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A MALDI-MSI approach to the characterization of radiation-induced lung injury and medical countermeasure development

Claire L. Carter^{*}, Jace W. Jones^{*}, Kory Barrow[†], Kaitlyn Kieta[†], Cheryl Taylor-Howell[†], Sean Kearney[†], Cassandra P. Smith[†], Allison Gibbs[†], Ann M. Farese[†], Thomas J. MacVittie[†], and Maureen A. Kane^{*}

^{*}University of Maryland, School of Pharmacy, Department of Pharmaceutical Sciences

[†]University of Maryland, School of Medicine, Department of Radiation Oncology, Baltimore, MD 21201

Abstract

Radiation-induced lung injury is highly complex and characterized by multiple pathologies, which occur over time, and sporadically throughout the lung. This complexity makes biomarker investigations and medical countermeasure screenings challenging. Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) has the ability to spatially resolve differences in molecular profiles within the lung following radiation exposure and can aid in biomarker identification and pharmaceutical efficacy investigations. MALDI-MSI was applied to the investigation of a whole-thorax lung irradiation model in non-human primates (NHP) for lipidomic analysis and medical countermeasure distribution.

Keywords

Biological indicators; imaging; radiation damage; respiratory system

Introduction

In 2004, the Radiation Nuclear Countermeasures Program (RNCP) was established through the National Institute of Allergy and Infectious Diseases (NIAID), of the National Institute of Health (NIH). The U.S Government financed program was tasked with providing funding in areas of basic and translational research to aid in the development of medical countermeasures (MCMs) in the event of a nuclear or terrorist attack (Rios *et al.* 2014). In order to develop MCMs, a clearer understanding of the mechanisms of action (MoA) of radiation-induced injury is needed, as currently these mechanisms remain ill-defined. Additionally, as human studies cannot be conducted, due to ethical reasons, medical countermeasures must be developed and approved under the FDA Animal Rule (FDA-AR) (Singh *et al.* 2015).

Correspondence: Maureen A. Kane, Ph.D, Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, 20 N. Pine Street, Room N706, Baltimore, MD 21201, Phone: 410-706-5097, Fax: 410-706-5017, mkane@rx.umaryland.edu.

Conflicts of Interest:

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Exposure to radiation results in dose- and time-dependent organ injury spanning the hematopoietic (H), gastrointestinal (GI) and central nervous systems (CNS), collectively known as the acute radiation syndromes (ARS). Those that survive the potentially lethal ARS go on to develop lung injury, a known delayed effect of acute radiation exposure (DEARE), as well as multi-organ injury, also termed radiation-induced multiple organ dysfunction syndrome (MODS) (Williams and McBride 2011).

During the past decade the RNCP funded, Medical Countermeasures Against Radiological Threats (MCART) consortium, has developed non-human primate (NHP) and murine models to study the acute and delayed radiation syndromes. MCART has established dose-response ranges and survival curves for H-ARS and GI-ARS following total body irradiation(Booth *et al.* 2012, Farese *et al.* 2012, MacVittie *et al.* 2012, Plett *et al.* 2012), lung-DEARE (a collaborative effort with the Biomedical Advanced Research and Development Authority (BARDA)) following whole-thorax lung irradiation(Jackson *et al.* 2012, Garofalo *et al.* 2014, Jackson *et al.* 2014) and multi-organ syndrome studies following partial-body irradiation(Booth *et al.* 2012, MacVittie *et al.* 2012, MacVittie *et al.* 2014), in both mouse and NHP models. The established models fulfill the requirements of the FDA-AR and medical countermeasure efficacy studies began in 2006(Farese *et al.* 2012, Farese *et al.* 2013, Chua *et al.* 2014). The FDA has recently approved the use of Neupogen® for the treatment of patients acutely exposed to myelosuppressive doses of radiation (Hematopoietic Syndrome of Acute Radiation Syndrome), based in part, on the large animal study performed within the consortium (Food and Drug Administration. FDA Advisory Committee Briefing Document: Safety and Efficacy of Currently Approved Leukocyte Growth factors (LGFs) as Potential Treatments for Radiation-induced Myelosuppression Associated with a Radiological/Nuclear Incident. 1–186; (Farese *et al.* 2013)).

As part of MCARTs continued effort to evaluate new compounds for efficacy, a study was designed to evaluate the therapeutic potential of an antioxidant, AEOL 10150, to mitigate radiation-induced lung injury. Long-term administration of the superoxide dismutase mimetic AEOL 10150 demonstrated a significant protective effect in a rodent model following lung irradiation(Rabbani *et al.* 2007). Recent results from a pilot NHP study performed within the MCART consortium demonstrated efficacy of AEOL 10150 to reduce radiation-induced pulmonary injury(Garofalo *et al.* 2014). Therefore, the current study evaluating optimal dosing schedule of AEOL 10150 in the NHP WTLI model of lung-DEARE was performed.

