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Low molecular weight catalytic metalloporphyrin antioxidant AEOL 10150 protects lungs from fractionated radiation

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Abstract

The objective of this study was to determine whether administration of a catalytic antioxidant, Mn(III) tetrakis(*N,N*′-diethylimidazolium-2-yl) porphyrin, AEOL10150, reduces the severity of long-term lung injury induced by fractionated radiation (RT). Fisher 344 rats were randomized into five groups: RT + AEOL10150 (2.5 mg/kg BID), AEOL10150 (2.5 mg/kg BID) alone, RT + AEOL10150 (5 mg/kg BID), AEOL10150 (5 mg/kg BID) alone and RT alone. Animals received five 8 Gy fractions of RT to the right hemithorax. AEOL10150 was administered 15 min before RT and 8 h later during the period of RT treatment (5 days), followed by subcutaneous injections for 30 days, twice daily. Lung histology at 26 weeks revealed a significant decrease in lung structural damage and collagen deposition in RT + AEOL10150 (5 mg/kg BID) group, in comparison to RT alone. Immunohistochemistry studies revealed a significant reduction in tissue hypoxia (HIF1α, CAIX), angiogenic response (VEGF, CD-31), inflammation (ED-1), oxidative stress (8-OHdG, 3-nitrotyrosine) and fibrosis pathway (TGFβ1, Smad3, p-Smad2/3), in animals receiving RT + AEOL10150 (5 mg/kg BID). Administration of AEOL10150 at 5 mg/kg BID during and after RT results in a significant protective effect from long-term RT-induced lung injury. Low dose (2.5 mg/kg BID) delivery of AEOL10150 has no beneficial radioprotective effects.

Keywords: *Radiation, pulmonary toxicity, hypoxia, oxidative stress, superoxide dismutase metalloporphyrin mimetic*

Introduction

Ionizing radiation (RT) is an important therapeutic modality in the treatment of thoracic tumours [1,2]. The tolerance of normal lung tissue continues to be an obstacle to the optimal use of radiation therapy in the treatment of cancer. Molecular and cellular changes that result in RT-induced lung damage commence during RT, but the clinical signs/symptoms and histological findings may not be revealed for months or even years after the treatment [1,2].

RT is associated with increased production of reactive oxygen/nitrogen species, which may damage lung cells either directly or indirectly via the action on parenchymal and infiltrating inflammatory cells [3–5]. These processes may overpower cellular antioxidant defenses and increase oxidative burden, which plays an important role in development of

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fibrosis [6–8]. In addition to an oxidant/antioxidant imbalance, RT results in induction and activation of important biological mediators, such as cytokines [4,5]. These cytokines perpetuate the inflammatory and fibrogenic processes associated with RT injury [4,5].

Recently, we have shown that, in irradiated lungs, hypoxia is associated with increased oxidative stress, inflammation and pro-fibrogenic/angiogenic cytokines activity [9,10]. Post-RT hypoxia, inflammation and oxidative stress could be modified by exogenous administration of superoxide dismutase mimetics (SOD mimetics) or transgenic over-expression of extracellular superoxide dismutase (ECSOD) [7,10,11]. Thus, AEOL 10150 (Mn(III) tetrakis (*N*,*N*´-diethylimidazolium-2-yl)porphyrin)), with SOD mimetic properties [12] reduces the severity of lung injury followed single dose RT of 28 Gy [10]. The objective of the current study was to determine whether administration of the same compound, AEOL10150, increases the tolerance of the lung to fractionated RT by reducing the severity of RT-induced pulmonary injury and also to determine the importance of drug dose on the extent of RT damage.

Materials and methods

Animals

Sixty female Fisher-344 rats weighing between 150–170 g were used in this study with prior approval from the Duke University Institutional Animal Care and Use Committee. Three animals were housed per cage and maintained under identical conditions with food and water provided *ad libitum*. Fisher 344 rats were randomized into five groups, receiving the following treatments: RT + AEOL10150 (2.5 mg/kg BID), AEOL10150 (2.5 mg/kg BID), RT + AEOL10150 (5 mg/kg BID), AEOL10150 (5 mg/kg BID) and RT alone. All rats were sacrificed at a pre-determined time of 26 weeks post-RT. At the time of sacrifice, the lungs were instilled with 10% neutral-buffered formalin, 2% glutaraldehyde and 0.085 m sodium cacodylate buffer prior to removal and fixed in 10% formalin.

Irradiation

RT consisted of daily fractions of 8 Gy for 5 consecutive days (40 Gy total dose) to the right hemithorax, using 150 kV x-rays (Therapax 320, Pantak Inc., East Haven, CT). A lead block was used to shield the left thorax and the rest of the body. All rats were anaesthetized before irradiation with an intraperitoneal (i.p.) injection of sodium pentobarbital (35 mg/kg).

AEOL 10150

The dosing schedule for low and high dose groups consisted of drug administration 15 min before RT, followed by a second injection 8 h later, each day during the 5 days of radiation treatment. Afterwards animals were injected subcutaneously, twice daily within an interval of 8 h, for 30 days. The drug was injected twice daily because of its short half-life [13]. AEOL10150 was supplied by Aeolus Pharmaceuticals (Laguna Niguel, CA, USA).

Histopathology and lung damage score

Five-micrometre thick sections of the right lung tissue embedded in paraffin were stained with haematoxylin and eosin and Masson's trichrome to visualize the extent of fibrosis and collagen deposition in the lung. Slides were systematically scanned under the microscope and eight-to-ten fields that contain the highest degree of fibrosis were selected. The extent of radiation induced fibrosis for each field was graded on a scale from 0 (normal lung) to 8 (total fibrous obliteration of the field), as previously described [14]. The average score was calculated for each animal and each group.

