Published in final edited form as:

Int J Radiat Oncol Biol Phys. 2007 February 1; 67(2): 573–580.

Long Term Administration of A Small Molecular Weight Catalytic Metalloporphyrin Antioxidant AEOL 10150 Protects Lungs From Radiation-Induced Injury

Zahid N. Rabbani, M.D.¹, Ines Batinic-Haberle, Ph.D.¹, Mitchell S. Anscher, M.D.¹, Jie Huang, M.D.², Brian J. Day, Ph.D.², Elaine Alexander, M.D., Ph.D.³, Mark W. Dewhirst, D.V.M., Ph.D.¹, and Zeljko Vujaskovic, M.D., Ph.D.^{1,*}

¹Department of Radiation Oncology, Duke University Medical Center, Durham, North Carolina, USA,

²Department of Medicine, National Jewish Medical & Research Center, Denver, CO, USA,

³Department of Aeolus Pharmaceuticals, Laguna Niguel, CA, USA

Abstract

Purpose—To determine whether administration of a catalytic antioxidant, Mn(III) tetrakis(N,N'-diethylimidazolium-2-yl) porphyrin, AEOL 10150, with SOD mimetic properties reduces the severity of radiation-induced injury to the lung from single dose irradiation (RT) of 28 Gy.

Materials/Methods—Animals were randomly divided into four different dose groups (0, 1, 10 and 30 mg/kg/day of AEOL 10150), receiving either short (1 week) or long-term (10 weeks) drug administration via osmotic pumps. Animals received single dose irradiation (RT) of 28 Gy to the right hemithorax. Breathing rates, body weights, blood samples, histopathology and immunohistochemistry were used to assess lung damage.

Results—There was no significant difference in any of the study endpoints between the irradiated controls and the 3 groups receiving RT and short-term administration of AEOL 10150. For the long term administration functional determinants of lung damage 20 weeks post radiation were significantly worse for RT+PBS and RT+1mg/kg/day of AEOL 10150 as compared to the irradiated groups treated with higher doses of AEOL 10150 (10 or 30mg/kg/day). Lung histology at 20 weeks revealed a significant decrease in structural damage and collagen deposition in animals receiving 10 or 30mg/kg/day after radiation in comparison to RT+PBS and 1mg/kg/day groups. Immunohistochemistry demonstrated a significant reduction in macrophage accumulation, oxidative stress and hypoxia in animals receiving AEOL 10150 (10 or 30mg/kg/day) after lung irradiation compared to RT+PBS and 1mg/kg/day groups.

Conclusion—The chronic administration of novel catalytic antioxidant, AEOL 10150, demonstrates a significant protective effect from radiation-induced lung injury. AEOL 10150 has its primary impact on the cascade of events following irradiation and adding drug prior to irradiation and its short-term administration have no significant additional benefits.

*Corresponding Author: Box 3455, DUMC, Durham, NC 27710 Tel: (919) 681-1675 FAX: (919) 684-8718 E-mail: vujask@radonc.duke.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest Notification: Dr. Alexander is an employee of Aeolus Pharmaceuticals. Dr. Day also holds an equity position and serves as a consultant for Aeolus Pharmaceuticals.

Keywords

Radiation; Pulmonary toxicity; Superoxide dismutase mimetic; AEOL10150; Rat

INTRODUCTION

Radiation (RT) induced lung toxicity remains a major factor which limits the ability to escalate radiation doses in the treatment of thoracic tumors [1]. The ability to optimize RT for tumors on the basis of relative risks for normal tissue injury has important implications in oncology, because higher doses of RT may improve both local tumor control and survival. For the radiation oncologist, a better understanding of the molecular events underlying normal tissue injury will allow a more logical approach to the prevention and treatment of this problem.

Advances in the tools of molecular and cellular biology have enabled researchers to develop a better understanding of underlying mechanisms responsible for RT-induced normal tissue injury. For decades ionizing radiation has been known to increase production of free radicals [2], which is reflected by the accumulation of oxidatively damaged cellular macromolecules. RT may impair lung cells either directly via generation of reactive oxygen species (ROS) [2] or indirectly via the action on parenchymal and inflammatory cells through biological mediators [3,4].

Since the discovery of superoxide dismutases (SOD) [5,6], it has become clear that these enzymes provide an essential line of defense against reactive oxygen species [2]. SODs act by catalyzing the dismutation of superoxide radical to hydrogen peroxide and oxygen [7,8]. They are localized intra/extracellularly, are widely expressed throughout the body, and are important in maintenance of redox status [9,10]. Previous studies have demonstrated that treating irradiated animal models with SOD delivered by injection of the enzyme, through liposome/viral-mediated gene therapy or insertion of human SOD gene can ameliorate RT-induced damage [11-18].

Recent evidence suggests that progressive oxidative stress might be, in part, the driving force behind chronic RT injury. Therefore prolonged treatment aimed at reducing chronic free radical generation might be beneficial. One promising approach to this problem involves the superoxide dismutase (SOD) pathway. Therefore, the objective of this study was to determine whether administration of a small molecular weight catalytic metalloporphyrin antioxidant, AEOL 10150, with SOD mimetic properties, and with the ability to nearly equally effectively eliminate ONOO^- [19,20] and $\text{CO}_3^{\cdot-}$ [19] also, increases the tolerance of the lung to ionizing RT by reducing the severity of RT-induced pulmonary injury, and to determine the importance of timing and duration of administration. This compound was selected because of its strong antioxidant properties, as well as its favorable toxicity profile.

