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UTL-5g Lowers Levels of TGF-β and TNF-α Elevated by Lung Irradiation and Does Not Affect Tumor-response to Irradiation

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Abstract

UTL-5g is a small-molecule TNF-α inhibitor having chemoprotective and liver radioprotective effects. We investigated the effects of UTL-5g on lung irradiated mice and whether UTL-5g affects tumor responses to radiotherapy. C57BL/6 mice were individually treated with UTL-5g at 15, 30, and 60 mg/kg and amifostine (200 mg/kg) by *i.p.* injection 30 min prior to lung irradiation of 6 Gy (daily x 5). Two mice in the amifostine group died within 8 wks. At the first time point (8 wks), the plasma levels of TGF-β elevated by irradiation were significantly reduced by UTL-5g at 60 mg/kg ($p<0.05$). For the second time point (5 months), elevated levels of TNF-α in lung tissue from the irradiated group were suppressed by UTL-5g in a dose-dependent manner and was statistically significant at 60 mg/kg ($p<0.05$). Amifostine also showed a similar effect but at a much higher dose, 200 mg/kg ($p<0.05$). Incidentally, it was observed that UTL-5g delayed the radiation-induced hair-discoloration significantly (>60 days after lung irradiation). The results indicate that UTL-5g may protect melanocytes against radiation-induced injury. Next, the effects of UTL-5g on tumor response were investigated in CD-1 nu/nu athymic nude mice bearing intramuscular A549 tumors (human non-small cell lung carcinoma) implanted in the right gastrocnemius muscle. Animals were treated with UTL-5g (30 mg/kg *i.p.*) 30 min prior to or after irradiation (5 Gy) daily for five days. The results showed that UTL-5g did not protect tumor from radiation damage. These observations suggest that UTL-5g may be lung radioprotective and thus, warranted further investigation.

Keywords: animal model, radiation, lung cancer, preclinical studies, radioprotector.

Introduction

Lung cancer is the leading cause of cancer-related mortality in the United States. In the U.S. alone, 228,190 new cases and 159,480 deaths were estimated (small cell and non-small cell lung cancer combined) in 2013 [1]. Depending on the extent of the disease, patients

with non-small cell lung cancer (NSCLC) have been treated by surgery, chemotherapy, and/or radiotherapy. Small cell lung cancer (SCLC) accounts for approximately 15% of bronchogenic carcinomas; without treatment, SCLC is the most aggressive type of pulmonary tumor, with median survival from diagnosis of only 2 to 4 months. Compared with other cell types of lung cancer, SCLC is more responsive to chemotherapy and radiotherapy; however, a cure is difficult to achieve because SCLC has a greater tendency to be widely disseminated by the time of diagnosis [2]. Overall, radiotherapy plays a critical role in the treatment of lung cancer, whether it is NSCLC or SCLC, irrespective of the stage. Unfortunately, radiotherapy can incur significant lung injury and other side effects [3, 4].

The expression of TGF-β, a fibrogenic cytokine, is the hallmark of pathological manifestation of radiation-induced lung injury. TGF-β plays a major role in pulmonary fibrosis, characterized by chronic scar formation and deposition of extracellular matrix, resulting in impaired lung function [5]. Another key mediator associated with radiation-induced lung injury is TNF-α, a major proinflammatory cytokine. TNF-α exerts various inflammatory effects on lung tissue and promotes fibrogenic effects by up-regulating TGF-β expression [6]. In addition, TNF-α has been implicated in the pathogenesis of radiation-induced pneumonitis.

Radiation-induced lung damage poses two major problems: For the patients, it represents a loss of lung function with shortness of breath and irritation leading to chronic coughing; for the clinicians, it represents a limitation of dose that can be delivered to the tumor and associated tumor-infiltrated lung tissue. This limitation of therapy has a consequence in terms of local cure rate for many tumors for which the lung is in the radiation field. Therefore, it is important to develop agents that protect (radioprotectors) or mitigate (radiomitigators) radiation-induced lung damage to improve survival, quality of life, and palliative care in lung cancer patients. For clinical radiotherapy applications, it is imperative to demonstrate that a radioprotector (a) protects normal tissue under radiotherapy and (b) does not protect cancer cells to radiotherapy. Although there are a number of potential radioprotectors under investigation, currently, there is not a lung radioprotector approved by the FDA. This is a serious problem that should be addressed and answered.

