

ORIGINAL ARTICLE

The Fatty Acid Amide Hydrolase Inhibitor URB937 Ameliorates Radiation-Induced Lung Injury in a Mouse Model

Rui Li,¹ Guo Chen,² Lin Zhou,¹ He Xu,¹ Fei Tang,¹ Jie Lan,¹ Ruizhan Tong,¹ Lei Deng,¹ Jianxin Xue,^{1,3} and You Lu^{1,3}

Abstract—Radiation-induced lung injury (RILI) is a potentially life-threatening complication of radiotherapy. In the current study, we examined the potential protective effects of URB937, an inhibitor of fatty acid amide hydrolase using a mouse model of RILI. Briefly, male C57BL/6 mice received 16Gy irradiation to the thoracic region and then intraperitoneal injection of either URB937 (1 mg/kg) or vehicle every 2 days for 30 days. The extent of the lung injury was evaluated histologically at the end of the drug treatment as well as 3 months after the cessation of the treatment. The data showed URB937 attenuated radiation-induced lung injury and increased endocannabinoid concentration in lung tissue. Treatment with URB937 decreased leukocyte migration and inflammatory cytokines in bronchoalveolar lavage fluid and plasma at day 30. Histopathological examination revealed URB937 could restore lung structure and restrain inflammatory cell and fibroblast accumulation caused by irradiation in lung tissue. URB937 also decreased radiation-induced pro-inflammatory (e.g., interleukin-1 β , interleukin-6, tumor necrosis factor- α) and pro-fibrotic cytokines (e.g., transforming growth factor- β 1) level in lung tissue, as well as lipid peroxidation in the lungs. Mouse survival examined in a separate group of experimental subjects indicated that URB937 could prolong animal survival. Experiments using a mouse bearing Lewis lung carcinoma cells showed that URB937 does not affect irradiation-induced inhibition of tumor growth. These results suggest that inhibiting fatty acid amide hydrolase could ameliorate RILI without compromising the efficacy of irradiation on tumor control.

KEY WORDS: radiation-induced lung injury; endocannabinoids; RILI; radiation pneumonia; pulmonary fibrosis.

Rui Li and Guo Chen contributed equally to this work.

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¹ Department of Thoracic Oncology, Cancer Center and State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China

² Laboratory of Anesthesiology & Critical Care Medicine, Translational Neuroscience Center, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China

³ To whom correspondence should be addressed at Department of Thoracic Oncology, Cancer Center and State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China. E-mails: xuejx@scu.edu.cn; radyoulu@hotmail.com

Abbreviations: RILI, Radiation-induced lung injury; MDA, Malondialdehyde; ROS, Reactive oxygen species; TGF- β , Transforming growth factor; TNF- α , Tumor necrosis factor- α ; IL-1 β , Interleukin-1 β ; IL-6, Interleukin-6; AEA, Anandamide; FAAH, Fatty acid amide hydrolase; H&E, Hematoxylin and eosin; BLAF, Bronchoalveolar lavage fluid; LLCs, Lewis lung carcinoma cells

INTRODUCTION

Irradiation is a major treatment modality for thoracic cancer but could damage healthy tissue through complex mechanisms that include reactive oxygen species (ROS), excessive inflammation, and fibrosis. Radiation-induced lung injury (RILI) is a major concern in the use of thoracic radiation [1, 2].

Acute RILI, typically manifesting as pneumonitis at 1–6 months after thoracic radiation [3], occurs in 9.4~28% of the patients receiving stereotactic thoracic irradiation [4]. Pulmonary fibrosis is the key feature of chronic RILI and evolves during a longer period of time [5, 6]. Exposure of lung tissue to irradiation leads to lung structure destruction, alveolar edema, fibroblast proliferation, and collagen deposition [7]. Ionizing radiation continuously induces inflammatory cascade and the resulting free radicals further promote activation of

fibroblasts in proliferation [8]. Previous studies have demonstrated that RILI could be alleviated by blocking ROS, pro-inflammatory cytokines, *e.g.*, tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6), as well as the pro-fibrotic cytokine transforming growth factor β 1 (TGF- β 1) [9, 10]. New strategies are needed to attenuate/prevent inflammatory and fibrosis without affecting the efficacy of radiotherapy.

