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Saponins in soy reduce NNK-induced lung cancer by increasing plasma isoflavone levels

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Recently, we found, using a cigarette carcinogen-induced lung cancer model, that soy protein isolate (SPI) was superior to casein in preventing lung cancer. In this study, we have attempted to identify the component(s) within SPI responsible for this chemopreventive effect. We fractionated the SPI using ethanol to separate the ethanol-soluble fraction (ESF) and the washed SPI and compared their efficacy to diets made with amino acids that comprise soy protein or casein, in preventing lung tumor formation in A/J mice. Only the ethanol-soluble fraction was as effective as SPI in preventing lung tumor formation. Since isoflavones and saponins are known ethanol-soluble bioactives from soy, we added isoflavones, or saponins or both to casein and found that isoflavones or saponins alone did not reduce lung nodule formation. However, when we combined soy saponins and isoflavones, we saw a significant ($P < 0.05$) reduction in NNK-induced lung nodules, and an increase in plasma isoflavone levels, suggesting that the saponins may enhance the bioavailability of the isoflavones in these mice. Taken together, we suggest that the superior efficacy of SPI over casein could be attributed, at least in part, to the synergistic effect of the soy saponins and isoflavones.

Keywords Isoflavones, Lung cancer, NNK, Saponins, Soy

The first hint that soy foods might lower the incidence of cancer came from population studies showing breast cancer rates were low in places where there was high consumption of soy[1,2]. Subsequent animal studies confirmed soy was indeed more effective than casein, a milk protein commonly used in rodent chows, at preventing mammary, prostate, liver, ovarian, colon, and cervical cancers[3–11].

However, despite decades of research, the active component(s) in soy responsible for its anti-cancer activity has not been completely elucidated. Historically, Bowman-Birk inhibitors (BBIs) were one of the first components of soy to be investigated for potential anti-carcinogenic activity. These small proteins (6–9 kDa), are potent serine protease inhibitors that bind and inactivate the digestive enzymes trypsin, chymotrypsin, and elastase, thus allowing the BBIs to enter the circulation[12]. Early reports in the 1980s and 90 s suggested that BBIs may be effective in both cancer prevention[13] and treatment, at least for prostate cancer[14]. Although BBIs subsequently received Investigational New Drug status from the FDA[13], they proved ineffective in decreasing oral lesions in a 2013 randomized, placebo-controlled trial with oral leukoplakia patients, perhaps due to stability issues[15].

In addition to BBIs, a number of other soy peptides and proteins have been studied. For example, soy peptides or soy protein hydrolysates, obtained using digestive enzymes, have been shown to suppress the growth of multiple cancer cell lines in vitro[16,17]. Of all the components within these hydrolysates, the most investigated has been lunasin, a 43 amino acid polypeptide[18] that has been shown to inhibit the carcinogen-induced transformation of cells and the proliferation of several cancer cell lines[19–25]. Lunasin has also been shown to inhibit tumor growth in vivo[26,27]. Mechanistically, it has been suggested that lunasin does this, at least in part, by preventing histone acetylation[28,29].

It should also be mentioned that plant proteins in general differ from animal proteins in essential amino acid content and this is especially true for methionine, which is significantly lower in soy proteins than in casein[30]. This could be important, given that methionine has been shown to promote cancer cell growth and metabolism[31].

In addition to peptides and proteins, soybeans are a rich source of isoflavones. Because of their high levels in soy, it was originally hypothesized that they were the components that reduced the risk of hormone-sensitive cancers such as breast and prostate cancers. This is because some of these ethanol-soluble compounds have been shown to bind estrogen receptors, potentially eliciting estrogenic activity at low concentrations and anti-

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estrogenic activities at high concentrations[32,33]. The three most abundant isoflavones in soy are genistin, daidzin, and glycetin, which are the glycosylated forms of the aglycones genistein, daidzein, and glycetein, respectively[34]. As well, daidzein can be metabolized by the gut microbiome to generate equol, which also has estrogenic activity[35]. A number of other components in soy with potential anti-cancer activity have been investigated, including soy saponins, which comprise approximately 5% of the dry weight of soybeans[36]. These saponins have been shown in vitro to reduce the growth of breast and colon cancer cell lines[37–39]. Tested in vivo, soy saponins have been shown to repress human MDA-MB-231 mammary tumor growth in immunocompromised mice[37] and reduce the number of carcinogen-induced aberrant crypt foci in rats[40].

We recently found that soy protein isolate (SPI), a protein-enriched (>90%) product from soybeans, devoid of fat and carbohydrate (CHO), was superior to casein in reducing the induction of lung cancer nodules by the cigarette smoke carcinogen, nicotine-derived nitrosamine ketone (NNK)[41]. This observation has given us the opportunity to investigate which component(s) within SPI is responsible for this effect. Our results are presented herein.

Methods

Mice

Female A/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME), and acclimatized to our specific pathogen-free facility in the British Columbia Cancer Research Centre. They were fed our standard Envigo code 2920 chow, ad libitum, until the mice reached 12 weeks of age, at which time they were randomly assigned to the various diets indicated below. The mice (10–15/cage) were housed in double-decker rat cages containing one exercise wheel and two huts per level instead of in standard mouse cages, so they could exercise freely as described previously[41]. Mice that reached humane endpoints before the experimental endpoints due to reasons unrelated to the study are excluded from the analysis. All animal experiments complied with the ARRIVE guidelines. All experiments were performed following guidelines and regulations and was approved by the University of British Columbia Animal Care Committee (A14-075/A18-0138/A22-0072).

