Original Article

The radioprotectant nano-genistein enhances radiotherapy efficacy of lung tumors in mice

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Background: Radiotherapy for non-small cell lung cancer (NSCLC) can be dose-limiting due to treatment-related toxicities. Genistein has been shown to be a robust radioprotective agent in preclinical models. A novel genistein oral nanosuspension formulation (nano-genistein) has demonstrated efficacy in mitigating radiation-induced lung damage in preclinical animal models. However, while those studies have confirmed that nano-genistein can protect normal lung tissue from radiation-induced toxicities, no studies have assessed the effect of nano-genistein on lung tumors. Here, we evaluated the impact of nano-genistein on the efficacy of radiation treatment of lung tumors in a mouse xenograft model.

Methods: Two separate studies were conducted utilizing human A549 cells implanted either dorsally within the upper torso or in the flank. Daily oral administration of nano-genistein (200 or 400 mg/kg/day) occurred prior to and after exposure to a single dose of thoracic or abdominal 12.5 Gy radiation. Tumor growth was monitored twice weekly, nano-genistein treatment continued for up to 20 weeks and histopathology of tissues was completed post euthanasia.

Results: Continuous nano-genistein dosing was safe across all study groups in both studies. Animals receiving nano-genistein better maintained body weight following irradiation compared to corresponding vehicle treated animals. Animals that received nano-genistein also had reduced tumor growth and improved normal lung histopathology compared to those receiving vehicle suggesting that nano-genistein does not protect tumors from radiotherapy but is radioprotective of the lungs. There were no treatment-related histopathological findings noted in the skin adjacent to the tumor, esophagus, or uterus.

Conclusions: These results, including the safety following extended dosing, support the continued evaluation of nano-genistein as an adjunctive treatment for patients with NSCLC undergoing radiotherapy and serve as the basis of a phase 1b/2a multicenter clinical trial.

Keywords: Genistein; non-small cell lung cancer (NSCLC); radiotherapy; pneumonitis; pulmonary fibrosis

Submitted Nov 30, 2022. Accepted for publication Apr 12, 2023. Published online May 09, 2023.
doi: 10.21037/tlcr-22-856
View this article at: https://dx.doi.org/10.21037/tlcr-22-856

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Introduction

Lung cancer affects nearly a quarter million people annually in the United States and accounts for more cancer-related deaths than any other cancer type (1). Over 85% of lung cancers are non-small cell lung cancer (NSCLC) (2). NSCLC treatment regimens are multi-modal and commonly involve surgical resection, chemotherapy and/or immunotherapy, and radiotherapy. Despite recent advances in radiation treatment technologies, damage to the surrounding normal tissue during NSCLC radiotherapy remains a common cause of dose-limiting toxicities (3,4). A significant adverse effect caused by NSCLC radiotherapy is radiation-induced damage to the normal lung tissue, manifesting as acute or subacute pneumonitis and late pulmonary fibrosis (5). These can result in respiratory declines and overall reduced quality of life following treatment. It is, therefore, necessary to evaluate treatment strategies that may mitigate the toxicities-associated with radiotherapy, without hindering the efficacy of the treatment.

Genistein oral nanosuspension (nano-genistein) is a promising radioprotectant and radiomitigator that has demonstrated efficacy in mitigating radiation-induced lung damage in mouse models (6). The nano-genistein formulation consists of synthetic genistein nanoparticles formulated into an aqueous suspension suitable for oral self-administration. Nano-genistein mitigates radiation-induced tissue damage via genistein's activity as a highly selective estrogen receptor beta (ERb) agonist, subsequently regulating gene expression and cell signaling to promote cell survival and anti-inflammatory effects (7-9). No data to date assessed differential radioprotection of normal tissues versus tumor, and the clinical utility of nano-genistein would be diminished if the radioprotective properties also extend to tumors. Therefore, we evaluated nano-genistein in a mouse xenograft model of NSCLC. Here, we present proof-of-concept preclinical studies confirming that nano-genistein does not protect a lung adenocarcinoma in mice from acute thoracic or abdominal radiation. This manuscript is written following the ARRIVE reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-856/rc).

Highlight box

Key findings

- In these preclinical studies using a NSCLC mouse xenograft model, nano-genistein was demonstrated to be radioprotective of normal lung tissue but not radioprotective of tumors. Nano-genistein appeared to also improve the efficacy of radiotherapy by sensitizing tumors.
- Nano-genistein was also shown to be safe following continuous oral dosing for 10–20 weeks.

What is known and what is new?

- Radiotherapy for NSCLC is often dose limiting due to therapy-related toxicities.
- In this study, we evaluated the therapeutic utility of radioprotectant, nano-genistein, for NSCLC by demonstrating that it is radioprotectant of lung tissue, but not tumors.

What is the implication, and what should change now?

- The positive safety and efficacy results, along with previous preclinical investigations of nano-genistein, provided the rationale to evaluate nano-genistein as a radioprotectant for patients with NSCLC undergoing chemoradiotherapy in a multicenter phase 1b/2a trial (NCT02567799).

Methods

Animals

Experiments were performed under Institutional Animal Care & Use Committee (IACUC) with Protocol No. 1222 granted by the Henry Ford Health System Research Administration, in compliance with national and institutional guidelines for the care and use of animals. All mice were housed and managed by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved animal facilities at Henry Ford Health System (Detroit, MI, USA). Female athymic CD1 nu/nu mice (Charles River Laboratories, Wilmington, MA, USA), 6–7 weeks old, were housed four per cage and provided food/water ad libitum. Mouse chow was phytoestrogen-free (not containing soy), Teklad 2914 rodent chow (Harlan Laboratories, Haslett, MI, USA). Rooms were maintained with a 12-hour light/12-hour dark cycle (on 5 am, off 5 pm) and kept at 72±2 °F with 40%±5% relative humidity. Treatment groups started with N=12 animals/group.

Nano-genistein and vehicle for animal studies

Nano-genistein (BIO 300 Oral Suspension, Humanetics Corporation, Minneapolis, MN, USA) is a wet-milled nanosuspension containing 325 mg/mL synthetic genistein with a d(50) particle-size distribution of less than 0.2 µm, 5% Povidone K17 (weight/weight; w/w), 0.2% Polysorbate 80 (w/w) in 50 mM phosphate buffered saline (61 mM sodium chloride). The control article ("vehicle") was nano-genistein without the active ingredient, synthetic genistein.

Nano-genistein was diluted with vehicle such that mice received either 200 mg/kg in volumes ranging from 0.07–0.10 mL or 400 mg/kg in volumes ranging from 0.14–0.20 mL, depending on animal weight. Drug dilutions were prepared weekly. Nano-genistein or vehicle were administered by oral gavage daily using 20-gauge × 1.5 inch oral animal feeding cannula (Cadence, Inc., Cranston, RI, USA). Individual animal dosing was determined based on the weekly average body weight of each experimental group.

