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(54) **Title**: SURFACTANT THERAPY FOR EXPOSURE TO IONIZING RADIATION

(57) **Abstract**: This invention relates to therapies for treatment or prevention of conditions resulting from direct or indirect exposure of pulmonary tissue to ionizing radiation. In particular, regimens for the treatment or prevention of conditions resulting from direct or indirect exposure of pulmonary tissue to ionizing radiation involve the administration of pulmonary surfactants. In certain embodiments, the pulmonary surfactant is KL4 surfactant.

FIG. 1A

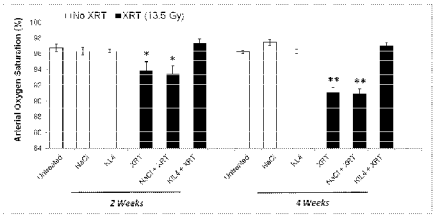
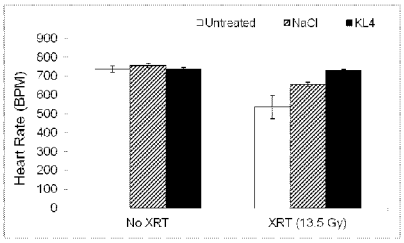


FIG. 1B



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TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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SURFACTANT THERAPY FOR EXPOSURE TO IONIZING RADIATION

Pursuant to 35 U.S.C. §202(c), it is acknowledged that the United States Government may have rights in the invention described herein, which was made in part with funding from the National Institute of Allergy and Infectious Disease, Grant No. 1R43AI102308-01.

FIELD OF THE INVENTION

This invention relates to therapies for treatment or prevention of conditions detrimental to pulmonary tissue resulting from exposure of any tissue, including but not limited to pulmonary tissue, to ionizing radiation. In particular, regimens for the treatment or prevention of conditions resulting from exposure to ionizing radiation involve the administration of pulmonary surfactants.

BACKGROUND

Various patents, patent publications, and scientific articles may be referred to throughout the specification. The content of each of these documents is incorporated by reference herein, in its entirety. The entire content of the priority documents, U.S. Provisional Application Nos. 61/642,856 and 61/794,036 is also incorporated by reference herein.

Exposure to ionizing radiation, which can occur, for example, through controlled therapeutic intervention (radiotherapy) generally to treat malignancies, or inadvertently following nuclear reactor accident, nuclear attack, or deliberate terrorist actions, including the detonation of "dirty bombs" (radiological dispersal devices (RDDs)), is a significant public health concern. The lung is particularly susceptible to ionizing radiation direct and indirect injury ("radiation induced injury"), be it from external sources or inhalation of radionuclides (radioactive particles), both soluble and insoluble, that can be incorporated in the lung. "Out-of-field" irradiation causing damage to the pulmonary tissue could include irradiation of other tissues or organs that are in close proximity or adjacent to the lungs but might also include tissue

and organs exposed to radiation that are not in close proximity and/or distal to the lung (Johnston CJ, et al.: *Int J Radiat Biol.* 2011: 87(8): 902–913).

With respect to incorporated radionuclides, the retention of the radionuclide can be very long, even the lifetime of the individual, especially for insoluble radioactive particles. Thus, accelerating clearance of the radionuclide can reduce the total radiation dose delivered to the individual and the health risk associated with exposure to the radionuclide. Radionuclides that have been most commonly identified in terms of availability and radiotoxicity, include, but are not limited to, ^{241}Am, ^{252}Cf, ^{137}Cs, ^{60}Co, ^{192}Ir, ^{238}Pu, ^{239}Pu, ^{226}Ra and ^{90}Sr. Radiotoxicity of each of these nuclides depends on its decay characteristics and chemical and biological properties in the body. One of the most important physicochemical properties of a radionuclide that determines its biological behavior is its chemical solubility *in vivo*. This characteristic determines the biological distribution of the radionuclide, both at the site of entry (*e.g.*, lung, GI tract, skin, wound) and the degree of systemic exposure.

In general, radionuclides that are reasonably soluble tend to be absorbed into the blood efficiently, and distributed to systemic organs, often times with selective uptake, making removal challenging. Chemical removal approaches, e.g., with chelating agents, are most effective for decorporating such soluble radionuclides. On the other hand, chelating agents are usually not effective for decorporating radionuclides that are insoluble *in vivo*, regardless of route of exposure. In this case, physical removal methods are required, e.g., surgical excision for wounds, surface decontamination for skin deposition, emetics and purgatives for ingestion, and lung lavage for inhalation of insoluble radioactive material.

There is extensive experience with lung lavage (BAL) to facilitate removal of inhaled particles from the lung. However, BAL using saline lavagate has been shown to be only moderately effective for decorporating insoluble inhaled radioactive particles. Since the early studies on BAL decorporation done in the late 1960s and 1970s, little advancement has been made over the past 30 years to improve on the nominal 50% removal efficiency of BAL, which has traditionally employed a series of lung lavages spaced over a 3-week period. Moreover, saline BAL washes out endogenous surfactant, which can lead to lung collapse and potentially reduce efficiency of the lavage technique, let alone lead to lung compromise, producing ARDS in some instances.

Radiation pneumonopathy can manifest with an acute radiation pneumonitis and/or delayed effects of acute radiation exposure (DEARE), which may lead to progressive, often fatal, pulmonary fibrosis many months or years later. Acute radiation pneumonitis generally presents with, but is not limited to, increased capillary permeability, interstitial and alveolar edema, protein leak into the alveolar space or lung tissue, influx of circulating inflammatory cells (such as white blood cells (leukocytes), which are classified into two main groups: granulocytes (neutrophils, eosinophils, and basophils) and nongranulocytes (lymphocytes and monocytes/macrophages; the latter being activated monocytes), leakage of red blood cells into the alveolar space and lung tissue, formation of hyaline membranes, and diffuse alveolar damage (Gross NJ:. *Lung* 1981, 159(3):115-125; Movsas B, Raffin TA, Epstein AH, Link CJ, Jr.: *Chest* 1997; 111(4):1061-1076). Activation of cytokines/chemokine pathways play a role in the pathogenesis of radiation pneumonopathy. Rubin and colleagues (*Int J Radiat Oncol Biol Phys* 1992: 24(1):93-101), and others (Morgan GW, Breit SN: *Int J Radiat Oncol Biol Phys* 1995: 31(2):361-369; Johnston CJ, et al.: *Int J Radiat Biol.* 2011: 87(8): 902–913) have hypothesized that the cytokines/chemokine activation begins immediately at the time of irradiation and persists up to the appearance of late injury that underlies the development of pulmonary fibrosis, which is characterized by inflammatory cell infiltrations, fibroblast proliferation, and excessive deposition of extracellular matrix proteins in lung parenchyma.

Clinically, the disease can manifest with the following signs and/or symptoms including but not limited to cough, and fullness in the chest, shortness of breath (dyspnea), low-grade fever, pleuritic chest pain, increased white blood cell count and hemoptysis, (Roswit and White, *Am J Roentgenol* 1997; 129:127-136; Roach et al., *J Clin Oncol* 1995; 13:2606-12; Girinsky et al., *J Clin Oncol* 2000; 18:981-6; Movsas et al., *Chest* 1997; 111:1061-76). Physiological evidence of acute and chronic pulmonary (lung) dysfunction can manifest with decreased arterial oxygenation (measured by arterial oxygen tension (PaO_2; sometimes described as arterial oxygen saturation), and/or peripheral oxygen saturation (SpO_2), abnormal respiration dynamics, increased pulmonary resistance, and/or decreased pulmonary compliance. The radiographic hallmarks of radiation pneumonopathy are a diffuse hazy infiltrate, changing to flocculent patchy infiltrates, and a straight edge effect, if the radiation field has been limited to a specific section of the lung. Radiation pneumonitis can be a dose-limiting side effect of radiation therapy and may

occur especially in patients with pre-existing lung diseases or in those receiving chemotherapy (Tsujino K, et al., *Int J Radiat Oncol Biol Phys.* 2003 ;55(1):110-15).

Whereas radiation pneumonitis is typically the result of direct irradiation of lung fields and direct injury to endothelial and epithelial cells, humoral factors may result in adjacent involvement including an "out-of-field" injury of the unirradiated lung (Roberts et al., *Ann Intern Med* 1993; 118:696-700; Johnston CJ, et al.: *Int J Radiat Biol.* 2011: 87(8): 902–913).

In radiation pneumonopathy, the role of endogenous pulmonary surfactant has not been fully understood (Rubin P, et al:. *Int J Radiat Oncol Biol Phys* 1980: 6(1):75-77; Rubin P, et al.: *Int J Radiat Oncol Biol Phys* 1983, 9(11):1669-1673; Shapiro DL, et al.: *Int J Radiat Oncol Biol Phys* 1982, 8(5):879-882; Shapiro DL, et al.: *Int J Radiat Oncol Biol Phys* 1984; 10(3):375-378; Hallman M, et al.: *Am Rev Respir Dis* 1990, 141(4 Pt 1):998-1005). Significant fluctuation in the natural pulmonary surfactant pool has been described, including reports of early surfactant insufficiency on the one hand, or transient early rises on the other hand, the latter having been attributed to a pseudo-physiological response of alveolar type II pneumocytes, which are particularly sensitive to ionizing radiation injury. Radiation-induced membrane lipid peroxidation and membrane permeabilization may lead to leakage of surfactant from lamellar bodies within these cells. Data are also limited on the functionality and composition of endogenous surfactant post irradiation. Moreover, exogenous surfactant therapies have not been evaluated to treat radiation pneumonopathy, either alone or in combination with other mitigating agents.

Natural pulmonary surfactants (PS) are protein/lipid compositions that are produced naturally in the lungs and are critical to the lungs' ability to absorb oxygen. They cover the entire alveolar surface of the lungs and the terminal conducting airways leading to the alveoli. Surfactants facilitate respiration by continually modifying the surface tension of the fluid normally present within the alveoli. In the absence of sufficient surfactant, or should the surfactant degrade or become inactivated, the alveoli tend to collapse and the lungs do not absorb sufficient oxygen. By lowering the surface tension of the terminal conducting airways, surfactant maintains patency, *i.e.,* keeps airways open. Loss of surfactant leads to loss of patency, obstruction of the airway, and compromised pulmonary function. Human surfactants primarily contain phospholipids, the major one being dipalmitoyl phosphatidyl-choline (DPPC),

and four surfactant polypeptides, A, B, C and D, with surfactant protein B (SP-B) being the most essential for respiratory function. Animal-derived and synthetic pulmonary surfactants are commonly used to treat respiratory distress syndrome in premature infants shortly after birth.