Although categorized into the earlier presentation of pneumonitis and the later onset of fibrosis, radiation-induced lung injury is highly complex and a combination of multiple pathologies, which occur over time and sporadically throughout the lung(Tsoutsou and Koukourakis 2006). These may include pleural effusion, edema, vascular leakage, inflammation, pneumonitis, and fibrosis(Rabbani *et al.* 2010, Williams *et al.* 2010, Jackson *et al.* 2011). Pulmonary fibrosis is irreversible scarring to the interstitium and, to date, there are no early diagnostic markers or effective therapeutic options(King Jr *et al.*, Ahluwalia *et al.* 2014). Molecular and histological investigations into radiation-induced lung injury are problematic because of the invasive nature of tissue sampling, and the complex nature of the disease, in which areas of heterogeneous tissue damage reside alongside normal tissue.

One technique that can greatly contribute to deciphering and understanding the MoA of radiation-induced tissue damage is matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI). This molecular imaging technique has been applied to the investigation of lipids, metabolites and proteins in a plethora of pathophysiological processes (Carter *et al.* 2011, Palmer *et al.* 2012, Steven and Bunch 2013, Berry *et al.* 2014, Djidja *et al.* 2014, Jackson *et al.* 2014, Jones *et al.* 2014, Manier *et al.* 2014, Seeley *et al.* 2014, Shariatgorji *et al.* 2014). By applying mass spectrometry to region-specific anatomical tissue architecture, MALDI-MSI offers another dimension in characterizing the natural history and development of these pathologies. The result is a powerful tool to aid in unraveling the complexity of damage and dysregulated repair pathways that are a result of radiation insult. Another unique aspect to MALDI-MSI is the ability of the technique to detect and localize drug compounds and their active metabolites, in organ sections and in whole-body animal sections, for toxicology and ADME investigations (Solon *et al.* 2010, Castellino *et al.* 2011, Prideaux and Stoeckli 2012).

The last decade has seen a huge shift in focus regarding the “omics” investigations into the pathogenesis of disease, from a mainly genomic and proteomic drive, to that of a lipidomic and metabolomic endeavor. Advancements in analytical technology and instrumentation have enabled more robust and sensitive methods for detection and identification of these small molecules, leading to a greater understanding of their involvement in disease pathology (Schmelzer *et al.* 2007, Loizides-Mangold 2013, O'Donnell *et al.* 2014). First coined a little over a decade ago, lipidomics is defined as the analysis of lipids in a given system, their molecular functions, and their interacting molecules (Kishimoto *et al.* 2001, Loizides-Mangold 2013). A profound body of literature has proven these molecules useful as markers for cell viability and integrity, biomarkers for disease and disease progression, and tumor grading (Blaho and Hla 2011, Fuchs *et al.* 2012, Doria *et al.* 2014, Eberlin *et al.* 2014, Zhang and Wakelam 2014). Lipids or proteins modifying and/or metabolizing lipids have been implicated in numerous pulmonary diseases, including cancers, cystic fibrosis, asthma, infections, and chronic obstructive pulmonary disease (Becker *et al.* 2011, Donnelly 2014, Guo *et al.* 2014, Kang *et al.* 2014, Telenga *et al.* 2014).

There is currently limited information on the spatial distribution of biologically active small molecules in the lung in a physiological state (Sparvero *et al.* 2012, Berry *et al.* 2014) and in the numerous pathologies that present following radiation insult, and the devastating pneumonitis and fibrosis that develop. Work presented in this study documents lipidomic distribution and dysregulation in the lung following whole-thorax lung irradiation, as well as the detection and localization of AEOL 10150 within the lung tissue following irradiation and treatment.

Material and Methods

Materials

Analytical grade solvents, isopropanol (IPA), tetrahydrofuran (THF), methanol (MeOH), and water were purchased from Fisher Scientific (Pittsburgh, PA, USA). AEOL 10150 (Manganese (III) meso-tetrakis(N, N'-diethylimidazolium-2-yl)porphyrin) was obtained from Aeolus Pharmaceuticals Inc. (Mission Viejo, CA, USA). 9-AA was purchased from

Sigma-Aldrich (St. Louis, MO, USA). DHB was purchased from Acros (New Jersey, USA). ITO-coated glass slides were obtained from Bruker Daltonics (Bruker Daltonics, Bremen, Germany). Hematoxylin and eosin (H&E) staining kit was purchased from Thermo Scientific (San Jose, CA, USA).