Murine xenograft studies

Study 1—thoracic irradiation model

In the first study, human A549 adenocarcinoma lung cancer (NSCLC) xenografts were implanted subcutaneously in mice dorsally within the upper torso, and the radiation field encompassed both the tumor xenograft and lung. For tumor cell implantation, 2×10^6 A549 cells were mixed 1:1 in Matrigel (100 mL total) and injected into each tumor site. Tumor volumes were recorded twice weekly by a researcher blinded to treatment groups using external measurements of the tumor dimensions. External measurements were used to calculate the tumor volume using the formula for the volume of an ellipse $[(4/3)pR_1 \bullet R_2 \bullet R_3]$ where R_1, R_2, and R_3 are the orthogonal radii of the tumor. This calculation was based on a modification and extension of a previously used method (10) and similar to other published ellipsoid formulas used to calculate tumor volumes (11). The third dimension (R_3) was estimated from an average of R_1 and R_2 [$R_3 = (R_2 + R_1)/2$] in order to avoid bias from choosing the smaller or larger of the two dimensions. Animals with ulcerated tumors were euthanized and excluded from the study from the time of ulceration. Tumor ulceration occurred unpredictably and independently of the tumor size. In addition, following tumor initiation the animals were randomized into treatment cohorts such that each cohort had a similar mean tumor volume and standard deviation.

Radiation was delivered once the mean tumor volume for each treatment group reached 400 mm^3. Thoracic irradiation was delivered using opposed lateral-beam geometry at a dose rate of 2.7 Gy/min. The radiation exposure conformed to a 2 cm wide field along the length of the mouse centered on the lung using a commercial collimator. A single 12.5 Gy radiation dose was delivered to anesthetized mice (60 mg/kg ketamine and 6 mg/kg xylazine, i.p.) using a 5000 Ci ^{137}Cs small animal irradiator (Mark I, J. L. Shepherd, San Fernando, CA, USA). Mice were irradiated in groups of 8. Radiation uniformity was maintained by delivering half the dose with the left side of the mouse closest to the source and half the dose with the right side of the mouse closest to the source. Nano-genistein or vehicle treatment was administered 1 h (±5 min) prior to radiation exposure. A single 12.5 Gy dose was chosen based on previous studies that indicated this dose can cause detectable lung tissue damage in addition to delaying tumor growth without completely ablating the tumor (6,12).

The 12.5 Gy dose accuracy was confirmed by measuring the radiation dose from the ^{137}Cs irradiator using 1 mm^3 LiF microcube dosimeters (thermo-luminescent dosimeters; TLDs) and Gafchromic radiation-sensitive film and comparing the dose to that from a calibrated clinical 6 MV linear particle accelerator (LINAC). For the ^{137}Cs irradiator, this dose corresponds to an exposure time of approximately 4.68 min. The dosimetry analysis confirmed the desired dose; film exposed to the ^{137}Cs source at 12.5 Gy had an optical density within 1% of film exposed to the same dose from a calibrated 6 MV clinical irradiator. The dose from the ^{137}Cs irradiator measured using TLDs were ±3% of that from the calibrated 6 MV clinical irradiator.

On the day of irradiation, mice were first administered their respective nano-genistein or vehicle treatment and then irradiated 1 h (±5 min) after dosing. At the time of irradiation, there was N=10–12 animals/group due to tumor ulcerations. In this first study, nano-genistein dosing started 7 days prior to A549 tumor cell implantation and continued for 10 weeks (5 days/week). The primary outcome measure was tumor growth. Body weights were collected twice weekly throughout the study. Lung, skin adjacent to the tumor, uterus, esophagus and tumor tissues were collected from animals at euthanasia and embedded in paraffin. Tissue samples from 5 animals/group were hematoxylin and eosin (H&E) stained and submitted to Consulting Tox/Path, LLC for independent histopathological review. Severity of microscopic findings in the tissues was graded on a 5-point Likert scale, and the distribution of the finding (focal, multifocal, diffuse) was reported.

Study 2—abdominal irradiation model

In this study, A549 cells were implanted subcutaneously in the dorsal rear flank to facilitate animal handling. Similar to the above experiment, 2×10^6 A549 cells were mixed 1:1 in Matrigel (100 mL total) and injected into each tumor site. Tumor volumes were recorded twice weekly, animals with ulcerated tumors were handled as described above and

animals were randomized into treatment groups so that each group had a similar mean tumor volume and standard deviation.

The delivered radiation field for this model was termed "abdominal radiation" because it was a 2 cm field that encompassed the subcutaneously implanted flank tumor, gastrointestinal (GI) tract and uterus. As described above, a single 12.5 Gy dose was delivered using the same irradiator, radiation geometry, and assessment of radiation accuracy. At the time of irradiation, there was N=9–12 animals/group due to tumor ulcerations. Mice received one of two treatment schedules for nano-genistein or vehicle dosing, (I) animals were dosed starting after A549 implantation when tumors reached a mean volume of 75 mm^3 or (II) animals were dosed starting 7 days prior to abdominal irradiation (tumors reached approximately 400 mm^3 on the day of irradiation). Nano-genistein dosing continued for 20 or 16 weeks, respectively (7 days/week). Body weights were collected twice weekly throughout the study.

As above in experiment 1, tumor growth was the primary outcome measure and body weights were collected twice weekly. To investigate the protective effects of nano-genistein, the skin directly above the tumor and the terminal ileum were collected at euthanasia and were submitted for independent histopathological review (Consulting Tox/Path, LLC). For these analyses, 3 skin and 3 terminal ileum tissue samples were H&E stained from each treatment group that received nano-genistein or vehicle starting at the time tumors reached 75 mm^3. Severity of findings in the tissues was graded on a 5-point Likert scale as described above.

Statistical analysis

For the xenograft studies, sample sizes were based on previously published studies in a similar model evaluating a related genistein product (10). Individual animal tumor volumes were compared relative to the tumor volume on the day of irradiation. Similarly, individual animal body weights were compared relative to the animal body weight on the day of tumor implantation. In order to avoid the limitations of statistically testing each individual timepoint in these studies, such as changes in sample size due to animal attrition, a mixed-effects analysis of variance (ANOVA), also known as a repeated measures ANOVA, was used. The mixed-effects ANOVA compared the changes in mean relative tumor volume or mean relative body weight over time between treatment groups. Survival was evaluated by Kaplan-Meier curves. Animals that were euthanized due to tumor ulcerations were censored for survival analyses. Kaplan-Meier curves were compared by log-rank test. All statistical analyses were completed using GraphPad Prism 9 (Dotmatics, Boston, MA, USA), and statistical significance was defined as $P<0.05$.