Diets

All diets were custom-made by Envigo (Madison, WI), vacuum-packed in 1 kg bags, irradiated, and stored at − 20 °C before and after opening. All diets used in this study were isocaloric low CHO diets and contained 15% (of total calories) as the resistant starch, Amylose, 35% as protein and 50% as fat, with the fat component being a mixture of fats normally consumed by humans on a Western diet[42]. Isoflavones (Novasoy400) was a gift from Archer Daniels Midland (Decatur, IL). The isoflavone levels within Novasoy and SPI were determined by N.P. Analytical Laboratories (St. Louis, MO). Isoflavone-free (< 0.3%) saponins (Saponin B-50), which contain a minimum of 59.9% soyasaponin group B and 12.3% soyasaponin group A, were a gift from J-Oil Mills (Japan).

Study #1

To investigate the role of soy proteins and isoflavones in preventing NNK-induced lung nodule formation, 12-week old mice (9–12 mice/group) were put on low CHO, (15% Amylose) diets containing either casein, SPI, or casein supplemented with isoflavones (Table 1). The isoflavones were added to casein at 3.6 g/kg, so that the level of genistein, the main isoflavone in SPI, was comparable to that present in SPI (Table 1).

Animal experiments

After acclimatizing to their diets for two weeks, mice were then intraperitoneally (IP) injected with NNK (50 mg/kg body weight) twice, one week apart, to initiate lung nodule formation. The mice were kept on their respective diets for 20 weeks and then anesthetized with isoflurane and euthanized by CO_2 asphyxiation, followed by cervical dislocation. Blood was obtained by cardiac puncture into heparin-coated tubes and centrifuged at 2500×*g* for 10 min at 4℃. Plasma was immediately frozen in dry ice and stored at − 80 °C for future biochemical analyses. Mouse lungs were rinsed with PBS and tumor nodules were counted by two blinded counters using a Leica MZ9.5 microscope (Meyer Instruments, Houston TX). Lungs were either immediately frozen or fixed in 10% formalin after inflating the lung with 1 mL of PBS. The fixed lungs were then stored in 70% ethanol, prior to paraffin embedding, sectioning (4 micron thickness) and H&E staining.

Study #2

To determine if non-isoflavone components in SPI were contributing to the anti-cancer activity of the SPI, the SPI was ethanol washed to remove the isoflavones and other ethanol soluble compounds as described in Supplementary Fig S1, using an extraction method adapted from Fukui et al.[43]. Briefly, SPI powder obtained from Envigo (Solae Supro® 661) was mixed with 70% ethanol (10 × v/w) for 16 h at 23℃. After vacuum filtration using a Whatman no. 1 filter, the filter cake was re-suspended in 70% ethanol (3 × v/w) and mixed for 15 min. The soy protein was allowed to settle for 30 min and then vacuum-filtered to obtain ethanol extract #2. The settled soy protein was extracted a third and final time with 99.5% ethanol (2 × v/w), stirred for 15 min before subjecting the mixture to a final vacuum filtration. The washed SPI was then spread out on a pre-weighed baking tray and air-dried for 16 h. Once the weight was stable, the washed SPI was stored at -20℃ until shipment to Envigo for incorporation into mouse chows. The ethanol solutions from the three extractions were pooled and evaporated to complete dryness using a Labconco lyophilizer (Kansas City, MO). This ethanol-soluble fraction (ESF) constituted approximately 8.1% of the SPI. This ESF of SPI was incorporated into the low CHO diet at a level comparable to that found in SPI. Since we did not find that isoflavones alone were effective in lowering lung nodule formation, we added the ESF to 15% Amylose/Isoflavones to explore the possibility that non-isoflavone components within the ESF may work synergistically with isoflavones.

	15% Amylose	15% Amylose/soy	15% Amylose/isoflavones
Carbohydrate			
High amylose corn starch	100	100	100
Maltodextrin	70	70	70
Sucrose	0	0	0
Cellulose	153	153	153
Protein			
Casein	396	0	396
Soy protein isolate	0	396	0
Fat			
Soybean oil	42.1	42.1	42.1
Milk fat	48.6	48.6	48.6
Olive oil	37.5	37.5	37.5
Lard	37.5	37.5	37.5
Beef tallow	33.2	33.2	33.2
Corn oil	22.1	22.1	22.1
Novasoy 400	0	0	3.6
Kcal/g	4	4	4
Carbohydrate (kcal %)	15.1	15.4	15.2
Protein (kcal %)	34.6	34.5	34.5
Fat (kcal %)	50.4	50.2	50.3
Total isoflavones (mg/kg	nd	800	932
Total daidzein (mg/kg diet)	nd	259	384
Total genistein (mg/kg diet)	nd	496	499
Total glycitein (mg/kg diet)	nd	44	50

Table 1. Diet formulation in Study #1 expressed as g/kg of diet. *Nd* not determined.

To determine if the superiority of SPI over casein at reducing NNK-induced lung nodules was due to differences in their amino acid composition, we formulated two diets containing amino acid mixtures that reflected the amino acid compositions of casein and soy protein. The diets used in this Study #2 are described in Table 2. The amino acid profile of the diets containing amino acids instead of intact proteins is described in Table 3. The casein containing 15% Amylose diet supplemented with isoflavones were used to confirm our findings in Study #1. The 15% Amylose diet served as controls. Animal experiments were performed as described in Study #1.

Study #3

Based on our findings from Study #2, we concluded that the tumor lowering activity was in the ESF of the SPI. Since soy saponins are ethanol soluble compounds previously reported to have anti-cancer effects[44], we tested in Study #3 the effect of 15% Amylose diets containing soy saponins at a level comparable to that in SPI, in the presence and absence of soy isoflavones. We describe the composition of the diets used in Study #3 in Table 4. The 15% Amylose diet served as controls. Animal experiments were performed as described in Study #1.