Endocannabinoids (*e.g.*, anandamide [AEA] and 2-arachidonoyl glycerol [2-AG]) are endogenous lipid-signaling molecules in many cells in a variety of tissues. Endocannabinoids have a broad range of biological properties that resemble the action of Δ -9-tetrahydrocannabinol [11]. Endocannabinoids have been shown to protect the heart from ischemia-reperfusion injury [12] and reduce the level of biomarkers for inflammation and fibrosis in pancreatic stellate cells [13]. Accumulating evidence suggests that increasing endocannabinoids, by inhibiting fatty acid amide hydrolase (FAAH), could inhibit tumor angiogenesis and decrease cancer cell migration [14–16].

We hypothesized that URB937 could attenuate RILI by suppressing pulmonary inflammation and lung fibrosis cascades, without affecting the efficacy of irradiation on tumor control. In the current study, we used a mouse model to examine whether URB937, a potent and selective peripherally-acting FAAH inhibitor [17], could attenuate RILI. Potential effects on tumor growth were also examined.

MATERIAL AND METHODS

Experimental Procedures

Adult male specific-pathogen-free C57BL/6J mice (8 weeks of age; from the Center of Experimental Animals, the Academy of Military Medical Sciences, Beijing, China) were maintained under a 12 h light/dark cycle with unlimited access to food and water. All animal experiments were performed in accordance with the guidelines set by the Care and Use of Laboratory Animals published by the US National Institutes of Health, and approved by the Animal Care and Use Committee of Sichuan University.

Mice received a single dose of X-ray irradiation (16Gy; 297.43 cGy/min) to the whole thorax under intraperitoneal (ip.) pentobarbital anesthesia (40 mg/kg). The head, abdomen, and extremities were shielded with lead strips. Starting from 2 h after the irradiation, mouse

received ip. injection of URB937 (1 mg/kg in 1:4 DMSO/PEG300; synthesized and characterized in our lab; chemical structure shown in Fig.1d, synthetic route shown in supplementary Fig.1) or vehicle, once every 2 days for 30 days. A group of mice receiving anesthesia but no irradiation was included as an additional control. At the end of the treatment, mice were sacrificed for histological examination. A separate group of mice was sacrificed 3 months after the cessation of the drug treatment to evaluate the extent of fibrosis. Mouse survival was monitored in a separate group of mice. In the survival experiments, mice were euthanized when losing 20% bodyweight compared to the baseline.

Malondialdehyde

Plasma malondialdehyde (MDA) concentration was determined using a commercial kit (Nanjing Jiancheng Bio-engineering Institute) based on thiobarbituric acid reactivity.

RNA Isolation and Real-Time PCR

Total RNA was extracted from the lungs, and reverse transcribed to cDNA using MMLV reverse transcriptase (Invitrogen). Primer sequence is listed in supplementary Fig. 2. Real-time quantitative PCR was carried out using the SYBR Premix Ex TaqTM II real-time PCR kit (Takara). Results were normalized against the internal GAPDH control. All experiments were performed in triplicate.

Histological Evaluation

Removed lungs were perfused routinely and immersed in 4% paraformaldehyde for 24 h, dehydrated through grade series of ethanol, and embedded in paraffin. Sections (4 μ m) were stained with hematoxylin and eosin (H&E) or Masson's triple stain and examined under a light microscope (Olympus CX41RF) [18, 19]. The extent of fibrosis was assessed by evaluating the thickness of alveolar and bronchiolar walls, as previously reported [20].

Bronchoalveolar Lavage Fluid Analysis

This set of experiments was conducted in a separate group of mice. Briefly, upon sacrifice of the mice, a small plastic tube was inserted into the trachea. The preparation was irrigated with 1-ml physiological saline for 3 times. The bronchoalveolar lavage fluid (BALF) was centrifuged at 400 g for 15 min. The pellet was suspended in 0.5 ml 1% glacial acetate to dissolve red blood cells and then