Results

Tumor growth

In the first study, mice received thoracic irradiation approximately 5–6 weeks after tumor implantation. At the time of irradiation, tumors were 392±248 mm^3 (vehicle), 429±245 mm^3 (nano-genistein, 200 mg/kg), and 440±316 mm^3 (nano-genistein, 400 mg/kg). Individual animal tumor volumes were normalized to the volume on the day of thoracic irradiation and plotted by treatment group versus days post-irradiation (*Figure 1A,1B*). Statistical comparisons are provided in *Table 1*. Nano-genistein (200 or 400 mg/kg/day) as a monotherapy had no effect on tumor growth (*Figure 1A,1B*; blue symbols). Radiation alone, or in combination with nano-genistein (200 mg/kg/day), had a similar inhibitory effect on tumor growth (*Figure 1A*). Radiation in combination with 400 mg/kg/day nano-genistein improved the efficacy of radiation compared to radiation alone (P=0.0025; *Figure 1B*; *Table 1*). Survival analysis during the BIO 300 dosing period post-irradiation shows minimal differences between the treatment groups (*Figure 1C,1D*). The lone exception are the animals that received nano-genistein (400 mg/kg/day) alone without radiation which have worse survival compared to the irradiated, nano-genistein (400 mg/kg/day) treated animals (log-rank test, P=0.021; *Figure 1D*). However, the animals that received nano-genistein (400 mg/kg/day) alone without radiation did not have significantly worse survival than the unirradiated vehicle-treated animals (log-rank test, P=0.084).

In the second study, all treatment groups received abdominal irradiation 31 days after the tumor volume reached 75 mm^3. The mean ± standard error of the mean (SEM) tumor volume across all study arms at the time of irradiation was 470±295 mm^3, and individual animal tumor volumes were normalized as described above. Nano-genistein treatment, in combination with radiation, at 200 mg/kg starting 24 h after tumor establishment (75 mm^3) or 7 days prior to irradiation significantly reduced tumor growth relative to radiation alone

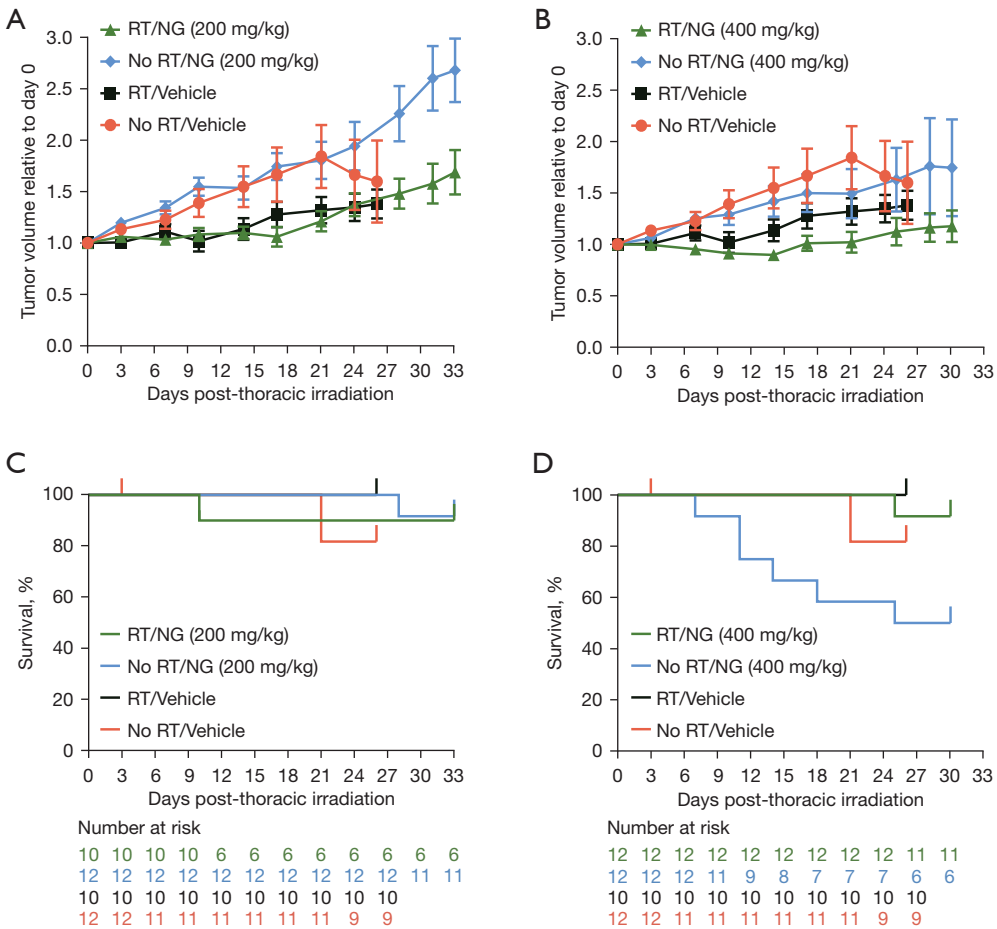

Figure 1 Growth of upper torso A549 tumors in mice treated with NG and exposed to a single dose of 12.5 Gy thoracic irradiation. Change in normalized tumor volume over time in study 1 animals treated with and without 12.5 Gy thoracic irradiation (radiation) and vehicle or (A) 200 mg/kg NG or (B) 400 mg/kg NG. Tumor volumes were normalized to the tumor volume on the day of irradiation (day 0). For groups that did not receive thoracic irradiation (no RT), tumor volumes were normalized to the corresponding radiation-treated group's day of irradiation. Statistical comparisons are found in *Table 1*. Kaplan-Meier survival curves are provided during the NG dosing period post-irradiation below each corresponding tumor volume graph for the (C) 200 mg/kg NG and the (D) 400 mg/kg NG dosing groups. The only significant difference in survival by log-rank test was between the no RT and RT 400 mg/kg NG groups (P=0.021). The number of animals at risk is provided below each graph. All symbols are the mean relative volume and error bars are SEM. RT, radiation; NG, nano-genistein; SEM, standard error of mean.

(*Figure 2A*, *Table 1*). Similar to the first study, nano-genistein (400 mg/kg) treatment alone did not impact tumor growth compared to vehicle treatment (P=0.9999; *Figure 2B*, *Table 1*). Nano-genistein treatment, in combination with radiation, at 400 mg/kg starting after tumor establishment synergized with radiation to significantly reduce tumor growth (*Figure 2B*, *Table 1*). Survival analysis reveal no significant differences between treatment groups and their respective controls (*Figure 2C*,*2D*).