MATERIALS & METHODS

Animals

Sixty four female Fisher-344 rats weighing between 150-170 grams were used in this study with prior approval from the Duke University Institutional Animal Care and Use Committee. Four animals were housed per cage and maintained under identical conditions with food and water provided *ad libitum*. All rats were sacrificed at a predetermined time of 20 weeks post-RT. At the time of sacrifice, the lungs were perfused and removed. Irradiated right lung was processed for histopathology and immunohistochemistry studies.

Irradiation

The animals were randomly divided into four duplicate groups for short and long-term drug administration experiments **a)** RT+PBS, **b)** RT+1mg/kg/day AEOL 10150, **c)** RT+10mg/kg/day AEOL 10150, and **d)** RT+30mg/kg/day AEOL 10150 (Figure 1A). The animals were anesthetized prior to irradiation with i.p. ketamine (67.5 mg/kg) and xylazine (4.5 mg/kg). Hemithoracic RT was delivered to the right lung using a single dorsal-ventral field with 4-MV photons. A single dose of 28 Gy was given. A 0.5 cm bolus was placed over the right lung, and the un-irradiated areas were shielded using lead blocks.

AEOL 10150

Metalloporphyrins act as scavengers of reactive species, primarily of superoxide [21,22], peroxyxynitrite [19,20], carbonate radical [19], lipid peroxy radicals [23], and hydrogen peroxide [24]. They are of low-molecular weight, and are water soluble and stable. The cationic Mn (III) porphyrin, AEOL 10150 [21,24] was 92.1% pure as determined by HPLC analysis and was provided by Aeolus Pharmaceuticals (Laguna Niguel, CA).

The short-term dosing schedule consisted of drug administration, beginning one day before RT, was given until one week following RT. In the long-term administration schedule, the drug was started one day after RT and continued for 10 weeks post-RT. The drug was given as a continuous infusion with an osmotic pump (Alzet mini osmotic pump, model 2002) implanted subcutaneously, because of its short half-life (Figure 1). Short term administration did not require changing the pumps, however, animals undergoing long term administration of the drug had the pumps changed every 2 weeks. Blood samples were drawn from the tail vein at 1 day, 1 and 7 weeks. Blood samples were centrifuged at 3000 rpm at 4° C for 30 minutes to collect plasma and aliquots were stored at -80° C till further evaluation.

Analytical analysis for rat plasma AEOL 10150 samples

Aliquots of rat plasma (12.5 µl, 7th week of treatment) were placed in microcentrifuge tubes along with 0.1N perchloric acid (81.5 µl) and 1 µM AEOL 10123 (6 µl) as an internal standard. Samples were mixed and stored on ice in the dark for 10 minutes. Precipitated proteins were removed by centrifugation (20,000 × g for 10 minutes). Supernatants were transferred to HPLC vials for analysis. HPLC conditions for analysis were as follows: mobile phase consisted of 85% A (20 mM triethylamine, 20 mM trifluoroacetic acid in water) and 15% B (100% acetonitrile); C18 column (YMC-Pack ODS-ATM, 4.6 × 150mm, 3 µm); oven temperature 45°C; flow rate of 1 ml/minute; 10 µl injection volume; detection at 446 nm (L2420UV-Vis, Hitachi). Under these conditions the retention time for AEOL 10150 was 3.5 minutes. AEOL 10150 and 10123 standards were prepared daily and responses were linear over the range of concentrations reported in these studies. Recovery of AEOL 10150 from plasma samples was found to be greater than 90%.

Functional assessment of lung injury

Breathing frequency using whole-body plethysmography (Model RM-80, Columbus Instruments, Columbus, Ohio) was measured as an indicator of pulmonary injury. Measurements were taken every two weeks for 20 weeks. The mean of five measurements, were recorded for each animal.

Histopathology & Lung damage score

All animals were followed for 20 weeks after irradiation. RT-induced lung damage was subsequently assessed histopathologically. Animals were euthanized with pentobarbital. Both lungs were fixed by tracheal instillation of 10% neutral-buffered formalin, 2% glutaraldehyde, and 0.085 M sodium cacodylate buffer and embedded in paraffin. Five-micrometer thick

sections were stained with hematoxylin and eosin and Masson's trichrome to visualize fibrosis and collagen. Slides were systematically scanned in a microscope and five representative fields were selected using a lower objective. 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 [25]. The average score was calculated for each animal and each group.

