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Leucoselect Phytosome Modulates Serum Eicosapentaenoic Acid, Docosahexaenoic Acid, and Prostaglandin E3 in a Phase I Lung Cancer Chemoprevention Study

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ABSTRACT

Grape seed procyanidin extract (GSE) has been shown to exert antineoplastic properties in preclinical studies. Recently, we reported findings from a modified phase I, open-label, dose escalation clinical study conducted to evaluate the safety, tolerability, MTD, and potential chemopreventive effects of leucoselect phytosome, a standardized GSE complexed with soy phospholipids to enhance bioavailability, in heavy active and former smokers. Three months of leucoselect phytosome treatment significantly decreased bronchial Ki-67 labeling index (LI), a marker of cell proliferation on the bronchial epithelium. Because GSE is widely used as a supplement to support cardiovascular health, we evaluate the impact of oral leucoselect phytosome on the fasting serum complex lipid metabolomics profiles in our participants. One month of leucoselect phytosome treatment significantly increased eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the omega-3 polyunsaturated fatty acids (n-3 PUFA) with well-established anticancer properties. Leucoselect phytosome also significantly increased unsaturated phosphatidylcholines (PC), likely from soy phospolipids in the phytosome and functioning as transporters for these PUFAs. Furthermore, 3-month leucoselect phytosome treatment significantly increased serum prostaglandin (PG) E_3 (PGE_3), a metabolite of EPA with anti-inflammatory and antineoplastic properties. Such increases in PGE_3 correlated with reductions of bronchial Ki-67 LI ($r = -0.9$; $P = 0.0374$). Moreover, posttreatment plasma samples from trial participants significantly inhibited proliferation of human lung cancer cell lines A549 (adenocarcinoma), H520 (squamous cell carcinoma), DMS114 (small cell carcinoma), and 1198 (preneoplastic cell line). Our findings further support the potential utility of leucoselect phytosome in reducing cardiovascular and neoplastic risks in heavy former and active smokers.

Prevention Relevance: In this correlative study of leucoselect phytosome for lung cancer chemoprevention in heavy active and former smokers, we demonstrate for the first time, favorable modulations of n-3PUFA and downstream PGE_3 in fasting serum, further supporting the chemopreventive potential of leucoselect phytosome against lung cancer.

Introduction

Preclinical studies demonstrate various antineoplastic effects of grape seed procyanidin extract (GSE) against lung cancer (1–5). To facilitate clinical translation, we have selected an inexpensive, over the counter GSE preparation (leucoselect), standardized to smaller size oligomeric procyanidins and complexed with soy phospholipids into phytosomes to improve bioavailability, for translation into a phase I lung cancer chemoprevention trial in heavy former or active smokers at high risk for lung cancer. This leucoselect phytosome has been shown to improve oxidative status, including the total antioxidant capacity of plasma, and reduce low density lipoprotein (LDL) susceptibility to oxidative stress in heavy smokers (6). We have recently reported the feasibility of leucoselect phytosome as a potential lung cancer chemopreventive agent from the study, including a significant reduction of bronchial Ki-67, a marker of cell proliferation on the bronchial epithelium and a key surrogate endpoint biomarker for lung cancer chemoprevention trials (7).

Because GSE is widely used to promote cardiovascular health, we evaluated the effects of oral leucoselect phytosome on the profiles of systemic complex lipid metabolomics by comparing matched pre- and posttreatment fasting serum samples from participants. Leucoselect phytosome treatment significantly increased serum eicosapentaenoic acid (EPA),

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docosahexaenoic acid (DHA), and unsaturated phosphatidylcholines (PC). Furthermore, leucoselect phytosome significantly increased fasting serum prostaglandin (PG) E_3 (PGE_3), a downstream eicosanoid derived from EPA, at the end of 3 months of treatment. Our findings support the continued investigation of leucoselect phytosome for lung cancer prevention and treatment.