Quantitation of saponins

Total saponins were quantified using a modified vanillin-sulfuric acid method, as described by Le et al.[45]. Briefly, ground rodent chows, the SPI, ethanol-washed SPI, ethanol-extract from SPI and Novasoy were reconstituted at 50 mg/mL in 70% ethanol for the extraction of saponins. Genistein was reconstituted at 0.1 mg/mL in 70% ethanol. The mixtures were sonicated for 2 min (40 Amp) twice and chilled on ice between rounds and after. The samples were centrifuged at 16,060×*g* for 10 min at 4℃, and the supernatants filtered using a 0.45 µm membrane filter. Then, 0.25 mL of each extract was combined with 0.25 mL of 8% vanillin made in ethanol and 2.5 mL of 72% sulfuric acid in a screw-capped glass tube. After mixing, the tubes were incubated in a 65℃ water bath for 15 min, immediately chilled on ice and the absorption read at 560 nm. Soyasaponin I (Cat# S9951, Sigma-Aldrich, St. Louis, MO) reconstituted in 70% ethanol at 1 mg/mL was used to generate a standard curve, and results were expressed as total Soyasaponin I equivalent.

Quantification of isoflavones in plasma

The genistein in mouse plasma was quantified using LC/MS, based on a method described by Twaddle et al.[46]. Briefly, 65 µL of plasma was mixed with an equal volume of d4-genistein (300 ng/mL in acetonitrile), vortexed and sonicated for 10 min. After centrifugation at 16,060×*g* for 5 min at 23℃, 100 µL of the supernatant was mixed with 900 µL of β-glucuronidase from *Helix pomatia* solution (G1512, 100 µg in 25 mM citrate buffer pH 5.0). After brief vortexing, the samples were placed on a heating block at 37℃ for 1 h. Upon completion, three

	15% Amylose	15% Amylose/soy	15% Amylose/isoflavones	15% Amylose/ washed SPI	15% Amylose/ EtOH washed product	15% Amylose/ casein amino acids	15% Amylose/ soy amino acids
Carbohydrate							
High amylose corn starch	100	100	100	100	100	100	100
Maltodextrin	70	70	70	70	70	70	70
Sucrose	0	0	0	0	0	0	0
Cellulose	153	153	153	153	153	153	153
Protein							
Casein	396	0	396	0	396	0	0
Soy protein isolate (SPI)	0	396	0	0	0	0	0
EtOH washed SPI	0	0	0	396	0	0	0
Casein amino acid	0	0	0	0	0	396	0
SPI amino acid	0	0	0	0	0	0	396
Fat							
Soybean oil	42.1	42.1	42.1	42.1	42.1	42.1	42.1
Milk fat	48.6	48.6	48.6	48.6	48.6	48.6	48.6
Olive oil	37.5	37.5	37.5	37.5	37.5	37.5	37.5
Lard	37.5	37.5	37.5	37.5	37.5	37.5	37.5
Beef tallow	33.2	33.2	33.2	33.2	33.2	33.2	33.2
Corn oil	22.1	22.1	22.1	22.1	22.1	22.1	22.1
Novasoy 400	0	0	3.6	0	3.6	0	0
Ethanol-washed product	0	0	0	0	32	0	0
Kcal/g	4	4	4	4	4	3.9	3.9
Carbohydrate (kcal %)	15.1	15.4	15.2	15.4	15.2	15.4	15.4
Protein (kcal %)	34.6	34.5	34.5	34.5	34.5	34.1	34.1
Fat (kcal %)	50.4	50.2	50.3	50.2	50.3	50.5	50.5

Table 2. Diet formulation in Study #2 expressed as g/kg of diet.

Amino acid	15% Amylose/casein amino acids (g/kg)	15% Amylose/soy amino acids (g/kg)
L-Alanine	10.88	11.33
L-Arginine	16.68	34.76
L-Asparagine	19.01	22.0
L-Aspartic acid	6.34	22.0
L-Cystine	4.01	7.92
L-Glutamic acid	45.62	36.3
L-Glutamine	30.41	36.3
Glycine	6.65	16.17
L-Histidine	14.15	13.53
L-Isoleucine	20.91	18.7
L-Leucine	33.48	30.91
L-Lysine	36.54	29.92
L-Methionine	9.61	4.84
L-Phenylalanine	18.37	19.58
L-Proline	37.59	19.14
L-Serine	20.91	19.58
L-Threonine	15.95	14.41
L-Tryptophan	4.22	5.28
L-Tyrosine	19.22	14.41
L-Valine	25.13	19.14

Table 3. Amino acid profile of the 15% Amylose/casein amino acids and 15% Amylose/soy amino acids diets.

	15% Amylose	15% Amylose/soy	15% Amylose/isoflavones	15% Amylose/saponins	15% Amylose/saponins + isoflavones
Carbohydrate					
High amylose corn starch	100	100	100	100	100
Maltodextrin	70	70	70	70	70
Sucrose	0	0	0	0	0
Cellulose	153	153	153	153	153
Protein					
Casein	396	0	396	396	396
Soy protein isolate (SPI)	0	396	0	0	0
Fat					
Soybean oil	42.1	42.1	42.1	42.1	42.1
Milk fat	48.6	48.6	48.6	48.6	48.6
Olive oil	37.5	37.5	37.5	37.5	37.5
Lard	37.5	37.5	37.5	37.5	37.5
Beef tallow	33.2	33.2	33.2	33.2	33.2
Corn oil	22.1	22.1	22.1	22.1	22.1
Novasoy 400	0	0	3.6	0	3.6
Soy saponin	0	0	0	9.6	9.6
Kcal/g	4	4	4	4	4
Carbohydrate (kcal %)	15.1	15.4	15.2	15.1	15.2
Protein (kcal %)	34.6	34.5	34.5	34.7	34.5
Fat (kcal %)	50.4	50.2	50.3	50.4	50.3

Table 4. Diet Formulation in Study #3 expressed as g/kg of diet.

rounds of solvent extraction were performed using 1 mL ethyl acetate. The ethyl acetate top layer was pooled, vacuum-evaporated and reconstituted in 50% methanol for LC/MS analysis.