Immunohistochemistry

Immunohistochemistry was carried out as described previously [26]. Briefly, paraffin embedded tissues were sectioned at 5 micron thickness, and antigen retrieval performed using citrate buffer from Biogenex (San Ramon, CA). Tissues were treated with primary antibodies against macrophages (1:100, mouse monoclonal, MCA341, Serotec, Oxford, UK), 8-OHdG (8-hydroxydeoxyguanine; marker for DNA oxidation and oxidative stress, dilution 1:2000, mouse monoclonal, JaICA, Japan) and CA9 (carbonic anhydrase 9, endogenous hypoxia marker, dilution 1:400, mouse monoclonal, M75, gift from Dr. Oosterwijk, Department of Urology, University Hospital Nijmegen, Netherlands). Secondary and tertiary antibodies were provided in a kit (314KLD, Innovex, Richmond, CA), and location of the reaction was visualized with 3, 3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO). Slides were counterstained with hematoxylin and mounted with coverslips. Normal mouse serum or mouse IgG, were used as controls.

Macrophage content was determined by counting the number per low power field. The same methodology was used to count 8-OHdG positively stained cells at high power field. Immunoreactivity of CA 9 was assessed semiquantitatively by using Adobe photoshop 7.0, and the extent of staining was assessed as the percentage of positively stained area per field under 100 × magnification. In addition, the intensity of staining was also scored as: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining.

Statistical Analysis

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

RESULTS

Short-term drug administration

There was no significant difference in any of the study endpoints between the irradiated controls and the 3 groups receiving short-term administration of AEOL 10150 (*data not shown*).

Long-term drug administration

Osmotic mini-pumps provided consistent and dose-dependent delivery of AEOL 10150 as evidenced by plasma levels of AEOL 10150 monitored at week 7 (Figure 1B). Animals treated with 10 and 30mg/kg/day AEOL 10150 showed 9 to 10 and 17 to 18 fold increases in plasma drug concentration compared to RT+PBS and RT+1mg/kg/day AEOL 10150. These data suggest a continuous availability of antioxidant via osmotic infusion pumps throughout the study. These data also suggest that the therapeutic window of AEOL 10150 in the protection against radiation-induced lung injury is between 200 ng/ml and 2400 ng/ml based on efficacy data from the dose response study.

All groups exhibited a steady gain in body weight throughout the treatment period. (Figure 2). RT+PBS and RT+1mg/kg/day AEOL 10150 animals, however, demonstrated significantly less weight gain beginning at week 14 compared to the higher doses (10 or 30mg/kg/day) animals

(*RT+PBS and RT+1mg/kg/day vs. RT+10mg/kg/day at 14-20 weeks, $p<0.05$, RT+PBS and RT+1mg/kg/day vs. RT+30mg/kg/day at 16-20 weeks, $p<0.05$, respectively*) (Figure 2).

The RT+PBS and RT+1mg/kg/day AEOL 10150 rats exhibited higher breathing rates compared with higher dose AEOL 10150 groups beginning at 8 weeks after radiation (Figure 3). There was a significant delay (up to 12-14 weeks) in the development of radiation-induced lung injury in animals treated with 10 and 30mg/kg/day AEOL 10150. The average breathing rates in irradiated groups treated with AEOL 10150 (10 or 30mg/kg/day) were significantly lower than the group receiving RT+PBS and RT+1mg/kg/day (*RT+PBS and RT+1mg/kg/day vs. RT+10mg/kg/day at 12-20 weeks, $p<0.05$, RT+PBS and RT+10mg/kg/day vs. RT+30mg/kg/day at 12-20 weeks, $p<0.05$, respectively*). Furthermore, the magnitude of the change in breathing rate was, on average, reduced by 25-30% indicating the ability of higher doses of AEOL 10150 to significantly reduce the severity of RT -induced lung injury

The RT+PBS and RT+1mg/kg/day AEOL 10150 groups exhibited more extensive lung damage than did the irradiated groups treated with higher doses AEOL 10150 (10 or 30mg/kg/day) (*RT+PBS and RT+1mg/kg/day AEOL 10150 vs RT+10 and 30mg/kg/day, $p=0.01$*) (Figure 4B). The damage in the RT+PBS and RT+1mg/kg/day AEOL 10150 rats was consistent with RT injury including areas of interstitial/intra-alveolar edema and higher alveolar macrophage content, which are suggestive of alveolitis, as well as focal areas of interstitial fibrosis. The RT+10 and 30mg/kg/day AEOL 10150 animals had areas of thickened alveolar walls, but there was remarkable reduction in interstitial edema, fibrosis, loss of tissue architecture, and minimal intra-alveolar cells. Neither higher dose groups had confluent damage consistent with chronic lung injury (Figure 4A).

The difference in collagen deposition among the groups was not as apparent as the difference in tissue architecture. However, the RT+PBS and RT+1mg/kg/day AEOL 10150 groups had greater number of focal areas with increased collagen deposition than did the RT+10 or 30 mg/kg/day AEOL 10150 groups (Figure 4A). This translated into a higher lung fibrosis score for the RT+PBS and RT+1mg/kg/day AEOL 10150 animals than the other groups, although no animals were found to have strong or intense staining for collagen (*RT+PBS vs RT+10 and 30mg/kg/day, $p=0.003$, 0.005 respectively; RT+1mg/kg/day vs RT+10 and 30mg/kg/day, $p=0.002$, 0.003 respectively*)(Figure 4C).