Materials and Methods

Leucoselect phytosome clinical study design

A single-arm, dose escalation, modified phase I lung cancer chemoprevention study of 3 months of oral leucoselect phytosome, comprised of standardized oligomeric procyanidin complexed with soy phospholipid or lecithin (1:2.6 w/w; Indena, supplied via Thorne Research), was conducted in high-risk heavy active or ex-smokers 21 years of age or older with a smoking history of at least 30 pack-years as described previously (7). Written informed consent was obtained in accordance with the New Mexico VA Health Care System (Albuquerque, NM) Institutional Review Board, following the guidelines of Declaration of Helsinki, Belmont Report, and U. S. Common Rules. Qualified participants were treated with one capsule, 450 mg/capsule once a day, escalating weekly to four capsules once a day for the rest of the treatment duration as tolerated. Fluorescence bronchoscopies with bronchial biopsies were performed at baseline and at the end of 3 months of treatment. Serial fasting blood samples were collected at baseline, at the end of month 1, and at the end of month 3 of treatment for comparative biomarker analysis. Blood samples were processed within 1 hour of collection, spun at 3,000 rpm, 15 minutes for serum and 10 minutes for heparinized plasma, aliquoted into cryovials, and stored at −80°C until analysis.

Lipidomics by charged surface hybrid column-electrospray quadrupole time of flight mass spectrometer tandem mass spectrometry

Extraction

Serum was extracted using a protocol described previously (8). One organic phase aliquot was resuspended in 100 μL of methanol:toluene [9:1, volume/volume (v/v)] mixture containing 50 ng/mL 12-[(cyclohexylamino) carbonyl]amino]-dodecanoic acid (Cayman Chemical). Samples were vortexed and sonicated for 5 minutes, centrifuged at 16,000 rcf, and prepared for lipidomic analysis. Method blanks and pooled human plasma (BioreclamationIVT) were included as quality control samples.

Chromatographic and mass spectrometric conditions for lipidomic reverse phase liquid chromatography-high-field quadrupole orbitrap mass spectrometer analysis

Using an Agilent 1290 Infinity Ultra-High-Performance Liquid Chromatography System, resuspended samples were injected at 3 and 5 μL for positive and negative electrospray (ESI) modes, respectively, onto a Waters Acquity UPLC Charged Surface Hybrid Column (CSH) C18 (100 mm length × 2.1 mm id; 1.7 μm particle size) with CSH C18 precolumn (5 mm × 2.1 mm id; 1.7 μm particle size). The column was maintained at 65°C. To improve lipid coverage, different mobile phase modifiers were used for positive and negative ESI mode analysis (9). For positive ESI mode, 10 mmol/L ammonium formate and 0.1% formic acid were used; for negative ESI mode, 10 mmol/L ammonium acetate (Sigma-Aldrich) was employed. Both positive and negative ESI modes used the same mobile phase composition of (A) 60:40 v/v acetonitrile:water (LC/MS grade) and (B) 90:10 v/v isopropanol:acetonitrile. The gradient started at 0 minute with 15% (B), 0–2 minutes 30% (B), 2–2.5 minutes 48% (B), 2.5–11 minutes 82% (B), 11–11.5 minutes 99% (B), 11.5–12 minutes 99% (B), 12–12.1 minutes 15% (B), and 12.1–15 minutes 15% (B). A flow rate of 0.6 mL/minute was used. For data acquisition, positively charged lipids, such as PC and lysoPC, were analyzed using an Agilent 6530 Quadrupole Time of Flight (QTOF) Mass Spectrometer at resolution R = 10,000, while negatively charged lipids, such as free fatty acids and phosphatidylinositols, were analyzed using an Agilent 6550 QTOF Mass Spectrometer at resolution R = 20,000.

Data processing using MS-DIAL

Untargeted lipidomic data processing was performed using MS-DIAL (10) for deconvolution, peak picking, alignment, and identification. The public LipidBLAST tandem mass spectrometry spectra database was used, validated by retention time and *m/z* matching to authentic standards (11). Detected lipids were used for statistical analysis when they were positively detected in at least 50% of all samples in each group. Data were normalized by the sum-norm of all identified lipids (mTIC; ref. 12), to scale each sample. Normalized peak heights were then submitted to R for statistical analysis.