Immunohistochemistry

Immunohistochemistry was carried out as described previously [15]. Briefly, paraffin-embedded tissues were sectioned at 5 micron thickness and antigen retrieval was performed using citrate buffer from Biogenex (San Ramon, CA). Tissues were treated with primary antibodies to activated macrophage marker ED1 (1:100, Serotec, Oxford, UK), CA IX (1:400, gift from Dr Oosterwijk, Department of Urology, University Hospital Nijmegen, Netherlands), 8-OHdG (1:2000, JaICA, Shizuoka, Japan), hypoxia inducible factor 1α (HIF1α, 1:100, Novus-Biologicals Inc, Littleton, CO), Nitrotyrosine (1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA),VEGF (1:100, Santa Cruz Biotechnology Inc.), active TGF-β1 (1:200, Santa Cruz Biotechnology Inc.), Smad-3 (1:200, Santa Cruz Biotechnology Inc.) and p-Smad2/3 (1:200, Santa Cruz Biotechnology Inc.) overnight at 4°C. Slides were then washed three times in phosphate-buffered saline for 5 min followed by the incubation with the appropriate secondary antibody (Jackson Immuno-Research, West Grove, PA) for 30 min at room temperature. Again slides were washed three times in phosphate-buffered saline for 5 min followed by incubation with ABC-Elite (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Reaction was localized by using DAB working solution (Laboratory Vision, Fremont, CA). Finally, the slides were counterstained with Harris haematoxylin (Fisher

Scientific, Pittsburgh, PA) and mounted with coverslips.

Image analysis was carried out as previously described [16]. Briefly, the slides were systematically scanned and 8–10 representative digital images were acquired from each slide using a 40 × objective. Digital images were quantified by image analysis with Adobe Photoshop (Version 7.0; Adobe Systems, San Jose, CA).

Statistical analysis

The two-tailed Student's *t*-test was used to test the significance of all differences between two groups with a single independent variable. $p < 0.05$ was considered statistically significant. All data are presented as mean ± standard error of the mean.

Results

Low dose drug administration (2.5 mg/kg BID)

There was no significant difference in lung damage and immunohistochemistry endpoints between the irradiated controls and the group receiving RT + AEOL 10150 (2.5 mg/kg BID) (data not shown).

High dose drug administration (5 mg/kg BID)

Haematoxylin and eosin and Masson's trichrome collagen staining of the rat lungs, 26 weeks after irradiation, showed focal interstitial fibrosis in the radiation-only treatment group. The extent and severity of lung damage was significantly reduced in the RT + AEOL 10150 (5 mg/kg BID) group compared with the RT alone animals (RT alone vs RT + AEOL 10150 (5 mg/kg BID), total damage $p = 0.003$; moderate-to-severe damage $p = 0.0004$; lung fibrosis score $p = 0.009$) (Figure 1B and C). The damage in RT alone rats was comprised of areas of interstitial/intra-alveolar oedema and higher alveolar macrophage content, which are suggestive of alveolitis, as well as focal areas of interstitial fibrosis (Figure 1A). The RT + AEOL 10150 (5 mg/kg BID) animals had areas of thickened alveolar walls, but there was remarkable reduction in interstitial oedema, fibrosis, loss of tissue architecture and minimal intra-alveolar cells (Figure 1A). Neither higher dose group had confluent damage consistent with chronic lung injury. The lungs of rats treated with drug alone showed no noticeable changes.

Twenty-six weeks after thoracic irradiation, the RT alone group exhibited strong HIF1α nuclear staining, mainly localized in damaged areas and inflammatory cells (Figure 2A). The intensity and extent of staining was significantly reduced in only the RT + AEOL 10150 (5 mg/kg BID) animals (RT alone vs RT + AEOL 10150 (5 mg/kg BID), $p = 0.006$) (Figure 2B). In AEOL 10150 alone lung tissue, the immunoreactivity of HIF1α was undetectable in the bronchial and alveolar epithelium.

CA IX is regulated by HIF1α and has been extensively investigated as an endogenous hypoxia marker. Animals that received RT alone expressed strong CA IX immunoreactivity, which was seen mainly in the irradiated, damaged tissue and inflammatory cells (Figure 2A). CA IX expression was markedly reduced in the group that received RT + AEOL 10150 (5 mg/kg BID) (RT alone vs RT + AEOL 10150 (5 mg/kg BID), $p = 0.003$) (Figure 2C).

VEGF is also under control of HIF1α transcriptional activity. It is a well known proangiogenic and vascular permeability factor. Animals in the RT alone group exhibited strong VEGF protein expression in the parenchymal and inflammatory cells (Figure 2A). VEGF expression was significantly reduced in groups that received RT + AEOL 10150 (5 mg/kg BID) (RT alone vs RT + AEOL 10150 (5 mg/kg BID), $p = 0.001$) (Figure 2D).

Mean vessel density was also significantly reduced in RT + AEOL 10150 (5 mg/kg BID) animals (Figure 2A). Mean vessel density averaged 56 ± 10 and 19 ± 5 vessels per low-power field in RT alone and RT + AEOL 10150 (5 mg/kg BID), respectively. This difference was statistically significant (RT alone vs RT + AEOL 10150 (5 mg/kg BID), $p = 0.009$) (Figure 2E).

Immunohistochemical staining for macrophages (ED1) demonstrated an increase in both the number and the activity of macrophages in the area of lung damage in irradiated animals (Figure 3A). A significant decrease in the macrophage count was found in the group receiving RT + AEOL 10150 (5 mg/kg BID) treatment compared with the RT alone (RT alone vs RT + AEOL 10150 (5 mg/kg BID), $p = 0.03$) (Figure 3B).

8-OHdG is a major biomarker for detection of oxidative stress (DNA oxidation). 8-OHdG immunohistochemistry revealed strong staining in irradiated lungs of RT alone animals (Figure 3A). A significant reduction in DNA oxidation was seen in RT + AEOL 10150 (5 mg/kg BID) animals (RT alone vs RT + AEOL 10150 (5 mg/kg BID), $p < 0.01$) (Figure 3C).