Immunohistochemical staining for macrophages demonstrated an increase in both the number and the activity of macrophages in the area of lung damage in irradiated animals (Figure 5A). A significant decrease in the alveolar macrophage count was found in the group receiving RT +10 or 30 mg/kg/day AEOL 10150 treatment compared with RT+PBS (*RT+PBS vs RT+10 and 30mg/kg/day, $p=0.0006$, 0.016 respectively; RT+1mg/kg/day vs RT+10mg/kg/day, $p=0.02$*). There was no difference between RT+1mg/kg/day AEOL 10150 compared with RT +PBS control and RT+30mg/kg/day ($p > 0.05$) (Figure5B).

8-OHdG is the major marker for the assessment of DNA oxidation and oxidative stress, being produced in vivo following the exposure to a number of free radical generating agents including ionizing radiation. Utilizing this marker to detect chronic oxidative stress revealed strong staining mainly in the macrophages and injured areas of irradiated lungs of RT+PBS and RT +1mg/kg/day AEOL 10150 animals (Figure 5A, C). A significant reduction in DNA oxidation was seen in RT plus high-dose AEOL 10150 treatment groups (*RT+PBS vs RT+10 and 30mg/kg/day, $p=0.006$, 0.014 respectively; RT+1mg/kg/day vs. RT+10mg/kg/day and 30mg/kg/day, $p=0.004$, 0.009 respectively*). (Figure 5C).

Carbonic anhydrase 9 is a HIF-target regulated gene that has been thoroughly investigated as an endogenous hypoxia marker [27,28]. RT+PBS and RT+1mg/kg/day AEOL 10150 animals expressed strong CA 9 immunoreactivity, which was seen mainly in the irradiated, damaged

tissue and inflammatory cells (Figure 5A, D). CA 9 expression was markedly reduced in groups that received radiation plus high-doses (10 or 30 mg/kg/day) AEOL 10150 (*RT+PBS* vs *RT+10* and *30mg/kg/day*, $p=0.001$, 0.0009 respectively; *RT+1mg/kg/day* vs. *RT+10mg/kg/day* and *30mg/kg/day*, $p=0.00001$, 0.00005 respectively). (Figure 5 D).

DISCUSSION

The data obtained in our study demonstrate that the post irradiation long term delivery of a novel catalytic antioxidant, AEOL 10150, may be protective against radiation-induced lung injury, as assessed by breathing frequency, histopathology, and immunohistochemistry. Oxidative stress, inflammation and hypoxia, which play important roles in the pathogenesis of radiation mediated fibrosis, were also shown to be reduced in animals treated with higher doses of AEOL 10150. Such findings support the concept that catalytic antioxidants with SOD activity may be useful as radioprotective agents.

Normal tissue response to RT is not only initiated by the generation of reactive species during RT therapy, it is also sustained by continuous production of ROS and their involvement in complex signaling and cytokine induction/activation [18,29-31]. As more has been learned about how oxidative stress promotes normal tissue toxicity, new opportunities for radioprotection have arisen. Radioprotectors are designed to interfere with the damaging reactive species cascades initiated by radiation [32]. They are often selectively beneficial for normal tissues, due to differences in either uptake or target sensitivity between normal and cancer cells [33,34]. 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 [35-37]. A series of studies by our group [17,18] and Epperly *et al.* [13, 15,16,38] have demonstrated the efficacy of SOD in preventing RT induced lung damage by EC-SOD or Mn-SOD based gene therapy.

Although, above mentioned studies generated promising results, the difficulties of delivering SOD to target tissues, with gene therapy or otherwise, have somewhat limited translation into the clinic. Intramuscular injections of liposomal Cu/Zn-SOD on 34 patients with palpable RT induced fibrosis, showed some clinical regression of fibrosis, with complete response in 17% of fibrotic areas [39]. Another study using liposomal Cu/Zn-SOD and Mn-SOD in pigs with subcutaneous RT fibrosis also demonstrated regression of fibrotic areas [40]. Using SOD mimetics via the intraperitoneal route, we found that pulmonary fibrosis, including excess collagen accumulation and profibrogenic cytokine TGF β activation, were greatly reduced in rodents after RT therapy [41]. The present study also showed that chemical radioprotection in form of catalytic antioxidants with SOD activity, offers the promise of reducing dose-limiting normal tissue radiotoxicity.

Inflammation is a driving force in the development of pulmonary fibrosis [3,42]. Inflammatory cells are a known source of ROS [43,44]. They also release inflammatory and fibrogenic cytokines [45]. In the presence of a potent anti-inflammatory stimulus, i.e., extracellular SOD, irradiated transgenic animals suffered significantly less RT-induced pulmonary damage [17, 18]. In a mice model of cerebral ischemia/reperfusion injury, treatment with AEOL 10150 attenuated the increase in expression of inflammatory genes such as heat shock protein, interleukin-6, and macrophage inflammatory protein-2 [46]. Intratracheal administration of AEOL 10150 caused marked reduction in tobacco smoke-induced inflammation and lung injury [47]. The ability of the catalytic antioxidant AEOL 10150 to decrease lung damage, is suggested through the inhibition of oxidant mediated activation of nuclear factor NF- κ B [47]. The inflammatory response of irradiated rats treated with higher doses of AEOL 10150 in current study was significantly reduced compared to controls. These observations further support the notion that the protective effect of AEOL 10150 is mediated, at least in part, by its

ability to modify the immune response as was reported by *Tse et. al.* [48]. These studies provide an important insight toward understanding how SOD mimetics might inhibit oxidant-induced signal transduction and cytokine production.