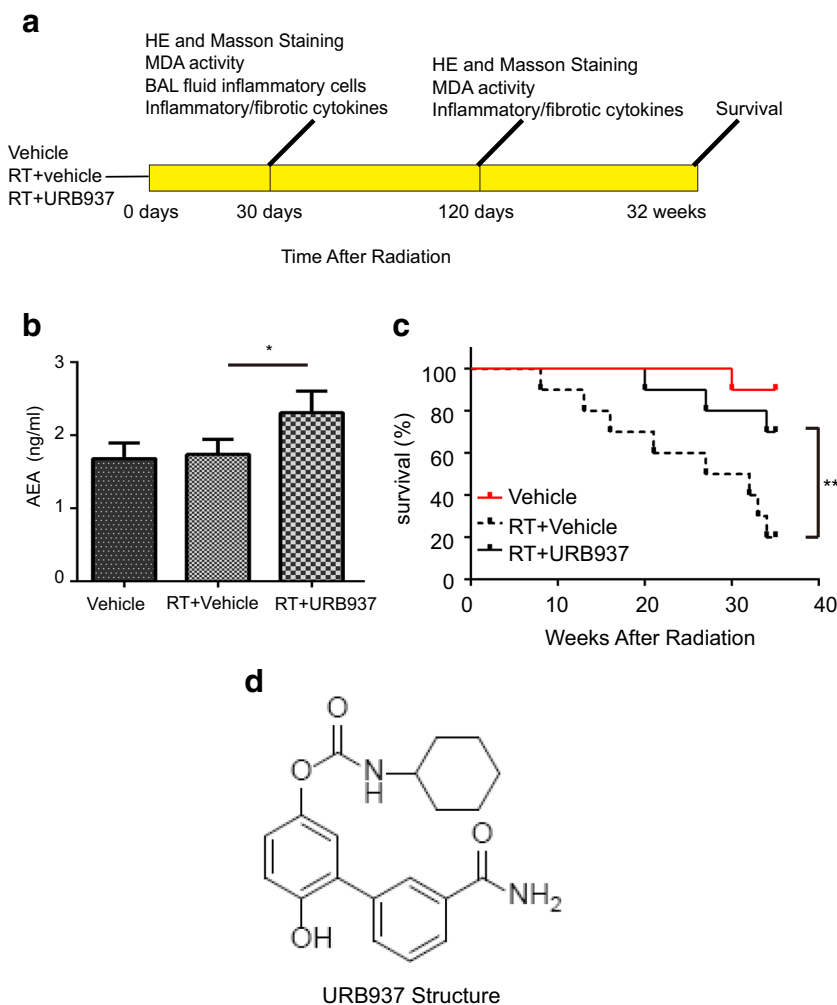


Fig. 1. The experimental design, survival curve, and endocannabinoids level. **a** A schematic diagram of the experimental design. **b** Endocannabinoids level. **c** Survival time. $n = 10$ for each group. **d** Chemical structure of URB937. $*p < 0.05$, $**p < 0.01$.

subjected to Wright-Giemsa staining. Inflammatory cells were counted by smear microscope.

Western Blot Analysis

Tissue samples were homogenized as previously described [3]. Samples containing equal amount of proteins were separated by 10% SDS-PAGE and transferred onto a PVDF membrane (Millipore). After blocking with 5% skim milk for 1 h, membranes were incubated with a rabbit TGF- β 1 antibody (1:500, Cell Signaling Technology) overnight at 4 °C. After extensive washing, membranes were incubated with HRP-conjugated anti-rabbit secondary antibody (1:5000, Santa Cruz) before visualization using an enhanced chemiluminescence kit (Millipore). β -

Actin (1:1000, Santa Cruz) was used as the internal control. Protein concentration was determined using a Bradford assay.

Plasma Cytokines

Plasma concentration of IL-1 β , IL-6, TNF- α , and TGF- β 1 was measured using Luminex® Screening (R&D Systems).

Liquid Chromatography Quantification for Endocannabinoids

Tissue samples were homogenized as previously described [21]. Homogenate (20 μ l) samples were