Despite the intensive research by many researchers and a number of radioprotectors discovered or under development, there is only one approved radioprotector in the US, amifostine (sold as Ethyol®), indicated to reduce xerostomia associated with radiotherapy of head-and-neck cancer. Other examples of radioprotectors incude soy isoflavones which protected the lungs against adverse effects of radiation including inflammation, pneumonitis and fibrosis [7]. Another example is wholegrain Flaxseed which was protective against radiation pneumonopathy *in vivo* [8].

We hereby present some preliminary studies of a potential lung radioprotector, UTL-5g (Fig. 1). UTL-5 series of compounds were based on a subtle modification on the molecular scaffold of a small-molecule anti-inflammatory/anti-arthritic drug, leflunomide (5-methylisoxazole-4-carboxamide) wherein the substitution on isoxazole ring was modified (Fig. 1). UTL-5 compounds were originally developed as potential anti-inflammatory agents. Although the modification on the molecular scaffold was subtle, the anti-inflammation effect (by a Carrageenan-induced edema rat model) was significantly improved and the acute toxicity (by LD_{50}) was significantly reduced as compared to leflunomide and other compounds based on the molecular scaffold of leflunomide [9]. In addition, UTL-5b, -5d, and -5g have shown reduced liver toxicity induced by liver-irradiation by lowering the elevated levels of AST/ALT in serum and levels of TNF-α levels in liver; TUNEL assay also showed the liver radioprotection by UTL-5g. Amongst them, UTL-5g showed the best liver radioprotection [10] and was selected as the lead compound for lung cancer radioprotection. Furthermore, UTL-5g also showed some chemoprotective effect against cisplatin-induced side effects by lowering the elevated levels of TNF-α, creatinine, and blood urea nitrogen (BUN) [11].

In this work, we investigated the effect of UTL-5g on the elevated levels of TNF-α and TGF-β in mice due to lung irradiation. In addition, we investigated the effect of UTL-5g on tumor response to irradiation in a CD-1 nu/nu athymic mouse model bearing intramuscular human A549 tumors (human non-small cell lung carcinoma).