3-Nitrotyrosine, associated with high levels of reactive nitrogen species (more specifically with peroxynitrite), was detected in the irradiated lung tissue (Figure 3A). Lung sections from radiation-exposed rats also displayed strong immunoreactivity for the presence of nitrotyrosine 26 weeks after the termination of exposure. Thus, diffusely positive cytoplasmic immunostaining for nitrotyrosine was evident in the injured and fibrotic areas of irradiated lungs. In contrast, only mild immunoreactivity for nitrotyrosine was detected in RT + AEOL 10150

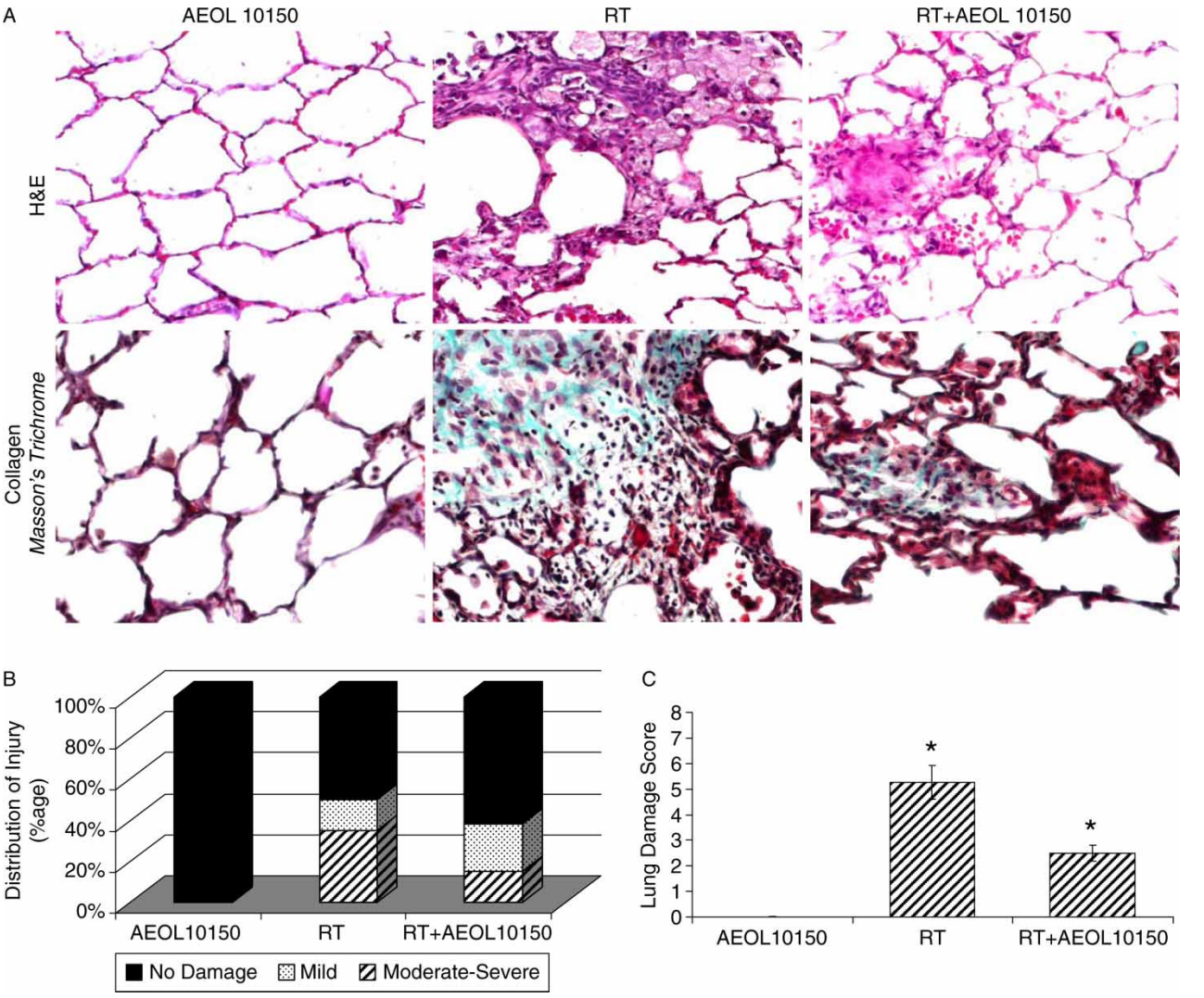

Figure 1. (A) Histologic comparison by H&E and Masson's Trichrome staining among AEOL 10150 alone, RT alone and RT + AEOL 10150 (5 mg/kg/day BID), at 26 weeks after 40 Gy (8 Gy × 5 days) of RT. Rats treated with AEOL 10150 after fractionated RT showed less pulmonary damage than rats that received irradiation alone. (B) Semi-quantitative analyses of lung histology at 26 weeks revealed a significant decrease in structural damage and its severity in animals receiving AEOL 10150 (5 mg/kg/day BID) after RT in comparison to RT alone (RT alone vs RT + AEOL 10150 (5 mg/kg BID), total damage $p = 0.003$; moderate-to-severe damage $p = 0.0004$). (C) RT alone group had greater number of focal areas with increased collagen deposition than did the RT + AEOL 10150 (5 mg/kg/day BID) animals. This translated into a higher lung fibrosis score for the RT alone animals than the other groups (RT alone vs RT + AEOL 10150 (5 mg/kg BID), $p = 0.009$).

(5 mg/kg BID) rats (RT alone vs RT + AEOL 10150 (5 mg/kg BID), $p = 0.01$) (Figure 3D).