Changes in body weight

In the second study involving abdominal irradiation, the field of radiation included the GI tract, and thus changes in body weight were also assessed. We found that both dose levels and both dose schedules of nano-genistein resulted in significant improvements in mean body weight over time compared to irradiated animals treated with vehicle alone (*Figure 3A*,*3B*). In the first xenograft study, significant weight loss was not anticipated due to the radiation field location; however, there

Table 1 Statistical comparisons for tumor growth in xenograft

Study	Radiation	Comparator 1	Comparator 2	P value
1	Thoracic	No RT/vehicle	12.5 Gy RT/vehicle	0.2201
1	Thoracic	No RT/vehicle	No RT/NG [200 mg/kg/day]	0.9385
		No RT/vehicle	12.5 Gy RT/NG [200 mg/kg/day]	0.0295
		RT/vehicle	No RT/NG [200 mg/kg/day]	0.0008
		RT/vehicle	12.5 Gy RT/NG [200 mg/kg/day]	0.0560
1	Thoracic	No RT/vehicle	No RT/NG [400 mg/kg/day]	0.7878
		No RT/vehicle	12.5 Gy RT/NG [400 mg/kg/day]	<0.0001
		RT/vehicle	No RT/NG [400 mg/kg/day]	0.2215
		RT/vehicle	12.5 Gy RT/NG [400 mg/kg/day]	0.0025
2	Abdominal	No RT/vehicle	12.5 Gy RT/vehicle	<0.0001
2	Abdominal	No RT/vehicle	12.5 Gy RT/NG [200 mg/kg/day]	<0.0001
		No RT/vehicle	12.5 Gy RT/NG [200 mg/kg/day], –7 d	<0.0001
		RT/vehicle	12.5 Gy RT/NG [200 mg/kg/day]	<0.0001
		RT/vehicle	12.5 Gy RT/NG [200 mg/kg/day], –7 d	<0.0001
2	Abdominal	No RT/vehicle	No RT/NG [400 mg/kg/day]	0.9999
		No RT/vehicle	12.5 Gy RT/NG [400 mg/kg/day]	<0.0001
		No RT/vehicle	12.5 Gy RT/NG [400 mg/kg/day], –7 d	<0.0001
		RT/vehicle	No RT/NG [400 mg/kg/day]	0.0021
		RT/vehicle	12.5 Gy RT/NG [400 mg/kg/day]	0.0013
		RT/vehicle	12.5 Gy RT/NG [400 mg/kg/day], –7 d	0.2155

All analyses were conducted using mixed-effects ANOVA. RT, radiation; NG, nano-genistein; –7 d, 7 days prior to irradiation; ANOVA, analysis of variance.

was an apparent decrease in mean body weight in each group following thoracic irradiation (*Figure 3C*). While all treatment groups had statistically different changes in mean body weight over time relative to vehicle (P<0.05), it appears that animals treated with 400 mg/kg maintained body weight better than vehicle treated animals in both studies.

Histopathology

In study 1 where animals received thoracic irradiation, all vehicle- and nano-genistein-treated animals displayed evidence of increased alveolar septal wall thickness with increased collagen, with the exception of the groups that received sham irradiation. The lungs of animals that received thoracic irradiation and vehicle treatment displayed clear signs of multifocal or diffuse congestion, alveolar macrophage infiltration, thickening of the septal wall, alveolar fibrin, pneumocyte hypertrophy, and overall reduction in alveolar space (*Figure 4*, *Table 2*). In contrast, animals that received nano-genistein (200 or 400 mg/kg/day) had markedly improved lung histology (*Figure 4*). The major finding in these animals was minimal thickening of the septal wall, and any instances of macrophage infiltration were minimal to mild and focal in distribution (*Table 2*). From a safety perspective, the uterus, esophagus, and skin of the sham-irradiated animals were also examined. Microscopic observations of the skin included minimal mixed cellular or neutrophilic inflammation and decreased or increased epidermal thickness, and in the esophageal lumen there was bacteria present in all groups. These findings were considered to be incidental with no relationship to any particular treatment group. Findings in tumor tissue

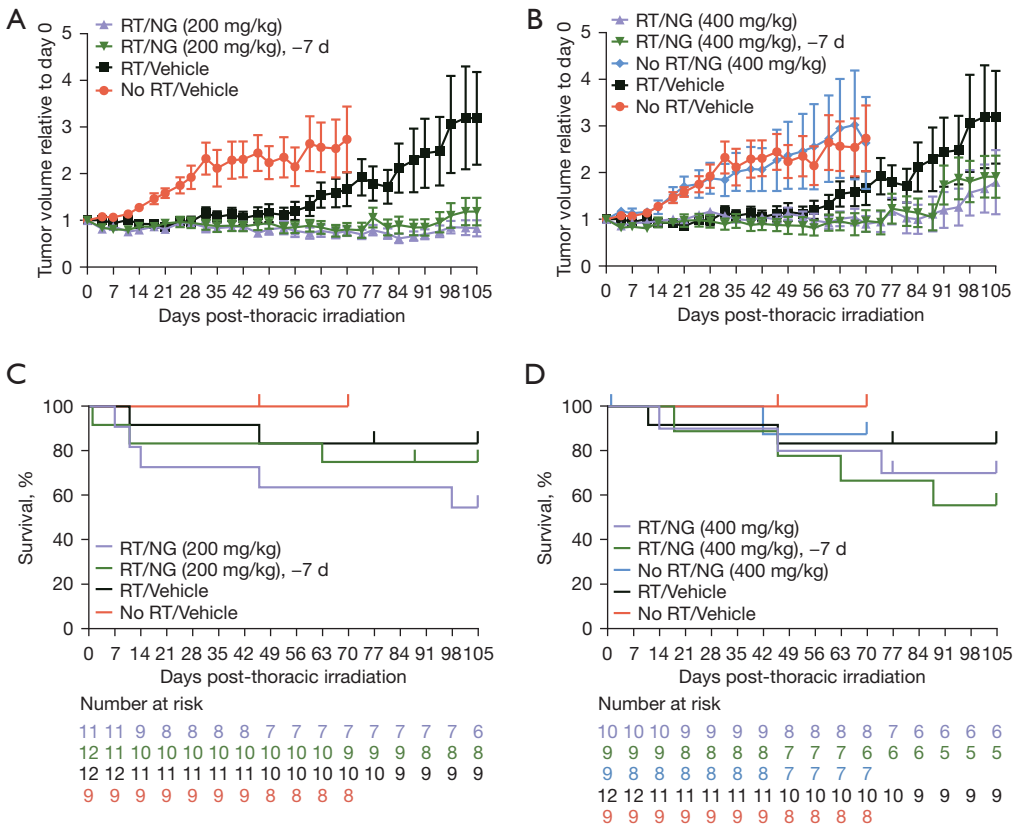

Figure 2 Growth of flank A549 tumors in mice treated with nano-genistein and exposed to a single dose of 12.5 Gy abdominal irradiation. Changes in normalized tumor volume over time in study 2 animals treated with and without 12.5 Gy abdominal irradiation (radiation). Tumor volumes were normalized to the tumor volume on the day of irradiation (day 0). Animals were treated with vehicle, (A) 200 mg/kg NG or (B) 400 mg/kg NG starting once tumors were established (75 mm^3) or 7 days prior to irradiation (−7 d). Statistical comparisons are found in *Table 1*. Kaplan-Meier survival curves are below each corresponding tumor volume graph for the (C) 200 mg/kg NG and the (D) 400 mg/kg NG dosing groups. No curves were significantly different by log-rank test amongst the various treatment groups and their respective controls. The number of animals at risk is provided below each graph. All symbols are the mean relative volume and error bars are SEM. RT, radiation; NG, nano-genistein; SEM, standard error of mean.