Blank plasma samples (60 µl) were spiked with genistein and d4-genistein (Cayman Chemical, Ann Arbor, MI) internal standard (IS) and precipitated with an equal volume of acetonitrile, centrifuged at 20,000 g for 2 min and 100 µL of supernatant added to 900 µL of enzyme stock. Following incubation they were extracted with ethyl acetate, the extract dried and the residue taken up in 100 µL of 67% acetonitrile. The resulting seven calibration samples had a final IS of 150 ng/mL with genistein ranging from 0–1500 ng/mL. Test samples were similarly prepared, omitting the genistein spike. All samples were analysed with a Waters Acquity LC coupled to a Waters Quattro Premier using a 2.1 × 50 mm BEH C18, 1.7µ column (Waters). Water (0.1% formic acid (FA)) and ACN (0.1% FA) were LC solvents A and B with 5% B, 0–0.2 min; 5–98% B, 0.2–2.5 min; 95% B, 2.5–3.0 min; 98–5% B, 3–3.1 min; with total run length 4 min at 0.33 mL/min. All MS data was collected in ES + at unit resolution with the following parameters: capillary, 3 kV; extractor and RF lens, 3 V and 0.1 V; source and desolvation temperatures, 120 ℃ and 350 ℃; desolvation and cone (N_2) flow, 1000 L/h and 50 L/h; collision gas (Ar) flow, 0.13 mL/min ($7.8e^{-3}$ m bar). Detection was by multiple reaction monitoring with m/z 271.1 > 153 and 271.1 > 91 for genistein and m/z 275.1 > 154.1 and 271.1 > 94.1 for d4-genistein (45 V/45 V and 45 V/40 V cone/collision respectively for both pairs) with 0.1 s dwell each and 1.9 min retention time (RT) for both. Quantitation was via the AUC ratio of genistein/d4-genistain using a linear fit with 1/X weighting with $R^2 > 0.99$ and accuracy within ± 15% across the entire range (5-1500 ng/ml) using Quanlynx (Waters) and exported to Excel for further analysis.

Statistics

Significant differences between treatment groups were performed using one-way ANOVA, using post-hoc Tukey's or Dunnett test to identify treatment groups that are significantly ($P < 0.05$) different when compared to the 15% Amylose group. Assessment for normality was performed with Shapiro–Wilk test. All statistical analyses were performed using Graphpad Prism 10.2.3.

Results

SPI but not isoflavone-supplemented casein reduces lung nodule numbers

In an earlier study, we found using a low CHO, fish oil-containing diet, that 35% soy protein isolate (SPI) was more effective than 35% casein, at reducing NNK-induced lung nodules[41]. To confirm the potential chemopreventive effect of soy protein, we investigated the effect of SPI versus casein in the absence of fish oil, since fish oil is a potent inhibitor of lung nodule formation, and obscures to some extent the effect of protein source. As shown in Fig. 1, replacing 35% casein with 35% SPI in a low CHO (15% Amylose) diet significantly lowered NNK-induced lung nodule numbers from an average of ~ 10 ± 1 to ~ 5 ± 1 (mean ± SEM). Since soy isoflavones, present in both soy and its protein-rich product, SPI, may be the bioactive component of soy responsible for this effect, we assessed the role of isoflavones in the prevention of NNK-induced lung nodule formation. Specifically, we compared the 15% Amylose diet containing casein supplemented with soy isoflavones at levels comparable to that found in SPI, the 15% Amylose with casein only and the 15% Amylose diet containing SPI only. Interestingly,