An unexpected finding from this study was that AEOL 10150 did not increase the tolerance of normal tissue to radiotherapy when it was delivered 1 day before RT. The fact that prolonged drug administration after RT sequencing was more effective than shorter administration, which included treatment before and during RT suggests that the relevant effects of AEOL 10150 in the irradiated tissue are exerted after RT therapy. These findings are consistent with recent data showing that free radicals might be important in the prolonged response to RT damage in normal tissue [49,50]. The present data validates our previous work in which constitutive overexpression of extracellular SOD has been shown to be particularly effective in a mouse model of lung injury [17,18]. Taken together, these studies suggest that long-term post RT treatment of SOD might be a particularly effective means of preventing lung injury.

The present study also showed a dose response for the protective effect AEOL 10150. Only AEOL 10150 at higher dosage of 10 or 30 mg/kg/day after RT for longer period led to significantly reduced lung damage, inflammation, oxidative stress and tissue hypoxia. SOD can be delivered in a reasonable dosing regimen that can attenuate lung dysfunction and pathology associated with animal models of RT and bleomycin induced pulmonary fibrosis [41,51]

In conclusion the prolonged administration of the novel catalytic antioxidant, AEOL 10150, after RT demonstrates a significant protective effect from RT-induced lung injury. AEOL 10150 appears to exert its primary therapeutic impact by influencing the cascade of events following irradiation. Giving the drug prior to and for a short time after RT had no significant benefits. Such findings support the concept that catalytic antioxidants that possess substantial SOD activity may be useful as radioprotective agents based on their ability to effectively scavenge ROS and reduce inflammation when administered chronically.

Acknowledgements

Supported by National Cancer Institute Grant R44 CA96245, ROI CA098452 and Aeolus Pharmaceuticals.

References

1. Stone HB, McBride WH, Coleman CN. Modifying normal tissue damage postirradiation. Report of a workshop sponsored by the Radiation Research Program, National Cancer Institute, Bethesda, Maryland September 6-8, 2000. *Radiation Research* 2002;157:204–223. [PubMed: 11835685]
2. Riley PA. Free radicals in biology: Oxidative stress and the effects of ionizing radiation. *International Journal of Radiation Biology* 1994;65:27–33. [PubMed: 7905906]
3. Rubin P, Finkelstein J, Shapiro D. Molecular biology mechanisms in the radiation induction of pulmonary injury syndromes: interrelationship between the alveolar macrophage and the septal fibroblast. *Int J Radiat Oncol Biol Phys* 1992;24:93–101. [PubMed: 1512168]
4. Rubin P, Johnston CJ, Williams JP, et al. A perpetual cascade of cytokines postirradiation leads to pulmonary fibrosis. *Int J Radiat Oncol Biol Phys* 1995;33:99–109. [PubMed: 7642437]
5. McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *Journal of Biological Chemistry* 1969;244:6049–6055. [PubMed: 5389100]
6. McCord JM, Fridovich I. The utility of superoxide dismutase in studying free radical reactions. I. Radicals generated by the interaction of sulfite, dimethyl sulfoxide, and oxygen. *Journal of Biological Chemistry* 1969;244:6056–6063. [PubMed: 4981789]
7. Fridovich I. Superoxide dismutases. *Annual review of biochemistry* 1975;44:147–159.