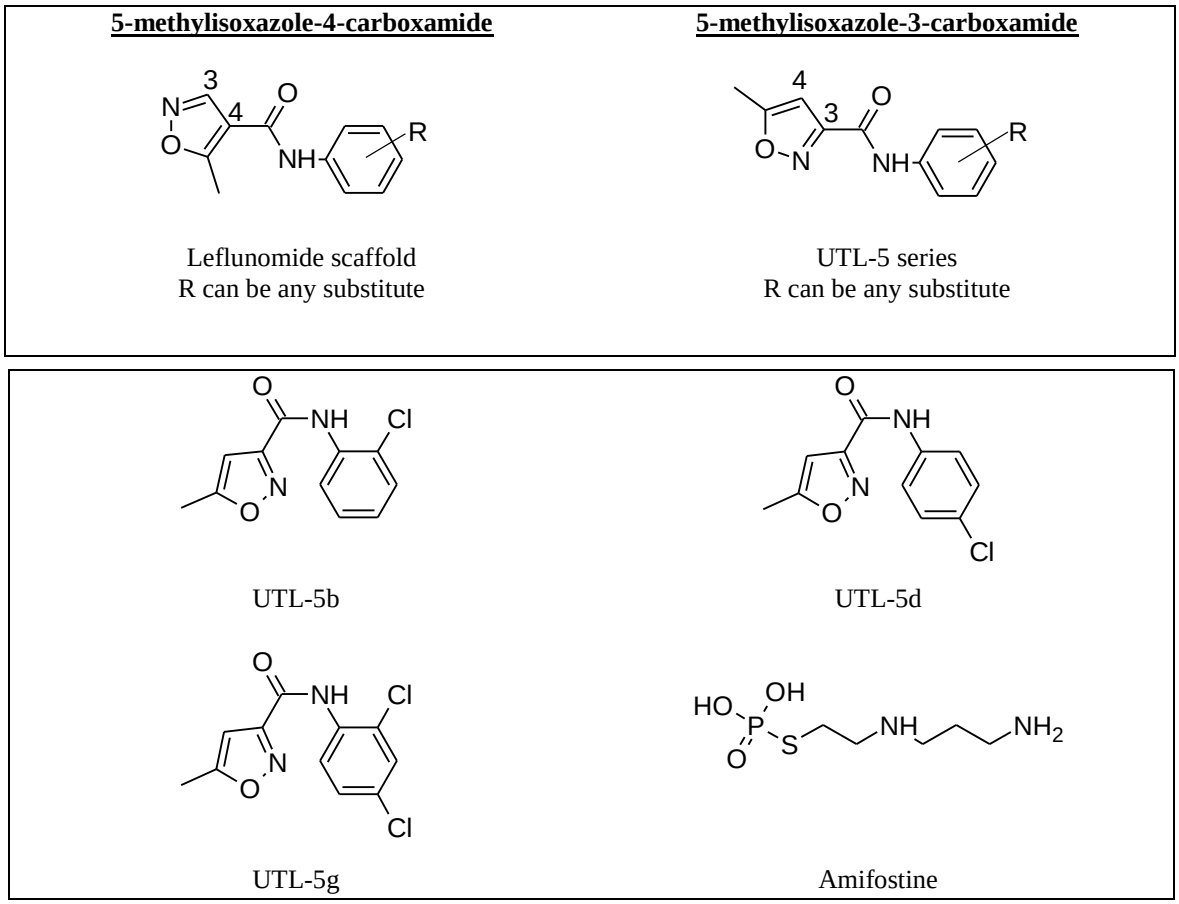

Figure 1 Structures of UTL-5 series of compounds, leflunomide, and amifostine.

Materials and methods

Chemicals, reagents and media

UTL-5g (Lot #1182-MEM-3D, Purity >99%) was manufactured by Kalexsyn (Kalamazoo, MI). Alpha-MEM was purchased from MediaTech (Manassas, VA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri). A549 cells (non-small cell lung carcinoma) were purchased from ATCC. Radiation was provided by a research-dedicated 5,000 Ci Cs-137 Shepherd Mark I irradiator and Faxitron orthovoltage X-ray unit.

Animals

All animal studies were in full compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines. C57BL/6, male mice (8-10 weeks) and CD-1 nu/nu athymic nude mice (male) were purchased from Charles River (Wilmington, MA).

Effects of UTL-5g pretreatment on Lung Injury Induced by Irradiation In vivo

Based on our previous results in liver radioprotection [12], 30 mg/kg of UTL-5g was

used as a benchmark dose and three doses (15, 30, and 60 mg/kg) were selected to study the pharmacological effect of UTL-5g *in vivo*. Vehicle control was made by mixing 1 mL of DMSO, 15 mL of saline, and 100 μL of Tween 20. UTL-5g test solution at 4.8 mg/mL was made by adding 15 mL saline into a mixture of 1 mL of UTL-5g stock (77 mg/mL in DMSO) and 100 μL of Tween 20. The same preparation was used to make UTL-5g at 2.4 and 1.2 mg/mL. For amifostine, 200 mg of amifostine was added in 12.5 mL saline to make a 16 mg/mL solution. All injections were made by *i.p.* and each injection volume is 0.25 mL.