Animals

All animal procedures were conducted in accordance with the NIH guidelines for the care and use of laboratory animals and experiments were performed with prior approval from the University of Maryland Institutional Animal Care and Use Committee (IACUC). Details of animal acclimation, housing, care and whole-thorax lung irradiation have been described in detail previously (Garofalo *et al.* 2014). Briefly, male rhesus macaques (*Macaca mulatta*, Chinese origin) were assigned to 1 of 4 cohorts encompassing a control (irradiated no drug treatment) and 3 experimental AEOL 10150-treated groups, to define the optimal administration schedule. AEOL 10150 was administered subcutaneously at 5 mg/kg daily as previously described (Garofalo *et al.* 2014); the dosing schedule for each cohort is out of the scope of this paper and will not be discussed. NHP were irradiated to the whole thorax at a midline tissue dose of 10.74 Gy, at a dose rate of $0.80 \pm 0.05 \text{ Gy min}^{-1}$. This was previously determined as an LD_(70/180). Animals were monitored twice daily by trained staff at least 6 h apart. Baseline and in-study CT scans for surveillance and assessment of radiation-induced lung injury were performed every 30 (± 5) days until the planned end of study [180 (± 5) days] or until euthanasia if the animal met the IACUC approved criteria prior to the end of study. Upon euthanasia lungs were excised and multiple samples were collected for MALDI-MSI. Samples were frozen in liquid nitrogen and stored at -80°C until analysis.

Computed Tomography

All computed tomography (CT) images were acquired using a GE Lightspeed 2.X multi-slice CT scanner (GE Medical Systems, Inc, 3000 Grandview Boulevard, Waukesha, WI 53188) without the aid of contrast media while NHP were anesthetized and secured in supine restraints. Scans were analyzed using the MIM software suite (MIM® Software Inc., 25200 Chagrin Boulevard, Suite 200, Cleveland, OH 44122) as described previously (Garofalo *et al.* 2014). Briefly, quantification of radiation-induced lung injury was determined using Hounsfield units (HU), in which each voxel is assigned a value based on the characteristic radiodensity scale of air (-1000) to bone ($+1000$), with water being 0. Volumes of pneumonitis/fibrosis (PF) were then calculated by subtracting normal lung volume from total lung volume using the MIM software's Boolean operations. Pleural effusions (PE) were identified by characteristic opacification and HU values close to 0.

Tissue Preparation for Mass Spectrometry

Lung samples from 2 different naïve (non-irradiated) and 3 different irradiated NHP (assigned 08094381, 08052001, and 098219) taken 180 days post-radiation, were removed from the -80°C freezer, transferred to a cryostat chamber and allowed to equilibrate for 1 hour prior to sectioning. Samples were mounted onto the cryostat chuck using water-ice slush. Frozen sections were taken at 10 μm using a Microm HM550 cryostat (Thermo Fisher

Scientific, MA, USA), thaw-mounted onto indium tin oxide-coated conductive glass slides (Bruker Daltonics, Bremen, Germany), and stored at -80°C until analysis. On the day of analysis the slides were removed from the freezer and dried in a vacuum desiccator for 20 minutes prior to matrix deposition. For high resolution imaging experiments of the AEOL 10150 compound within lung tissue following irradiation, lung biopsy sections were inflated using an optimized method (manuscript in preparation), embedded in gelatin on dry ice and processed as described above. Technical replicates ($n=3$) were carried out on select samples to ensure reproducibility of matrix deposition along with instrument stability.

Matrix Deposition

For negative ion mode analysis 9-AA at 10 mg/mL in IPA:THF:water (4.5:4.5:1, v/v/v) was deposited over the slides in 3 horizontal passes using the HTX Sprayer, (HTX Technologies, North Carolina, USA). Spray parameters were; flow rate - 150 $\mu\text{L}/\text{min}$, pressure - 10 psi, track spacing - 3 mm, velocity - 1250 mm/min, nozzle temperature - 65°C , and dry time - 0.5 min. For positive ion mode analysis, DHB at 20 mg/mL in 70% MeOH was deposited over the tissue in 24 cycles using the HTX Sprayer, flow rate was 80 $\mu\text{L}/\text{min}$, 10 psi, 3 mm track spacing and 1333 velocity, the nozzle temperature was maintained at 70°C and deposition was alternating horizontal and vertical passes.

Mass Spectrometry Imaging

Imaging experiments were performed using an UltrafleXtreme TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a smartbeam II Nd:YAG laser, 355 nm, operated with the medium spot size ($\sim 50\text{ }\mu\text{m}$) at 1 kHz. The instrument was operated within the mass range of m/z 200–1000 for negative ion mode and m/z 300–2000 for positive ion mode. Images were acquired at a spatial resolution of 100 μm summing 300 laser shots per position using the random walk setting. For high resolution imaging experiments the laser was operated with the minimum spot size at a repetition rate of 1 kHz, acquisition was in the positive ion mode at a resolution of 20 μm , summing 200 laser shots per position. External calibration was performed using a lipid/peptide standard mix; internal calibration was performed using known matrix and lipid masses. For positive ion mode calibration was performed using DHB $[\text{M}+\text{H}]^{+}$ and $[\text{M}+\text{Na}]^{+}$ ions, and the $[\text{M}+\text{H}]^{+}$ ions of LysoPC (16:0), PC 32:0), angiotensin I, angiotensin II, substance P and bombesin. Negative ion mode calibration was performed using the $[\text{M}-\text{H}]^{-}$ ions of 9-AA, cholesterol sulfate, PI(34:1), PI(36:2) and PI(38:4). For MS/MS analysis LIFT was carried out on selected ions with a mass window of $\pm 1\text{ Da}$. Identification was based on a combination of fragmentation data, literature and database searches using LipidMaps and Metlin. Following analysis sections were washed with solvents to remove the matrix and stained with hematoxylin and eosin (H&E). Slides were then scanned using the Aperio slide scanner (Leica Microsystems Inc., IL, USA) and images were co-registered in FlexImaging version 4.0, (Bruker Daltonics, Bremen, Germany) for analysis.