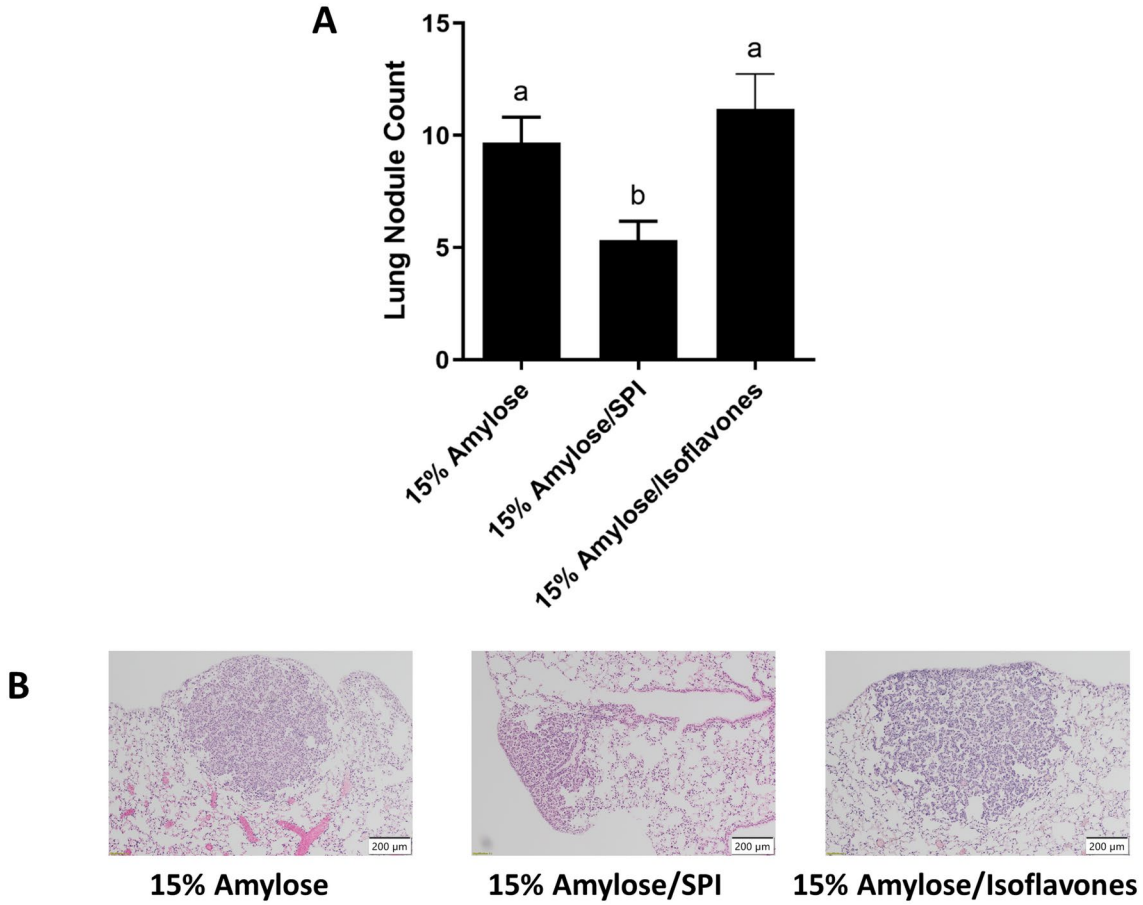

Fig. 1. The efficacy of SPI in reducing NNK-induced lung nodule formation in A/J mice is not due to soy isoflavones. Female A/J mice (n = 12) were acclimatized to either a 15% Amylose/30% casein/50% fat diet, a 15% Amylose/30% SPI/50% fat diet or a 15% Amylose/30% casein/50% fat + isoflavone diet for 2 weeks before IP injection of NNK (50 mg/kg body weight) twice, one week apart. The mice were then kept on their respective diets for 20 weeks, euthanized and lung tumor nodules counted by two blinded counters using a Leica MZ9.5 microscope. (**A**) Data shown are the biological replicates of lung nodule counts per mouse as mean ± SEM. Significant differences between treatment groups were identified using one-way ANOVA followed with Tukey's multiple comparison test. Different letters denote significant ($P < 0.05$) differences between treatments. (**B**) Representative H&E images of the tumors in the lung. Images were taken using Olympus BX46 microscope at × 100 magnification.

the low CHO diet containing isoflavone-supplemented casein was not effective in lowering lung nodule numbers compared to the 15% Amylose diet with casein alone (Fig. 1).

The efficacy of soy protein isolate is not due to the protein component of the SPI

To determine if the superiority of SPI over casein was due to the proteins themselves, we carried out two studies. First, we asked if the difference in amino acid composition between soy and casein could be responsible for the superior efficacy of SPI in reducing NNK-induced lung nodules. This is possible, since certain amino acids such as methionine, which is at a significantly higher level in casein than in soy proteins[30], have been shown to promote cancer cell growth and metabolism[31]. To test this, we replaced SPI and casein in the diets with cocktails of amino acids at levels and compositions similar to that found in casein and soy protein. As shown in Fig. 2, the cocktail of soy amino acids was not superior to casein amino acids in reducing NNK-induced lung nodules. Secondly, we treated the SPI with ethanol to separate the proteins from the ethanol-soluble components, such as soy isoflavones, saponins, and other ethanol-soluble compounds. Ethanol washing of SPI produced an ethanol-insoluble SPI protein preparation and an ethanol-soluble fraction, the latter being approximately 8.1% (w/w) of the SPI. We then tested both the pure protein preparation (ethanol-washed SPI) and the ESF and found that the protein component, i.e., the ethanol-washed SPI, did not contribute to the chemopreventive effect of the SPI (Fig. 2). Since bioactive peptides, such as lunasin, reported to have anti-cancer properties[18], will be in the ethanol-washed protein fraction, we conclude that the chemopreventive effect of SPI is also unlikely due to bioactive peptides.