Immunohistochemical detection of TGFβ1, Smad3 and p-Smad2/3 demonstrated similar expression patterns (Figure 4A), with strongly stained signals mainly in the macrophages and fibrotic foci. A significant reduction in the expression of all of these components of the TGFβ–mediated fibrosis pathway was seen in the RT + AEOL 10150 (5 mg/kg BID) treatment group compared with RT alone (TGFβ1, $p = 0.01$; Smad3; $p = 0.02$ and p-Smad2/3, $p = 0.008$) (Figure 4 B, C and D). In AEOL 10150 alone lung tissue, the immunoreactivity of TGFβ1, Smad3 and p-Smad2/3 were undetectable in the bronchial and alveolar epithelium. Thus, RT not only increased TGFβ1 expression and activation, but also increased signal transduction down the fibrosis pathway. This effect was significantly attenuated by exogenous SOD (Figure 4A).

Discussion

This study demonstrates that AEOL 10150, previously shown to protect normal lung tissue from single dose radiation (28 Gy) in rodents [10], may also protect the lungs from fractionated RT. These findings suggest that AEOL 10150 could significantly increase the therapeutic index of RT therapy. Moreover, this study improves our understanding of the role of oxidative stress in regulating the normal tissue response to ionizing RT.

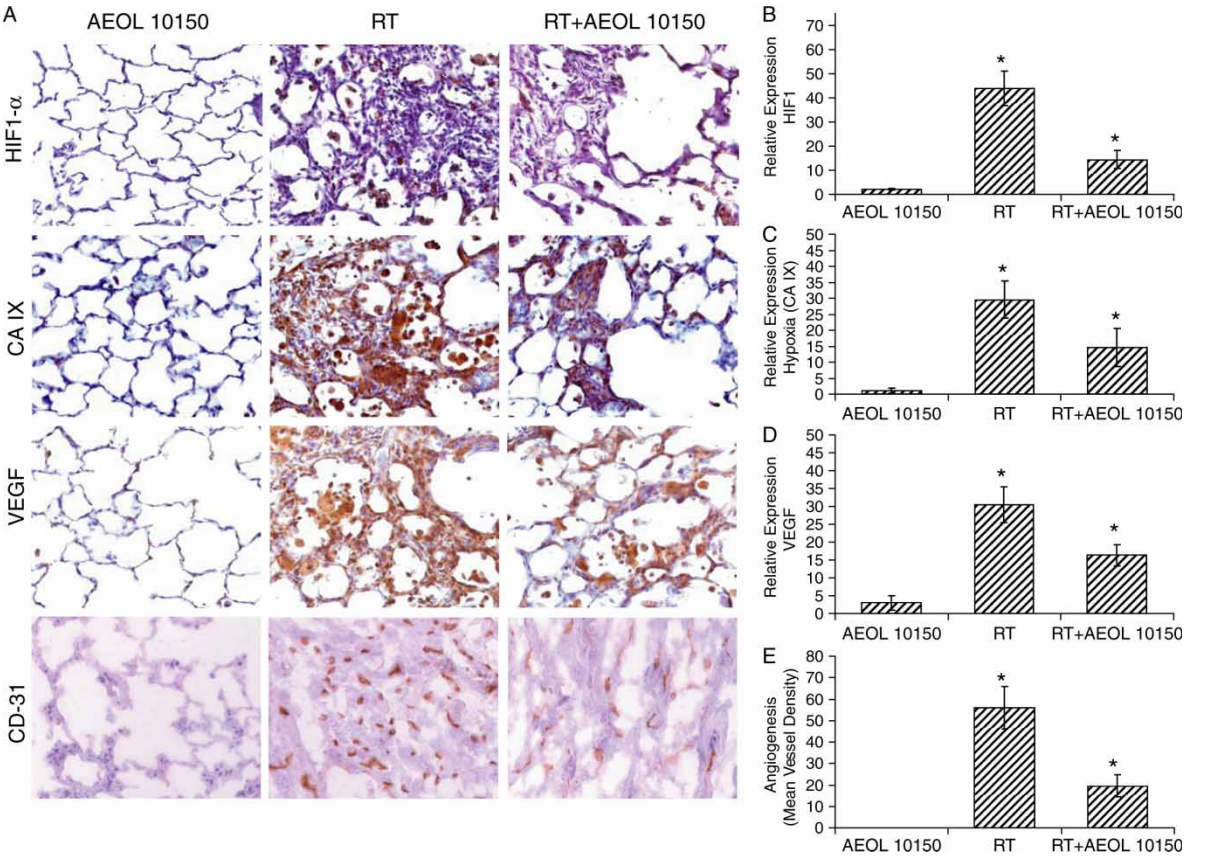

Figure 2. (A) Panel of HIF1α, CA IX, VEGF and CD31 immunohistochemistry: effect of AEOL 10150 on expression of HIF1α, CA IX, VEGF and CD 31 by immunohistochemistry in the lungs of rats 6 months after 40 Gy of fractionated RT. For HIF1α, CA IX and VEGF, positive staining (brown) was most intense in macrophages and fibrotic foci. However, these proteins were expressed to a lesser extent in the lungs of rats treated with RT+AEOL 10150 (5 mg/kg/day BID), compared to RT alone. Similarly a smaller number of blood vessels was documented in RT+AEOL 10150 (5 mg/kg/day BID). These photomicrographs are representative of results obtained from five animals in each group. Each image is 400 $\times$ magnification. (B) Semi-quantitative analysis of HIF1α in the lungs of rats 26 weeks after 40 Gy of fractionated dose RT with or without AEOL 10150 treatment. The RT alone group had significantly more intense nuclear staining than RT+AEOL 10150 (5 mg/kg/day BID) (RT alone vs RT+AEOL 10150 (5 mg/kg BID), $p=0.006$). (C) RT alone animals expressed strong CA IX immunoreactivity, which was seen mainly in the irradiated damaged tissue and inflammatory cell. The tissue protein expression of CA IX was markedly reduced in the groups that received RT+AEOL 10150 (5 mg/kg/day BID) (RT alone vs RT+AEOL 10150 (5 mg/kg BID), $p=0.003$). (D) VEGF immunohistochemistry also displayed a strong expression in RT alone rats. The lung tissue expression of VEGF was significantly reduced in the groups that received RT+AEOL 10150 (5 mg/kg/day BID) (RT alone vs RT+AEOL 10150 (5 mg/kg BID), $p=0.001$). (E) Mean vessel density analysis of irradiated lung tissue displayed a significant reduction in angiogenesis in RT+AEOL 10150 (5 mg/kg/day BID) treatment group (RT alone vs RT+AEOL 10150 (5 mg/kg BID), $p=0.009$) when compared with RT alone animals. Error bars represent $\pm$SEM.