8. Okado-Matsumoto A, Fridovich I. Subcellular distribution of superoxide dismutases (SOD) in rat liver. Cu,Zn-SOD in mitochondria. *Journal of Biological Chemistry* 2001;276:38388–38393. [PubMed: 11507097]
9. Fattman CL, Schaefer LM, Oury TD. Extracellular superoxide dismutase in biology and medicine. *Free Radical Biology and Medicine* 2003;35:236–256. [PubMed: 12885586]
10. Noor R, Mittal S, Iqbal J. Superoxide dismutase - Applications and relevance to human diseases. *Medical Science Monitor* 2002;8
11. Malaker K, Das RM. Effect of superoxide dismutase on early radiation injury of lungs in the rat. *Molecular and Cellular Biochemistry* 1988;84:141–145. [PubMed: 3231220]
12. Breuer R, Tochner Z, Conner MW, et al. Superoxide dismutase inhibits radiation-induced lung injury in hamsters. *Lung* 1992;170:19–29. [PubMed: 1732683]
13. Epperly MW, Bray JA, Krager S, et al. Intratracheal injection of adenovirus containing the human MnSOD transgene protects athymic nude mice from irradiation-induced organizing alveolitis. *International Journal of Radiation Oncology Biology Physics* 1999;43:169–181.
14. Stickle RL, Epperly MW, Bray JA, et al. Prevention of irradiation-induced esophagitis by plasmid/liposome delivery of the human manganese superoxide dismutase transgene. *Radiation Oncology Investigations* 1999;7:204–217. [PubMed: 10492161]
15. Epperly M, Bray J, Kraeger S, et al. Prevention of late effects of irradiation lung damage by manganese superoxide dismutase gene therapy. *Gene Therapy* 1998;5:196–208. [PubMed: 9578839]
16. Epperly MW, Epstein CJ, Travis EL, et al. Decreased pulmonary radiation resistance of manganese superoxide dismutase (MnSOD)-deficient mice is corrected by human manganese superoxide dismutase-plasmid/liposome (SOD2-PL) intratracheal gene therapy. *Radiation Research* 2000;154:365–374. [PubMed: 11023599]
17. Rabbani ZN, Anscher MS, Folz RJ, et al. Overexpression of extracellular superoxide dismutase reduces acute radiation induced lung toxicity. *BMC Cancer* 2005;5:59. [PubMed: 15949035]
18. Kang SK, Rabbani ZN, Folz RJ, et al. Overexpression of extracellular superoxide dismutase protects mice from radiation-induced lung injury. *Int J Radiat Oncol Biol Phys* 2003;57:1056–1066. [PubMed: 14575837]
19. Ferrer-Sueta G, Vitturi D, Batinic-Haberle I, et al. Reactions of manganese porphyrins with peroxynitrite and carbonate radical anion. *J Biol Chem* 2003;278:27432–27438. [PubMed: 12700236]
20. Ferrer-Sueta G, Hannibal L, Batinic-Haberle I, et al. Reduction of manganese porphyrins by flavoenzymes and submitochondrial particles and the catalytic redox cycle of peroxynitrite. *Free Radic Biol Med*. 2006Submitted
21. Batinic-Haberle I, Spasojevic I, Stevens RD, et al. New class of potent catalysts of O₂.-dismutation. Mn(III) ortho-methoxyethylpyridyl- and di-ortho-methoxyethylimidazolylporphyrins. *Dalton Trans* 2004:1696–1702. [PubMed: 15252564]
22. Bloodworth A, O'Donnell VB, Batinic-Haberle I, et al. Manganese-porphyrin reactions with lipids and lipoproteins. *Free Radic Biol Med* 2000;28:1017–1029. [PubMed: 10832063]
23. Batinic-Haberle I, Spasojevic I, Stevens RD, et al. New PEG-ylated Mn(III) porphyrins approaching catalytic activity of SOD enzyme. *Dalton Trans* 2006:617–624. [PubMed: 16402149]
24. Kachadourian R, Johnson CA, Min E, et al. Flavin-dependent antioxidant properties of a new series of meso-N,N'-dialkyl-imidazolium substituted manganese(III) porphyrins. *Biochem Pharmacol* 2004;67:77–85. [PubMed: 14667930]
25. Ashcroft T, Simpson JM, Timbrell V. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *J Clin Pathol* 1988;41:467–470. [PubMed: 3366935]
26. Vujaskovic Z, Anscher MS, Feng QF, et al. Radiation-induced hypoxia may perpetuate late normal tissue injury. *Int J Radiat Oncol Biol Phys* 2001;50:851–855. [PubMed: 11429211]
27. Kim SJ, Rabbani ZN, Dewhirst MW, et al. Expression of HIF-1 α , CA IX, VEGF, and MMP-9 in surgically resected non-small cell lung cancer. *Lung Cancer* 2005;49:325–335. [PubMed: 15935515]
28. Kim SJ, Rabbani ZN, Vollmer RT, et al. Carbonic anhydrase IX in early-stage non-small cell lung cancer. *Clin Cancer Res* 2004;10:7925–7933. [PubMed: 15585626]