A total of 56 mice (C57BL/6, male) were randomly divided into 5 groups (10 mice/group) with additional 6 mice in non-irradiated vehicle control group. Individual mice in each group received *i.p.* injection with vehicle, UTL-5g (15, 30, 60 mg/kg), or amifostine (200 mg/kg) at 30 min prior to each fractionated dose of lung irradiation (6 Gy/day for 5 days). Mice were anesthetized with dose collimation to the lung to spare other major organs. Half of the mice from each group were euthanized 8 wk and the other half were euthanized 5 months after irradiation. The 8 wk point was planned because it was reported that 8 weeks may be a sufficient time to show the lung damage using C57BL/6 mice [13]. Blood samples were drawn by cardiac puncture followed by plasma separation; lung tissues were removed and stored at -70 °C. TNF-α and TGF-β levels in homogenized lung tissue or plasma were measured by commercially available ELISA detection kits (eBioscience).

Effect of UTL-5g on tumor response to radiation in vivo

Because this is a tumor model, CD-1 nu/nu mice were used instead of C57BL/6 mice, which were used in the lung injury study. In this study, male CD-1 nu/nu athymic nude mice, bearing A549 intramuscular tumors (human non-small cell lung carcinoma) implanted in the right gastrocnemius, were used. The A549 cells were inoculated as described previously [14]. Briefly, $2x10^6$ cells suspended in 0.1 mL of normal saline were injected intramuscularly into the right gastrocnemius muscle. When average tumor volumes reached 0.35 cm^3, mice were randomly assigned to four groups: (a) vehicle alone, (b) irradiation plus vehicle, (c) UTL-5g at 30 mg/kg 30 min prior to irradiation, and (d) UTL-5g at 30 mg/kg 30 min after irradiation. A total radiation exposure of 25 Gy under the fractionated schedule of 5 Gy per day for 5 consecutive days was used; radiation exposures were limited to the tumored leg using a custom plastic jig. On each day of irradiation, vehicle and UTL-5g (2.4 mg/mL) was injected *i.p.* (0.25 mL) 30 min prior to and after irradiation. An additional group of mice were treated with amifostine (200 mg/kg) prior to irradiation as positive control.

Results and discussion

At the first time point (8 wks), 5 mice per group were euthanized. Blood sample were collected by cardiac puncture (for cytokine analysis), each lung was washed with saline, removed, and then stored in -20 ºC until needed. After 5 months (the second time point), the same procedure was performed on the mice. Two mice died in the amifostine group (group F), presumably due to the complication from anesthesia and/or the toxicity of amifostine; thus four mice were euthanized at each time point. No mice died in any other groups, and 5 mice were euthanized in each (groups A - E) at the second time point.

Inflammatory cytokine analysis

Eight weeks after irradiation, blood levels of TGF-β were elevated significantly by irradiation (Table 1, group B). For those animals pretreated with UTL-5g (15, 30, or 60 mg/kg by *i.p.* injection) or amifostine (200 mg/kg by *i.p.* injection), blood levels of TGF-β were lowered. The lowering is statistically significant for UTL-5g at the dose of 60 mg/kg (from 3.67 in group B lowered to 2.50 ng/mL in group E). Although 30 mg/kg was selected a benchmark dose from previous studies, the results in Table 1 indicate that it may not be the optimal dose for lowering TGF-β levels. Also, the high standard deviations may have obscured the dose dependency and animal number should be increased in the future studies for levels of TGF-β. Since TGF-β is a

viable target to attenuate radiation-induced lung toxicity [5, 15], these results indicate that UTL-5g may be potentially lung radioprotective. Amifostine provided similar reduction on TGF-β but at a much higher dose, 200 mg/kg.

Table 1. Effects of UTL-5g pretreatment on blood levels of TGF-β

Group	Treatment	TGF-β (ng/mL) ± S.D.
A	Non-irradiated control	$1.94^{a} \pm 0.42^{*, \#}$
B	6 Gy, daily x 5	$3.67^{b} \pm 0.78^{*}$
C	UTL-5g (15 mg/kg by *i.p.* injection) before irradiation, daily x 5	$2.42^{c} \pm 0.58$
D	UTL-5g (30 mg/kg by *i.p.* injection) before irradiation, daily x 5	$3.22^{c} \pm 0.95$
E	UTL-5g (60 mg/kg by *i.p.* injection) before irradiation, daily x 5	$2.50^{b} \pm 0.32^{\#}$
F	Amifostine (200 mg/kg by *i.p.* injection) before irradiation, daily x 5	$2.71^{c} \pm 0.59$

[a] Average of 3 mice; [b] Average of 5 mice; [c] Average of 4 mice
The p value is <0.05 for Groups A and B (*) and <0.05 for groups B and E ([#]) by Student's t-test.