Results and Discussion

MALDI-MSI analysis of lipids in the lung 180 days post radiation

Results from the CT images taken prior to euthanasia from the 3 NHP used for comparison in this study show differences in the degree of injury for each NHP, as shown in figure 1. As these are not high resolution computed tomography (HRCT) images the exact type or characteristic lung presentations cannot be discerned, thus the regions are divided into damaged and normal, based on their opacities and Hounsfield units. All CT findings of lung damage were assigned pneumonitis/fibrosis, or PF. Pneumonitis/fibrosis was more severely documented in NHP 08094381 shown in figure 1A, followed by NHP 08052001 figure 1B, NHP 098219 presented with the least damage on CT as shown in figure 1C.

Lipidomic analysis of the lung sections revealed differences in lipid profiles following radiation insult. An increase in intensity was observed for the ion at m/z 465 in NHP 08094381, as demonstrated in figure 2B, compared to the naïve samples (to avoid repetition only one naïve imaging data set is shown) and the other IR samples (08052001 and 098219) analyzed, figure 2A, C and D respectively. For structural elucidation this ion was subject to tandem MS investigations and was then identified as cholesterol sulfate, based on the detection and abundance of the fragment ion at m/z 97, attributed to $[\text{HSO}_4]^-$, combined with a LipidMaps database search of the precursor ion. As these sections were analyzed on separate slides an image of a separate lung section analyzed from NHP 08093931 taken 101 days post-IR, which was prepared and analyzed on the same slide and same day as NHP 08094381, is shown in supplementary figure 1. The need to run the sections on the same slide to avoid variability caused by different amounts of matrix solution being deposited on different slides is not something of concern using an automated deposition device, if used by an experienced user, and combined with the use of technical replicates. This figure is included to give the reader an additional perspective of the increase in intensity observed for cholesterol sulfate in this lung section, as the lung section from NHP 08093931 showed normal lung anatomy and cholesterol sulfate intensities similar to those observed from the naïve samples analyzed to date.

Several ions were detected in the lungs following radiation insult that were not detected in the naïve lung sections and IR 098219 section, which demonstrated no obvious histological damage, and was taken from the NHP that presented with less damage on CT. The ions at m/z 498 and 509 were detected in two of the irradiated lung samples analyzed, samples 08094381 and 08052001. Furthermore, as these ions have not been detected in irradiated samples that demonstrate minimal-to-no injury on histological analysis, as demonstrated by the absence of a signal for these ions in figure 2C (NHP098219), they are believed to be injury specific ions. The ions at m/z 498 and 509 are displayed in figure 2B and C, for NHP 08094381 and 098219 respectively. Upon comparison to histology it can be seen that these ions show increased intensity in areas of lung parenchyma that contain a substantial amount of immune cell infiltration within the alveolar space, as can be seen by comparing the ions at m/z 498 and 509 in figures 2B and C, with their respective H&E stained section (figure 2). These ions were not detected in the samples analyzed from IR 098219, which showed clear alveoli space and normal appearing lung parenchyma, as demonstrated by figure 2D and the

respective histological section for this sample. The distribution of these and another IR-specific ion at m/z 507 is further demonstrated in figure 3 for NHP 08094381, which shows the MSI images of m/z 498, 507 and 509 in figure 3A–C, respectively, the H&E section with a magnified region showing the immune infiltrations correlate to the regions of high intensity for these ions (figure 3D), followed by an overlaid image of the ion at m/z 498 and the H&E section, figure 3E. Example spectra demonstrating sample quality and lipid species away from the infiltrate region in shown in figure 3F, along with a zoomed in region of a spectrum taken from the immune infiltration region, showing the ions presented in figures 3A–C. These lipids are yet to be definitively identified; however, as these signals appear to be localized to regions of immune infiltration they may be useful as markers of inflammation.