One available type of exogenous surfactant is a peptide-based synthetic surfactant, KL4 surfactant, such as SURFAXIN® (lucinactant, Discovery Laboratories, Inc., Warrington, PA). KL4 surfactant can be delivered to the lungs via a variety of means, including as an intratracheal instillate, as a lung lavage to subjects on ventilatory support, or as an aerosol to spontaneously breathing subjects. KL4 surfactant is more resistant to inactivation by plasma proteins and oxidants present in the inflamed lung than natural and other animal-derived exogenous surfactants and can thus potentially function in an inflammatory milieu. Further, KL4 surfactant has been shown to be generally safe and well-tolerated studied in preterm infants at risk for RDS and other populations, including children under age two with viral-induced hypoxic respiratory failure, as well as in adults.

There remains a need for improved therapies for treatment or prevention of conditions resulting from exposure of pulmonary tissue to ionization radiation, be it direct, or "out-of-field".

SUMMARY OF INVENTION

In one aspect, the invention features a method of using an exogenous pulmonary surfactant to treat an individual exposed or at risk of exposure to ionizing radiation. The method comprises the steps of: (a) identifying an individual who (i) has radiation induced injury resulting from prior exposure to ionizing radiation, or (ii) who is at risk of radiation induced injury resulting from exposure to ionizing radiation, wherein the radiation induced injury includes at least one of lung injury and multi-organ injury; and (b) administering the exogenous pulmonary surfactant to the individual in an amount and for a time effective to treat the individual's lungs. In one embodiment, the exposure to ionizing radiation comprises direct exposure of the individual's lungs to the ionizing radiation. In another embodiment, the exposure to ionizing radiation comprises exposure of the individual's whole body to the ionizing radiation. In yet another embodiment, the exposure to ionizing radiation comprises exposure of portions of the individual's body excluding the lungs to the ionizing radiation. In certain embodiments, the exogenous pulmonary surfactant is administered to the lungs of the individual.

Various embodiments comprise detecting or monitoring one or more signs or symptoms of lung injury before and/or after the exogenous pulmonary surfactant is administered. In these embodiments, a favorable result of treatment, as compared with an equivalent untreated individual, or as compared with the individual prior to treatment, is indicated by any one or more of: improved respiration dynamics, pulmonary resistance, or pulmonary compliance; maintaining oxygenation in a normal physiological range; reduced leak of protein or profibrotic and pro-inflammatory cytokine, including IL-6, TNF, TGF ß, MCP-1, IL-1ß, or RANTES, in the alveolar space or lung tissue; reduced neutrophil or macrophage migration into the alveolar space or lung tissue; reduced signs of pulmonary injury on biochemical analysis of bronchoalveolar fluid or lung tissue; reduced lipid peroxidation of lung tissues; reduced blood levels and or tissue and or biological fluid levels of 8-oxo-dGuo; reduced ratio of secretory proteins to surfactant proteins; reduced levels of biomarkers of oxidative stress; reduced alterations in lung architecture; reduced fibrosis and tissue oxidative stress; and reduced lung damage based on semiquantitative and or quantitative histopathologic assessment and histopathologic injury score. In particular a reduction of at least one, two, three, four or five percent of any of the following criteria, as compared to an equivalent untreated individual or as compared with the same individual prior to treatment, indicates a favorable treatment result: protein leak; neutrophil/macrophage migration; IL-6, TNF, IL-1 ß, MCP-1, RANTES, or TGFß mRNA or protein production; the plasma ratio of secretory proteins to surfactant proteins; assessment of fibrosis utilizing a quantitative image analysis technique looking at collagen deposition or the ratio of tissue to airspace. In other embodiments, an improvement of at least six, seven, eight, nine or ten percent of any of the criteria, as compared to an equivalent untreated individual or the same individual prior to treatment, indicates a favorable treatment result.

In certain embodiments, the exogenous pulmonary surfactant is administered in combination with additional components including therapeutic agents or radio-protectants. Those additional components can be administered simultaneously or in series with each other and/or with the exogenous pulmonary surfactant.

The exogenous pulmonary surfactant can administered as one or more of: a liquid instillate, a dry powder or an aerosol. In certain embodiments, the exogenous pulmonary surfactant is administered at a dosage of 5 to 1000 mg total phospholipid (TPL) per kilogram

body weight. More particularly, the exogenous pulmonary surfactant is administered at a dosage of 5 to 200 mg TPL/kg body weight.

In one embodiment, the individual identified as a treatment candidate has been exposed to the ionizing radiation and has lung injury. The lung injury can comprise, among others, at least one of acute radiation pneumonitis (ARP) or delayed effects of acute radiation exposure (DEARE). In these instances, one regimen involves the administration of exogenous pulmonary surfactant beginning not more than about 24-48 hours after the exposure to ionizing radiation. In another regimen, the administration of exogenous pulmonary surfactant begins not more than about 24 hours to about 2 weeks after onset of clinical symptoms of DEARE.

In another embodiment, the individual identified as a treatment candidate is at risk of future exposure to ionizing radiation. In this instance, the administration of exogenous pulmonary surfactant begins at least about 2 weeks prior to the anticipated future exposure to ionizing radiation. In another regimen, the administration of exogenous pulmonary surfactant begins at least about 12 hours prior to the anticipated future exposure to ionizing radiation.

In another embodiment, the individual identified as a treatment candidate has been exposed previously to ionizing radiation but does not yet exhibit symptoms of lung injury. In this instance, the treatment can commence at any time following the exposure or following the ascertainment that such exposure has occurred. Preferably, the treatment commences immediately or shortly after the exposure has occurred, *e.g.*, within 24-48 hours.

In one embodiment, the exposure to ionizing radiation involves inhalation of radionuclides. If an individual has been exposed to this type of ionizing radiation, the exogenous pulmonary surfactant can be administered by bronchoalveolar lavage, by segmental bronchoscopic pulmonary lavage, or by aerosol. In a particular embodiment, the exogenous pulmonary surfactant comprises KL4 (SEQ ID NO:1) and is administered at a concentration of about 1mg/mL to about 50 mg/mL TPL.

Another aspect of the invention features a method of using exogenous pulmonary surfactant to treat a subject who is exposed to ionizing irradiation of at least 1 Gy and who exhibits lung dysfunction measured by a decrease of arterial oxygenation of at least two percent (2 %), wherein the use comprises administering the exogenous pulmonary surfactant in an amount of 0.5 mg to 2000 mg TPL per kilogram of body weight to the subject at the time of

exposure or within 30 minutes to six months after exposure to the irradiation. In one embodiment, the exposure to ionizing radiation comprises direct exposure of the individual's lungs to the ionizing radiation. In another embodiment, the exposure to ionizing radiation comprises exposure of the individual's whole body to the ionizing radiation. In yet another embodiment, the exposure to ionizing radiation comprises exposure of portions of the individual's body excluding the lungs to the ionizing radiation. In certain embodiments, the exogenous pulmonary surfactant is administered to the lungs of the individual.

Another aspect of the invention features a method of using an exogenous pulmonary surfactant to treat an individual exposed to ionizing radiation, wherein ionizing radiation has caused or could cause lung injury, and wherein the lung injury can cause lung injury-induced multi-organ injury. The method comprises the steps of: (a) identifying an individual who (i) has lung injury resulting from prior exposure to ionizing radiation, or (ii) who is at risk of lung injury resulting from exposure of the individual to ionizing radiation; and (b) administering the exogenous pulmonary surfactant to the individual's lungs in an amount and for a time effective to treat the individual's lungs, resulting in treatment of the multi-organ injury. In certain embodiments, the multi-organ injury includes one or more of myocardial injury, renal injury and liver injury. In particular, the individual may exhibit decreased heart rate as a symptom of the myocardial injury.

In any of the aspects and embodiments of the invention set forth above, the exogenous pulmonary surfactant comprises KL4 (SEQ ID NO:1).

Other features and advantages of the invention will be understood from the drawings, detailed description and examples that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 A and 1 B are graphs depicting effect of KL4 surfactant treatment on animals exposed to ionizing radiation: Arterial oxygenation, described as arterial oxygen saturation in Figure 1A; and sometimes referred to as PaO_2 or SpO_2 is expressed in percent (%). Figure 1B shows heart rate expressed in beats per minute (BPM). Measurements were made at day 14 (week 2) and 30 (week 4) post irradiation as described in Example 1. A statistically significant reduction in arterial oxygenation is observed in untreated irradiated (XRT) animals and NaCl-

treated irradiated animals at day 14 (week 2) and 30 (week 4) compared with non-irradiated control animals (untreated and NaCl-treated); KL4 surfactant-treated irradiated animals showed preservation of arterial oxygenation. Similarly, a reduction in heart rate is observed at day 14 (week 2) and 30 (week 4) in untreated and NaCl-treated irradiated animals compared with non-irradiated control animals (untreated and NaCl-treated); KL4 surfactant-treated animals showed no appreciable change in heart rate relative to untreated controls. KL4 = KL4 surfactant-treated animals; NaCl = Sodium Chloride-treated animals; * $p \leq 0.05$; ** $p < 0.001$.

Figures 2A and 2B are graphs depicting concentration of total white blood cells (WBC) (A) and neutrophils (PMN) (B) in bronchoalveolar fluid (BAL) (cells/ml BAL) measured at day 21 (week 3) post irradiation as described in Example 1. Statistically significant lower WBC counts in BAL as well as a trend towards lower PMN counts were observed in KL4 surfactant-treated animals compared with untreated and NaCl-treated non-irradiated control animals. KL4 = KL4 surfactant-treated animals; NaCl = sodium chloride-control treated animals). In Figure 2A: * $p \leq 0.05$ for XRT vs. no XRT; $^{\$}$ and $^{\#}$ are $p \leq 0.05$ for KL4+XRT vs. XRT, and KL4 + XRT vs. NaCl+XRT, respectively. In Figure 2B: * $p \leq 0.05$ for XRT vs. No XRT. Note: KL4+XRT vs. XRT = $p > 0.05$ but < 0.1.