For the plasma samples taken at 5 months post-irradiation, supernatants from homogenized left lung tissue samples, exhibited elevated TNF-α levels as compared to non-irradiated group (Fig. 2). The increase of TNF-α (group B vs group A) was statistically significant, $p<0.05$. TNF-α levels were lowered by UTL-5g in a dose dependent manner (15, 30, and 60 mg/kg) and lowering of TNF-α levels by UTL-5g at 60 mg/kg (Group D), is statistically significant vs. Group B, $p<0.05$. UTL-5g at 60 mg/kg (Group D) and amifostine at 200 mg/kg (Group E) showed similar effects in lowering TNF-α. Since UTL-5g (MW 271) at 60 mg/kg is equivalent to 221 μM/kg while amifostine (MW 214) at 200 mg/kg is equivalent to 934 μM/kg, it can be concluded that UTL-5g is more potent than amifostine with respect to lowering TNF-α levels *in vivo*. In addition, in the amifostine group (Group F), two out of 5 animals died, but no animal died in all groups treated by UTL-5g. Therefore, UTL-5g appears to have a lower acute toxicity than amifostine under current experimental condition.

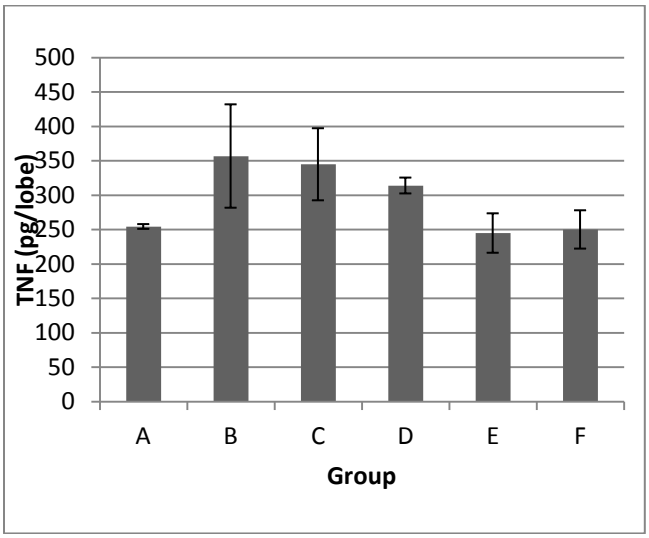

Figure 2 Effect of UTL-5g pretreatment on TNF-α level per lobe of lung (5 months after irradiation). (A) Vehicle injection only without irradiation (medium control); (B) Vehicle 30 min prior to irradiation; (C) UTL-5g at 15 mg/kg 30 min prior to irradiation; (D) UTL-5g at 30 mg/kg 30 min prior to irradiation; (E) UTL-5g at 60 mg/kg 30 min prior to irradiation; (F) Amifostine at 200 mg/kg 30 min prior to irradiation. The vehicle control group consisted of 3 mice; each treatment groups consisted of 5 mice except in Group F wherein one died in early stage and only 4 mice left. Levels of TNF-α in Group D (UTL-5g at 60 mg/kg) is statistically significant vs. Group B ($p<0.05$); so is Group F vs. Group B ($p<0.05$).