Oxidative damage to the lung develops for several months following the initial RT exposure [7,9,10]. Previous studies have provided direct proof that ROS/RNS are significantly increased after RT [17], providing further support for the hypothesis that oxidative stress might play a key role in the delayed effects of ionizing RT [7,9,10]. This chronic oxidative stress might be involved in regulation of different signalling pathways. This paradigm would provide a major role for chemical radioprotection such as SOD mimetics in influencing RT outcome.

Oxidative stress, in particular superoxide (O_2^-) and its progeny, is involved in the aetiology of RT-induced lung injury [7,9,10]. O_2^- formation directly removes NO by producing $ONOO^-$, which acts as a potent oxidant [18] and also elicits vasoconstriction [19]. $ONOO^-$ can induce cell damage by sulphydryl oxidation, lipid peroxidation and nitration of tyrosine [20,21]. Hypoxia-induced rat diaphragm dysfunction is associated with elevated nitrotyrosine levels [22] and it is reversed by antioxidant therapy [23,24]. We have shown in several models of oxidative stress that Mn porphyrin-based SOD mimetics, in addition to scavenging superoxide, can also significantly decrease peroxynitrite-mediated damage through decreasing levels of 3-nitrotyrosine [25,26] (see below also).

Inducible NOS (iNOS) is strongly expressed in pneumocytes and inflammatory cells in pulmonary fibrosis and in areas where higher levels of peroxynitrite are detected [27]. In bleomycin-induced fibrosis, iNOS expression is highly increased in alveolar and bronchiolar epithelia and in inflammatory cells [28]. Exposure of endothelial cells to RT results in a 9-fold increase in the expression of