included cystic areas of degeneration and necrosis with or without inflammation in all animals (Table S1). These findings were also not related to any particular treatment group. Importantly, no treatment-associated adverse effects were observed following 10-week of nano-genistein dosing for either the 200 or the 400 mg/kg/day groups.

In the second study, histopathology was limited to the skin and terminal ileum from animals that received nano-genistein or vehicle starting when tumors reached 75 mm^3. Histopathology review concluded that there were no nano-genistein related effects of the skin adjacent to the tumor or the terminal ileum (Table S2, Figure S1). Minimal to mild and multifocal to diffuse amyloid-like material was observed in the lamina propria of the ileum in all mice in all groups, but otherwise it was normal in appearance. Amyloid deposition is a common finding in various organs, especially the ileum, in mice and may be related to age and/or inflammation.

Discussion

The soy isoflavone, genistein, has been studied for decades as a potential therapeutic for various cancers and inflammatory diseases, such as asthma, because of its anti-inflammatory, antioxidant and radioprotective properties (13-15). Despite its intriguing biological activity, genistein has yet to be translated into use as a clinical therapeutic. This is in large part due to its poor water solubility and oral bioavailability (16). In animal studies, genistein is often

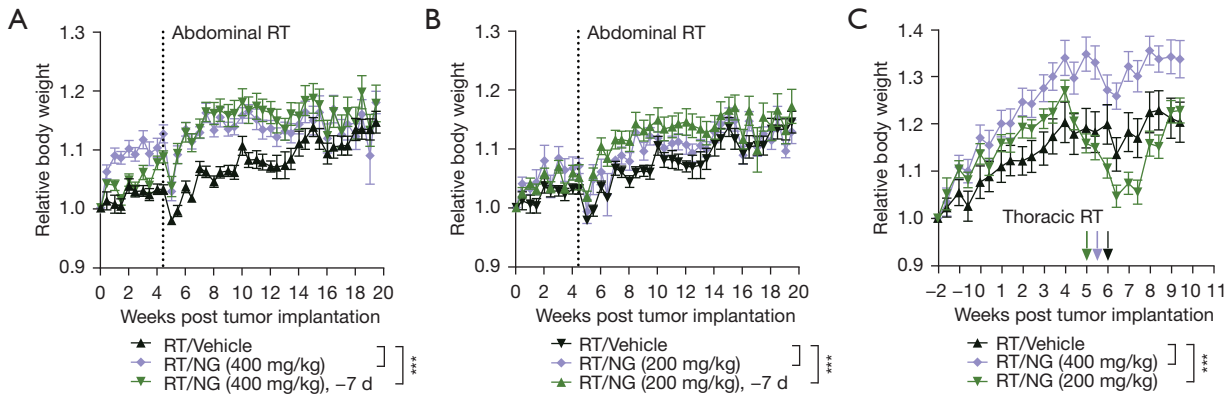

Figure 3 Relative changes in body weight of mice treated with nano-genistein and exposed to a single dose of 12.5 Gy abdominal irradiation. Mice with implanted A549 cells had body weights, in grams, measured weekly. (A,B) Animals in xenograft study 2 (abdominal radiation) were treated once daily with vehicle, 200 mg/kg NG or 400 mg/kg NG starting once tumors were established (75 mm^3) or 7 days prior to irradiation (–7 d). Animal body weights were normalized relative to the day tumors were established (day 0). The day of irradiation in this study (31 days after tumors reached 75 mm^3) is marked by a dotted line. Statistical significance was determined by mixed-effects ANOVA (***, P<0.05). (C) Animals in xenograft study 1 (thoracic irradiation) were treated with vehicle, 200 mg/kg NG or 400 mg/kg NG starting 1 week prior to tumor implantation (week –1). Animal body weights were normalized relative to week –2, prior to animals receiving treatment or tumor cells. Arrows indicate the day of irradiation for the color corresponding treatment groups. Statistical significance was determined by mixed-effects ANOVA (***, P<0.05). Symbols are the mean relative volume and error bars are SEM. RT, radiation; NG, nano-genistein; ANOVA, analysis of variance; SEM, standard error of mean.

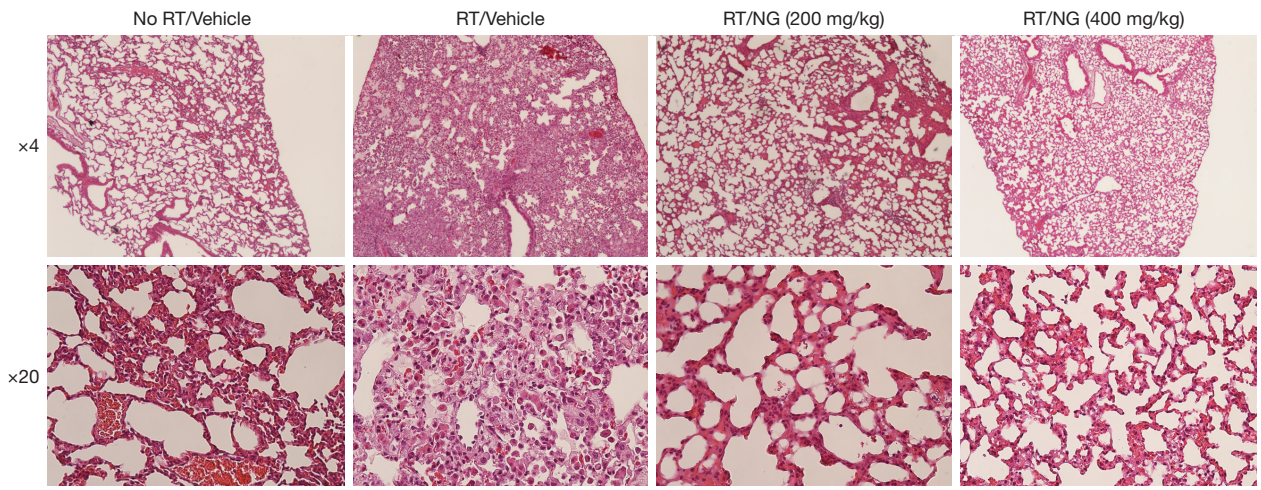

Figure 4 Lung images from animals that received thoracic irradiation. Representative images of H&E stained lung sections from mice at euthanasia that were treated with vehicle with or without 12.5 Gy thoracic irradiation and animals treated with 200 mg/kg or 400 mg/kg NG and exposed to 12.5 Gy thoracic irradiation. Photomicrographs are ×20 or ×4 magnification as specified. RT, radiation; NG, nano-genistein; H&E, hematoxylin and eosin.