Figure 3 is a graph depicting percent change in μmol malondialdehyde (MDA)/g lung measured at day 21 (week 3) post irradiation. MDA is a marker indicating oxidative tissue damage. Irradiated KL4 surfactant-treated animals have significantly less oxidative damage compared with untreated and NaCl-treated irradiated animals.

Figure 4 is a graph depicting neutrophil population density in lung tissue assayed by immunostaining and image analysis after a dose 5Gy whole body irradiation (WBI) and an additional 11Gy to the lung only (total 16Gy). Neutrophil population density was observed to be lower in the irradiated KL4 surfactant-treated animals versus the irradiated saline-treated (NaCl) control animals.

DETAILED DESCRIPTION

Unless otherwise specified, all medical procedures described or referred to herein are performed in accordance with current standards of care understood by physicians and/or other healthcare practitioners.

It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a peptide" includes a combination of two or more peptides, and the like.

Additionally, as used herein, "comprising" is to be interpreted as specifying the presence of the stated features, integers, steps, or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps, or components, or groups thereof. Thus, for example, a sample comprising one surfactant may contain additional surfactants or other components. Additionally, the term "comprising" is intended to include examples encompassed by the terms "consisting essentially of" and "consisting of." Similarly, the term "consisting essentially of" is intended to include examples encompassed by the term "consisting of."

"About" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20%, ±10%, ±5%, ±1%, or ±0.1% from the specified value, as such variations are appropriate to perform the disclosed methods.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred materials and methods are described herein. In describing and claiming the present invention, the following terminology will be used.

As used herein, "irradiation" or "exposure to ionizing radiation" includes exposure of an individual's tissue to ionizing radiation in any form, whether directly or indirectly. By way of example, ionizing radiation includes inhalation of radionuclides, including those that have been most commonly identified in terms of availability and radiotoxicity, such as, but not limited to, ^{241}Am, ^{252}Cf, ^{137}Cs, ^{60}Co, ^{192}Ir, ^{238}Pu, ^{239}Pu, ^{226}Ra and ^{90}Sr.

As used herein "radiation induced injury" is injury induced by exposure to ionizing radiation and includes lung injury and multi-organ injury.

As used herein, "lung injury" is a disease or condition that manifests as a radiation induced lung injury and includes radiation pneumonopathy (both acute pneumonitis and/or DEARE) in various stages induced by direct or indirect exposure to ionizing radiation which can be manifested even after several months following the exposure.

As used herein, "signs or symptoms of lung injury" include but are not limited to coughing; chest pain; difficulty breathing; decreased respiration dynamics, pulmonary resistance, or pulmonary compliance; decrease in arterial oxygenation (measured by arterial oxygen tension (PaO_2; sometimes described as arterial oxygen saturation), and/or peripheral oxygen saturation (SpO_2)); increased leak of protein into the lung and or systemic circulation, and/or upregulation of profibrotic and pro-inflammatory cytokines/chemokines in the lung and or systemic circulation, including but not limited to IL-6, TNF, TGF ß, MCP-1, IL-1ß, or RANTES, and/or down-regulation of antifibrotic and anti-inflammatory cytokines/chemokines in the lung and/or systemic circulation; increased neutrophil or macrophage migration as an example of white cells but not limited to, into the alveolar space or lung tissue; increased signs of pulmonary injury on biochemical analysis of bronchoalveolar fluid or lung tissue; increased lipid peroxidation of lung tissues; increased blood levels and/or tissue and/or biological fluid levels of 8-oxo-dGuo; increased secretory proteins to surfactant protein (SP; e.g., SP-A, AP-B, SP-C, and or SP-D) ratios; increased levels of biomarkers of oxidative stress; alterations in lung architecture; increased fibrosis and tissue oxidative stress; and lung damage based on semiquantitative and or quantitative histopathologic assessment and histopathologic injury score.

As used herein, "signs or symptoms of multi-organ injury" include but are not limited to myocardial injury, renal injury, or liver injury as an example are: (a) for myocardial injury: brady- and/or tachycardia (measured by examination of the pulse and or on electrocardiogram as an example but not limited to), decreased cardiac output and/or ejection fraction (as measure by echocardiogram; and/or fast scan cardiac computed axial tomography (CT); and/or cardiac magnetic resonance imaging (MRI); and/or MUGA scanning, as an example but not limited to); for renal injury: decrease urine output; rises in serum creatinine and/or serum blood urea nitrogen; decreased glomerular filtration rate (measure by creatinine clearance as an example but not limited to); and /or urine protein leak (as measure semiquantitatively on dipsticks or by collection of urine samples for quantitative analysis of protein concentration as an example but

not limited to); and for liver injury, rise in total, direct or indirect serum bilirubin levels; and/or serum liver enzymes such as AST (Aspartate transaminase) also called SGOT (Serum Glutamic Oxalocetic Transaminase); ALT (Alanine Transaminase) also called SGPT (Serum Glutamic Pyruvic Transaminase); ALP (Alkaline phosphatase); GGT (Gamma Glutamyl Transpeptidase); and/or LDH (Lactic Acid Dehydrogenase), as an example.

"Polypeptide," "peptide," and "protein" are used interchangeably to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

In certain embodiments, a peptide may include other peptides that are conservative variations of those peptides specifically exemplified herein. "Conservative variation" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include, but are not limited to, the substitution of one hydrophobic residue such as isoleucine, valine, leucine, alanine, cysteine, glycine, phenylalanine, proline, tryptophan, tyrosine, norleucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. Neutral hydrophilic amino acids that can be substituted for one another include asparagine, glutamine, serine and threonine. "Conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide. Such conservative substitutions are within the definition of the classes of the peptides of the invention. "Cationic" as used herein refers to any peptide that possesses a net positive charge at pH 7.4. The biological activity of the peptides can be determined by standard methods known to those of skill in the art and described herein.

Peptides of the invention can be synthesized by methods known in the art. For example, in certain embodiments, commonly used methods such as t-BOC or FMOC protection of alpha-amino groups can be used. Both methods involve stepwise syntheses whereby a single amino

acid is added at each step starting from the C terminus of the peptide (See, Coligan *et al.*, *Current Protocols in Immunology*, Wiley Interscience, 1991, Unit 9). Peptides of the invention can be synthesized, for example, by the well known solid phase peptide synthesis methods described in Merrifield, *J. Am. Chem. Soc.* 85: 2149, 1962, and Stewart and Young, 1969, *Solid Phase Peptides Synthesis*, pp. 27-62, using a copoly(styrene-divinylbenzene) containing 0.1-1.0 mMol amines/g polymer. On completion of chemical synthesis, the peptides can be deprotected and cleaved from the polymer by treatment with liquid HF-10% anisole for about 1/4-1 hours at 0°C. After evaporation of the reagents, the peptides are extracted from the polymer with 1% acetic acid solution, which is then lyophilized to yield the crude material. This can normally be purified by such techniques as gel filtration on Sephadex G-15 using 5% acetic acid as a solvent. Lyophilization of appropriate fractions of the column will yield the homogeneous peptide or peptide derivatives, which can then be characterized by such standard techniques as amino acid analysis, thin layer chromatography, high performance liquid chromatography, ultraviolet absorption spectroscopy, molar rotation, solubility, and quantitated by the solid phase Edman degradation. KL4 peptide can be prepared as described in U.S. Patent Nos. 6,013,764 and 6,492,490.

"Treating" or "treatment" refers to any indicia of success in the amelioration of the disease or condition as relates to radiation induced injury; i.e., lung injury and /or multi-organ injury, or in the reduction in incidence or severity of the disease or condition. As used herein, the term "treating" includes prophylactic or therapeutic treatment. Prophylactic treatment is directed to an individual who may be exposed to ionizing radiation in the future, and/or to an individual who has been exposed to ionizing radiation but has not developed or displayed signs or symptoms of lung injury or possible sequelae. In a prophylactic treatment, the outcome is directed to preventing, or delaying the onset of, or reducing the incidence or severity of the disease or condition that does occur. A prophylactic treatment also can be directed to decreasing the toxicity of ionizing radiation, for example, so that an individual can withstand a larger radiation dose than would otherwise be possible without incurring lung injury, and/or multi-organ injury, or possible sequelae. Therapeutic treatment is directed to individuals who have been exposed to ionizing radiation and who have developed and/or display at least one sign or symptom of lung injury, or at least one sign or symptom of multi-organ injury, or possible

sequelae. In a therapeutic treatment, the outcome is directed to reducing or ameliorating the lung injury, or multi-organ injury, or possible sequelae.

"Surfactant activity" refers to the ability of a substance, such as an organic molecule, protein or polypeptide, either alone or in combination with other molecules, to lower surface tension at an air/water interface. The surface tension measurement can be made with a Wilhelmy balance or pulsating bubble surfactometer by an *in vitro* assay. See, for example King et *al, Am. J. Physiol.* 1972, 223:715-726, or *Enhorning, J. Appl. Physiol.*, 1977, 43:198-203, each of which is incorporated herein by reference in its entirety. Briefly, the Enhorning Surfactometer (Surfactometer International, Toronto, Ontario) measures the pressure gradient (δP) across a liquid-air interface of a bubble that pulsates at a rate of 20 cycles/min between a maximal (0.55 mm) and minimal (0.4 mm) radius. The bubble, formed in a 37°C, water-enclosed, 20-µl sample chamber, is monitored through a microscopic optic while the pressure changes are recorded on a strip chart recorder calibrated for 0 and -2 cm H_2O. In addition, *in vivo* measurements of increases of compliance or airflow at a given pressure of air entering the lung can be readily made, such as in the assay of Robertson, *Lung,* 1980, 158:57-68, incorporated herein by reference in its entirety. In this assay, the sample to be assessed is administered through an endotracheal tube to fetal rabbits or lambs delivered prematurely by Caesarian section. These "preemies" lack their own pulmonary surfactant, and are supported on a ventilator. Measurements of lung compliance, blood gases and ventilator pressure provide indices of activity. *In vitro* assays of surfactant activity, which is assessed as the ability to lower the surface tension of a pulsating bubble, and *in vivo* assays utilizing fetal rabbits are described in detail by Revak *et al, Am. Rev. Respir. Dis.*, 1986, 134:1258-1265.