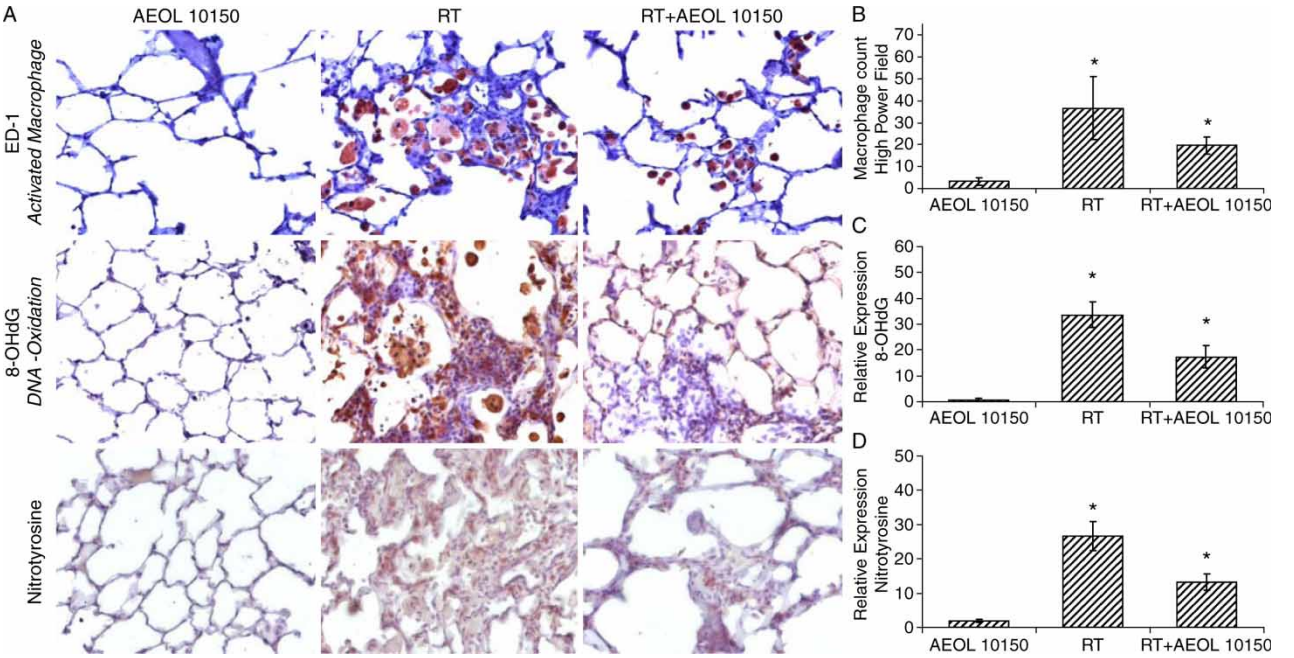

Figure 3. (A) Panel of ED-1, 8-OHdG and Nitrotyrosine immunohistochemistry: lung tissues were immunostained for macrophage activation (ED1), oxidative stress (8-OHdG) and nitrotyrosine. The lungs of rats treated with RT+AEOL 10150 (5 mg/kg/day BID) exhibited much fewer activated macrophages and less oxidative stress than rats treated with RT alone. These photomicrographs are representative of results obtained from five animals in each group. (B) Semi-quantitative analysis for macrophages demonstrated an increase in number of macrophages in the areas of lung damage in RT alone animals. A significant decrease in the alveolar macrophage count was found in the group receiving RT+AEOL 10150 (5 mg/kg/day BID) treatment compared with the RT alone (RT alone vs RT+AEOL 10150 (5 mg/kg BID), $p=0.03$). (C) 8-OHdG expression revealed strong staining in irradiated lungs of RT alone animals. A significant reduction in DNA oxidation was seen in RT+AEOL 10150 (5 mg/kg BID) group (RT alone vs RT+AEOL 10150 (5 mg/kg BID), $p=0.0004$). (D) Lung sections from RT-exposed rats also displayed strong immunoreactivity for the presence of nitrotyrosine at 26 weeks after the termination of exposure. Thus, diffusely positive cytoplasmic immunostaining for nitrotyrosine was evident in the injured and fibrotic areas of irradiated lungs. In contrast, only significantly mild immunoreactivity for nitrotyrosine was detected in RT+AEOL 10150 (5 mg/kg BID).

iNOS and release of NO [29]. Recent data have also shown that NO induces stabilization of HIF1α through a mechanism involving reactive species, so that upregulation of NOS by radiation may subsequently trigger the HIF/VEGF molecular cascade [30]. These findings are consistent with current evidence showing that ROS/RNS might be important in the delayed response to RT damage in normal tissue and extended administration of AEOL 10150 can scavenge them successfully (Figure 5).

Oxidative stress has been implicated in upregulating HIF1α [31–34], which in turn induces a variety of cytokines [35]. In irradiated tissue, ROS also stabilize HIF1α, ultimately leading to the development of a hypoxia-like tissue response. Hypoxia itself is also known to generate ROS, increase leukocyte migration and vascular permeability, up-regulate TGFβ and promote collagen formation, which are all important processes in the development of fibrosis [36,37]. Our group and others have recently demonstrated that chronic hypoxia might be a contributing factor in the development of RT-induced normal tissue injury [9,10,15,38]. Apart from the HIF-1 pathway, oxidative stress might also be involved in stimulating other transcriptional factors such as NF-κB and AP-1 [39,40].

Recent investigations have highlighted inhibitory role of Mn porphyrin-based catalytic antioxidants on transcription factors HIF1α, AP-1 and NF-κB signalling [17,41,42]. Such effect may be the consequence of antioxidant ability of Mn porphyrins to remove signalling ROS and RNS [13,18,43] and/or to directly interact with signalling proteins [42]. It has been reported that, following RT, the generation of ROS was blocked by a potent pyridylporphyrin-based SOD mimetic, MnTE-2-PyP^{5+} (Mn(III) tetrakis (*N*-ethylpyridinium-2-yl) porphyrin, AEOL10113)) resulting in HIF-1α inactivation and down-regulation of VEGF/angiogenesis pathway [17]. In a rat model of tobacco smoke-induced lung injury, the ability of the catalytic antioxidant AEOL 10150 to decrease lung damage is suggested by inhibition of oxidant-mediated activation of NF-κB [43].

We have previously shown that Mn porphyrin can substitute for and protect mitochondrial MnSOD enzyme. Namely, we reported that pyridyl analogue, MnTE-2-PyP^{5+} substituted for MnSOD in skin cancer model that had been performed with MnSOD heterozygous DBA/2 MnSOD knock-out mice (*sod2*-/+) [42]. It is further displayed that the lipophilic