solubilized in polyethylene glycol (PEG) 400 to improve oral bioavailability, but this is not a feasible option for human dosing given the strong laxative properties of PEG 400. To overcome the inherently poor oral pharmacokinetic properties of genistein, a proprietary process for nanomilling synthetic genistein was developed to produce a genistein nanosuspension suitable for oral administration (nano-genistein) (6,17). This formulation has a reduced

Table 2 Histopathological effects in the lungs of animals treated with thoracic irradiation

Lung microscopic observations and time of euthanasia	Treatment group																									
	No RT/vehicle					12.5 Gy RT/vehicle					No RT/NG (200 mg/kg)					12.5 Gy RT/NG (200 mg/kg)					No RT/NG (400 mg/kg)					12.5 Gy RT/NG (400 mg/kg)				
Euthanasia (weeks post-RT)	5.4	2.5	2.9	4.4	3.7	8.0	11.4	6.0	8.7	8.7	4.7	4.7	4.7	7.0	9.0	1.9	7.0	9.7	1.9	9.0	1.9	1.9	3.4	4.7	2.7	3.1	5.3	5.3	5.3	5.3
Alveolar hemorrhage					1M										1M															
Alveolar edema							2M													1M										
Alveolar fibrin							1M																							
Congestion						1D	1D	1D		1D																1D				
Increased alveolar macrophages						1M	3D											2F								1F		1M	1M	1M
Increased septal thickness						2D	3D	1M	1D	2D						1D	1M	2M	1D	1D						1M	1M	1M	1D	2M
Mixed cellular infiltrate																													1M	
Pneumocyte hyperplasia							3M																							
Unremarkable (no findings)	0	0	0	0							0	0	0	0							0	0	0	0	0					

Each column represents an individual animal. Severity: 0, normal; 1, minimal; 2, mild; 3, moderate. Distribution: F, focal; M, multifocal; D, diffuse. RT, radiation; NG, nanogenistein.

average particle size of approximately 100-fold compared to standard genistein, allowing for improved bioavailability.

On the molecular level, genistein has been shown to regulate proteins such as NF-kB, IL-1b, COX2, p53, and p21 (9,18-20). These effects can be attributed to genistein's activation of ERb which promote anti-inflammatory effects, DNA damage response, and cell cycle regulation (21-23). Radiation-induced pneumonitis is caused by damage to the alveolar epithelium and vascular endothelium resulting in the release of pro-inflammatory and pro-fibrotic cytokines (5). Oxidative stress, DNA damage and cytokine release leads to hypoxia, pulmonary edema and an increase in lung density. If the injury is not managed, it can progress to pulmonary fibrosis. Genistein has the potential to mitigate the development of these pathologies by counteracting oxidative stress and reducing inflammation. Further, the differential function and expression of DNA damage response proteins (e.g., p53) and pro-inflammatory pathways (e.g., NF-kB), likely allow genistein to prevent or mitigate radiation damage of normal tissues without protecting tumors. Genistein has even been shown to have anti-tumor effects, including synergizing with radiation to kill cancer cells (20,24-26). Nano-genistein was also shown to inhibit growth of prostate cancer tumors alone or in combination with radiation in mouse xenograft models (27).

The present study demonstrated that daily nano-genistein doses of 200–400 mg/kg did not protect lung tumors from radiation treatment; on the contrary, significant radiosensitization was observed when tumors were grown in the flank (a location that allowed tumors to grow for several months). Nano-genistein was also shown to protect the normal lung tissues at both dose levels (200 and 400 mg/kg/day), as evidenced by the reduction in pulmonary congestion, inflammation and collagen deposition. However, future studies will need to be carried out for a longer period in order to better evaluate the drug's effect on lung fibrosis, which can take up to 6 months to develop in mice (28). Importantly, nano-genistein was well-tolerated by animals in both studies, and no toxic effects were observed in animals at nano-genistein doses up to 400 mg/kg/day administered up to 7 days/week for 20 weeks. The mortality observed in the thoracic irradiation study in unirradiated animals that received 400 mg/kg/day nano-genistein was not considered treatment-related since the results were not reproduced in unirradiated animals that received the same dose, but for a longer duration, in the abdominal irradiation study. This supports the safety of extended continuous daily dosing of nano-genistein. Animals treated with nano-genistein appeared to better maintain body weight following irradiation, particularly in the second xenograft study where the radiation field included the GI tract. Translationally, a >5% weight loss can result in worse clinical outcomes for patients with NSCLC treated with concurrent chemoradiotherapy (29).

Limitations to this study are the use of a single cell line, a single fixed radiation dose instead of fractionated radiation, and the use of a single stain (H&E) to assess histological tissue damage. Future studies with a syngeneic mouse model (e.g., LLC1 cells) should be considered. Studies in using animals with an intact immune system will be important in evaluating the drugs mechanism. Stains such as Masson's trichrome stain for collagen should also be considered. Additionally, there are several caveats in translating these findings to the clinic, including starting nano-genistein dosing prior to or immediately following tumor implantation and only exposing animals to a single dose of radiation when NSCLC is clinically treated with fractionated radiotherapy. However, previous preclinical studies demonstrated that nano-genistein protects lung tissue from radiation exposure (6) and showed that nano-genistein can also sensitize multiple prostate xenografts to radiation-induced tumor killing (27).

Conclusions

In conclusion, this study demonstrated that daily oral administration of nano-genistein is safe and not radioprotective of NSCLC tumors. Nano-genistein also sensitized tumors to acute radiation exposure and promoted stable body weight following irradiation. Taken together, this body of evidence supports the continued investigation of nano-genistein as a radioprotectant candidate for use during radiotherapy for patients with NSCLC, and these findings served as the basis of a multicenter phase 1b/2a trial of nano-genistein with concurrent chemoradiation for locally advanced NSCLC (NCT02567799).

Acknowledgments

The authors thank John Zenk, MD for his role in study conception and interpretation. John Zenk, MD passed away prior to the publication of this article. The authors also thank the veterinary pathology expertise provided by Roger E. Wells, DVM, MS, DACVP, Toxicologic Pathologist at Consulting Tox/Path, LLC, and Melissa Ingram, PhD, for providing the initial draft of the manuscript.

