL-1β did increase expression of Ptx3 in murine primary dermal fibroblasts (Figure 3).

To further elucidate possible mechanisms for enhanced expression of Mt3 and/or Ptx3 in the tumor microenvironment of mice receiving the pPLC conjugate feed, we examined changes in murine inflammatory cytokines and receptors again using a RT²Profiler™ PCR array. Analyses revealed upregulation of four cytokines tested on the array (Table III). These findings suggest that pPLC treatment may significantly alter the inflammatory profile of the tumor microenvironment and further influence tumor cell growth. However, further experiments are necessary to determine the types of inflammatory cells that may be recruited to pPLC-treated tumors and if this indeed influences tumor growth.

### Discussion

The National Cancer Institute recognizes cancer preventive therapy as the reduction of cancer mortality via reduction in the incidence of cancer. Cancer prevention involves lifestyle changes, such as smoking cessation, reduced alcohol consumption, avoiding exposure to known carcinogens, and enhanced surveillance to allow early detection and surgical resection of precursor lesions. Cancer prevention also includes chemoprevention that was demonstrated over 30 years ago, as well as the more recent concept of angioprevention (1,16,34,35). Chemoprevention and angioprevention involve dietary or pharmacologic interventions that disrupt the carcinogenic and/or angiogenic processes associated with cancer development and progression.

In this study, we examined the antitumorigenic activity of a compound phytochemical agent pPLC using a rapidly growing syngeneic murine model of lung carcinoma. The aim of these studies was to use this fast growing, highly aggressive murine lung cancer model to rapidly determine the antitumor activity of pPLC before initiating more complex prevention studies. Our results demonstrate that pPLC reduced tumor-induced angiogenesis and enhanced tumor cell apoptosis, which are consistent with the reported effects of the soy sterols, ferulic acid.

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Table II. Fold change in gene expression in tumors from mice fed a pPLC diet compared to tumors from control mice using the Murine Hypoxia Signaling Pathway RT² Profiler™ PCR Array

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
<th>Fold change</th>
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<tr>
<td>Pentraxin-related gene</td>
<td>Ptx3</td>
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<tr>
<td>Metallothionein-3</td>
<td>Mt3</td>
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<td>Interleukin 6</td>
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<td>Proteasome subunit, beta 3</td>
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<tr>
<td>IQmotif containing GTPase</td>
<td>Iqgap1</td>
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</table>

*Table quantitated by counting the number of vessels stained positive for CD-31 within 20 high-power (×400) fields per animal in each group and the mean number of vessels per high-power field. (Figure 3 lower panels) Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay was used to measure cell death in tumor samples from mice on a diet with 5% weight percent pPLC (upper panel) or vehicle control (lower panel). (D lower panel) Quantification of staining was assessed by calculating the mean number of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling positive cell nuclei within 10 high-power (×200) fields per animal in each group. The bar graph represents the mean number of apoptotic cells counted per high-power field (B, right). * indicates P < 0.05.
pPLC decreases tumor growth and angiogenesis

PLC decreases tumor growth and angiogenesis and lipoic acid constituents of pPLC. However, it is presently unclear if these effects are related, in that reduced blood flow to the tumor further enhances hypoxic conditions that promote tumor cell apoptosis.

Rapidly growing tumors and tumors with reduced angiogenesis often encounter hypoxic conditions. Our quantitative RT-PCR analysis of differential gene expression demonstrated enhanced levels of Mt3 and Ptx3. Mt3 is a cysteine-rich cytosolic protein that regulates intracellular metal atom homeostasis. Low cytoplasmic Mt3 levels are associated with poor patient outcome in non–small cell lung cancer patients (36). Mt3 expression is reduced in gastric and esophageal carcinomas by methylation of key CpG islands in the promoter region (37). In contrast, Mt3 levels are increased in breast cancer tissues but not normal breast tissue (38). Apo-forms of Mt3 can bind zinc where oxidized zinc-binding cysteine residues of Mt3 will cause a release of zinc leading to cell death (13,16). Therefore, the oxidative status can influence whether Mt3 acts as a zinc buffer or induces zinc cytotoxicity within cells. Augmented Mt3 gene expression in pPLC-treated lung tumors may initially act as an antioxidant, but increased oxidative stress that may be associated with reduced tumor angiogenesis would increase intracellular zinc concentrations contributing to enhanced tumor cell apoptosis.

The elevation of Ptx3 levels in non-tumor cells was confirmed in vitro using IL-1β-stimulation and in vivo using immunohistochemistry. Ptx3 is a soluble pattern recognition receptor, also referred to as tumor necrosis factor–stimulated gene 14 and is part of the long pentraxin subfamily (11,39). Ptx3 is synthesized at sites of inflammation by mononuclear phagocytes, myeloid dendritic cells, fibroblasts, adipocytes, granulosa cells, mesangial cells, smooth muscle cells and endothelial cells (9,12). Extensive studies on potential Ptx3 ligands indicate that Ptx3 binds complement component C1q; apoptotic cells;