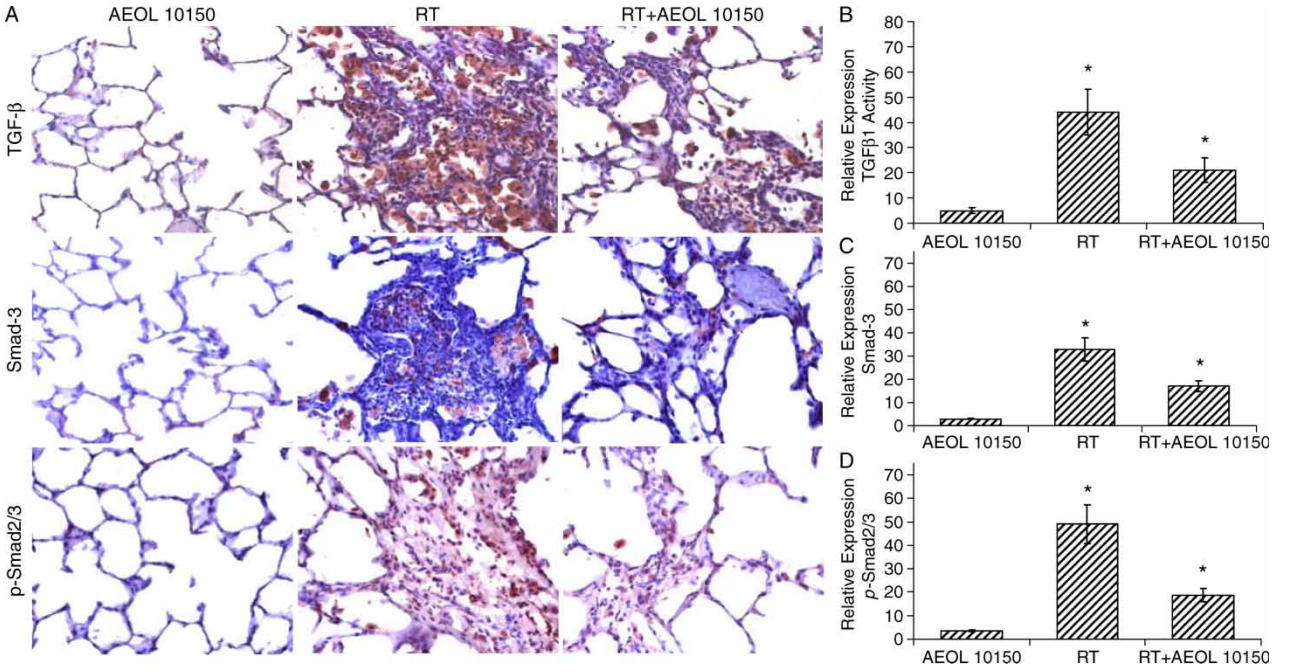

Figure 4. (A) Panel of TGFβ1, Smad-3 and p-Smad-2/3 immunohistochemistry: the lungs of rats treated with RT+AEOL 10150 (5 mg/kg/day BID) exhibited less expression for TGFβ1, Smad-3 and p-Smad-2/3 than rats treated with RT alone. RT not only increased TGFβ1 expression and activation, but also increased signal transduction down the fibrosis pathway. This effect was significantly attenuated by exogenous SOD. These photomicrographs are representative of results obtained from five animals in each group. Each image is 400 × magnification. (B) Semi-quantitative analysis of TGFβ1 in the lungs of rats after 40 Gy of fractionated RT with or without AEOL 10150 treatment. RT alone group had significantly more intense staining than RT+AEOL 10150 (5 mg/kg/day BID) (RT alone vs RT+AEOL 10150 (5 mg/kg BID), TGFβ1, $p=0.01$). (C) RT alone animals expressed strong Smad-3 immunoreactivity, which was seen mainly in the irradiated, damaged tissue and inflammatory cell. The tissue protein expression of Smad-3 was markedly reduced in the groups that received RT+AEOL 10150 (5 mg/kg/day BID) (RT alone vs RT+AEOL 10150 (5 mg/kg BID), $p=0.02$). (D) p-Smad2/3 expression also displayed a strong immunoreactivity in RT alone rats. The lung tissue expression of p-Smad2/3 was significantly reduced in the groups that received RT+AEOL 10150 (5 mg/kg/day BID) (RT alone vs RT+AEOL 10150 (5 mg/kg BID), $p=0.008$). Error bars represent ± SEM.

pyridyl analogue of higher bioavailability but equal antioxidant potency, MnTnHex-2-PyP^{5+} [44], was able to fully preserve renal MnSOD activity, resulting from ischemia-reperfusion, when only a single dose of 50 μg/kg was administered intravenously 24 hbefore insult [26]. Yet, in a control experiment (no ischemia-reperfusion) MnTnHex-2-PyP^{5+} did not increase basal MnSOD antioxidant capacity, indicating that the effects may be due to the additional actions of porphyrin on the protein expression level. Finally, in a mouse stroke model, MnTE-2-PyP^{5+} and its imidazolyl analogue AEOIL10150 were both able to preserve activity of a critical enzyme, mitochondrial aconitase [13,45]. All of these data also indicate that MnTE-2-PyP^{5+} was able to translocate into mitochondria. Indeed follow-up study was performed and showed that MnTE-2-PyP^{5+} enters mouse heart mitochondria after only a single 10 mg/kg ip dose [46]. We have reported that in submitochondrial particles ≥ 3 μM MnTE-2-PyP^{5+} prevents ONOO$^-$ injury [47]. Given the high and similar rate constants for $O_2^{\cdot -}$ dismutation (log $k_{cat}=7.76$, 25°C) and ONOO$^-$ reduction (log $k_{red}=7.53$, 37°C), MnTE-2-PyP^{5+} is able to efficiently remove both $O_2^{\cdot -}$ and ONOO$^-$, the later presumably being its major function *in vivo* [48,49]. The same is true for AEOL10150 as both have nearly identical constants for the reactions with $O_2^{\cdot -}$ (log $k_{cat}=7.83$, 25°C) [12] and ONOO$^-$ (log $k_{red}=7.00$, 37°C) (Ferrer-Sueta and Batinic-Haberle, unpublished data).

The current study provides further insight toward understanding how SOD mimetics might inhibit oxidant-induced signal transduction and cytokine production. In late RT-induced lung injury, sustained oxidative stress acts via positive feedback mechanism by cytokine induction and activation, mainly via TGFβ. Prior studies have shown that TGFβ1, if released soon after injury, acts primarily as a proinflammatory agent [50]. Later, TGFβ1 function switches to resolution of inflammation and initiation of repair. There are several possible mechanisms of interaction between TGFβ1 and oxidants in the lung. First, TGFβ1 induces differentiation of myofibroblasts, which can themselves serve as a producer of reactive species [51]. Secondly, ROS can increase the release of TGFβ1 from pulmonary epithelial cells [52] and also directly activate TGFβ [53]. TGFβ has been shown to activate NADPH oxidase in human fibroblasts, leading to increased production of ROS [54]. Thus, oxidants and TGFβ1 may interact to enhance and sustain the fibrotic response in irradiated tissue (Figure 5). These observations