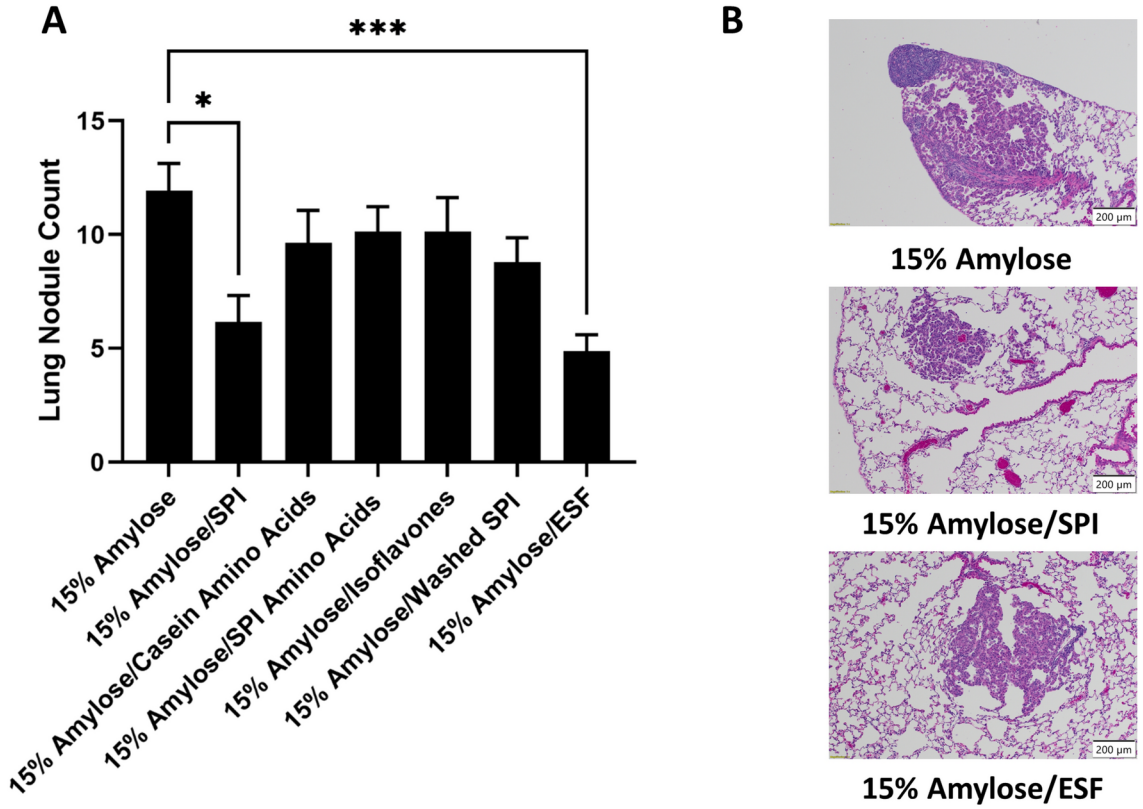

Fig. 2. The efficacy of SPI in reducing NNK-induced lung nodule formation in A/J mice (n = 8–9) is not due to the difference in amino acid composition between SPI and casein, nor to the soy proteins but to the ESF within the SPI. (**A**) Values are biological replicates and the data are shown as the mean ± SEM. Significant differences between treatment groups and the 15% Amylose group were identified using one-way ANOVA followed with Dunnett's multiple comparison test. Asterisks denote a significant difference between mice fed the different diets, *P ≤ 0.05, ***P ≤ 0.001. (**B**) Representative H&E images of the tumors in the lung from groups that were significantly impacted by the diet, relative to the 15%Amylose. Images were taken using Olympus BX46 microscope at × 100 magnification.

The efficacy of soy protein isolate to prevent lung nodules may be attributable to saponins

Given that the protein fraction of SPI was not responsible for the superiority of SPI over casein, we formulated a 15% Amylose, 35% casein + ESF diet that contained a level of ESF equal to that present in unfractionated SPI. This diet composed of casein supplemented with the ESF significantly lowered lung nodule formation (Fig. 2). Since we showed that isoflavones did not contribute to the prevention of lung nodule formation, we hypothesized that other ethanol-soluble components, such as the saponins, might be responsible for the lower lung nodule numbers in SPI-fed mice.

To test this hypothesis, we quantified the total saponin content of the low CHO diets containing casein, SPI, isoflavones only, the ethanol-washed soy protein, and the ESF. As shown in Fig. 3, ethanol washing of the SPI efficiently transferred the saponins from the SPI to the ESF. We also tested Novasoy, which is the isoflavone mixture used to formulate the isoflavone-supplemented diet, for its saponin content. It is clear that while Novasoy does contain saponins, the level identified in the product was relatively minor compared to that found in SPI.

When the saponin content in mouse chows was compared, the saponin level in the diets corresponded closely with the efficacy of the diet to reduce lung nodule counts, in that the diets with SPI and ESF had comparable, high levels of saponins (Fig. 3). It is thus possible that soy saponins, rather than isoflavones, may be the bioactive compounds contributing to the chemopreventive activity of the SPI.

Soy saponins reduce NNK-induced lung cancer by increasing plasma isoflavone levels

To confirm that saponins were responsible for the superior effects of SPI, we compared diets containing SPI, casein + saponins, casein + isoflavones, and casein + saponins + isoflavones for their ability to lower the number of NNK-induced lung nodules. As shown in Fig. 4A, B, only the casein supplemented with both saponins and isoflavones significantly reduced the number of lung nodules, compared to casein alone. This suggested these two ethanol-soluble components worked together to lower lung cancer nodules.

To determine how these two agents worked together to lower NNK-induced lung nodules, we postulated that perhaps the saponins, given their amphipathic "soapy" nature, might be loosening the tight junctions between colonocytes and/or damaging the colonocytes[47], allowing more isoflavones to enter the circulation. To test this, we quantified the level of the isoflavone, genistein, in mice fed 15% Amylose/SPI, 15% Amylose/isoflavones and

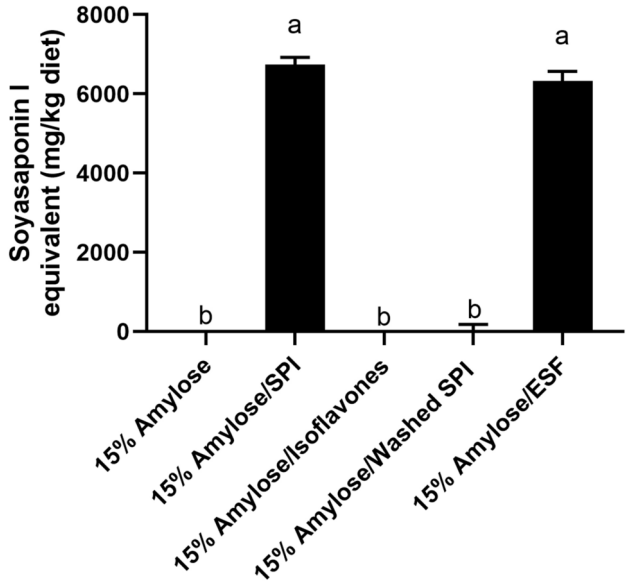

Fig. 3. Saponin content of the chows. The 15% Amylose/ESF chow was supplemented with ESF at saponin levels equal to that in unfractionated SPI. Values are technical replicates and results are expressed as soyasaponin I equivalents. The data (n = 3) are shown as mean ± SD. Significant differences between treatment groups were identified using one-way ANOVA followed with Tukey's multiple comparison test. Different letters denote significant ($P < 0.05$) difference between treatments.