![Fig. 2. Photomicrographs of Ptx3 immunostaining 21 days posttumor cell inoculation. An increase in the number of distinct, solitary Ptx3 positive cells was observed in LLC tumors from mice fed the pPLC conjugate diet compared with vehicle controls (A–D, upper and middle panels). The bar graphs represent quantification of staining assessed by calculating the mean number of Ptx3 positive cells in 10 high-power (×200) fields per animal in each group (Control versus pPLC; E and F, lower panel). Graph pad Prism 6 t-test was used to compare mean values. * indicates P < 0.05.](https://academic.oup.com/carcin/article-abstract/35/7/1556/378752)
Fig. 3. Expression of Ptx3 is not increased in Lewis lung carcinoma cells in vitro. Lewis lung carcinoma (black) and primary dermal fibroblasts (gray) were plated and grown until 80% confluent prior to stimulation with 0.1 μg/ml IL-1β, 10 μg/ml IL-1β, or 1 μg/ml transforming growth factor-β for 24 h. mPtx3 expression was measured by RT-PCR using endogenous glyceraldehyde 3-phosphate dehydrogenase expression to normalize and the ΔΔCt method to calculate fold change in expression. Error bars represent standard error of the mean from three experiments.

Table III. Fold change in gene expression in tumors from mice fed a pPLC diet compared to tumors from control mice using the Murine Inflammatory Cytokines and Receptors RT² Profiler™ PCR Array

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokine (C-X-C motif) ligand 13</td>
<td>BCA1/CCL13</td>
<td>4.8</td>
</tr>
<tr>
<td>Interleukin 10</td>
<td>IL10</td>
<td>3.2</td>
</tr>
<tr>
<td>Interleukin 13</td>
<td>IL13</td>
<td>3.2</td>
</tr>
<tr>
<td>Stromal-derived factor</td>
<td>SDF1/CXCL12</td>
<td>2.9</td>
</tr>
<tr>
<td>Monocyte chemotactic protein 2</td>
<td>MCP2/CCL8</td>
<td>2.4</td>
</tr>
<tr>
<td>ATP-binding cassette, subfamily D</td>
<td>ABCD1</td>
<td>2.0</td>
</tr>
<tr>
<td>Macrophage inflammatory protein-3 alpha</td>
<td>MIP3α</td>
<td>-4.3</td>
</tr>
</tbody>
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Extracellular matrix components, such as tumor necrosis factor-α-induced protein 6, and angiogenic growth factors, Ptx3 has a high affinity for fibroblast growth factor-2 (FGF2) (9,12,40). The Ptx3/FGF2 interaction prevents FGF2 from binding its receptor and has been shown to inhibit FGF2-dependent endothelial cell proliferation in vitro and in vivo, in addition specific Ptx3 peptides have been proposed as novel therapeutics for cancer treatment (10,12). Thus, augmented Ptx3 may be responsible for the observed decrease in tumor-associated angiogenesis. In vivo data suggest that IL-1β stimulation does not enhance Ptx3 expression in LL2 tumor cells but induces a significant increase of Ptx3 expression in dermal fibroblasts. Thus, our data would suggest that pPLC treatment influences gene expression in both tumor cells (Mt3) and the host compartment (Ptx3). The identification of Ptx3 producing cells, as well as their contribution to observed reduction in tumor angiogenesis, is currently under further study in this model.

Hypoxic conditions can increase SDF1/CXCL12 expression and accumulation of SDF1/CXCL12 inhibits tumor growth and increases leukocyte recruitment (41,42). Additionally, increased expression of MCP2/CCL8 has been shown to have an antitumor effect by increasing migration of monocytes and T-lymphocytes within the tumor (43,44). Increased BCA1/CXCL13 expression is associated with stroma-rich tumors, which retain malignant cells and result in decreased metastasis (45). Taken together, these data presented do not reveal a specific polarized microenvironment attributable to M1/M2 macrophages or N1/N2 neutrophils. Further studies are in progress to examine pPLC effects on the tumor-associated cellular infiltrates. Results of additional gene expression profiling experiments indicate that pPLC dietary supplementation alters the cytokine production and that this may alter the composition of the cellular compartment in the tumor microenvironment, leading to decreased tumor growth.

Future studies are needed to investigate the long-term effects of pPLC on prevention of tumor initiation, growth and angiogenesis, as well as the cellular composition of the tumor microenvironment. In conclusion, using a rapid growing in vivo, syngeneic murine lung cancer model (Lewis lung carcinoma), we demonstrate that pPLC has a significant effect in slowing tumor growth, reducing tumor-associated angiogenesis and increasing apoptosis. These effects are consistent with the reported effects of the soy sterols, ferulic acid and lipoic acid present in pPLC. Furthermore, the effects of pPLC are mediated by decreased tumor angiogenesis and a direct effect on tumor cell gene expression (Mt3). These compounds present in pPLC have little or no reported toxicity, which makes pPLC an excellent candidate for long-term administration as a cancer preventive agent via its combined chemopreventive and angiopreventive activities.

Funding
National Cancer Institute, Center for Cancer Research Intramural Program (NCI Intramural Project # ZIA SC009179-23 to W.G.S.-S.).

Conflict of Interest Statement: G.V. is President of Conjugated Functional Foods, 235 Prospect Ave., Hackensack, NJ 07601, manufacturer of pPLC used in these experiments, but has not contributed financially to funding of this research and all other coauthors participated as United States Federal Employees and received no financial benefit or other considerations from Conjugated Functional Foods.

References
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Received September 3, 2013; revised January 7, 2014; accepted January 29, 2014