In one aspect, the invention contemplates the therapeutic administration of pulmonary surfactant (PS) to individuals exposed to direct or indirect irradiation of lung tissue and exhibiting signs or symptoms of lung injury. In another aspect, the invention contemplates the therapeutic administration of pulmonary surfactant to individuals exposed to irradiation in areas other than the lung tissue and exhibiting signs or symptoms of lung injury. In a further aspect, the therapeutic administration of pulmonary surfactant is intended reduce or decrease the undesirable side effects of radiation therapy involving direct or indirect irradiation of lung tissue or direct irradiation of other tissues.

In yet another aspect, the therapeutic administration of pulmonary surfactant is intended to decrease toxicity of the radiation to the lungs to prevent or decrease injury of other organ systems (i.e., lung injury-induced multi-organ injury), such as the heart, kidney and/or the liver resulting from upregulation/production of proinflammatory cytokines/chemokines in the lung after exposure of an individual to ionization radiation, the use comprising administering an exogenous pulmonary surfactant to the individual's lungs in an amount and for a time effective to treat or prevent the lung injury and subsequent multi-organ injury, such as myocardial injury, renal injury, liver injury as an example. In connection with this aspect of the invention, it has been shown that exposure of individuals to ionizing radiation results in a slowing of heart rate, believed to be indicative of myocardial stress or damage resulting as a downstream effect of lung injury. Surprisingly, treatment with exogenous pulmonary surfactant following exposure to ionizing radiation has been shown to reverse this change in heart rate, indicating a reduction or prevention in downstream myocardial damage as a result of irradiation delivered to the lungs.

In yet another aspect, the prophylactic or therapeutic administration of pulmonary surfactant is intended to decrease toxicity of the radiation treatment and therefore allow an increased dose of radiation to be administered.

In still another aspect, the invention provides prophylactic administration of pulmonary surfactant to individuals not exhibiting signs or symptoms of lung injury. Such prophylactic administration can take place prior to exposure to the ionizing radiation, or after the exposure but prior to the onset of any signs or symptoms of lung injury.

As discussed above, signs and symptoms of lung injury can include but not be limited to coughing; chest pain; difficulty breathing; decreased respiration dynamics, pulmonary resistance, or pulmonary compliance; decrease in arterial oxygenation; increased leak of protein into the lung and or systemic circulation, and/or upregulation of profibrotic and pro-inflammatory cytokines/chemokines in the lung and or systemic circulation, including but not limited to IL-6, TNF, TGF ß, MCP-1, IL-1ß, or RANTES, and/or down-regulation of antifibrotic and anti-inflammatory cytokines/chemokines in the lung and/or systemic circulation; increased neutrophil or macrophage migration as an example of white cells but not limited thereto, into the alveolar space or lung tissue; increased signs of pulmonary injury on biochemical analysis of bronchoalveolar fluid or lung tissue; increased lipid peroxidation of lung tissues; increased blood

levels and/or tissue and/or biological fluid levels of 8-oxo-dGuo; increased secretory proteins to surfactant protein (SP; e.g., SP-A, AP-B, SP-C, and or SP-D) ratios; increased levels of biomarkers of oxidative stress; alterations in lung architecture; increased fibrosis and tissue oxidative stress; and lung damage based on semiquantitative and or quantitative histopathologic assessment and histopathologic injury score.

In particular embodiments, pulmonary surfactant can be delivered to the lungs as an intratracheal instillate. In other embodiments, pulmonary surfactant can be delivered as a lung lavage. In further embodiments, pulmonary surfactant can be delivered as an aerosol. In particular examples, pulmonary surfactant can be delivered as a lung lavage to subjects receiving ventilatory support. In other examples, pulmonary surfactant can be delivered as an aerosol to spontaneously breathing subjects or those receiving ventilatory support.

In yet another aspect, the invention contemplates administering pulmonary surfactant to patients prior to, contemporaneously with or after administration of certain cytotoxic drugs, principally chemotherapeutic agents such as erlotinib, to prevent, alleviate or treat radiation pneumonitis triggered by the administration of chemotherapeutic agents in conjunction with radiation treatment.

In yet another aspect, the invention contemplates administering pulmonary surfactant to patients in combination with cyto-protective agents, such as, for example, amifostine (see U.S. Patent No. 6573253), CBLB502 (see U.S. Patent Application Publication 20090246303) and MAXY-G34 (see U.S. Patent Application Publication 20100183543) to decrease toxicity to radiation treatment.

In yet another aspect, the invention contemplates administering pulmonary surfactant to patients in combination with cyto-protective agents, such as, for example, flaxseed or flaxseed lignan metabolite, including secoisolariciresinol diglucoside (*see, e.g.,* Melpo Christofidou-Solomidou, *et al.*,: Dietary Flaxseed Administered Post-Thoracic Radiation Treatment Improves Survival And Mitigates Radiation-Induced Pneumonopathy In Mice. BMC Cancer. 11:269, 2011).

In another aspect, KL4 surfactant as a lavagate (lung wash) will improve the efficiency of decorporating inhaled radioactive particles (radionuclides; both soluble and insoluble) by: (1). augmenting the contact efficiency (wetting) of the lavage fluid in the lung parenchyma given its

surface-tension-lowering properties; (2) decreasing adhesiveness of alveolar macrophages containing radioactive particles, making them more amenable to the lung wash; and (3) allowing more efficient distal lavage of the lung parenchyma given the ability of surfactant to expand terminal airways and alveoli.

In another aspect, residual KL4 surfactant in the lung is used to facilitate natural mucociliary clearance (*e.g.,* through expectoration and swallowing of cleared material and subsequent elimination in the feces) of terminal airway and alveolar macrophages that have phagocytized radioactive particles, thereby reducing the radioactive body burden.

In particular embodiments, pulmonary surfactant can be delivered to the lung as a lung lavage or aerosol to decorporate inhaled radionuclides (radioactive particles) that are soluble and or insoluble. In certain examples, the pulmonary surfactant concentration ranges between 1mg/mL to 50 mg/mL TPL. In yet another aspect, the invention contemplates administering pulmonary surfactant to lavage/wash out alveolar macrophages that have phagocytized and contain the radionuclide-containing particles. The lavage procedure can be used as described in U.S. Pat. No. 6,013,619 to Cochrane et al.

The rationale for using pulmonary surfactant, preferably, KL4 surfactant together with BAL to significantly increase the efficacy of BAL (versus BAL alone) is based on the supposition that the current saline BAL technique does not efficiently deploy lavage fluid into all or even a large majority of terminal airways and the approximate 4.5×10^7 alveoli present in a normal human lung, where most of the particle-containing macrophages reside. Furthermore, modifying the technique by employing segmental bronchoscopic lavage may further enhance efficacy of removal of radioactive-laden macrophages. When lung lavage is not feasible and a non-invasive approach is under consideration to remove inhaled insoluble radioactive particles, aerosolized KL4 surfactant may facilitate lung clearance through acceleration of the natural mucociliary clearance pathway.

In essence, lavage containing KL4 surfactant improves the efficiency of decorporating inhaled insoluble radioactive particles by: (1). augmenting the contact efficiency (wetting) of the lavage fluid in the lung parenchyma given its surface-tension-lowering properties; (2) decreasing adhesiveness of alveolar macrophages containing radioactive particles, making them more amenable to the lung wash; and (3) allowing more efficient distal lavage of the lung parenchyma

given the ability of surfactant to expand terminal airways and alveoli. Moreover, residual KL4 surfactant in the lung should facilitate natural mucociliary clearance of terminal airway and alveolar macrophages that have phagocytized radioactive particles, and through expectoration and swallowing of cleared material and subsequent elimination in the feces, reduce the radioactive body burden.

In one embodiment, pulmonary surfactant administration is initiated following acute and/or chronic exposure of lung tissue to radiation. In certain embodiments, treatment is initiated about 0.5 hours, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 4 months, 5 months, 6 months, or more after exposure.

In other embodiments, pulmonary surfactant administration is initiated prior to radiation exposure. In certain embodiments, treatment is initiated about 0.5 hours, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or14 days prior to exposure.

In certain embodiments, treatment is continued for a period of time deemed by a physician or other medical practitioner as appropriate to achieve a therapeutic or prophylactic effect. Treatment can be continued, for example, for 30 minutes, 1 hour, 2 hours, 6 hours 12 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 4 months, 5 months, 6 months, or until the individual is discharged from his or her physician's care. In various embodiments, the pulmonary surfactant is administered periodically or continuously throughout the treatment period, at dosages and utilizing protocols in accordance with standard and/or manufacturer's instructions or an alternative dosing regimen as set forth herein.

In certain embodiments, the administration can comprise multiple separate prophylactic or therapeutic treatment regimens, for example 2, 3, 4, 5, 6, 7, 8, 9, 10, or more separate administration regimens. In certain examples, the treatment regimens are given at least about 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 14 hours, 16 hours, 18 hours, 20 hours, 22 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 4 months, 5 months, 6 months, or more apart from one another. In certain other examples, the treatment regimens are given not more than about 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 14 hours, 16 hours, 18 hours, 20 hours, 22 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 4 months, 5 months, or 6 months apart from one another.

In other embodiments, a treatment regimen can comprise multiple doses. In certain examples, the treatment regimen can comprise at least about 2 doses per day, 3 doses per day, 4 doses per day, 5 doses per day, 6 doses per day, 7 doses per day, 8 doses per day, 9 doses per day, 10 doses per day, 2 doses per week, 3 doses per week, 4 doses per week, 5 doses per week, 6 doses per week, 7 doses per week, or more. In certain other examples, the treatment regimen can comprise not more than about 2 doses per day, 3 doses per day, 4 doses per day, 5 doses per day, 6 doses per day, 7 doses per day, 8 doses per day, 9 doses per day, 10 doses per day, 2 doses per week, 3 doses per week, 4 doses per week, 5 doses per week, 6 doses per week, or 7 doses per week. In further examples, the treatment regimen can comprise continuous administration of pulmonary surfactant for 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 4 months, 5 months, or 6 months.

In certain embodiments, the patient can be dosed more frequently early in the treatment regimen, and with decreasing frequency later in the treatment regimen, *e.g.*, once every other day for one week, followed by twice weekly until the end of the treatment period. Depending on the dosage form, *e.g.,* aerosol or dry powder as compared with liquid instillate, the patient can be dosed continuously for part or all of the treatment period.