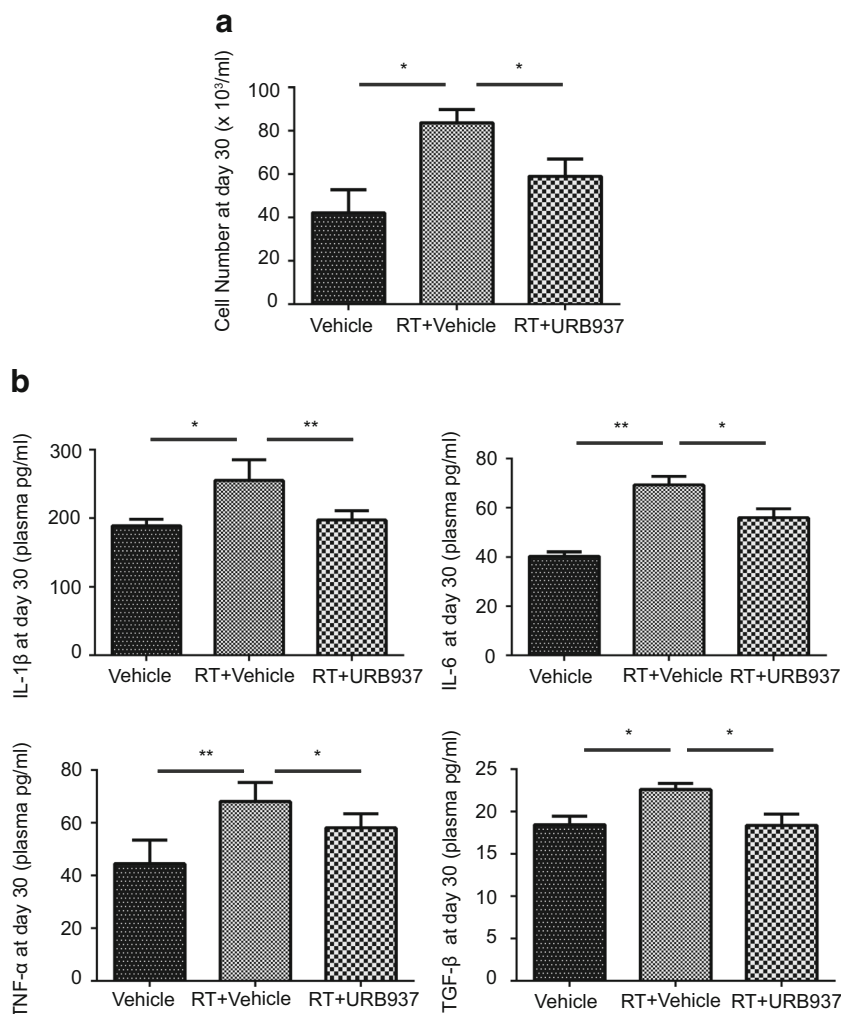


Fig. 2. Inflammatory cell infiltration in BALF and plasma cytokines in plasma at day 30. **a** The numbers of inflammatory cells. **b** Plasma cytokine level. * $p < 0.05$, ** $p < 0.01$.

added to a polypropylene plastic tube containing 2.0 mL chloroform and 1.0 mL methanol. After adding aliquots of 10 pmol d5-2-arachidonoylglycerol and 5 pmol d8-arachidonoyl-ethanolamide, the mixture was vortexed for 30 s, and centrifuged at 1400g for 10 min at 10 °C. The aqueous phase was dried using a stream of nitrogen gas. The extraction was repeated twice. The product was re-constituted in 0.1 ml 2:1 CHCl₃:CH₃OH and purified on silica columns. The eluate from the column with 9:1 CHCl₃:CH₃OH was dried for analysis of AEA with a liquid chromatography coupled with an atmospheric pressure-chemical ionization-single quadrupole mass spectrometer using

positive ion analysis mode. The amount of endocannabinoids was determined against deuterated internal standards.

Xenograft Assays

Lewis lung carcinoma cells (LLCs; from American Type Culture Collection) were cultured in RPMI-1640 medium (Hyclone) supplemented with 10% fetal bovine serum (Hoffmann-La Roche) and 1% penicillin/streptomycin at 37 °C in 5% CO₂. Mice were subcutaneously injected with 1×10^6 LLC cells into the right proximal hind leg. When the tumor volume reached

70 mm³, mice received irradiation (16Gy at 297.43 cGy/min; directed to the lesions), followed by URB937 (1 mg/kg, ip., once every 2 days) or vehicle until sacrifice (when tumor volume reached 2000 mm³). Tumour volume was calculated according to the following formula: volume (V) = length \times width \times width \times 0.5.

Statistical Analyses

Data are represented as mean \pm SEM, with at least three independent experiments. All variables were analyzed by one-way ANOVA followed by Dunnett's t test for pairwise comparisons. Statistical significance was defined at $p < 0.05$.