29. Griendling KK, Ushio-Fukai M. Reactive oxygen species as mediators of angiotensin II signaling. *Regulatory Peptides* 2000;91:21–27. [PubMed: 10967199]
30. Schieffer B, Luchtefeld M, Braun S, et al. Role of NAD(P)H oxidase in angiotensin II-induced JAK/STAT signaling and cytokine induction. *Circulation Research* 2000;87:1195–1201. [PubMed: 11110778]
31. Schmidt-Ullrich RK, Dent P, Mikkelsen RB, et al. Signal transduction and cellular radiation responses. *Radiation Research* 2000;153:245–257. [PubMed: 10669545]
32. Stewart FA. Keynote address: Modification of normal tissue response to radiotherapy and chemotherapy. *International Journal of Radiation Oncology Biology Physics* 1989;16:1195–1200.
33. Zhang X, Lai PP, Taylor YC. Differential radioprotection of cultured human diploid fibroblasts and fibrosarcoma cells by WR1065. *International Journal of Radiation Oncology Biology Physics* 1992;24:713–719.
34. Quinones HI, List AF, Gerner EW. Selective exclusion by the polyamine transporter as a mechanism for differential radioprotection of amifostine derivatives. *Clinical Cancer Research* 2002;8:1295–1300. [PubMed: 12006551]
35. Petkau A. Radiation protection by superoxide dismutase. *Photochemistry and Photobiology* 1978;28:765–774. [PubMed: 366641]
36. Petkau A, Chelack WS. Radioprotection by superoxide dismutase of macrophage progenitor cells from mouse bone marrow. *Biochemical and Biophysical Research Communications* 1984;119:1089–1095. [PubMed: 6712667]
37. Petkau A, Chelack WS, Pleskach SD. Protection by superoxide dismutase of white blood cells in x-irradiated mice. *Life Sciences* 1978;22:867–881. [PubMed: 642702]
38. Epperly MW, Defilippi S, Sikora C, et al. Intratracheal injection of manganese superoxide dismutase (MnSOD) plasmid/liposomes protects normal lung but not orthotopic tumors from irradiation. *Gene Therapy* 2000;7:1011–1018. [PubMed: 10871749]
39. Delanian S, Baillet F, Huart J, et al. Successful treatment of radiation-induced fibrosis using liposomal Cu/Zn superoxide dismutase: Clinical trial. *Radiotherapy and Oncology* 1994;32:12–20. [PubMed: 7938674]
40. Lefaix JL, Delanian S, Leplat JJ, et al. Successful treatment of radiation-induced fibrosis using Cu/Zn-SOD and Mn-SOD: An experimental study. *International Journal of Radiation Oncology Biology Physics* 1996;35:305–312.
41. Vujaskovic Z, Batinic-Haberle I, Rabbani ZN, et al. A small molecular weight catalytic metalloporphyrin antioxidant with superoxide dismutase (SOD) mimetic properties protects lungs from radiation-induced injury. *Free Radic Biol Med* 2002;33:857–863. [PubMed: 12208373]
42. Ward PA, Hunninghake GW. Lung inflammation and fibrosis. *Am J Respir Crit Care Med* 1998;157:S123–129. [PubMed: 9563771]
43. Hagiwara SI, Ishii Y, Kitamura S. Aerosolized administration of N-acetylcysteine attenuates lung fibrosis induced by bleomycin in mice. *Am J Respir Crit Care Med* 2000;162:225–231. [PubMed: 10903246]
44. MacNee W, Rahman I. Oxidants/antioxidants in idiopathic pulmonary fibrosis. *Thorax* 1995;50 (Suppl 1):S53–58. [PubMed: 7570466]
45. Johnston CJ, Williams JP, Okunieff P, et al. Radiation-induced pulmonary fibrosis: examination of chemokine and chemokine receptor families. *Radiat Res* 2002;157:256–265. [PubMed: 11839087]
46. Bowler RP, Sheng H, Enghild JJ, et al. A catalytic antioxidant (AEOL 10150) attenuates expression of inflammatory genes in stroke. *Free Radic Biol Med* 2002;33:1141–1152. [PubMed: 12374626]
47. Smith KR, Uyeminami DL, Kodavanti UP, et al. Inhibition of tobacco smoke-induced lung inflammation by a catalytic antioxidant. *Free Radic Biol Med* 2002;33:1106–1114. [PubMed: 12374622]
48. Tse HM, Milton MJ, Piganelli JD. Mechanistic analysis of the immunomodulatory effects of a catalytic antioxidant on antigen-presenting cells: Implication for their use in targeting oxidation-reduction reactions in innate immunity. *Free Radical Biology and Medicine* 2004;36:233–247. [PubMed: 14744635]

49. Rugo RE, Secretan MB, Schiestl RH. X radiation causes a persistent induction of reactive oxygen species and a delayed reinduction of TP53 in normal human diploid fibroblasts. *Radiation Research* 2002;158:210–219. [PubMed: 12105992]
50. Clutton SM, Townsend KMS, Walker C, et al. Radiation-induced genomic instability and persisting oxidative stress in primary bone marrow cultures. *Carcinogenesis* 1996;17:1633–1639. [PubMed: 8761419]
51. Oury TD, Thakker K, Menache M, et al. Attenuation of bleomycin-induced pulmonary fibrosis by a catalytic antioxidant metalloporphyrin. *Am J Respir Cell Mol Biol* 2001;25:164–169. [PubMed: 11509325]

Figure 1.

Figure 1(A) Experimental Design: Animals were randomly divided into four duplicate groups for short (one day before through one week following irradiation) and long-term (from one day after through 10 weeks post-radiation) drug administration experiments. AEOL 10150 was delivered as a continuous infusion with an osmotic pump implanted subcutaneously, at three different concentrations of 1, 10 and 30 mg/kg/day.

Figure 1(B) Plasma concentrations of AEOL 10150 at 7th week of infusion. AEOL 10150 was delivered as a continuous infusion with an osmotic pump implanted subcutaneously, at three different concentrations of 1, 10 and 30mg/kg/day. Significant steady state plasma levels of

AEOL 10150 were achieved at both the 10 and 30mg/kg/day treatments. Error bars represent $\pm$ SEM.

Figure 2.

All groups exhibited steady gain in body weight throughout the treatment period. Both the RT +PBS and RT+ 1mg/kg/dayAEOL 10150 animals, however, exhibited significantly less weight gain beginning at week 14 compared with RT+10 or 30 mg/kg/day AEOL 10150 groups (*RT +PBS and RT+1mg/kg/day vs. RT+10mg/kg/day at 14-20 weeks, # $p < 0.05$, RT+PBS and RT +1mg/kg/day vs. RT+30mg/kg/day at 16-20 weeks, * $p < 0.05$, respectively*). Error bars represent $\pm$ SEM.