In certain particular examples, the pulmonary surfactant is administered 2-3 times per day for 2 to 4 weeks for initial therapy and then again 2-3 times per day for 6 weeks to 6 months. pulmonary surfactant therapy can be provided alone, sequentially, or concomitantly with other forms of respiratory support or with other compounds. In certain embodiments, pulmonary surfactant administration is performed on spontaneously breathing individuals, meaning individuals who are breathing without any external assistance. In such individuals, administration of the pulmonary surfactant may be, for example, by metered dose inhaler (for example, for a liquid formulation) or dry powder inhaler (for example, for a lyophilized or micronized formulation). In certain embodiments, pulmonary surfactant administration is performed on individuals who are intubated and maintained on ventilation, either conventional ventilation or high frequency ventilation, for a period of time or for the entire duration of the pulmonary surfactant treatment. In other embodiments, alternative modes of administration can be utilized, as well as alternative pulmonary surfactant formulations. For example, pulmonary surfactant can be formulated for aerosolization (nebulization) and administered *via* nasal CPAP, nasal or naso-pharyngeal prongs in combination with low-flow oxygen, or *via* face mask or oxygen hood.

In certain embodiments, aerosolized pulmonary surfactant can be administered as provided in U.S. patent publication US 2006-0078506 A1 and U.S. patent publication US 2011-0011395 A1 incorporated herein by reference their entireties and for all purposes. Administration can be in conjunction with another noninvasive pulmonary respiratory therapy involving the administration of positive airway pressure. The term "noninvasive pulmonary respiratory therapy" refers to respiratory therapy which does not use mechanical ventilation and can include CPAP, bilevel positive airway pressure (BiPAP), synchronized intermittent positive airway pressure (SIPAP), and the like. The employment of such therapies involves the use of various respiratory gases, as would be appreciated by the skilled artisan. Respiratory gases used for noninvasive pulmonary respiratory therapy are sometimes referred to herein as "CPAP gas," "CPAP air," "nCPAP", "ventilation gas," "ventilation air," or simply "air." However, those terms are intended to include any type of gas normally used for noninvasive pulmonary respiratory therapy, including but not limited to gases and gaseous combinations listed above for use as the conditioning gas. In certain embodiments, the gas used for noninvasive pulmonary

respiratory therapy is the same as the conditioning gas. In other embodiments, the respective gases are different from one another.

In certain embodiments, the pulmonary delivery methods of this invention are employed in conjunction with CPAP. It has been shown that use of CPAP allows for an increase in functional residual capacity and improved oxygenation. The larynx is dilated and supraglottic airway resistance is normal. There is also an improvement of the synchrony of respiratory thoracoabdominal movements and enhanced Hering-Breuer inflation reflex following airway occlusion. CPAP has been shown to be useful in treating various conditions such as sleep apnea, snoring, ARDS, IRDS, and the like.

In order to effect administration of CPAP, a pressure source and a delivery device or delivery apparatus are required. CPAP-producing airflow is typically generated in the vicinity of the nasal airways by converting kinetic energy from a jet of fresh humidified gas into a positive airway pressure. A continuous flow rate of breathing gas of about 5 to about 12 liters/minute generates a corresponding CPAP of about 2 to about 10 cm H_2O. Various modifications can be applied to the CPAP system, which include sensors that can individualize the amount of pressure based on the patient's need.

Typically, flow rates and pressures suitable for achieving CPAP are based upon the characteristics of the patient being treated. Suitable flow rates and pressures can be readily calculated by the attending clinician. The present invention encompasses the use of a variety of flow rates for the ventilating gas, including low, moderate and high flow rates. Alternatively, the aerosol can be supplied without added positive pressure, *i.e.*, without CPAP as a simultaneous respiratory therapy.

Preferably, the CPAP-generating airflow being delivered to the patient has a moisture level that will prevent unacceptable levels of drying of the lungs and airways. Thus, the CPAP-generating air is often humidified by bubbling through a hydrator or the like to achieve a relative humidity of preferably greater that about 70%. More preferably, the humidity is greater than about 85% and still more preferably 98%.

A suitable source of CPAP-inducing airflow is the underwater tube CPAP (underwater expiratory resistance) unit. This is commonly referred to as a bubble CPAP. Another preferred source of pressure is an expiratory flow valve that uses variable resistance valves on the

expiratory limb of CPAP circuits. This is typically accomplished via a ventilator. Other CPAP systems including those that contain similar features to systems just discussed are also contemplated by the present invention.

Any type of nebulizer or aerosol generator may be used. For instance, the aerosol generator can be an ultrasonic nebulizer or vibrating membrane nebulizer or vibrating screen nebulizer. Typically, jet nebulizers are not employed although the present methods can be adapted to all types of nebulizers or atomizers. In one embodiment, the aerosol generator is an Aeroneb® Professional Nebulizer (Aerogen Inc., Mountain View, CA, USA). In another embodiment, the aerosol generator is a capillary aerosol generator, an example of which is a soft-mist generator by Philip Morris USA, Inc. Richmond, VA (see, for example, U.S. Patent Nos. 5,743,251 and 7,040,314).

The aerosol stream generated in accordance with aerosolized delivery is preferably delivered to the patient via a nasal delivery device which can involve, for example masks, single nasal prongs, binasal prongs, nasopharyngeal prongs, nasal cannulae, and the like. The delivery device is chosen so as to minimize trauma, maintain a seal to avoid waste of aerosol, and minimize the work the patient must perform to breathe. An adaptor as described in a co-pending U.S. patent application No. 12/922,981 by Mazela *et al.*, can be used in conjunction with the source of aerosol, ventilation gas and patient interface to improve delivery of aerosol.

When used as an aerosol preparation, the surfactant composition can be supplied in finely divided form, optionally in combination with a suitable propellant. Useful propellants are typically gases at ambient conditions and are condensed under pressure including, for example, lower alkanes and fluorinated alkanes, such as freon. In certain embodiments wherein the surfactant composition is delivered as an aerosol, the aerosol can be packaged in a suitable container under pressure.

Suitable dosage of the surfactant, whether aerosolized or delivered as a liquid or dry powder will be dependent on numerous factors and will be readily ascertainable by an attending clinician, physician, or healthcare worker. The factors affecting proper dosage of pulmonary surfactant include the extent of exposure and particular status of the subject (*e.g.*, the subject's age, size, fitness, extent of symptoms, susceptibility factors, and the like). By "effective dose" herein is meant a dose that produces effects for which it is administered.

In certain embodiments, a dose of surfactant may comprise at least about 0.5 mg total phospholipid (TPL)/kg body weight, 1 mg TPL/kg body weight, 2 mg TPL/kg body weight, 3 mg TPL/kg body weight, 4 mg TPL/kg body weight, 5 mg TPL/kg body weight, 6 mg TPL/kg body weight, 7 mg TPL/kg body weight, 8 mg TPL/kg body weight, 9 mg TPL/kg body weight, 10 mg TPL/kg body weight, 15 mg TPL/kg body weight, 20 mg TPL/kg body weight, 25 mg TPL/kg body weight, 30 mg TPL/kg body weight, 35 mg TPL/kg body weight, 40 mg TPL/kg body weight, 45 mg TPL/kg body weight, 50 mg TPL/kg body weight, 60 mg TPL/kg body weight, 70 mg TPL/kg body weight, 80 mg TPL/kg body weight, 90 mg TPL/kg body weight, 100 mg TPL/kg body weight, 125 mg TPL/kg body weight, 150 mg TPL/kg body weight, 175 mg TPL/kg body weight, 200 mg TPL/kg body weight, 300 mg TPL/kg body weight, 400 mg TPL/kg body weight, 500 mg TPL/kg body weight, 600 mg TPL/kg body weight, 700 mg TPL/kg body weight, 800 mg TPL/kg body weight, 900 mg TPL/kg body weight, 1000 mg TPL/kg body weight, 1200 mg TPL/kg body weight, 1400 mg TPL/kg body weight, 1600 mg TPL/kg body weight, 1800 mg TPL/kg body weight, or 2000 mg TPL/kg body weight. In other embodiments, a dose of surfactant may comprise not more than about 0.5 mg TPL/kg body weight, 1 mg TPL/kg body weight, 2 mg TPL/kg body weight, 3 mg TPL/kg body weight, 4 mg TPL/kg body weight, 5 mg TPL/kg body weight, 6 mg TPL/kg body weight, 7 mg TPL/kg body weight, 8 mg TPL/kg body weight, 9 mg TPL/kg body weight, 10 mg TPL/kg body weight, 15 mg TPL/kg body weight, 20 mg TPL/kg body weight, 25 mg TPL/kg body weight, 30 mg TPL/kg body weight, 35 mg TPL/kg body weight, 40 mg TPL/kg body weight, 45 mg TPL/kg body weight, 50 mg TPL/kg body weight, 60 mg TPL/kg body weight, 70 mg TPL/kg body weight, 80 mg TPL/kg body weight, 90 mg TPL/kg body weight, 100 mg TPL/kg body weight, 125 mg TPL/kg body weight, 150 mg TPL/kg body weight, 175 mg TPL/kg body weight, 200 mg TPL/kg body weight, 300 mg TPL/kg body weight, 400 mg TPL/kg body weight, 500 mg TPL/kg body weight, 600 mg TPL/kg body weight, 700 mg TPL/kg body weight, 800 mg TPL/kg body weight, 900 mg TPL/kg body weight, 1000 mg TPL/kg body weight, 1200 mg TPL/kg body weight, 1400 mg TPL/kg body weight, 1600 mg TPL/kg body weight, 1800 mg TPL/kg body weight, or 2000 mg TPL/kg body weight.

When the surfactant is supplied as a liquid, an aliquot of the surfactant composition is delivered to provide an effective dose of pulmonary surfactant in the lungs of the treated patient.

In certain examples, a single surfactant dose ranges, for example, from about 20 to about 300 mg TPL/kg body weight, or from about 60 to about 175 mg TPL/kg body weight. In certain embodiments wherein the surfactant composition is delivered as an aerosol, the effective dose of pulmonary surfactant can be, for example, from about 1 mg TPL/kg body weight to about 1000 mg TPL/kg body weight, or from about 2 mg TPL/kg body weight to about 175 mg TPL/kg body weight. In certain embodiments wherein the surfactant composition is delivered as a dry powder formulation, the effective dose of pulmonary surfactant can be, for example, from about 1 mg TPL/kg body weight to about 1000 mg or more TPL/kg body weight, preferably from about 2 mg TPL/kg body weight to about 175 mg TPL/kg body weight. Other methods of delivery include lavage (see, for example, U.S. Patent No. 6,013,619), lung wash, and the like. When so employed, dose ranges are well within the skill of one in the art.