RESULTS

URB937 Prolonged the Survival of Irradiated Mice and Increased Pulmonary Endocannabinoid Concentration

Figure 1a is a schematic illustration of the *in vivo* experiments. URB937 increased AEA in mice receiving irradiation (Fig. 1b). URB937 increased the mouse survival: 70 vs. 30% in the vehicle control at 32 weeks (Fig. 1c).

URB937 Attenuated Inflammatory Infiltration to the Lungs and Decreased Plasma Concentration of Representative Pro-inflammatory Cytokines

URB937 attenuated irradiation-induced increase of inflammatory cells in the BALF (Fig. 2a) and plasma concentration of representative cytokines, including IL-1 β , IL-6, TNF- α , and active TGF- β 1 (Fig. 2b).

URB937 Attenuated Acute RILI (30 Days After Irradiation)

Irradiation increased the number of inflammatory cells outside the alveolar septa and alveolar thickness. Interstitial edema was also apparent. Such change was attenuated by URB937. Masson's triple stain did not show a difference in fibrosis of alveolar walls at day 30 (Fig. 3a, b).

URB937 decreased MDA content by 31% compared with radiation only group (Fig. 3c). Irradiation increased messenger RNAs (mRNAs) for pro-inflammatory and pro-fibrotic cytokines, including IL-1 β , IL-6, TNF- α , and TGF- β 1 in the lungs. URB937 significantly reduced lung

concentration of these cytokines (Fig. 3d). Western blot showed a decrease of TGF- β 1 in the lungs (Fig. 3e).

URB937 Alleviated Lung Fibrosis

Histopathologic examination at 120 days after radiation revealed significant fibrosis of the lungs, including dramatic interstitial hyperplasia, vacuolization of the blood vessels, and irregular thickening of the muscular layer. Masson's trichrome staining revealed collagen deposition in alveolar septa and bronchiolar tissue. URB937 attenuated these changes (Fig. 4a, b).

URB937 attenuated the elevation of MDA levels by 41% at day 120 (Fig. 4c). Irradiation-induced increases in mRNA level of pro-inflammatory cytokines and pro-fibrotic cytokines in lung tissues were also attenuated by URB937 (Fig. 4d). Western blot analysis demonstrated that irradiation-induced increase of TGF- β 1 in the lungs was decreased by URB937 (Fig. 4e).

URB937 Did Not Affect the Antitumor Effects of Irradiation

Irradiation resulted in a dramatic decrease in tumor growth. URB937 did not alter the effects of the irradiation (Fig. 5).

DISCUSSION

Radiotherapy is recommended by the current guidelines for patients with thoracic carcinoma. However, RILI not only affects the life quality of patients but also limit the doses of radiotherapy [22]. To improve tumor locoregional control and patient survival, new strategies that could prevent this severe side effect without interfering with anticancer treatments are needed.

Cannabinoids have been used clinically for a variety of disease conditions, for example to alleviate nausea and vomiting induced by chemotherapy. Cannabinoids have been shown to attenuate oxidative stress, inflammation, cell death, and fibrosis in both preclinical and clinical researches [23, 24]. Cannabis-based pharmacological agents, however, face legal and ethical concerns, but also generate physical and psychological dependence symptom. Targeting endocannabinoids clearly represents a strategy that could circumvent such barriers.

The present study showed that inhibiting fatty acid amide hydrolase with URB937 could attenuate lung injury