15% Amylose/saponins + isoflavones, and found that the mice on the combination of saponins and isoflavones had significantly higher genistein levels than the mice on the other two diets (Fig. 4C). We also measured the levels of genistein in the plasma of mice fed either casein (15% Amylose) or casein + saponins (15% Amylose/saponins) as negative controls and, as expected, found no detectable genistein in their plasmas (Fig. 4C). Representative mass spectra of genistein and its fragments are depicted in Fig. 4D.

Discussion

Lung cancer continues to be the world's leading cause of cancer deaths[48]. Observational studies, however, suggest that soy foods may be helpful in preventing lung cancer since its consumption is inversely correlated with lung cancer incidence and mortality[49,50]. The anti-cancer properties of soy have also been demonstrated in several animal models[3,9,10], which show that soy is superior to other protein sources in slowing cancer growth[3–8]. We recently confirmed the anti-cancer properties of soy by showing that low CHO diets containing SPI were significantly more effective than the same low CHO diets containing casein, a milk protein commonly formulated in standard rodent chow, in preventing lung tumors in cigarette carcinogen-injected A/J mice[41]. Despite the numerous reports of the anti-cancer properties of soy, the component(s) responsible for this activity has not been completely elucidated. In the current study, we used the NNK-induced lung cancer mouse model to try and identify these components.

One of the soy components thought to contribute to its anti-cancer properties is the isoflavones, which is a family of phytoestrogenic compounds naturally occurring in soy. While genistin, daidzin and glycetin, which are the glycosylated versions of genistein, daidzein, and glycetein, respectively, are the most commonly found isoflavones in soy foods, they typically are hydrolyzed to their aglycones prior to absorption[34,51]. These aglycones are typically comparable or more anti-proliferative than their glycosylated counterparts, likely due to differences in their water solubility[52,53]. Genistein, typically the most abundant isoflavone, for example, has been shown to suppress non-small-cell lung cancer progression in a xenograft model[54]. There are also multiple reports suggesting the efficacy of isoflavones in slowing the growth of breast[55], colon[56], and bladder[57] cancers in mice. In a meta-analysis that found a reduced risk of cancer incidence with higher consumption of soy foods, the intake of isoflavones was inversely associated with cancer incidence[58]. We were thus surprised when we found that isoflavones, added to a standard low CHO diet containing casein, at a comparable level to that found in SPI, had no impact on lung tumorigenesis.

There have also been reports suggesting that the protein component, and not the isoflavones, is the primary bioactive agent within soy foods[59]. Lunasin, for example, is a bioactive peptide derived from the digestion of soy protein that has been shown to have anti-proliferative effects against lung cancer cell lines in vitro and in vivo[23,60]. BBIs are another family of proteins present in soy that has been reported to have anti-cancer effects[61]. We show here that isoflavone-poor-soy protein was ineffective at preventing NNK-induced lung tumor formation. This rules out the possibility that soy proteins or their breakdown products such as lunasin, BBIs, or other bioactive peptides are significantly chemopreventive, at least in this model system.

Since the amino acid profile of soy protein is distinct from casein, and different amino acids could play different roles in cancer development, we decided to test if this difference in amino acid profile could contribute