Measurements of serum PGE_3 and LTB_5

PGE_3 and leukotriene B_5 (LTB_5) levels in matched fasting serum collected pre- and posttreatment were measured using specific ELISA Kit (MyBioSource per the manufacturer's instructions.

Cell cultures

As models to evaluate the antineoplastic bioactivity of oral leucoselect phytosome against lung cancer, the human non–small cell lung cancer cell lines, A549 and H520, small-cell lung cancer cell line, DMS114 (purchased from ATCC, in 2019, 2015, and 2016, respectively), and the bronchial preneoplastic cell line, 1198 (generously provided by Dr. Andres Klein-Santos, Fox Chase Cancer Center, Philadelphia, PA, received in 2013) were cocultured *in vitro* with matched, pre- and post 3 months treatment fasting heparinized plasma from six study participants. Experiments involving A549 were initiated within 6 months of purchase and the cell line was not further authenticated. ATCC uses short tandem repeat profiling for cell line authentication. H520, DSM114, and 1198 cells were not further authenticated. Cells were last tested for *Mycoplasma*

in 2017. Cells were maintained as monolayers in an atmosphere of 5% CO_2 in air at 37°C in 25 cm^2 tissue culture flasks containing cell line–specific culture medium, as described previously (4, 13). Only cells within passages 3–6 at 70%–80% confluence were used. Aliquots (100 μL) of 6×10^4 cells/mL of A549, 10×10^4 cells/mL of H520, 12×10^4 cells/mL of DMS114, or 10×10^4 cells/mL of 1198 were plated in 96-well plates and incubated at 37°C for 2 hours, followed by addition of 10, 10, 5, or 5 μL heparinized plasma, respectively, and incubated for 44 hours. Cells were then subjected to PrestoBlue cell viability/proliferation assay.

PrestoBlue cell viability/proliferation assay

To quantify cellular proliferation in conditioned cells, PrestoBlue HS Cell Viability Reagent (Invitrogen) at 1/10th of cell culture volume was added to conditioned cells per well, and then incubated for 20 minutes at 37°C, 5% CO_2, to measure the reduction of the reagent by metabolically active cells according to the manufacturer's instructions.

Statistical analysis

The effects of leucoselect phytosome treatment on complex lipid metabolomics were determined by comparing baseline values with those obtained at 1 month of treatment using Wilcoxon signed-rank test to assess the difference between treatment effect on each compound. Benjamini–Hochberg procedure (14) was used to control the FDR. Fold changes, defined as median average of posttreatment divided by the median average of pretreatment, were calculated for each compound. Chemical similarity enrichment analysis (15) was performed using the raw *P* value and fold change of each compound. Kolmogorov–Smirnov test was used to test the difference at compound set enrichment level. All statistical analyses were conducted using R.

The effects of leucoselect phytosome treatment on PGE_3 and LTB_5 and plasma cocultures with human lung cancer and preneoplastic cells were determined by comparing matched pre- and post 3 months treatment values from each of the six participants who completed 3 months treatment. Fold or percentage change of each biomarker from each participant was calculated first by normalizing matched posttreatment to baseline pretreatment values, followed by paired *t* tests. Data were expressed as the mean ± SEM in all circumstances where mean values were compared. Differences were considered significant when $P < 0.05$. Pearson correlation coefficients were computed for modulations of serum EPA, DHA, and PGE_3 with modulations of bronchial Ki-67 by leucoselect phytosome from these participants.

Results

Effects of 1 month of oral leucoselect phytosome treatment on complex lipid metabolomics profiles in fasting serum

To determine the effects of oral leucoselect phytosome treatment on the systemic metabolomics profiles of complex lipids, matched pre- and post 1 month treatment fasting serum samples were compared. A total of eight sets of paired samples were available for evaluation. Among the 23 clusters of compounds, three clusters were found to be significantly altered: (i) unsaturated fatty acid, (ii) unsaturated PC, and (iii) carnitine (**Table 1A**; **Fig. 1A**).