Funding: Research reported in this study was supported by the National Cancer Institute Small Business Innovation Research award (No. HHSN261201200078C). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute.

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-856/rc

Data Sharing Statement: Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-856/dss

Peer Review File: Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-856/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-856/coif). CBS serves as an unpaid editorial board member of *Translational Lung Cancer Research* from July 2017 to June 2024. MDK, AAS and BM are current employees of Humanetics Corporation and inventors on 4 Humanetics Patents and 3 Humanetics PCT applications. The research presented in this manuscript was financially support by SBIR grant (No. HHSN261201200078C), of which MDK was the PI on the award. MDK and AAS are supported by 5 additional grants that were awarded to Humanetics Corporation; however, none of these grants supported, including financially, the research presented in this manuscript. BM currently receives payments to support research unrelated to this manuscript from 3 other companies. BM was formerly the President of American Radium Society, Inc., and this role is unrelated to the current manuscript. SLB received funding support from the NIH grant that supported the studies in the manuscript and receives royalty payments from Humanetics Corporation for intellectual property unrelated to the manuscript. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were performed under Institutional Animal Care & Use Committee (IACUC) with Protocol No. 1222 granted by the Henry Ford Health System Research Administration, in compliance with national and institutional guidelines for the care and use of animals.

References

1. National Cancer Institute Surveillance, Epidemiology and End Results Program. Available online: https://seer.cancer.gov/statistics/interactive.html
2. Miller KD, Siegel RL, Lin CC, et al. Cancer treatment and survivorship statistics, 2016. CA Cancer J Clin 2016;66:271-89.
3. Simone CB 2nd. Thoracic Radiation Normal Tissue Injury. Semin Radiat Oncol 2017;27:370-7.
4. Verma V, Simone CB 2nd, Werner-Wasik M. Acute and Late Toxicities of Concurrent Chemoradiotherapy for Locally-Advanced Non-Small Cell Lung Cancer. Cancers (Basel) 2017;9:120.
5. Yan Y, Fu J, Kowalchuk RO, et al. Exploration of radiation-induced lung injury, from mechanism to treatment: a narrative review. Transl Lung Cancer Res 2022;11:307-22.
6. Jackson IL, Zodda A, Gurung G, et al. BIO 300, a nanosuspension of genistein, mitigates pneumonitis/fibrosis following high-dose radiation exposure in the C57L/J murine model. Br J Pharmacol 2017;174:4738-50.
7. Landauer MR, Harvey AJ, Kaytor MD, et al. Mechanism and therapeutic window of a genistein nanosuspension to protect against hematopoietic-acute radiation syndrome. J Radiat Res 2019;60:308-17.
8. Jones JW, Jackson IL, Vujaskovic Z, et al. Targeted Metabolomics Identifies Pharmacodynamic Biomarkers for BIO 300 Mitigation of Radiation-Induced Lung Injury. Pharm Res 2017;34:2698-709.
9. Ha CT, Li XH, Fu D, et al. Genistein nanoparticles protect mouse hematopoietic system and prevent proinflammatory factors after gamma irradiation. Radiat

Res 2013;180:316-25.
10. Gallo D, Zannoni GF, De Stefano I, et al. Soy phytochemicals decrease nonsmall cell lung cancer growth in female athymic mice. J Nutr 2008;138:1360-4.
11. Rodallec A, Vaghi C, Ciccolini J, et al. Tumor growth monitoring in breast cancer xenografts: A good technique for a strong ethic. PLoS One 2022;17:e0274886.
12. Storozhuk Y, Sanli T, Hopmans SN, et al. Chronic modulation of AMP-Kinase, Akt and mTOR pathways by ionizing radiation in human lung cancer xenografts. Radiat Oncol 2012;7:71.
13. Goh YX, Jalil J, Lam KW, et al. Genistein: A Review on its Anti-Inflammatory Properties. Front Pharmacol 2022;13:820969.
14. Rasheed S, Rehman K, Shahid M, et al. Therapeutic potentials of genistein: New insights and perspectives. J Food Biochem 2022;46:e14228.
15. Singh VK, Seed TM. BIO 300: a promising radiation countermeasure under advanced development for acute radiation syndrome and the delayed effects of acute radiation exposure. Expert Opin Investig Drugs 2020;29:429-41.
16. Yang Z, Kulkarni K, Zhu W, et al. Bioavailability and pharmacokinetics of genistein: mechanistic studies on its ADME. Anticancer Agents Med Chem 2012;12:1264-80.
17. Salem AM, Jackson IL, Gibbs A, et al. Interspecies Comparison and Radiation Effect on Pharmacokinetics of BIO 300, a Nanosuspension of Genistein, after Different Routes of Administration in Mice and Non-Human Primates. Radiat Res 2022;197:447-58.
18. Zhu Q, Zhang W, Mu D, et al. Effects of genistein on lipopolysaccharide-induced injury of mouse alveolar epithelial cells and its mechanism. Biosci Biotechnol Biochem 2020;84:544-51.
19. Zhang Z, Wang CZ, Du GJ, et al. Genistein induces G2/M cell cycle arrest and apoptosis via ATM/p53-dependent pathway in human colon cancer cells. Int J Oncol 2013;43:289-96.
20. Jiang H, Fan J, Cheng L, et al. The anticancer activity of genistein is increased in estrogen receptor beta 1-positive breast cancer cells. Onco Targets Ther 2018;11:8153-63.
21. Sareddy GR, Li X, Liu J, et al. Selective Estrogen Receptor β Agonist LY500307 as a Novel Therapeutic Agent for Glioblastoma. Sci Rep 2016;6:24185.
22. Gong P, Madak-Erdogan Z, Li J, et al. Transcriptomic analysis identifies gene networks regulated by estrogen receptor α (ERα) and ERβ that control distinct effects of different botanical estrogens. Nucl Recept Signal 2014;12:e001.
23. Warner M, Huang B, Gustafsson JA. Estrogen Receptor β as a Pharmaceutical Target. Trends Pharmacol Sci 2017;38:92-9.
24. Zhang Z, Jin F, Lian X, et al. Genistein promotes ionizing radiation-induced cell death by reducing cytoplasmic Bcl-xL levels in non-small cell lung cancer. Sci Rep 2018;8:328.
25. Yan H, Jiang J, Du A, et al. Genistein Enhances Radiosensitivity of Human Hepatocellular Carcinoma Cells by Inducing G2/M Arrest and Apoptosis. Radiat Res 2020;193:286-300.
26. Liu X, Wang Q, Liu B, et al. Genistein inhibits radiation-induced invasion and migration of glioblastoma cells by blocking the DNA-PKcs/Akt2/Rac1 signaling pathway. Radiother Oncol 2021;155:93-104.
27. Jackson IL, Pavlovic R, Alexander AA, et al. BIO 300, a Nanosuspension of Genistein, Mitigates Radiation-Induced Erectile Dysfunction and Sensitizes Human Prostate Cancer Xenografts to Radiation Therapy. Int J Radiat Oncol Biol Phys 2019;105:400-9.
28. Bertho A, Dos Santos M, Braga-Cohen S, et al. Preclinical Model of Stereotactic Ablative Lung Irradiation Using Arc Delivery in the Mouse: Is Fractionation Worthwhile? Front Med (Lausanne) 2021;8:794324.
29. Kim YJ, Song C, Eom KY, et al. Combined Chemoradiotherapy-induced Weight Loss Decreases Survival in Locally Advanced Non-small Cell Lung Cancer Patients. In Vivo 2019;33:955-61.