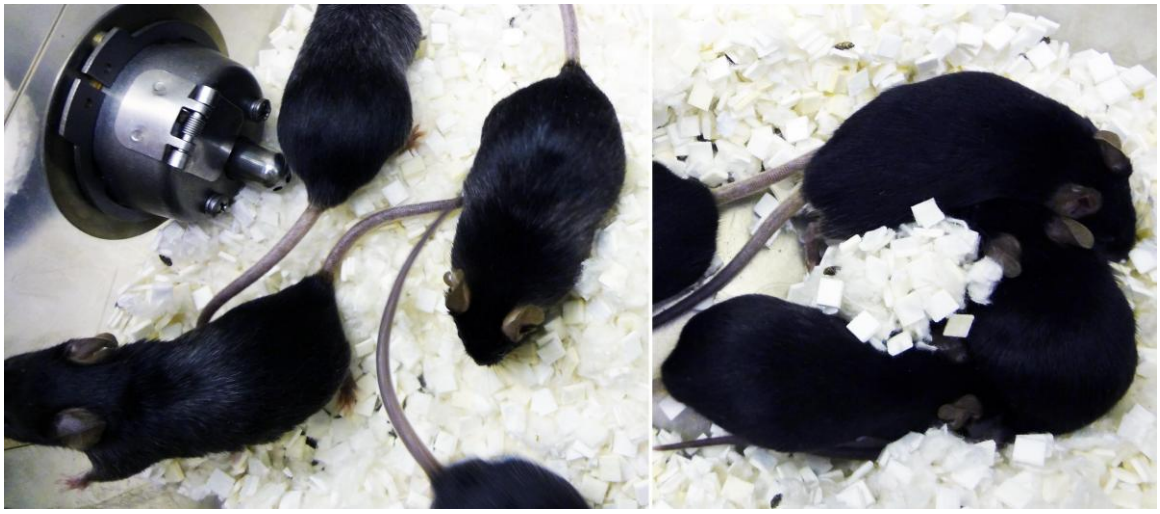

Figure 3 Photographs of animals with discolored hair around the chest area where it was irradiated (left panel) and animals with hair color essentially unchanged or just beginning to change (right panel)