Table 1A. Systemic effects of 1 month of leucoselect phytosome treatment on complex lipid metabolomics ($n = 8$).

Cluster name	Cluster size	*P*	FDR
Unsaturated FA	14	0.000014	0.00032
Unsaturated PCs	75	0.000049	0.00038
Carnitine	6	0.000037	0.00038
Cholesterol esters	9	1	1
Diglycerides	8	1	1
Galactosylceramides	6	1	1
Lactosylceramides	4	1	1
Lysophospholipids	3	1	1
NewCluster_14	6	1	1
NewCluster_18	5	1	1
NewCluster_19	4	1	1
Phosphatidylethanolamines	16	1	1
Phospholipid ethers	5	1	1
Plasmalogens	11	1	1
Saturated ceramides	3	1	1
Saturated FA	7	1	1
Saturated_lysophosphatidylcholines	8	1	1
Saturated_PCs	7	1	1
Saturated triglycerides	5	1	1
Sphingomyelins	23	1	1
Unsaturated ceramides	12	1	1
Unsaturated lysophosphatidylcholines	14	1	1
Unsaturated_triglycerides	51	1	1

Abbreviation: FA, fatty acid.

Effects of 1 month of oral leucoselect phytosome treatment on EPA, DHA, and unsaturated PC in fasting serum

Key compounds significantly altered/increased by leucoselect phytosome treatment in the fasting serum of study participants included EPA and DHA in the unsaturated fatty acid cluster. In addition, leucoselect phytosome treatment significantly increased a total of three unsaturated PC, with PC(36:5) A as the key compound within that cluster. Moreover, plasmalogen PC(p-18:1/18:3) was also significantly increased (**Table 1B**). Pearson correlation of modulations of EPA and DHA with modulations of bronchial Ki-67 labeling index (LI) showed a trend toward statistical significance (**Table 1C**).

Effects of 3 months of oral leucoselect phytosome treatment on fasting serum PGE_3 and LTB_5

EPA can function as a substrate for cyclooxygenases (COX) to synthesize unique 3-series PG compounds, especially PGE_3, which tends to have antiproliferative and anti-