In other embodiments, the individual can be treated with other therapeutic, prophylactic or complementary agents, such as steroids, nitric oxide, antioxidants or reactive oxygen scavengers, bronchodilators, diuretics, antimicrobial or anti-infective agents, anti-hypertensive agents, or anti-inflammatory agents (*e.g.*, PLA_2 inhibitors, protease or elastase inhibitors, PDE-4 inhibitors, to name a few), as would be appreciated by one of skill in the art. Such treatment can include concomitant administration of the pulmonary surfactant with other therapeutic, prophylactic or complementary agents. Concomitant administration can involve concurrent (*i.e.* at the same time, either separately or admixed), prior, or subsequent administration of the complementary agent with respect to the administration of the pulmonary surfactant. When administered in combination, each component can be administered at the same time or sequentially in any order at different points in time. Thus, each component can be administered separately but sufficiently closely in time so as to provide the desired therapeutic effect. Concomitant administration of a pulmonary surfactant with other therapeutic, prophylactic or complementary agents means administration of the pulmonary surfactant and other agents at such time that both will have a therapeutic effect. A person of ordinary skill in the art would be able to readily determine the appropriate timing, sequence and dosages of administration for particular drugs of the present invention.

Any pulmonary surfactant currently in use or hereafter developed may be suitable for use in the present invention. In certain aspects, a pulmonary surfactant of the present invention

comprises a cationic peptide that can be derived from animal sources or synthetically. Exemplary peptides for use herein include naturally and non-naturally occurring pulmonary surfactant polypeptides, such as, for example, one or a combination of animal-derived SP-A, SP-B, SP-C, or SP-D polypeptides; recombinant SP-A, SP-B, SP-C, or SP-D polypeptides; synthetically derived SP-A, SP-B, SP-C, or SP-D polypeptides; SP-A, SP-B, SP-C, and SP-D analogs; SP-A, SP-B, SP-C, and SP-D polypeptide mimics; conservatively modified variants thereof retaining activity; and fragments thereof retaining activity. A pulmonary surfactant polypeptide mimic is generally a polypeptide that is engineered to mimic the essential attributes of human surfactant protein. In certain embodiments, the pulmonary surfactant polypeptide comprises a cationic peptide that comprises at least about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, or 80 amino acid residues. In other embodiments, the pulmonary surfactant polypeptide comprises a cationic peptide that comprises not more than about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, or 80 amino acid residues.

Exemplary amino acid sequences of pulmonary surfactant polypeptides for use herein, methods of isolating them, and producing them by genetic engineering techniques are known in the art. See for example, U.S. Patent Nos. 5,874,406; 5,840,527; 4,918,161; 5,827,825; 6,660,833, 5,006,343; 5,455,227; 5,223,481; 5,753,621; 5,891,844; 4,861,756; 5,272,252; 5,024,95; 5,238,920; 5,302,481; 6,022,955; 5,874,406; 5,840,527; 5,827,825; 6,013,619; 6,660,833; and International Publication Nos, WO8603408 and WO8904326, the disclosures of each of which are hereby incorporated by reference in its entirety. A preferred pulmonary surfactant peptide for use herein is a SP-B or SP-C polypeptide, or polypeptide mimic.

In certain embodiments, the pulmonary surfactant comprises one or more phospholipids and a polypeptide, in which the polypeptide, when admixed with a phospholipid, forms a synthetic pulmonary surfactant having a surfactant activity greater than the surfactant activity of the phospholipid alone. In particular embodiments, the pulmonary surfactant polypeptide for use herein is a SP-B polypeptide or polypeptide mimic. SP-B is the protein in natural pulmonary surfactant known to be the most important surfactant protein for surface tension lowering and promoting oxygen exchange. SP-B polypeptide mimics are small hydrophobic polypeptides, generally less than about 80 amino acids in size. Many SP-B polypeptide mimics possess a

repeating hydrophobic cationic motif. Like natural SP-B polypeptide, SP-B mimics, preferably, lower surface tension of the terminal conducting airways and promote oxygen exchange.

In specific examples, an SP-B mimetic for use in the present invention is KL4 peptide, which is a cationic peptide containing repeating lysine and leucine residues. KL4 is representative of a family of pulmonary surfactant polypeptide mimetics that are described, for example, in U.S. Patent 5,260,273, 5,164,369, 5,407,914 and 6,613,734, each of which is hereby incorporated by reference in its entirety and for all purposes. Methods of preparing the KL4 peptide can be found in U.S. Patent 5,164,369.

In certain embodiments, pulmonary surfactant polypeptide mimics refer to polypeptides with an amino acid residue sequence that has a composite hydrophobicity of less than zero, preferably less than or equal to -1, more preferably less than or equal to -2. The composite hydrophobicity value for a peptide is determined by assigning each amino acid residue in a peptide its corresponding hydrophilicity value as described in Hopp *et al.*, *Proc. Natl. Acad. Sci.* 78: 3824-3829, 1981, which disclosure is incorporated by reference. For a given peptide, the hydrophobicity values are summed, the sum representing the composite hydrophobicity value. These hydrophobic polypeptides typically perform the function of the hydrophobic region of SP18. Accordingly, in certain embodiments, the amino acid sequence of the pulmonary surfactant polypeptide mimic mimics the pattern of hydrophobic and hydrophilic residues of SP18 and performs the function of the hydrophobic region of SP18. SP18 is a known pulmonary surfactant apoprotein, more thoroughly described in Glasser *et al., Proc. Natl. Acad. Sci.* 84: 4007-4001, 1987, which is hereby incorporated by reference in its entirety and for all purposes. It should be understood, however, that polypeptides and other surfactant molecules of the present invention are not limited to molecules having sequences like that of native SP18. To the contrary, some preferred surfactant molecules of the present invention have little resemblance to SP18 with respect to a specific amino acid residue sequence, except that they have similar surfactant activity and alternating charged/uncharged (or hydrophobic/hydrophilic) residue sequences.

In certain embodiments, exemplary polypeptides for use herein have alternating hydrophobic and hydrophilic amino acid residue regions and are characterized as having at least 10 amino acid residues represented by the formula:

$$(Z_aU_b)_cZ_d$$

Z and U are amino acid residues such that at each occurrence Z and U are independently selected. Z is a hydrophilic amino acid residue, preferably selected from the group consisting of R, D, E and K. U is a hydrophobic amino acid residue, preferably selected from the group consisting of V, I, L, C, Y, and F. The letters, "a," "b,", "c" and "d" are numbers which indicate the number of hydrophilic or hydrophobic residues. The letter "a" has an average value of about 1 to about 5, preferably about 1 to about 3. The letter "b" has an average value of about 3 to about 20, preferably about 3 to about 12, most preferably, about 3 to about 10. The letter "c" is 1 to 10, preferably, 2 to 10, most preferably 3 to 6. The letter "d" has an average value of about 0 to 3, preferably 1 to 2.

In certain embodiments, surfactant polypeptides include a sequence having alternating groupings of amino acid residues as represented by the formula:

$$(Z_aJ_b)_cZ_d$$

wherein Z is an amino acid residue independently selected from the group consisting of R, D, E, and K; J is an α-aminoaliphatic carboxylic acid; a has an average value of about 1 to about 5; b has an average value of about 3 to about 20; c is 1 to 10; and d is 0 to 3.

In certain embodiments, polypeptides of the present invention have alternating groupings of amino acids residue regions as represented by the formula:

$$(B_aU_b)_cB_d$$

wherein B is an amino acid residue independently selected from the group consisting of H, 5-hydroxylysine, 4-hydroxyproline, and 3-hydroxyproline; and U is an amino acid residue independently selected from the group consisting of V, I, L, C, Y, and F. In one preferred variation, B is an amino acid derived from collagen and is preferably selected from the group consisting of 5-hydroxylysine, 4-hydroxyproline, and 3-hydroxyproline; a has an average value of about 1 to about 5; b has an average value of about 3 to about 20; c is 1 to 10; and d is 0 to 3.

In certain embodiments, surfactant polypeptides of the present invention include a sequence having alternating groupings of amino acid residues as represented by the formula:

$$(B_aJ_b)_cB_d$$

wherein B is an amino acid residue independently selected from the group consisting of H, 5-hydroxylysine, 4-hydroxyproline, and 3-hydroxyproline; and J is an α-aminoaliphatic carboxylic

acid; a has an average value of about 1 to about 5; b has an average value of about 3 to about 20; c is 1 to 10; and d is 0 to 3.

In various embodiments including "J" in the relevant formula, J is an α-aminoaliphatic carboxylic acid having four to six carbons, inclusive. In other variations, J is an α-aminoaliphatic carboxylic acid having six or more carbons, inclusive. In yet other variations, J is selected from the group consisting of α-aminobutanoic acid, α-aminopentanoic acid, α-amino-2-methylpropanoic acid, and α-aminohexanoic acid.

In certain embodiments, surfactant polypeptides of the present invention comprise a sequence having including a sequence having alternating groupings of amino acid residues as represented by the formula:

$$(Z_aU_b)_cZ_d$$

wherein Z is an amino acid residue independently selected from the group consisting of R, D, E, and K; and U is an amino acid residue independently selected from the group consisting of V, I, L, C, Y and F; from the group consisting of V, I, L, C and F; or from the group consisting of L and C; a has an average value of about 1 to about 5; b has an average value of about 3 to about 20; c is 1 to 10; and d is 0 to 3.

In the foregoing formulas, Z and U, Z and J, B and U, and B and J are amino acid residues that, at each occurrence, are independently selected. In addition, in each of the aforementioned formulae, a generally has an average value of about 1 to about 5; b generally has an average value of about 3 to about 20; c is 1 to 10; and d is 0 to 3.

In certain embodiments, Z and B are charged amino acid residues. In other preferred embodiments, Z and B are hydrophilic or positively charged amino acid residues. In one variation, Z is preferably selected from the group consisting of R, D, E and K. In a related embodiment, Z is preferably selected from the group consisting of R and K. In yet another preferred embodiment, B is selected from the group consisting of H, 5-hydroxylysine, 4-hydroxyproline, and 3-hydroxyproline. In one preferred embodiment, B is H. In another preferred embodiment, B is a collagen constituent amino acid residue and is selected from the group consisting of 5-hydroxylysine, (δ-hydroxylysine), 4-hydroxyproline, and 3-hydroxyproline.