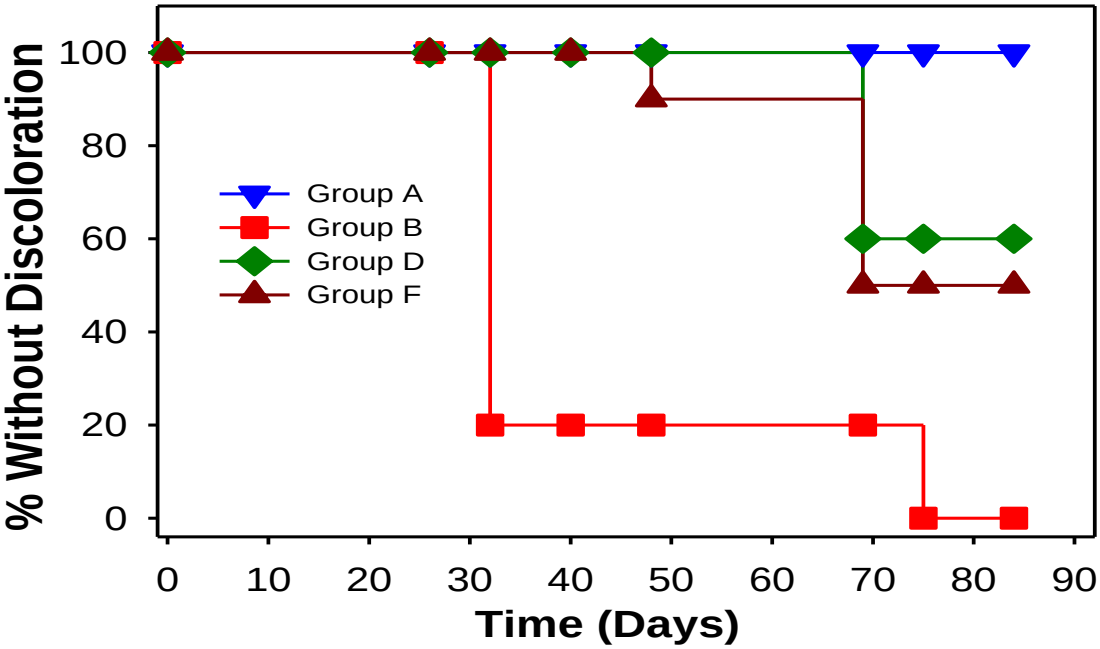

Figure 4 Effect of UTL-5g on the discoloration of hair around the chest area. Group A: Non-irradiated mice; Group B: Irradiation alone: 30 Gy total given as 6 Gy x 5 once daily for 5 days; Group D: Irradiation with UTL-5g pretreatment at a dose of 30 mg/kg; Group F: Irradiation with amifostine pretreatment at 200 mg/kg. Note 1: Amifostine appeared toxic; 20% of the mice died from the combination of drug and anesthesia. Note 2: At days 57-61, mouse numbers in all groups were reduced by half. Note 3: All injections were *i.p.*

Based on the observed up-regulations of TGF-β and TNF-α, which are known to promote fibrogenic effects, it was expected to see significant fibrosis. Unfortunately, observed fibrogenic effect by irradiation was not significant. This may be due to factors including (a) the radiation dose was not optimized, and/or (b) the time period may not be long enough. We admit that the protocol design was not perfect and

suitable modifications will be needed in further investigation.

Hair discoloration

During the study, local hair discoloration was detected 30 days after irradiation. Significant protection of local hair discoloration by UTL-5g from irradiation was observed (Fig. 3) indicating that UTL-5g may be protecting melanocytes from radiation injury; this may be part of overall skin radioprotection. As shown in Fig. 4, pretreatment of UTL-5g, protected irradiated hair from discoloration. The optimal dose appears to be around 30 mg/kg by *i.p.* injection, which protected hair color for >60 days after lung irradiation (30 days longer than group B, which was subject to irradiation alone). Amifostine showed similar protection against discoloration induced by irradiation, but not to the same extent as UTL-5g. First, 2 out of 10 mice receiving amifostine died in the first week. Furthermore, a much higher dose of amifostine (200 mg/kg or 934 μM/kg) as compared to UTL-5g (30 mg/kg or 111 μM/kg) was used in this study. Therefore, UTL-5g is significantly more efficacious than amifostine as radioprotector for melanocytes