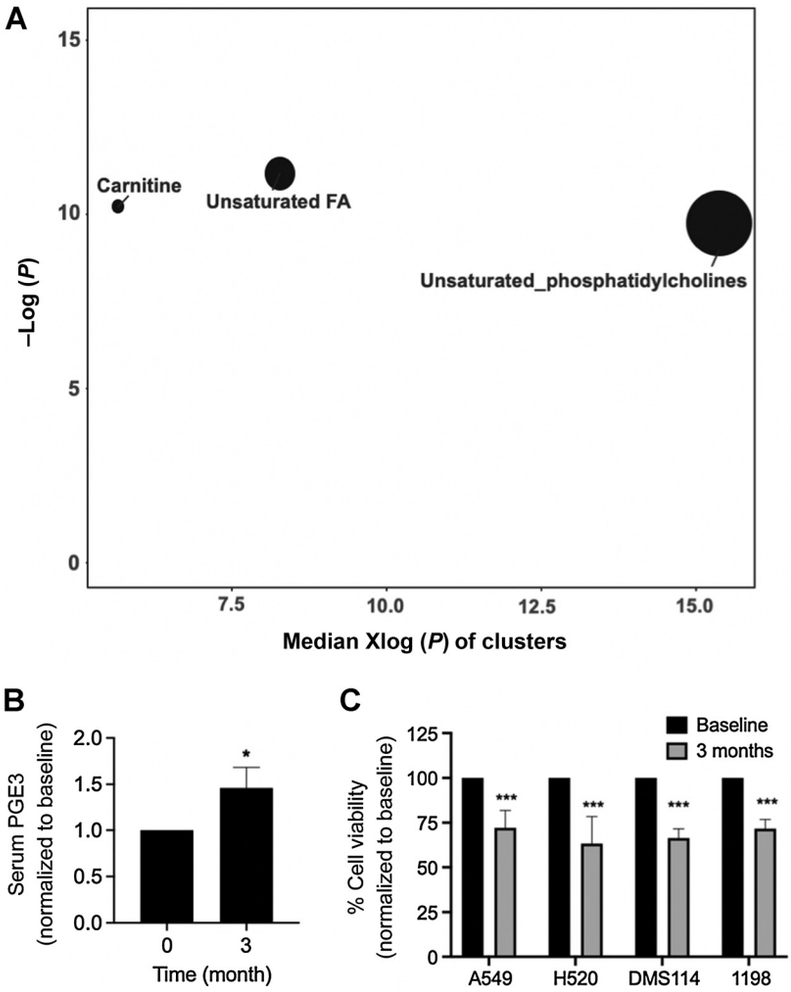

Figure 1.

A, Impact plot of modulations of complex lipidomics in fasting serum by leucoselect phytosome treatment. Oral leucoselect phytosome treatment significantly altered unsaturated fatty acid (FA), unsaturated PC, and carnitine clusters. **B,** Three months of oral leucoselect phytosome treatment significantly increased PGE_3 levels in fasting serum of study participants by an average of 26% (66.2 ± 10.3 pg/mL at baseline vs. 96.3 ± 17.0 pg/mL at 3 months). Fold change of each biomarker from each participant was calculated first by normalizing matched posttreatment to baseline pretreatment values. Columns, mean; bars, SEM ($n = 6$). *, $P < 0.05$. **C,** To determine the systemic, antineoplastic bioactivity of oral administration of leucoselect phytosome, A549, H520, DMS114, and 1198 cells were treated with matched fasting heparinized plasma obtained from subjects pre- and post 3 months of oral leucoselect phytosome treatment (plasma: culture medium = 1:10 for A549 and H520, and 1:20 for DMS114 and 1198). Posttreatment plasma significantly reduced proliferation of A549, H520, DMS114, and 1198 cells in comparison with pretreatment plasma. Percentage change of cell viability from each participant was calculated first by normalizing matched posttreatment to baseline pretreatment values. Mean; bars, SEM ($n = 6$; ***, $P < 0.001$).

inflammatory activities. To determine whether the increase in systemic EPA by leucoselect phytosome treatment might lead to an increase in PGE_3, the levels of PGE_3 in matched pre- and posttreatment fasting serum samples were measured. Leucoselect phytosome treatment significantly increased PGE_3 levels by an average of 45% (**Fig. 1B**). As EPA could be a precursor for LTB_5, the levels of LTB_5 were also measured. Leucoselect phytosome treatment did not significantly change LTB_5 levels. Increases of PGE_3 significantly correlated with decreases of bronchial Ki-67 LI (**Table 1C**).

Posttreatment fasting plasma samples inhibit proliferation of human lung cancer and preneoplastic cells

To evaluate the systemic, antineoplastic effects from oral leucoselect phytosome treatment, A549, H520, DMS114, and 1198 human cell lines were cocultured with matched pre- and post 3 months treatment fasting plasma. Posttreatment plasma samples significantly inhibited cell proliferation in comparison with pretreatment plasma (**Fig. 1C**).

Discussion

In this correlative study of our recently published, modified phase I lung cancer chemoprevention study with leucoselect phytosome in heavy former and active smokers, we demonstrated that once a day oral leucoselect phytosome results in significant increases of omega-3 polyunsaturated fatty acids (n-3 PUFA), EPA and DHA, unsaturated PC, and PGE_3 in fasting serum. Posttreatment fasting plasma samples also significantly inhibited proliferation of human lung cancer and preneoplastic cell lines *in vitro*.