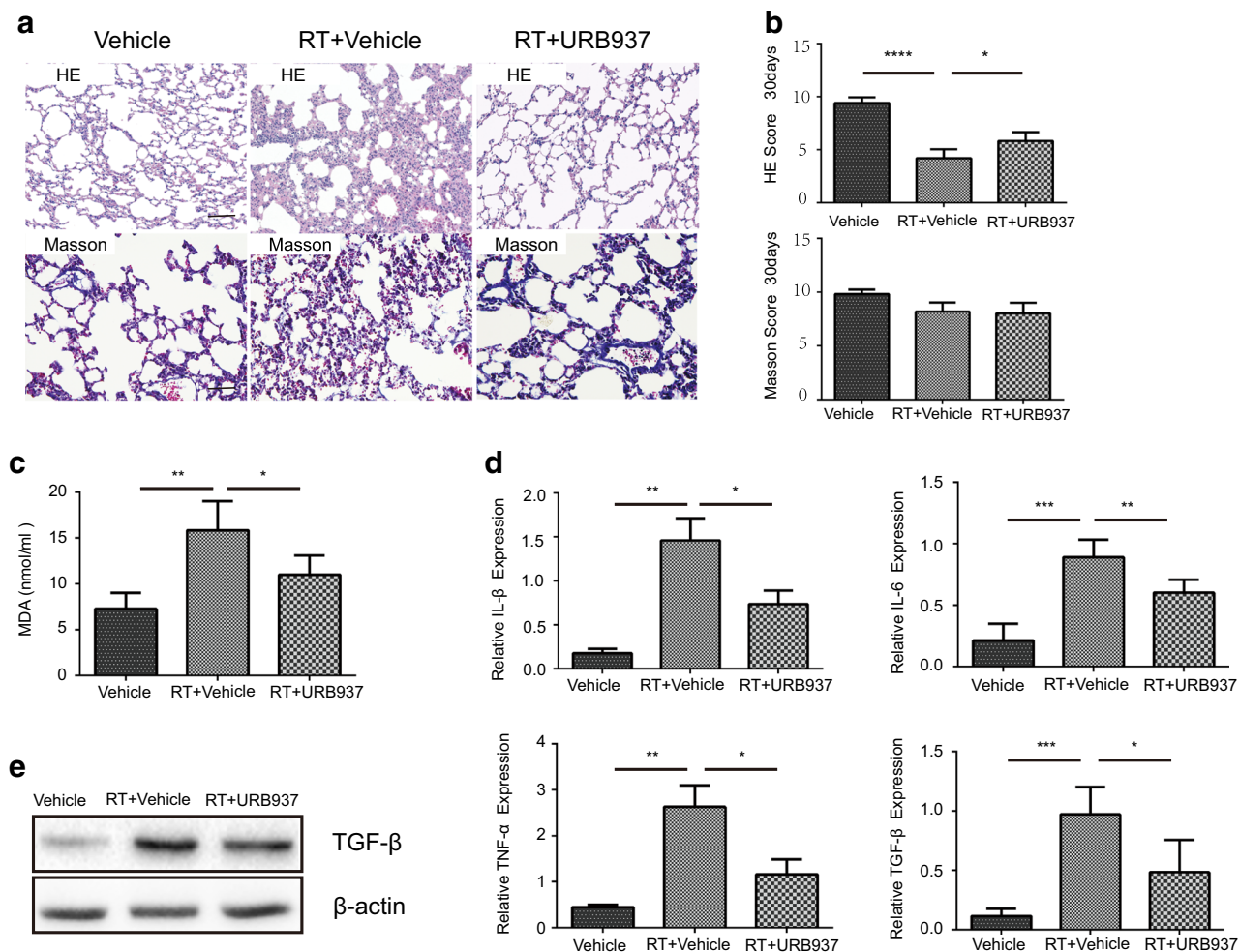


Fig. 3. Effects of URB937 at day 30. **a** Representative H&E (upper panel) and Masson staining (lower panel). Bar = 50 μm. **b** Summary histopathologic alterations. **c** Plasma MDA concentration. **d** Cytokine mRNA levels. **e** Western bolt analysis of TGF-β protein expression. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

in both acute and chronic stages of RILI. URB937 also significantly prolonged the survival time of mice with RILI. Mechanistic experiments showed that URB937 decreased inflammatory cell infiltration in BALF and cytokine expression in plasma at early RILI and decreased oxidative stress and pro-inflammatory/fibrotic cytokines in lung tissue at the entire process of radiation-induced lung injury. Importantly, URB937 did not alter tumor growth inhibition caused by irradiation.

The observed increase of endocannabinoids upon URB937 treatment in the current study supported the notion that FAAH is the pharmacological target of URB937. Radiation-induced lung injury is typified by lung structure pathological changes such as pulmonary interstitial edema

and excessive collagen deposition [7, 25]. Pathologic examination in the current study showed that the severity of lung structure damage induced by irradiation could be attenuated by URB937, supporting the beneficial action of URB937 in the radiation lung injury.