In certain embodiments, U and J are, preferably, uncharged amino acid residues. In another preferred embodiment, U and J are hydrophobic amino acid residues. In one embodiment, U is preferably selected from the group consisting of V, I, L, C, Y, and F. In another preferred embodiment, U is selected from the group consisting of V, I, L, C, and F. In yet another preferred embodiment, U is selected from the group consisting of L and C. In various preferred embodiments, U is L.

Similarly, in certain embodiments, B is an amino acid preferably selected from the group consisting of H, 5-hydroxylysine, 4-hydroxyproline, and 3-hydroxyproline. Alternatively, B can be selected from the group consisting of collagen-derived amino acids, which includes 5-hydroxylysine, 4-hydroxyproline, and 3-hydroxyproline.

In certain embodiments, charged and uncharged amino acids are selected from groups of modified amino acids. For example, in one preferred embodiment, a charged amino acid is selected from the group consisting of citrulline, homoarginine, or ornithine, to name a few examples. Similarly, in various preferred embodiments, the uncharged amino acid is selected from the group consisting of α-aminobutanoic acid, α-aminopentanoic acid, α-amino-2-methylpropanoic acid, and α-aminohexanoic acid.

In certain embodiments of the present invention, items "a", "b", "c" and "d" are numbers which indicate the number of charged or uncharged residues (or hydrophilic or hydrophobic residues). In various embodiments, "a" has an average value of about 1 to about 5, preferably about 1 to about 3, more preferably about 1 to about 2, and even more preferably, 1.

In various embodiments, "b" has an average value of about 3 to about 20, preferably about 3 to about 12, more preferably about 3 to about 10, even more preferably in the range of about 4-8. In one preferred embodiment, "b" is about 4.

In various embodiments, "c" is 1 to 10, preferably 2 to 10, more preferably in the range of 3-8 or 4-8, and even more preferably 3 to 6. In one preferred embodiment, "c" is about 4.

In various embodiments, "d" is 0 to 3 or 1 to 3. In one preferred embodiment, "d" is 0 to 2 or 1 to 2; in another preferred embodiment, "d" is 1.

By stating that an amino acid residue is independently selected, it is meant that at each occurrence, a residue from the specified group is selected. That is, when "a" is 2, for example, each of the hydrophilic residues represented by Z will be independently selected and thus can

include RR, RD, RE, RK, DR, DD, DE, DK, and the like. By stating that "a" and "b" have average values, it is meant that although the number of residues within the repeating sequence (*e.g.*, Z_aU_b) can vary somewhat within the peptide sequence, the average values of "a" and "b" would be about 1 to about 5 and about 3 to about 20, respectively.

For example, using the formula $(Z_aU_b)_cZ_d$ for the peptide designated "KL8" in Table 1 below, the formula can be rewritten as $K_1L_8K_1L_8K_1L_2$, wherein the average value of "b" is six [*i.e.*,(8+8+2)/3=6], c is three and d is zero.

Polypeptides of the present invention can also be subject to various changes, such as insertions, deletions and substitutions, either conservative or non-conservative, where such changes provide for certain advantages in their use.

Additional residues can be added at either terminus of a polypeptide of the present invention, such as for the purpose of providing a "linker" by which such a polypeptide can be conveniently affixed to a label or solid matrix, or carrier. Labels, solid matrices and carriers that can be used with the polypeptides of this invention are known in the art.

Amino acid residue linkers are usually at least one residue and can be 40 or more residues, more often 1 to 10 residues. Typical amino acid residues used for linking are tyrosine, cysteine, lysine, glutamic and aspartic acid, or the like. In addition, a polypeptide sequence of this invention can differ from the natural sequence by the sequence being modified by terminal-NH_2 acylation, *e.g.*, acetylation, or thioglycolic acid amidation, terminal-carboxlyamidation, *e.g.*, ammonia, methylamine, and the like.

In certain embodiments, exemplary SP-B polypeptide mimics that can be used in the present invention include, but are not limited to, those shown in the Table 1.

Table 1: Pulmonary Surfactant Mimetic Peptides

Designation[1]	SEQ ID NO	Amino Acid Residue Sequence
KL4	1	KLLLLKLLLLKLLLLKLLLLK
DL4	2	DLLLLDLLLLDLLLLDLLLLD
RL4	3	RLLLLRLLLLRLLLLRLLLLR
RL8	4	RLLLLLLLLRLLLLLLLLRLL
R2L7	5	RRLLLLLLLRRLLLLLLLRRL
	6	RLLLLCLLLRLLLLLCLLLR

Designation[1]	SEQ ID NO	Amino Acid Residue Sequence
	7	LLLLLCLLLRLLLLCLLLRLL
	8	RLLLLCLLLRLLLLCLLLRLLLLCLLLR DLLLDLLLDLLLDLLLDLLLD
RCL1	9	RLLLLCLLLRLLLLCLLLR
RCL2	10	RLLLLCLLLRLLLLCLLLRLL
RCL3	11	RLLLLCLLLRLLLLCLLLRLLLLCLLLR
KL8	12	KLLLLLLLLKLLLLLLLLKLL
KL7	13	KKLLLLLLLKKLLLLLLLKKL

[1] The designation is an abbreviation for the indicated amino acid residue sequence.

The present invention contemplates a variety of surfactant molecules, including proteins, polypeptides, and molecules including amino acid residues, as well as a variety of surfactant compositions. A wide variety of other molecules, including uncommon but naturally occurring amino acids, metabolites and catabolites of natural amino acids, substituted amino acids, and amino acid analogs, as well as amino acids in the "D" configuration, are useful in molecules and compositions of the present invention. In addition, "designed" amino acid derivatives, analogs and mimics are also useful in various compounds, compositions and methods of the present invention, as well as polymers including backbone structures composed of non-amide linkages.

For example, in addition to the L-amino acids, amino acid metabolites such as homoarginine, citrulline, ornithine, and α-aminobutanoic acid are also useful in pulmonary surfactants. Thus, in the various formulas described above, "Charged", Z, or B can comprise homoarginine, citrulline, or ornithine, as well as a variety of other molecules as identified herein. Similarly, J can comprise α-aminobutanoic acid (also known as α-aminobutyric acid), α-aminopentanoic acid, α-aminohexanoic acid, and a variety of other molecules identified herein.

Further, substituted amino acids which are not generally derived from proteins, but which are known in nature, are useful as disclosed herein, include the following examples: L-canavanine; 1-methyl-L-histidine; 3-methyl-L-histidine; 2-methyl L-histidine; α, ε-diaminopimelic acid (L form, meso form, or both); sarcosine; L-ornithine betaine; betaine of histidine (herzynine); L-citrulline; L-phosphoarginine; D-octopine; o-carbamyl-D-serine; γ-aminobutanoic acid; and β-lysine. D-amino acids and D-amino acid analogs, including the

following, are also useful in proteins, peptides and compositions of the present invention: D-alanine, D-serine, D-valine, D-leucine, D-isoleucine, D-alloisoleucine, D-phenylalanine, D-glutamic acid, D-proline, and D-allohydroxyproline, to name some examples. The foregoing can also be used in surfactant molecules according to the present invention; particularly preferred for use accordingly are those corresponding to the formula $\{(\text{Charged})_a\ (\text{Uncharged})_b\}_c(\text{Charged})_d$.

An extensive variety of amino acids, including metabolites and catabolites thereof, can be incorporated into molecules which display a surfactant activity. For example, molecules such as ornithine, homoarginine, citrulline, and a-aminobutanoic acid are useful components of molecules displaying surfactant activity as described herein. Surfactant molecules according to the present invention can also comprise longer straight-chain molecules; α-aminopentanoic acid and α-aminohexanoic acid are two additional examples of such useful molecules.

It should also be appreciated that the present invention encompasses a wide variety of modified amino acids, including analogs, metabolites, catabolites, and derivatives, irrespective of the time or location at which modification occurs. In essence, one can place modified amino acids into three categories: (1) catabolites and metabolites of amino acids; (2) modified amino acids generated via posttranslational modification (*e.g.*, modification of side chains); and (3) modifications made to amino acids via non-metabolic or non-catabolic processes (*e.g.*, the synthesis of modified amino acids or derivatives in the laboratory).

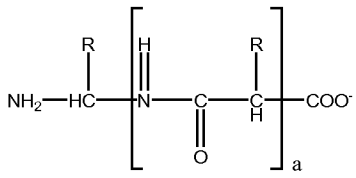
The present invention also contemplates that one can readily design side chains of the amino acids of residue units that include longer or shortened side chains by adding or subtracting methylene groups in either linear, branched chain, or hydrocarbon or heterocyclic ring arrangements. The linear and branched chain structures can also contain non-carbon atoms such as S, O, or N. Fatty acids can also be useful constituents of surfactant molecules herein. The designed side chains can terminate with (R') or without (R) charged or polar group appendages.

In addition, analogs, including molecules resulting from the use of different linkers, are also useful as disclosed herein. Molecules with side chains linked together via linkages other than the amide linkage *e.g.*, molecules containing amino acid side chains or other side chains (R- or R'-) wherein the components are linked via carboxy- or phospho-esters, ethylene, methylene, ketone or ether linkages, to name a few examples, are also useful as disclosed herein. In essence, any amino acid side chain, R or R' group-containing molecule can be useful as disclosed herein,

as long as the molecule includes alternating hydrophilic and hydrophobic residues (*i.e.*, component molecules) and displays surfactant activity as described herein.

The present invention also contemplates molecules comprising peptide dimers joined by an appropriate linker, *e.g.*, peptide dimers linked by cystine molecules. Such linkers or bridges can thus cross-link different polypeptide chains, dimers, trimers, and the like. Other useful linkers which can be used to connect peptide dimers and/or other peptide multimers include those listed above *e.g.*, carboxy- or phospho-ester, ethylene, methylene, ketone or ether linkages, to name a few examples.