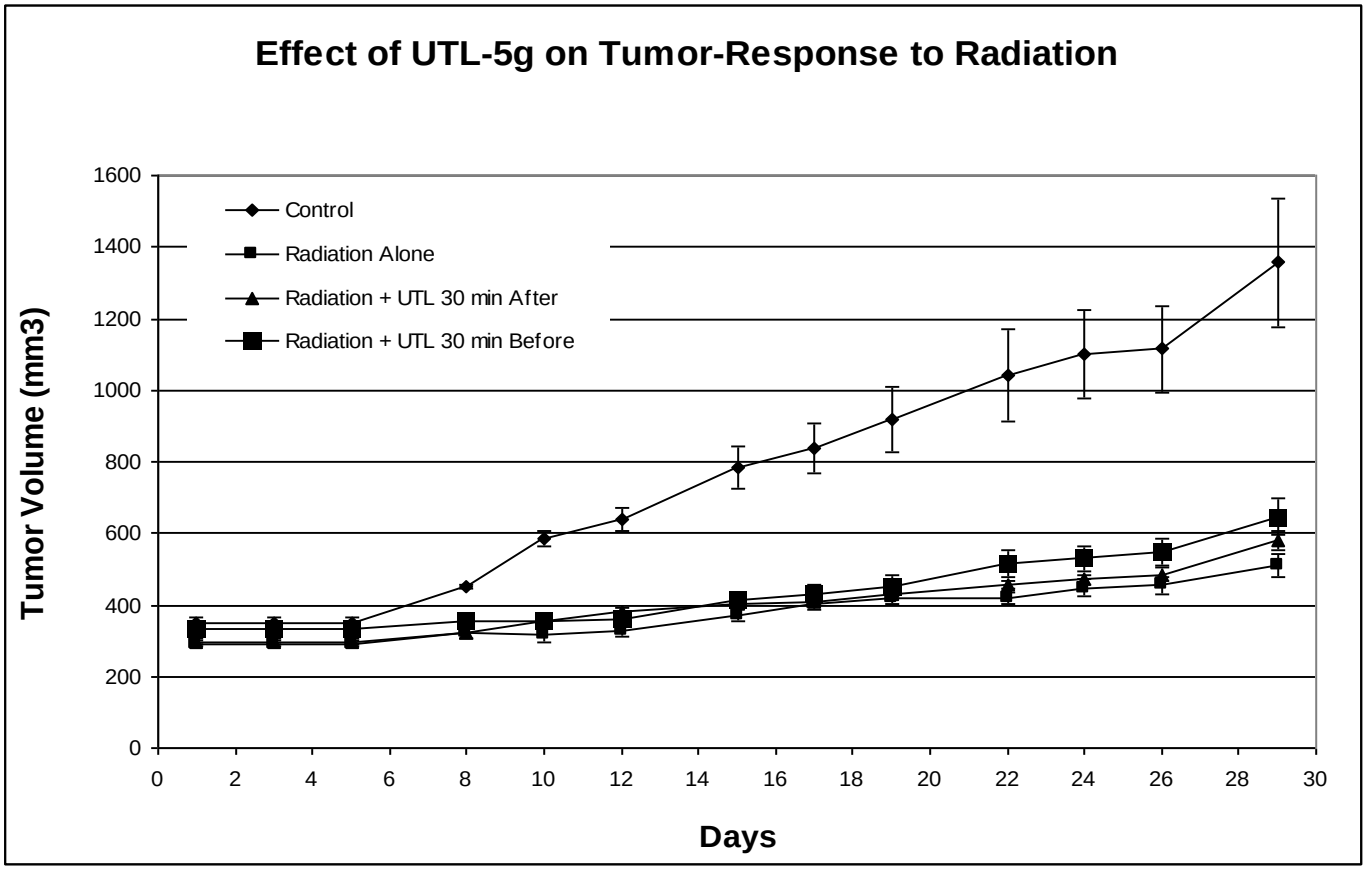

Figure 5 Tumor growth delay assay showing the tumor volume as a function of time for 4 groups (5 mice per treatment group, 4 mice in vehicle control group): vehicle alone (Control), irradiation plus vehicle, irradiation plus UTL-5g at 30 mg/kg at 30 min after irradiation, and irradiation plus UTL-5g at 30 mg/kg at 30 min prior to irradiation. Irradiation at 5 Gy per day for 5 days was given starting on day 0 when tumors were approximately 0.35 cubic cm in volume. Agents (drug or vehicle) were administered 30 minutes prior to (or after) the radiation exposure. Tumor volumes shown are average of 4 for groups and average of 5 for groups c and d; standard error bars are shown.

Tumor-response to radiation

A critical criterion of a radioprotector is that it can protect normal tissue but does not protect tumor cells under radiotherapy. We therefore investigated whether UTL-5g affected tumor response to radiotherapy using the athymic CD-1 mouse model transplanted with human A549 tumor cells. As shown in Fig. 5, animals

treated with UTL-5g 30 min prior to irradiation showed essentially the same tumor growth delay and there was no significant effect on tumor-response to radiation. In addition, treatment of UTL-5g 30 min after irradiation also showed no effect on tumor response to irradiation. The effect of UTL-5g (whether it was 30 min prior to or after radiation) on radiation response was not statistically different from that of irradiation plus vehicle (radiation alone control). Likewise, amifostine treatment did not affect tumor response to irradiation (data not shown).

Our results from this work showed that the small-molecule TNF-α inhibitor, UTL-5g, may be a potential lung radioprotector. Our finding is corroborated by a recent study by Huang et al. who showed that antisense oligonucleotides for TNF-α receptor could protect radiation-induced liver injury [16]. Because there were only 5 mice used per group, these results are considered preliminary and further investigations are needed in order to conclude that UTL-5g is a lung radioprotector.

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Disclosure Statement

No competing financial interests exist.

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