Whilst m/z 715 and 794 were not detected from IR 08052001, as shown in figure 2C compared to the other samples analyzed, this is not a result of radiation injury. The lack of signal for these ions is due to differences in the anatomical features within this section compared to the other sections investigated. This highlights the extreme care that needs to be taken when analyzing lung biopsy-type samples from humans and larger mammals. On comparison to histology it can be seen that there are bronchi present (notable by the presence of cartilage, as seen by the blueish stained chondrocytes, which support the larger conducting airways) in the naïve, IR 08094381 and IR 098219 samples analyzed, demonstrated by the magnified images of the conductive airways presented in figures 2A, B and D respectively. These ions are distributed along the smooth muscle tissue lining the vessels and bronchi, m/z 715, and the cartilage itself, m/z 794, and thus were not detected in IR 08052001 as these features are not present. To enable the reader easier comparison to their respective histological sections, the images for m/z 794 have the tissue regions analyzed outlined, to show the anatomical localization of this signal and that it does indeed map to the regions of cartilage. Tandem MS investigations of the ions at m/z 715 resulted in fragment ions corresponding the a phosphocholine head-group with a loss of CH₃, at m/z 168, and thus was identified as SM(d18:1/18:0) corresponding to the [M-CH₃][−] ion. Fragmentation studies of m/z 794 identified this ion as C16(OH) sulfatide, based on the abundance of the sulfate ion at m/z 97, and a LipidMaps search of the precursor mass.

The ion at m/z 687 is also shows regional specificity and again the differences in intensity and distribution reflect differences in anatomy and are not a result of radiation injury. Identified as SM(d18:1/16:0), [M-CH₃][−], this lipid also displayed higher relative intensity within the smooth muscle regions surrounding blood vessels and bronchioles. A list of select lipids detected is presented in table 1. An example of LIFT fragmentation and peak assignment for one of the lipids identified is shown in supplementary figure 2.

MALDI-MSI detection and localization of AEOL 10150 in lung tissue

Before tissue imaging studies may be performed on a new compound of interest, sample preparation must be optimized and the ionization characteristics of the drug elucidated. A standard of AEOL10150 was analyzed using a range of matrices and solvent systems to establish the ionization efficiency and characteristics for MALDI-MS investigations. DHB in 70 % methanol was chosen as the matrix and solvent system of choice as this not only

enabled the sensitive detection of the compound but it is also the matrix and solvent system of choice in our laboratory for the analysis of phospholipids in the positive ion mode. Meaning we can obtain information on the spatial localization of the drug compound whilst mapping changes in endogenous lipid and metabolite species. It should be stressed that sometimes this is less desirable as this method may cause ion suppression and thus impact sensitivity; however, this was not the case here. The overarching aim of the analysis of all lung samples was to detect AEOL 10150, if present, as well as endogenous small molecules that could be useful as biomarkers for injury type (diagnostic), severity (prognostic), and mitigation.

AEOL 10150 is a manganese (III) meso-tetrakis(N, N'-diethylimidazolium-2-yl)porphyrin that is formulated as a pentachloride salt, $C_{48}H_{56}Cl_5MnN_{12}$, with a monoisotopic mass of 1032.2574 Da (Garofalo *et al.* 2014). An example spectrum showing the typical ionization pattern detected from the AEOL 10150 standard is shown in figure 4A. A cluster of ions were detected between m/z 1008 and 1325, relating to AEOL 10150, of these the $[M+H]^+$ ion was not detected. Synthetic cationic porphyrins have been shown to produce multiple different ions depending on the ionization source used for analysis, and their structure, metal complex, and counter ions (Batini -Haberle *et al.* 2000, Schäfer and Budzikiewicz 2001, Ramos *et al.* 2008). These can include but are not limited to, deprotonation, one to four electron reduction of the cations, loss of the metal complex, loss of alkyl groups, and matrix adduct/complex formation (MALDI/fast atom bombardment (FAB)) (Batini -Haberle *et al.* 2000, Kachadourian *et al.* 2001, Schäfer and Budzikiewicz 2001, Ramos *et al.* 2008, Ramos *et al.* 2014). The ion at m/z 1008 was the most abundant peak in all spectra acquired and arises from a DHB adduct of the compound without the chloride salts. The structure of AEOL 10150 without the chlorides is shown in figure 4A along with the spectrum of the standard. The ion at m/z 1008 is thus attributed to $[M^{5+}+4e^-+DHB]^+$, with a molecular formula of $C_{55}H_{61}MnN_{12}O_4$ and a theoretical m/z 1008.4293, agreeing previously published ion formations of similar porphyrins (Kachadourian *et al.* 2001, Ramos *et al.* 2008). All additional ions in the spectra are believed to be associated with porphyrin-matrix complexes with varying losses of the ethyl groups. This is further supported by the laser desorption ionization (LDI) spectrum acquired from the same standard (supplementary figure 3A). As expected no matrix-porphyrin adduct/complex peaks were detected in the LDI spectrum, evident by the lack of peaks above m/z 832, compared to the MALDI spectrum of the same sample (supplementary figure 3A and B respectively). Several ions were detected for both ionization methods as demonstrated by peaks at m/z 242, 699, 744, 768, 780 and 832. The elucidation of the gas phase characteristics of AEOL 10150 from desorption/ionization methods is out of the scope of this manuscript thus these ions were not investigated further.