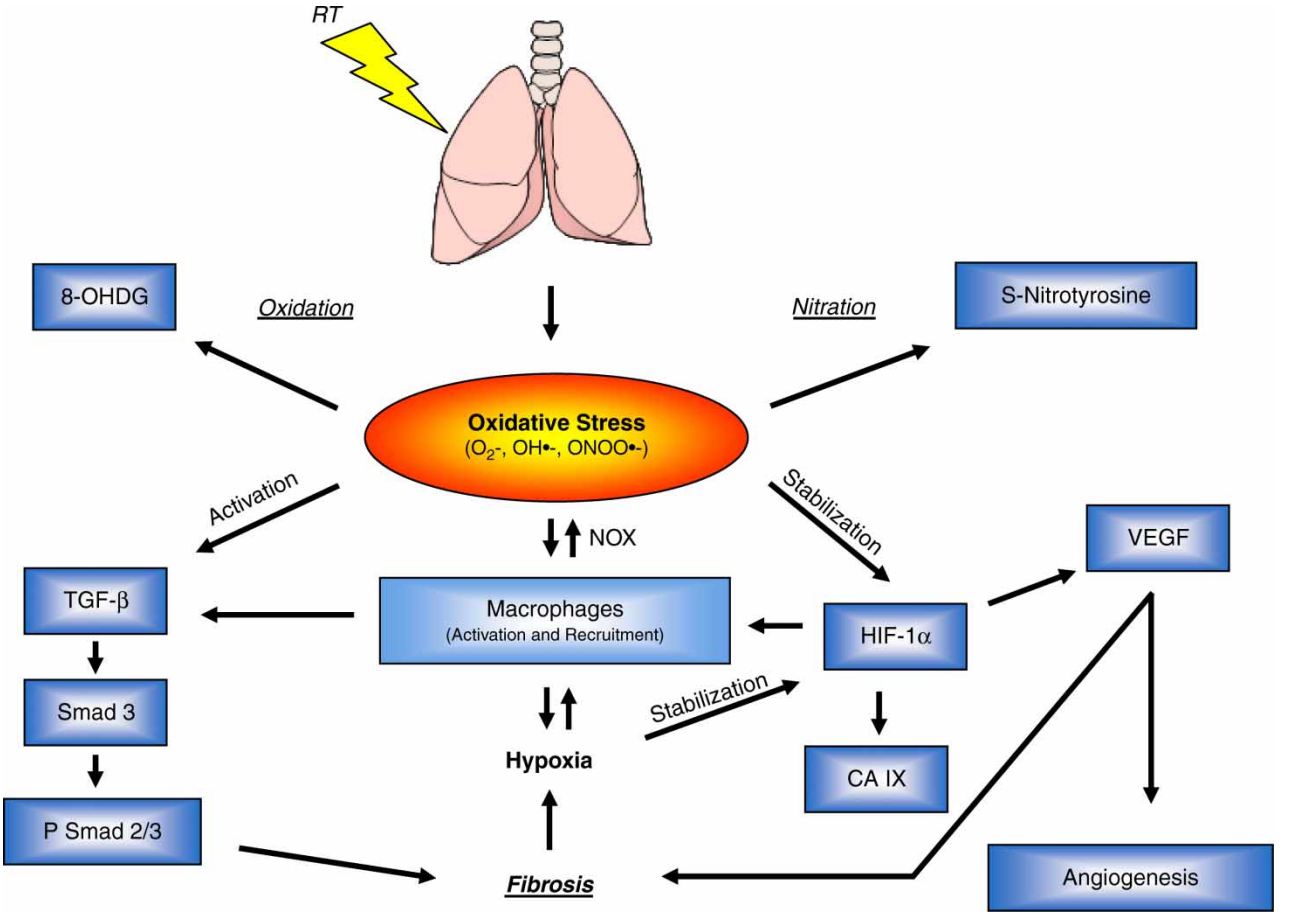

Figure 5. RT-induced activation of HIF1α and TGFβ signalling pathways: reactive oxygen and nitrogen species (oxidative stress) are significantly increased after RT therapy, providing further support for the hypothesis that oxidative stress might play a key role in the delayed effects of ionizing RT. HIF1α stabilizes through a mechanism involving reactive species, which may subsequently trigger the HIF1α molecular cascade. In irradiated lung, oxidative stress after upregulating HIF1α leads to the development of hypoxia-like tissue response. This RT-induced normal tissue hypoxia itself is also known to promote inflammation, vascular permeability, angiogenesis, increased TGFβ activity and its signalling proteins, which are all important processes in the development of pulmonary fibrosis.

further support the notion that the protective effect of AEOL 10150 is mediated, at least in part, by its ability to inhibit the TGFβ1 activation and its signalling.

SOD inhibits RT induced changes in a number of biological end points, including enzyme activity, membrane integrity, DNA damage, cell transformation and cell and organism survival [55–57]. Previous studies by our group and others [7,11,58–61] suggest the efficacy of SOD in ameliorating RT induced lung damage by EC-SOD or Mn-SOD-based gene therapy. When SOD mimetics were delivered by intraperitoneal route or subcutaneously by osmotic pump, we found that pulmonary fibrosis was significantly reduced in rodents after RT therapy [10,62]. In the present study, subcutaneous delivery of AEOL 10150 also showed that AEOL10150 is a potent radioprotector. These results offer the promise of minimizing dose-limiting normal tissue radiotoxicity in a clinical setting.

The present study showed a dose response for the radioprotective effect of AEOL 10150. Only AEOL 10150 at higher dosage (5 mg/kg BID) significantly decreased tissue hypoxia, inflammation, oxidative stress and signalling along the TGFβ1 pathway. The present data validates our previous work in which AEOL 10150 at higher dosage of 10 or 30 mg/kg/day after RT (28Gy Single Dose) for longer periods led to significantly reduced lung damage, inflammation, oxidative stress and tissue hypoxia [10]. This study also suggests that AEOL 10150 did not increase the tolerance of normal tissue to radiotherapy when it was delivered at a lower concentration (2.5 mg/kg BID). Despite the ineffectiveness of the lower dose, SOD mimetics can be delivered in a reasonable dosing regimen that can attenuate lung dysfunction and pathology associated with RT and bleomycin-induced pulmonary fibrosis [10,63]. Taken together, these studies suggest that long-term post-RT treatment with SOD mimetic might be a particularly effective means of preventing lung injury.

The extended administration of 5 mg/kg BID of the novel catalytic antioxidant, AEOL 10150, during and after RT demonstrates a significant protective effect from RT-induced lung injury. These results support

the concept that catalytic antioxidants that possess strong SOD activity act as beneficial radioprotecive agents depending on their efficacy to scavenge reactive oxygen/nitrogen species and reduce lung damage. These data also support the use of AEOL 10150, in a clinical trial designed to prevent radiation-induced lung injury.

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