There are two major classes of PUFAs: the n-3 and the n-6. Three n-3s have been most studied: alpha-linolenic acid (ALA, containing 18 carbons), EPA (20 carbons), and DHA (22 carbons). ALA is an essential fatty acid that needs to be obtained from the diet. ALA can be converted into EPA and then to DHA, but the conversion is limited (reported rate of 15% primarily in the liver). Therefore, consumption of EPA and DHA from foods or dietary supplements is required to practically increase their levels in the body. ALA is present in plant oils, such as soybean, flaxseed, and canola oils. Whereas

Table 1B. Key compounds in the unsaturated FA, the unsaturated PC clusters, and a plasmalogen that were significantly increased by leucoselect phytosome treatment.

Compound name	Cluster name	PubChem. ID	Fold change	*P*
FA (20:5) (EPA)	Unsaturated FA	446284	1.2	0.031
FA (22:6) (DHA)	Unsaturated FA	445580	1.3	0.047
PC(39:6)	Unsaturated_PCs	52922637	1.3	0.031
PC(p-18:1/18:3)	Plasmalogen	53480747	1.4	0.047

Abbreviation: FA, fatty acid.

Table 1C. Pearson correlation coefficients for modulations of PGE_3, EPA, or DHA with modulations of bronchial Ki-67 LI by leucoselect phytosome ($n = 6$).

Ki-67 LI	*r*	*P*
PGE_3	−0.900	0.037
EPA	−0.794	0.109
DHA	−0.709	0.178

DHA and EPA are present in fish, fish oils, and Krill oils, but are originally synthesized by microalgae, then consumed by fish (16).

EPA and DHA are long-chain n-3 PUFAs with anti-inflammatory and immunomodulatory properties. They are believed to benefit cardiac, musculoskeletal, gastrointestinal, and immune systems in humans (17). Epidemiologic and preclinical findings also support their anticancer properties. For example, n-3 fatty acids reduce onset of different cancers and protect against late-stage cancers in carcinogen-induced mouse tumors, human tumor xenografts on mouse, and spontaneous mouse tumors induced by transgenes. A higher intake of n-3 PUFAs is linked to a reduced risk of skin, colorectal, lung, prostate, and breast cancers in humans (18).

The anticancer activities of EPA and DHA are partially associated with their effects on modulating eicosanoid metabolism (19), including inhibition of PGE_2 production. PGE_2 is derived from the n-6 arachidonic acid precursors liberated from phospholipids in the cell membrane and converted into PG by COX. Ample studies have implicated proinflammatory and procancerous effects of the inducible COX-2/PGE_2 pathways in lung cancer (20). We have previously demonstrated that GSE might simultaneously function as a natural COX-2 inhibitor and prostacyclin (PGI_2) inducer. PGI_2 is known for its antineoplastic and antiplatelet properties, capable of improving endobronchial dysplasia in former smokers (21). In this study, we demonstrated that oral leucoselect phytosome significantly increases serum PGE_3, likely through the increase of precursor EPA. EPA can function as a selective COX-2 inhibitor through competitive inhibition of the n-6 arachidonic acid binding to COXs, resulting in a decreased production of PGE_2, while concomitantly generating PGE_3. PGE_3 has antiproliferative and anti-inflammatory activities, can potentially antagonize tumor promoting effects of PGE_2 in tumorigenic cells. Whereas