MALDI-MSI investigation of lung tissue detected the AEOL 10150 compound within several lung sections analyzed. Due to the complex nature of tissue many of the lower abundant ions that were detected from the analysis of the AEOL 10150 standard were not detected from tissue. The most abundant ion at m/z 1008 and a lower abundant ion at m/z 1162 were detected during imaging analysis, as shown in the spectrum presented in figure 4B. For further validation MS/MS investigations were carried out on the precursor ions at m/z 1008 and 1162 from the AEOL10150 standard and from tissue regions following

imaging investigations. Example spectra demonstrating the fragmentation pattern of the precursor ion at m/z 1008 detected from the standard and from lung tissue is shown in figure 4C and D respectively. The main product ions arise from the loss of ethyl groups at m/z 979 and 950.

To investigate the localization of AEOL 10150 within lung tissue following irradiation, high resolution imaging experiments were carried out at 20 μm , on an adjacent section of lung tissue. The MS image of AEOL10150, m/z 1008, is shown in the top left of the figure 5, as labeled. The compound shows higher accumulation around the lower portion of the bronchiole and within regions of the respiratory airways, demonstrated by the increased intensity (refer to intensity scale), and correlation to the H&E section. The compound again showed decreased signal in the larger and smaller vessel regions, with little to no signal in the tissue surrounding the larger vessel. The image of heme, m/z 616, was included to highlight the vasculature/blood in order to demonstrate the differential distribution compared to the drug compound, indicating AEOL 10150 passes from the circulation into the target organ. This is easier to visualize in the overlaid or merged ion images of the two compounds presented in the bottom left of the figure, AEOL 10150 is shown in green and heme in red, the intensity scales are presented to the right of the image. Collectively these results demonstrate the SOD mimetic is passing from the circulation into the target tissue.

Discussion

MALDI-MSI has the ability to provide simultaneous label-free localization of molecular changes representative of the different pathologies that can present during the course of radiation injury. To the best of the authors' knowledge, this is the first report documenting changes in the relative abundance of cholesterol sulfate within the lung parenchyma as a result of radiation-insult. In addition to stabilizing cell membranes, cholesterol sulfate has been proven to play a significant regulatory role in a plethora of processes, whose activity includes the regulation of serine proteases, specific protein kinase C isoforms, as well as the modulation of phosphatidylinositol-3-kinase activity(Strott and Higashi 2003). In addition to its enzyme-like properties, cholesterol sulfate is a regulator of gene expression, as demonstrated by a number of publications over the past two decades(Kawabe *et al.* 1998, Hanyu *et al.* 2012, Zenri *et al.* 2012, Shi *et al.* 2014). Of key importance was the publication by Okano *et al.*, 2001, showing cholesterol sulfate induces a conformational change in high-mobility group protein 1 (HMG1), a step thought to be essential for its phosphorylation. HMG1 is an abundant component of chromatin and is involved in the modulation of transcription, DNA integration and re-combination(Okano *et al.* 2001). This suggests increased cholesterol sulfate will likely have a profound effect on gene expression and downstream signaling within lung following irradiation. Several reports have also demonstrated a role of cholesterol sulfate in epithelial differentiation(Rearick *et al.* 1987) and, more recently, MSI investigations have localized the molecule to tumor and pre-tumor regions in prostate tissue samples(Eberlin *et al.* 2010, Cooks *et al.* 2011).

There is currently little-to-no literature on the detection and spatial localization of specific lipid species during an active inflammatory response within the respiratory airways. The detection of lipids specific to immune infiltration within the alveolar space may prove

pivotal in identifying new signaling molecules. Whilst the ions at m/z 498, 507, and 509 are yet to be identified, they fall into the mass range of lysoPLs, DGs and sterol lipids, all of which are involved in regulatory processes. Indeed, these hormone-like molecules are now the focus of many research groups, due to the increasing identification of their receptors and thus knowledge of their regulatory pathways(Fuchs *et al.* 2012, Makide *et al.* 2014). For example, LPA has been shown to mediate fibroblast migration and recruitment as well as increasing vascular permeability in a bleomycin mouse model of fibrosis, highlighting the impact these small molecular weight lipids have on disease progression(Tager *et al.* 2008). Being able to localize specific lipid signatures to activated immune cells is of great importance as prolonged inflammatory insult following irradiation is believed to contribute to the onset of fibrosis. Additionally, the ability to map specific lipid species to distinct inflammatory cell accumulation for the given pathological presentation will enable us build a profile of markers reflective of the phenotype for injury severity, and resolution.