Figure 3.

Time course of breathing rates after 28 Gy of single dose irradiation to the right hemithorax with or without AEOL 10150 treatment at dose of 1, 10 and 30 mg/kg. Breathing rates increased beginning at 8 weeks and reached a peak 18 weeks after irradiation in animals treated with PBS and 1mg AEOL 10150. The average breathing rates in irradiated groups treated with AEOL 10150 (10 or 30mg/kg/day) were significantly lower than the group receiving RT+PBS and RT+1mg/kg/day (*RT+PBS and RT+1mg/kg/day vs. RT+10mg/kg/day at 12-20 weeks, # $p < 0.05$, RT+1mg and RT+10mg/kg/day vs. RT+30mg/kg/day at 12-20 weeks, * $p < 0.05$, respectively*). Error bars represent $\pm$ SEM.

B)

Figure 4.

Figure. 4(A) Histologic comparison by hematoxylin and eosin staining among RT+PBS, RT +1mg mg/kg/day and RT+10 or 30mg/kg/day of AEOL 10150 at 20 weeks after 28 Gy of irradiation. Rats treated with 10 or 30 mg/kg/day AEOL 10150 after single dose irradiation showed less pulmonary damage than rats received irradiation plus PBS or AEOL 10150 1mg/kg/day.

Figure. 4 (B) Semiquantitative analyses of lung histology at 20 weeks revealed a significant decrease in structural damage and its severity in animals receiving 10 and 30 mg/kg/day after radiation in comparison to RT+PBS group or RT+1mg/kg/day (*RT+10 and 30mg/kg/day vs. RT+PBS and RT+1mg/kg/day AEOL 10150, $p=0.01$*).

Figure. 4(C) RT+PBS and RT+1mg/kg/day AEOL 10150 groups had greater number of focal areas with increased collagen deposition than did the RT+10 or 30 mg/kg/day AEOL 10150 groups. This translated into a higher lung fibrosis score for the RT+PBS and RT+1mg AEOL 10150 animals than the other groups, although no animals were found to have strong or intense staining for collagen (*RT+PBS vs. RT+10 and 30mg/kg/day, * $p=0.003, 0.005$ respectively: RT+1mg/kg/day vs. RT+10 and 30mg/kg/day, # $p=0.002, 0.003$ respectively*). Error bars represent $\pm$ SEM.

(A)

Figure 5.

Figure. 5(A) *Panel of ED-1, 8-OHdG and CA9 immunohistochemistry:* Lung tissues were immunostained for macrophage activation (ED1), oxidative stress (8OHdG) and tissue hypoxia (CA 9) (brown staining), in RT+PBS, RT+1mg AEOL 10150, RT+10or 30 mg/kg/day AEOL 10150 groups. The lungs of rats treated with high-doses of AEOL 10150 (10 or 30 mg/kg/day) exhibited much fewer activated macrophages, less oxidative stress and less hypoxia than rats treated with RT+PBS and RT+1 mg/kg/day AEOL 10150. These photomicrographs are representative of results obtained from 6-8 animals in each group. For ED1 and 8OHdG, each image is 400× magnification and CA9 100x.

Figure. 5(B) Semi quantitative analysis of activated macrophages in the lungs of rats 20 weeks after 28 Gy of single dose irradiation with or without AEOL 10150 treatment. RT+PBS and RT+1 mg/kg/day AEOL 10150 groups had significantly more activated macrophages per field of lung tissue than high-doses (RT+PBS vs. RT+10 and 30mg/kg/day, * $p=0.0006$, 0.016 respectively; RT+1mg/kg/day vs. RT+10mg/kg/day, # $p=0.02$). There was no significant difference between group receiving RT+1mg/kg/day AEOL 10150 compared with RT+PBS and RT+30mg/kg/day ($p > 0.05$). Error bars represent $\pm$ SEM.

Figure. 5(C) Semi quantitative analysis for the expression of 8-OHdG marker of DNA oxidation and oxidative stress, after 28 Gy of single dose irradiation. A significant reduction in the expression of DNA oxidation was seen in radiation plus high-dose AEOL 10150 treatment groups (RT+PBS vs. RT+10 and 30mg/kg/day, * $p=0.006$, 0.014 respectively; RT+1mg/kg/day vs. RT+10mg/kg/day and 30mg/kg/day, # $p=0.004$, 0.009 respectively). Error bars represent $\pm$ SEM.

Figure. 5(D) RT+PBS and RT+1mg/kg/day AEOL 10150 animals expressed strong CA 9 immunoreactivity, which was seen mainly in the irradiated, damaged tissue and inflammatory cell. The tissue protein expression of CA 9 was markedly reduced in the groups that received radiation plus high doses of AEOL 10150 (10 and 30 mg/kg/day) (RT+PBS vs. RT+10 and 30mg/kg/day, * $p=0.001$, 0.0009 respectively; RT+1mg/kg/day vs. RT+10mg/kg/day and 30mg/kg/day, # $p=0.00001$, 0.00005 respectively). Error bars represent $\pm$ SEM.