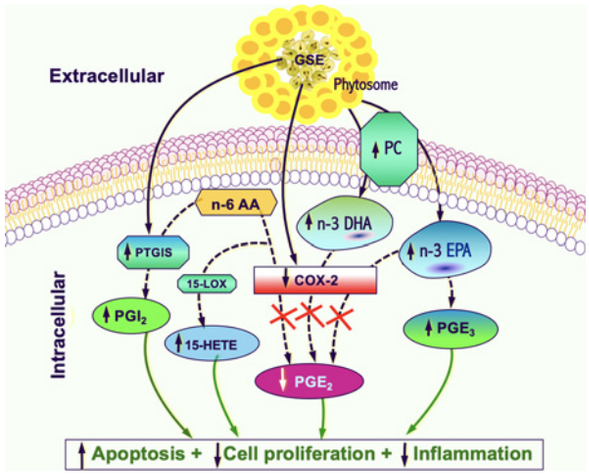

Figure 2.
Proposed mechanistic diagram of the effects of leucoselect phytosome treatment on EPA, DHA, PC, and major eicosanoids against lung cancer. The phytosome portion of leucoselect phytosome likely contains high levels of PC and serves as transporters for n-3 PUFAs, EPA and DHA. Increases in EPA and DHA function as competitive inhibitors of COX-2. In addition, an increase in EPA results in an increase in downstream PGE_3. Other GSE-mediated modulations of major eicosanoids signaling pathways described previously by our group are also depicted [a decrease in PGE_2 due to COX-2 inhibition by GSE, while an increase in prostacyclin synthase (PTGIS) by GSE results in an overall increase in PGI_2]. Furthermore, an increase in 15-HETE is likely due to shunting of the n-6 arachidonic acid (AA) precursor toward the 15-LOX pathway in the setting of COX-2 inhibition. Collectively, through modulations of these eicosanoids and n-3 PUFAs, leucoselect phytosome increases apoptosis, decreases cell proliferation and inflammation, and thereby reduces the driving forces of cancerization.

the modulations of serum PGE_3 significantly correlated with the modulations of Ki-67 LI, the modulations of EPA and DHA only showed a trend toward significant correlations with modulations of bronchial Ki-67 LI, likely due to insufficient sample sizes.

Because our participants were specifically instructed to not alter their dietary intake nor start new dietary supplements during the course of the study, we speculate that the increases in DHA and EPA post-leucoselect phytosome treatment likely are associated with the phytosome or lecithin component of leucoselect phytosome. Lecithin is comprised of mixtures of glycerophospholipids, including PC (22). It is conceivable that the phytosome increased systemic PC, which functions as transporters that enhanced absorption and bioavailability of EPA and DHA from the usual diet of the participants. Such a notion is supported by a study showing that combined intake of dietary crude lecithin with DHA increases systemic availability of DHA in rats (23). Long-chain n-3 PUFAs may also be synthesized from ALA by a progressive series of enzymatic desaturation and chain elongation steps (24). To this end, significant increases in the carnitine clusters of metabolites in the lipidomics likely reflect increases in carbon input from phytosome and transport of long-chain fatty acids into mitochondria for subsequent β-oxidation. The mechanisms involved in modulations of the carnitine cluster of metabolites

and their potential anticancer roles in leucoselect phytosome treatment remain to be elucidated. Significant change in the plasmalogen PC(p-18:1/18:3) also indicates differences in peroxisome metabolism, an organelle that is specifically active in PUFA metabolism that generates plasmalogen precursors (25).

Our findings illustrate the novel, additive effects of phytosome in leucoselect phytosome, beyond its original intended purposes of enhancing absorption, intercellular transport, and systemic bioavailability of GSE. To our knowledge, this is the first report demonstrating the additional, potential benefits of the carrier phytosome in humans. These findings, along with the significant reduction of Ki-67 LI in the proximal bronchi, and favorable modulations of a variety of other eicosanoids from our previous reports (7), further support the notion that oral administration of leucoselect phytosome is capable of dampening the driving forces of cancerizations systemically and in the lungs (**Fig. 2**). Our findings support the continued clinical investigations of leucoselect phytosome as an antineoplastic and chemopreventive agent against lung cancer.

Authors' Disclosures

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Authors' Contributions

J.T. Mao: Conceptualization, data curation, formal analysis, supervision, funding acquisition, investigation, methodology, writing–original draft, project administration. **B. Xue:** Investigation, methodology, writing–review and editing. **S. Fan:** Data curation, formal analysis, methodology, writing–review and editing. **P. Neis:** Methodology, writing–review and editing. **C. Qualls:** Data curation, formal analysis, writing–review and editing. **L. Massie:** Investigation, writing–review and editing. **O. Fiehn:** Data curation, formal analysis, investigation, methodology, writing–review and editing.

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