This is, to the best of the authors' knowledge, the first MALDI-MSI report of sulfatide species localized within lung cartilage. Whilst at this stage significant differences between naïve and irradiation samples have not been established, these findings are still of importance. Cartilage provides vital support for bronchi, which would collapse upon exhalation if damaged or weakened, a condition known as bronchomalacia(Austin and Ali 2003). Currently there is no literature pertaining to the effects of radiation on the molecular profile of cartilage, thus it is not known how the chondrocytes and extracellular matrix are affected. There has been a report of bronchomalacia in a patient that received high-dose thoracic radiation therapy, providing evidence that these regions may be damaged as a result of irradiation(Dechambre *et al.* 1998). As diagnosis of bronchomalacia is carried out by bronchoscopy, this would go undetected in laboratory models as it is highly unlikely this type of examination would be performed, thus the ability to detect alterations in molecular profiles across the chondrocytes and extracellular matrix is pertinent.

AEOL 10150 is a small molecule catalytic metalloporphyrin rationally designed to be a superoxide dismutase mimetic(Kachadourian *et al.* 2004), which is being trialed as a therapeutic antioxidant to reduce oxidative stress. Following IR, reactive oxygen species and reactive nitrogen species are known to increase as a result of DNA damage, the inflammatory response that follows further increases the levels of ROS/RNS, resulting in a cyclic cascade of reactive species. By acting as an anti-oxidant, AEOL10150 is believed to scavenge free radicals that accumulate following irradiation, thereby reducing oxidative stress and radiation damage. An in-depth discussion of AEOL 10150 and other SOD mimetic is beyond the scope of this paper, however, the interested readers are directed to the following review articles (Batinic-Haberle *et al.* 2010, Miriyala *et al.* 2012, Batinic-Haberle *et al.* 2014). MALDI-MSI was successful in the detection and localization of AEOL 10150 within the lung tissue following radiation insult. Work presented demonstrated that AEOL 10150 did indeed leave the circulation and pass into the target tissue, that it was localized to specific anatomical areas, and that there were no signs of drug accumulation that would lead to target organ toxicity.

Conclusion

Herein, we have demonstrated the first MSI investigation into the spatial distribution of lipids within lung sections following IR. Additionally, we have shown the detection and localization of AEOL 10150 within lung tissue following treatment. This compound is currently being evaluated by MCART and BARDA for efficacy to mitigate lung-DEARE. Preliminary data identified several lipid species localized to areas of immune infiltration, which may be important markers of inflammation and/or specific immune cell accumulation, and increases in a regulatory lipid, cholesterol sulfate. Combining MSI findings with the quantification techniques applied to plasma by Jones *et al* (in this issue) will identify tissue- and pathology-specific circulating markers of radiation-injury. Linking MALDI-MSI data to the longitudinal in-study imaging techniques of CT and echocardiogram, (also in this issue) will provide associations between molecular abnormalities and clinical presentations. Lastly, the ability to detect and localize drug compounds under evaluation for efficacy studies is of vital importance, particularly with regard to approval through the FDA-AR. Not only can MALDI-MSI offer the capability to investigate the drug reaching the target organ, as demonstrated here, but the technique has the ability to evaluate whether it is reaching the diseased regions needed, such as penetration into pre-neoplastic areas or fibrotic foci, for example. The MCART consortium possesses multi-faceted expertise in animal models, technology and instrumentation and is proving to be a true driving force in determining both the mechanism of action of radiation-induced injury and medical countermeasures, and the sequence and interplay for multi-organ injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

CT images taken prior to euthanasia from the 3 NHP used for comparison in this study. Damage severity can clearly be observed from figure A–C. Pneumonitis/fibrosis (PF) was more severely documented in the NHP shown in figure A (PF = 40.35 mL) and B (PF = 10.33 mL) compared to C (PF = 3.36 mL).

Figure 2.

Representative MALDI-MS images of lipid species detected in naïve (non-irradiated) and irradiated lung samples (08094381, 08052001 and 098219). The images presented show changes in the relative intensity and distribution of ions at m/z 465, 498, 509, 687, 715, and 794. Images were acquired at 100 μm . The scale bar is shown in white and represents 2mm. Differences in lipid profiles as a result of radiation can be seen for the ions at m/z 465, 498 and 509, which are only present in the samples from NHP 08094381 and 08052001 (figures B and C), these ions were not detected in the naïve sample (figure A) and the third IR sample analyzed (NHP 098219, figure 1D). The ions at m/z 687, 715 and 794 are localized to specific anatomical regions and the differences observed are a result of different anatomy in each section, and not due to the effects of radiation, as shown by the magnified H&E regions presented on the right.

Figure 3.

Images and representative spectra taken from NHP 08094381 demonstrating the injury specific ions at m/z 498, 507 and 509 (figures A–C); H&E section with a magnified region showing alveolar infiltration within the regions the former ions accumulate (figure D); H&E and m/z 498 overlaid image to further demonstrate the increased intensity within the infiltrate regions (figure E); and representative mass spectra taken away from the infiltrate, larger spectrum, and from the infiltrate regions, smaller zoomed spectrum (figure F).

Figure 4.