Tissues exposed to irradiation increase cytokine production at early stage, which in turn induces inflammatory cells in BLAF. In the current study, URB937 decreased inflammatory cells in BLAF and cytokines in plasma. Such a finding is consistent with the effects of URB937 in mouse staphylococcal enterotoxin B-induced lung injury model [26].

Irradiation increases lipid peroxidation which in turn produces a variety of damages to tissues and cells [27].

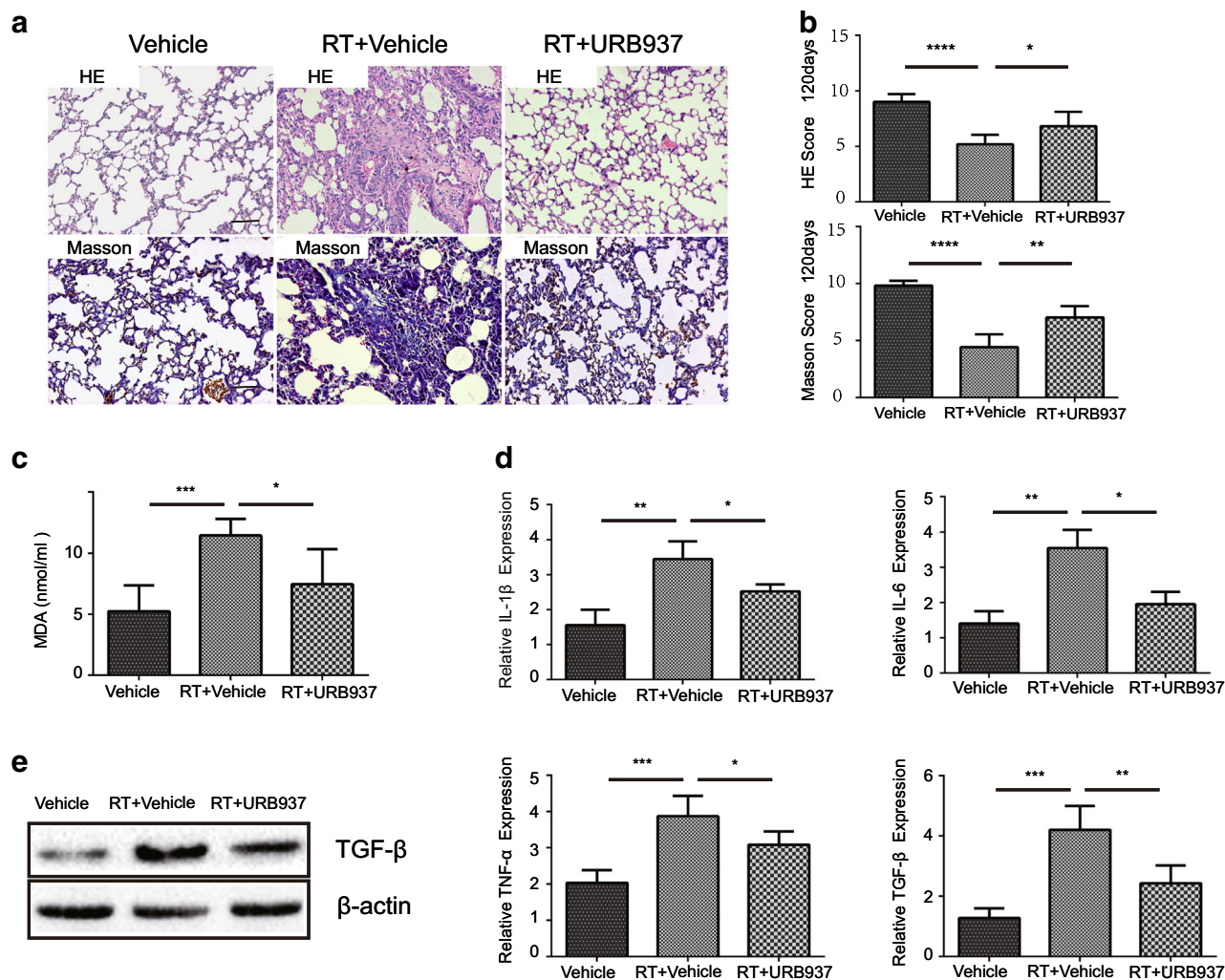


Fig. 4. Effects of URB937 at day 120. **a** Represented H&E staining (*upper panel*) and Masson staining (*lower panel*). Bar = 50 μ m. **b** Summary histological changes. **c** Plasma MDA concentration. **d** Cytokine mRNA levels. **e** Western blot of TGF- β . * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

Decreasing lipid peroxidation product (*e.g.*, MDA) could protect lung tissue from radiation injury [28]. A previous study showed that endocannabinoids could decrease the MDA level in the gastric mucosa and colitis [29]. Our study suggests that the protective effect of URB937 on RILI involves reduction of the plasma lipid peroxidation.