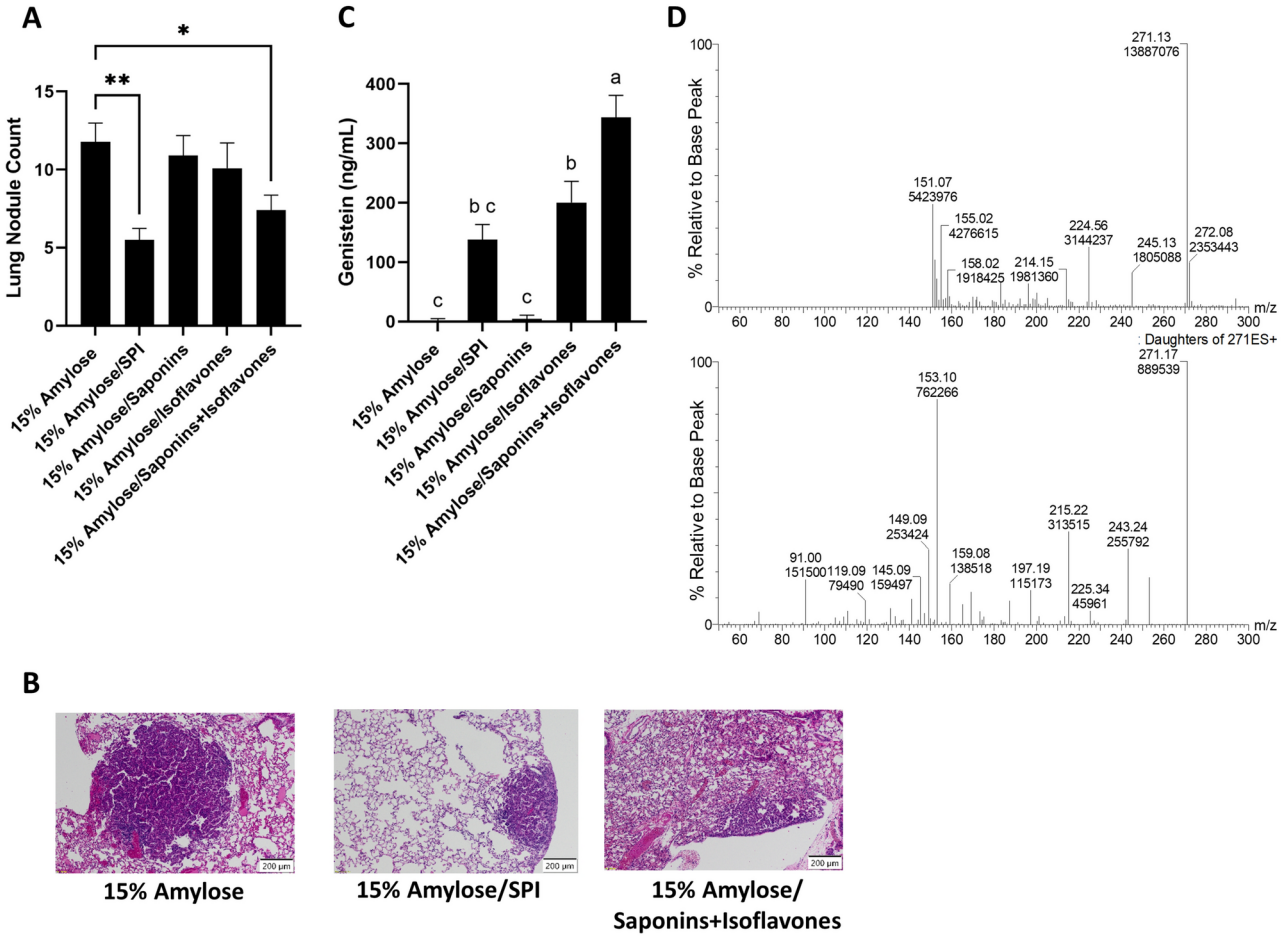

Fig. 4. (**A**) Soy saponins plus soy isoflavones significantly reduce NNK-induced lung nodules. The data (n = 5–12) are biological replicates and shown as the mean ± SEM. Significant differences between treatment groups and the 15% Amylose group were identified using one-way ANOVA followed with Dunnett's multiple comparison test. Asterisks denote a significant difference between mice fed the different diets, *$P \leq 0.05$, ** $P \leq 0.01$. (**B**) Representative H&E images of the tumors in the lung from groups that were significantly impacted by the diet, relative to the 15%Amylose. Images were taken using Olympus BX46 microscope at 100 × magnification. (**C**) Soy saponins increase plasma levels of genistein in mice. The data (n = 5–12) are biological replicates and shown as the mean ± SEM. Significant differences between treatment groups were identified using one-way ANOVA followed with Tukey multiple comparison test. Groups not sharing the same letter denote significant ($P < 0.05$) differences between treatments. (**D**) Representative mass spectra of genistein (m/z 271.13, top panel) and its fragments (bottom panel).

to the anti-cancer effects of soy. We, however, found that the low CHO diets made with a set of amino acids corresponding to that in casein or soy protein made no difference in lung tumor numbers. This further confirmed that the chemopreventive activity of soy is unlikely attributable to the soy protein within the SPI.

Saponins are co-extracted with the isoflavones during the ethanol washing of SPI. Soyasaponins are naturally occurring triterpenoid glycosides with an oleanane-type aglycone that are structurally diverse depending on the number, position and type of polysaccharides attached to the aglycone backbone[62]. The two major types of saponins are the group A soyasaponins, which are glycosylated at C-3 and C-22 positions, and group B soyasaponins, which are glycosylated or conjugated to a 2,3-dihydro-2,5-dihydroxy-6-methyl-4-pyrone (DDMP) moiety at the C-3 position [44]. Saponins are amphiphilic due to the presence of the lipid-soluble aglycone and the water-soluble polysaccharide chain, giving them a soap-like property[63]. These soy saponins have recently been reported to have anti-proliferative effects[37,39,44]. When saponins were added to our low CHO diet, however, we saw no evidence of it being effective in reducing the development of lung nodules in our A/J mice. However, when the saponins were combined with isoflavones at levels present in SPI, they were almost as effective as SPI. This is consistent with saponins + isoflavones being the major contributors responsible for the ability of SPI to reduce NNK-induced lung nodules. The fact that the combination of isoflavones and saponins are effective at reducing lung nodule numbers, but not when they are present independently, could also explain a number of publications that reported a greater efficacy of SPI or concentrate when compared to pure isoflavones[57,64,65]. Our finding that this combination was not quite as effective as unfractionated SPI, could suggest that there are as of yet unidentified components within SPI that contribute to its efficacy in reducing NNK-induced lung nodules.

Since saponins are detergent-like molecules that can permeabilize cell membranes, we hypothesized during this study that the soy saponins might be enhancing the absorption of isoflavones through the gastrointestinal barrier, which leads to higher circulating isoflavone levels. We showed that this was indeed the case, suggesting that these soy saponins appear to act, at least in part, by increasing plasma levels of isoflavones. It is also possible that the saponins and the isoflavones work to prevent lung tumour formation via a synergistic mechanism that we have not currently identified.

Since genistein has previously been shown to be oxidized by CYP4501A2, a cytochrome P450 reported to be involved in converting the pro-carcinogen NNK to its active metabolites, it is conceivable that genistein may help reduce the formation of lung nodule formation by interfering with the biotransformation of NNK[66,67]. However, future work is needed to confirm this potential mechanism of action. To conclude, we have identified the combination of isoflavones and saponins as the primary components of SPI that contribute to the efficacy of soy in preventing cigarette carcinogen-induced lung tumor formation. However, we cannot completely rule out that other components within the SPI contribute to the lowering of NNK-induced lung nodules in our mouse model of lung cancer.

Data availability

Reasonable requests for data can be made to the corresponding author.

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Author contributions
IE and GK conceived and designed the study, interpreted data and wrote the manuscript. IE, MY, SK, AW, CD, SW, VC, HA, acquired and analyzed data, and revised the manuscript. All authors approved the final version of the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

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