MALDI mass spectra from the analysis of a standard of AEOL 10150, figure A, and the molecule directly from a treated lung section, figure B. The base peak detected from the standard and from the lung section is at m/z 1008, resulting from the formation of a porphyrin-DHB complex, assigned $[M^{5+}+4e^{-}+DHB]^{+}$. Tandem MS spectra acquired from the dissociation of the precursor ion at m/z 1008, from the AEOL10150 standard and from the lung tissue is shown in figures C and D respectively. The spectra demonstrate similar fragmentation patterns resulting from the loss of ethyl groups at m/z 979 and 950, confirming the molecule detected in tissue is the drug. A structure of AEOL 10150 is shown in figure 4A.

Figure 5. MALDI-MS images acquired at 20 μm resolution from an irradiated + AEOL10150 treated lung section. The H&E stained section for this lung is shown in the bottom right of the figure. Higher accumulation of AEOL 10150, m/z 1008, can be observed surrounding the bronchiole, as indicated by the change in intensity in relation to the intensity scale next to the image, followed by increased signal across the lung parenchyma. AEOL 10150 shows a differential distribution to that of heme, m/z 616 (top right image), which maps the blood/vasculature, thus indicating the compound passes from the circulation into the target organ. This is further demonstrated by the merged ion images of AEOL 10150 (green) and heme (red) presented in the bottom right of the figure.

Table 1

A list of select lipid species detected in lung sections. Where fragmentation studies have been carried out directly from tissue the diagnostic fragment ion has been included. For lipids presented without validation by MS/MS, identification was based on the precursor mass and literature. Lipids in *italics* demonstrated an increase following IR, lipids in **bold** font were only detected in tissue following IR that presented with histological evidence of immune infiltration.

m/z	Assignment	Diagnostic Fragment Ion	Ion	Distribution
465.3	Cholesterol Sulfate	m/z 97 [HSO ₄] ⁻	[M-H] ⁻	Ubiquitous
498.3	Unassigned		[M-H] ⁻	Inflammatory Infiltrate
507.3	Unassigned		[M-H] ⁻	Inflammatory Infiltrate
509.3	Unassigned		[M-H] ⁻	Inflammatory Infiltrate
687.5	SM(d18:1/16:0)	m/z 168 [C ₄ H ₁₂ NO ₄ P] ⁻	[M-CH ₃] ⁻	Bronchi and Vessels
715	SM(d18:1/18:0)	m/z 168 [C ₄ H ₁₂ NO ₄ P] ⁻	[M-H] ⁻	Bronchi and Vessels
718.5	PE(34:0)		[M-H] ⁻	Respiratory Airways
742.5	PE(36:2)		[M-H] ⁻	Ubiquitous
744.6	PE(36:1)		[M-H] ⁻	Ubiquitous
747.5	PG(34:1)		[M-H] ⁻	Respiratory Airways
788.5	PS(36:1)		[M-H] ⁻	Respiratory Airways
794.5	C16(OH) Sulfatide	m/z 97 [HSO ₄] ⁻	[M-H] ⁻	Cartilage
797.7	SM(d18:1/24:1)	m/z 168 [C ₄ H ₁₂ NO ₄ P] ⁻	[M-CH ₃] ⁻	Respiratory Airways
799.7	SM(d18:1/24:0)	m/z 168 [C ₄ H ₁₂ NO ₄ P] ⁻	[M-CH ₃] ⁻	Respiratory Airways
833.5	PI(34:2)	m/z 241 [C ₆ H ₁₀ O ₈ P] ⁻	[M-H] ⁻	Respiratory Airways
835.5	PI(34:1)	m/z 241 [C ₆ H ₁₀ O ₈ P] ⁻	[M-H] ⁻	Respiratory Airways
857.5	PI(36:4)		[M-H] ⁻	Ubiquitous
859.5	PI(36:3)	m/z 241 [C ₆ H ₁₀ O ₈ P] ⁻	[M-H] ⁻	Ubiquitous
861.5	PI(36:1)	m/z 241 [C ₆ H ₁₀ O ₈ P] ⁻	[M-H] ⁻	Ubiquitous
863.5	PI(36:0)	m/z 241 [C ₆ H ₁₀ O ₈ P] ⁻	[M-H] ⁻	Ubiquitous
881.5	PI(38:6)		[M-H] ⁻	Ubiquitous
883.5	PI(38:5)		[M-H] ⁻	Ubiquitous
885.5	PI(38:4)	m/z 241 [C ₆ H ₁₀ O ₈ P] ⁻	[M-H] ⁻	Ubiquitous
906.7	C24(OH) Sulfatide	m/z 97 [HSO ₄] ⁻	[M-H] ⁻	Cartilage
909.5	PI(40:6)		[M-H] ⁻	Respiratory Airways
911.5	PI(40:5)	m/z 241 [C ₆ H ₁₀ O ₈ P] ⁻	[M-H] ⁻	Respiratory Airways