Cite this article as: Kaytor MD, Serebrenik AA, Lapanowski K, McFall D, Jones M, Movsas B, Simone CB 2nd, Brown SL. The radioprotectant nano-genistein enhances radiotherapy efficacy of lung tumors in mice. Transl Lung Cancer Res 2023;12(5):999-1010. doi: 10.21037/tlcr-22-856

Supplementary

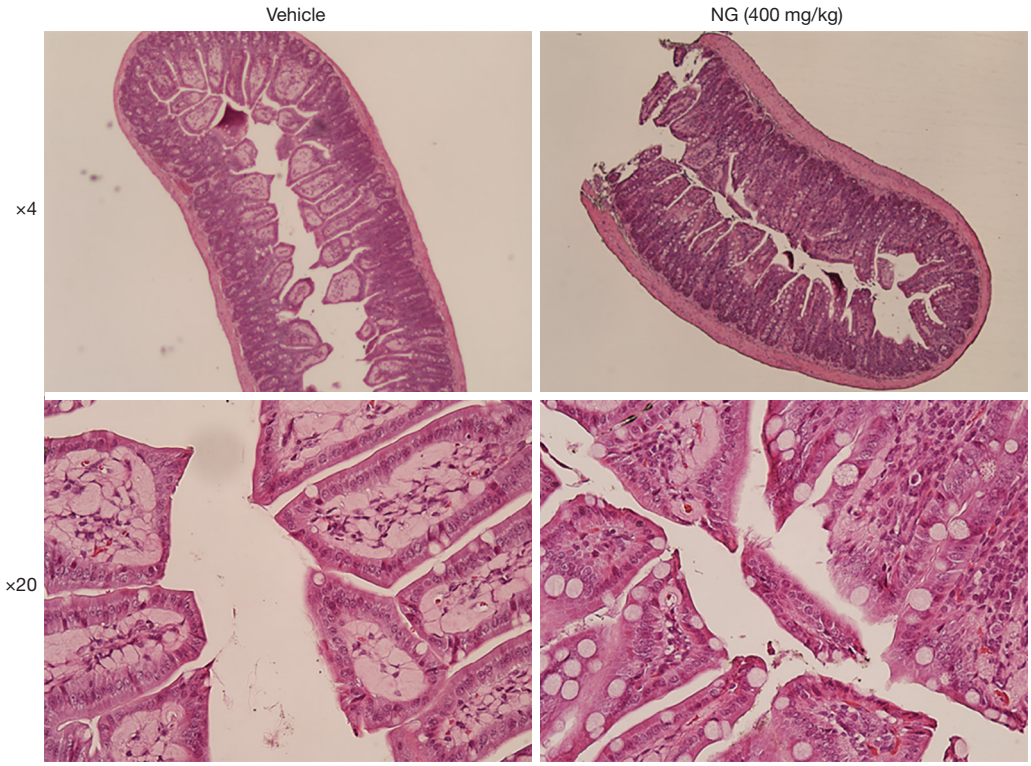

Figure S1 Terminal ileum images from xenograft study 2. Representative images of H&E stained terminal ileum sections from mice at euthanasia that were treated with vehicle or 400 mg/kg/d NG without abdominal irradiation. Photomicrographs are ×20 or ×4 magnification as specified. H&E, hematoxylin and eosin; NG, nano-genistein.

https://dx.doi.org/10.21037/tlcr-22-856

Table S1 Histopathological effects of the tumor from animals treated with thoracic irradiation

Tumor implant microscopic observations	Treatment group																													
	No RT/vehicle					12.5 Gy RT/vehicle					No RT/NG (200 mg/kg)					12.5 Gy RT/NG (200 mg/kg)					No RT/NG (400 mg/kg)					12.5 Gy RT/NG (400 mg/kg)				
Lymphocytic infiltrate											2M	2M		1M																
Mixed cellular exudate											2M	1M	1M	1M					3M											
Mixed cellular infiltrate			3M	3M	2M	2M	1M	1M	1M	2M							1M	1M					2M		1M	1M	1M	2M	1M	
Multinodular mass with cystic degeneration/necrosis		4M	3M	3M	4M	3M		2M	1M	1M	1M	2M	3M	1M	3M	1M	3M	3M	2M		1M	3M	3M	3M	3M		2M	3M	1M	3M	2M
Multinodular mass with diffuse degeneration/necrosis	4D																													
Multinodular mass with large central area degeneration/necrosis							3M																							
Multinodular mass with total degeneration/necrosis																			3M					4M						
Neutrophilic infiltrate		4M																			4F									
Purulent exudate	4M																		4F					4F						
Skin ulcer over tumor	P	P																	P					P						

Each column represents an individual animal. Severity: 0, normal; 1, minimal; 2, mild; 3, moderate; 4, severe. Distribution: F, focal; M, multifocal; D, diffuse; P, present. RT, radiation; NG, nano-genistein.

Table S2 Histopathological effects in the skin and terminal ileum of animals treated with abdominal irradiation

Skin and terminal ileum microscopic observations	Treatment group																	
	No RT/vehicle			12.5 Gy RT/vehicle			12.5 Gy RT/NG (200 mg/kg)			No RT/NG (400 mg/kg)			12.5 Gy RT/NG (400 mg/kg)					
Amyloid, lamina propria (ileum)	1M	2D	2D	2D	2D	2D	1M	1M	1M	1M	2M	2D	2D	2D	2M			
Mixed cellular infiltrate, deep hypodermis, skeletal muscle (skin)											1M							
Mixed cellular infiltrate, sebaceous gland (skin)	1F				1F													
Unremarkable (no findings) (skin)		0	0	0		0	0	0	0	0		0	0	0	0			

Each column represents an individual animal. Severity: 0, normal; 1, minimal; 2, mild; 3, moderate; 4, severe. Distribution: F, focal; M, multifocal; D, diffuse; P, present. RT, radiation; NG, nano-genistein.

https://dx.doi.org/10.21037/tlcr-22-856