Cytokines are essential for both inflammation establishment and phagocyte activation. Previous studies have demonstrated the critical role of inflammatory cytokines in the radiation injury process [30]. The cannabinoid-2 (CB2) receptors are expressed in immune cells and have been shown to reduce IL-1 β and TNF- α RNA expression in lung tissue [31, 32], whereas AEA has been shown to

decrease IL-6 expression and protect against the development of intestinal radiation injury [33]. In the current study, we observed a reduction in pro-inflammatory cytokine (IL-1 β , IL-6, TNF- α) in irradiated mice receiving URB937; such a reduction might have contributed to attenuated radiation pneumonia and fibrosis.

Irradiation accelerates the production of pro-fibrotic cytokine TGF- β 1 in lung tissue, forming a perpetual cascade of inflammatory cytokines. TGF- β 1 overexpression after radiation injury promotes the formation and inhibits the breakdown of connective tissue [34, 35]. Recently, Gonzalez and colleagues showed that endocannabinoids could downregulate TGF- β 1 and restrain fibrogenesis by

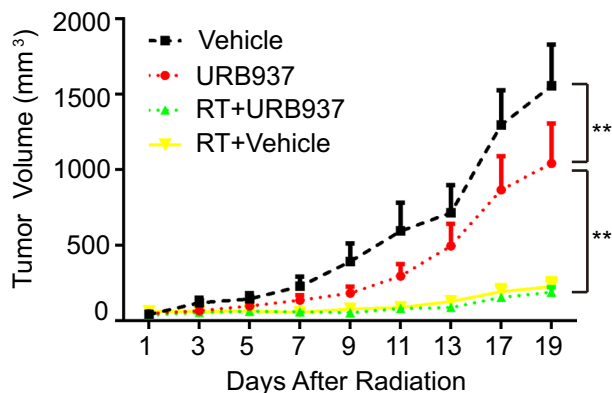


Fig. 5. Effects of URB937 on the antitumor action of irradiation. No difference in tumor growth was noted between radiation alone and radiotherapy combined with URB937 group. URB937 could control tumor growth compared to control group. * $p < 0.05$, ** $p < 0.01$.

stimulating PPAR- γ signaling [36]. Our study showed that increasing endocannabinoids by URB937 could suppress radiation-induced elevation of TGF- β 1 expression.

Ideal radioprotective agents should alleviate radiation pneumonia and fibrosis without compromising the antitumor effects of radiotherapy. The anti-inflammatory and analgesic therapeutic effects of FAAH inhibitors have been shown in some clinical test [37]. Inhibiting FAAH attenuate tumor proliferation, migration, and invasiveness and was used in preclinical cancer treatment [38, 39]. In current the study, we found URB937 did not alter tumor growth inhibition caused by irradiation, confirming the selectivity of the protective effects to healthy but not tumor cells.

The current study has several limitations. First, URB937 is a chemical agent with limited selectivity. We are now planning experiments using molecular approaches such as gene knockout. Also, despite a preventive role of endocannabinoids in early inflammation in most disease models [40, 41], endocannabinoids have been shown to produce opposite effects (either pro- or anti-fibrogenic action), possibly due to involvement of distinct receptor types [42, 43].

CONCLUSIONS

Increasing endogenous endocannabinoids by inhibiting FAAH with URB937 could alleviate RILI in a mouse model. These findings implicated endocannabinoids in controlling inflammation and fibrosis. The results also encourage future study of inhibiting FAAH as a target in the treatment of RILI.

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COMPLIANCE WITH ETHICAL STANDARDS

The use of C57BL/6J mice was approved by Animal Care and Use Committee of Sichuan University.

Conflict of Interest. The authors declare that they have no conflicts of interests.

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