While it is appreciated that many useful polypeptides disclosed herein comprise naturally-occurring amino acids in the "L" form which are joined via peptide linkages, it should also be understood that molecules including amino acid side chain analogs, non-amide linkages (*e.g.*, differing backbones) can also display a significant surfactant activity and can possess other advantages, as well. For example, if it is desirable to construct a molecule (*e.g.*, for use in a surfactant composition) that is not readily degraded, one may wish to synthesize a polypeptide molecule comprising a series of D-amino acids. Molecules comprising a series of amino acids linked via a "retro" backbone, *i.e.*, a molecule that has internal amide bonds constructed in the reverse direction of carboxyl terminus to amino terminus, are also more difficult to degrade and can thus be useful in various applications, as described herein. For example, the following illustrates an exemplary molecule with a "retro" bond in the backbone:



In another variation, one may wish to construct a molecule that adopts a more "rigid" conformation; one means of accomplishing this would be to add methyl or other groups to the a carbon atom of the amino acids.

As noted above, other groups besides a CH_3 group can be added to the carbon atom, that is, surfactant molecules of the present invention are not limited to those incorporating a CH_3 at

the α carbon alone. For example, any of the side chains and molecules described above can be substituted for the indicated CH_3 group at the α carbon component.

As used herein, the terms "analogs" and "derivatives" of polypeptides and amino acid residues are intended to encompass metabolites and catabolites of amino acids, as well as molecules which include linkages, backbones, side-chains or side-groups which differ from those ordinarily found in what are termed "naturally-occurring" L-form amino acids. The terms "analog" and "derivative" can also conveniently be used interchangeably herein. Thus, D-amino acids, molecules which mimic amino acids and amino acids with "designed" side chains (*i.e.*, that can substitute for one or more amino acids in a molecule having surfactant activity) are also encompassed by the terms "analogs" and "derivatives" herein.

A wide assortment of useful surfactant molecules, including amino acids having one or more extended or substituted R or R' groups, is also contemplated by the present invention. Again, one of skill in the art should appreciate from the disclosures that one can make a variety of modifications to individual amino acids, to the linkages, and/or to the chain itself, which modifications will produce molecules falling within the scope of the present invention, as long as the resulting molecule possesses surfactant activity as described herein.

In certain methods of the present invention, a pulmonary surfactant comprises one or more lipids. In these embodiments, the surfactant composition can comprise, for example, from as little as about 0.05 to 100 % weight percent lipid, so long as the resulting composition has surfactant activity. By weight percent is meant the percentage of a compound by weight in a composition by weight. Thus, a composition having 50-weight percent lipid contains, for example, 50 grams lipids per 100 grams total composition. The term "lipid" as used herein refers to a naturally occurring, synthetic or semi-synthetic (*i.e.*, modified natural) compound which is generally amphipathic. The lipids typically comprise a hydrophilic component and a hydrophobic component. Exemplary lipids include, but are not limited, phospholipids, fatty acids, fatty alcohols, neutral fats, phosphatides, oils, glycolipids, surface-active agents (surfactants), aliphatic alcohols, waxes, terpenes and steroids. The phrase semi-synthetic (or modified natural) denotes a natural compound that has been chemically modified in some fashion. Preferably, the lipids of are fatty acids, alcohols, esters and ethers thereof, fatty amines, or combinations thereof.

Examples of phospholipids include native and/or synthetic phospholipids. Phospholipids that can be used include, but are not limited to, phosphatidylcholines, phospatidylglycerols, phosphatidylethanolamines, phosphatidylserines, phosphatidic acids, phosphatidylinositols, sphingolipids, diacylglycerides, cardiolipin, ceramides, cerebrosides and the like. Exemplary phospholipids include, but are not limited to, dipalmitoyl phosphatidylcholine (DPPC), dilauryl phosphatidylcholine (DLPC) (C12:0), dimyristoyl phosphatidylcholine (DMPC) (C14:0), distearoyl phosphatidylcholine (DSPC), diphytanoyl phosphatidylcholine, nonadecanoyl phosphatidylcholine, arachidoyl phosphatidylcholine, dioleoyl phosphatidylcholine (DOPC) (C18:1), dipalmitoleoyl phosphatidylcholine (C16:1), linoleoyl phosphatidylcholine (C18:2), myristoyl palmitoyl phosphatidylcholine (MPPC), steroyl myristoyl phosphatidylcholine (SMPC), steroyl palmitoyl phosphatidylcholine (SPPC), palmitoyloleoyl phosphatidylcholine (POPC), palmitoyl palmitooleoyl phosphatidylcholine (PPoPC), dipalmitoyl phosphatidylethanolamine (DPPE), palmitoyloleoyl phosphatidylethanolamine (POPE), dioleoylphosphatidylethanolamine (DOPE), dimyristoyl phosphatidylethanolamine (DMPE), distearoyl phosphatidylethanolamine (DSPE), dioleoyl phosphatidylglycerol (DOPG), palmitoyloleoyl phosphatidylglycerol (POPG), dipalmitoyl phosphatidylglycerol (DPPG), dimyristoyl phosphatidylglycerol (DMPG), distearoyl phosphatidylglycerol (DSPG), dimyristoylphosphatidylserine (DMPS), distearoylphosphatidylserine (DSPS), palmitoyloleoyl phosphatidylserine (POPS), soybean lecithin, egg yolk lecithin, sphingomyelin, phosphatidylinositols, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidic acids, and egg phosphatidylcholine (EPC).

Examples of fatty acids and fatty alcohols include, but are not limited to, sterols, palmitic acid, cetyl alcohol, lauric acid, myristic acid, stearic acid, phytanic acid, dipalmitic acid, and the like. Preferably, the fatty acid is palmitic acid and preferably the fatty alcohol is cetyl alcohol.

Examples of fatty acid esters include, but are not limited to, methyl palmitate, ethyl palmitate, isopropyl palmitate, cholesteryl palmitate, palmityl palmitate sodium palmitate, potassium palmitate, tripalmitin, and the like.

An example of a semi-synthetic or modified natural lipid is any one of the lipids described above which has been chemically modified. The chemical modification can include a number of modifications; however, a preferred modification is the conjugation of one or more

polyethylene glycol (PEG) groups to desired portions of the lipid. Polyethylene glycol (PEG) has been widely used in biomaterials, biotechnology and medicine primarily because PEG is a biocompatible, nontoxic, nonimmunogenic and water-soluble polymer. Zhao and Harris, *ACS Symposium Series* 680: 458-72, 1997. In the area of drug delivery, PEG derivatives have been widely used in covalent attachment (*i.e.*, "PEGylation") to proteins to reduce immunogenicity, proteolysis and kidney clearance and to enhance solubility. Zalipsky, *Adv. Drug Del. Rev.* **16**: 157-82, 1995.

Lipids that have been conjugated with PEG are referred to herein as "PEG-lipids." Preferably, when PEG-lipids are used, they are present in alcohols and/or aldehydes.

The pulmonary surfactant can comprise other excipients, including, but not limited to, various sugars such as dextrose, fructose, lactose, maltose, mannitol, sucrose, sorbitol, trehalose, and the like, surfactants such as, for example, polysorbate-80, polysorbate-20, sorbitan trioleate, tyloxapol and the like, polymers such as PEG, dextran and the like, salts such as NaCl, $CaCl_2$ and the like, alcohols, such as cetyl alcohol, and buffers.

Exemplary surfactant compositions can be prepared using methods known in the art (see U.S. Patent Nos. 7,863,241 to Cochrane, 6,120,795 to Klimchak, 5,952,303 to Bornstein and 7,582,312 to Johnson). For example, in certain embodiments, an exemplary surfactant composition comprising lipids and polypeptides can be prepared by admixing a solution of a surfactant polypeptide with a suspension of liposomes, or by admixing the surfactant polypeptide with a suspension of liposomes, or by admixing the surfactant polypeptide and phospholipids directly in the presence of organic solvent.

Preferably, the pulmonary surfactant comprises phospholipids and free fatty acids or fatty alcohols, *e.g.,* DPPC (dipalmitoyl phosphatidylcholine), POPG (palmitoyl-oleyl phosphatidylglycerol) and palmitic acid (PA). See, for example, U.S. Patent No 5,789,381 the disclosure of which is incorporated herein by reference in its entirety and for all purposes.

In certain preferred embodiments, the pulmonary surfactant is lucinactant or another pulmonary surfactant formulation comprising the synthetic surfactant protein KLLLLKLLLLKLLLLKLLLLK (KL4; SEQ ID NO:1). Lucinactant, is a combination of DPPC, POPG, palmitic acid (PA) and the KL4 peptide (weight ratio of approximately 7.5 : 2.5 : 1.35 : 0.267). In certain embodiments, the drug product is formulated at concentrations of, for example,

10, 20, and 30 mg/ml of phospholipid content. In certain other embodiments, the drug product is formulated at greater concentrations, *e.g.*, 40, 50, 60, 90, 120 or more mg/ml phospholipid content, with concomitant increases in KL4 concentration. In certain embodiments, pulmonary surfactant is one of the compositions described in U.S. Pub. No. US 2006-0286038 A1 to Rairkar.

Any pulmonary surfactant currently in use or hereafter developed may be suitable for use in the present invention. These include naturally occurring and synthetic pulmonary surfactant. Synthetic pulmonary surfactant, as used herein, refers to both protein-free pulmonary surfactants and pulmonary surfactants comprising synthetic peptides, including peptide mimetics of naturally occurring surfactant protein. Current pulmonary surfactant products include, but are not limited to, lucinactant (Surfaxin®, Discovery Laboratories, Inc., Warrington, PA), bovine lipid surfactant (BLES®, BLES Biochemicals, Inc. London, Ont), calfactant (Infasurf®, Forest Pharmaceuticals, St. Louis, MO), natural bovine surfactant (Alveofact®, Thomae, Germany), bovine surfactant (Surfactant TA®, Tokyo Tanabe, Japan), poractant alfa (Curosurf®, Chiesi Farmaceutici SpA, Parma, Italy), pumactant (Alec®, Britannia Pharmaceuticals, UK), beractant (Survanta®, Abbott Laboratories, Inc., Abbott Park, IL) and colfosceril palmitate (Exosurf®, GlaxoSmithKline, plc, Middlesex, U.K.). In a preferred embodiment, the pulmonary surfactant is lucinactant or another pulmonary surfactant formulation comprising the synthetic surfactant protein KLLLLKLLLLKLLLLKLLLLK (KL4; SEQ ID NO:1).

The following examples are provided to describe the invention in greater detail. They are intended to illustrate, not to limit, the invention.

EXAMPLES

PROPHETIC EXAMPLE 1:

Pulmonary irradiation in mouse model

Experimental Approach:

Approximately 165 C57BL/6 wild type mice at 6-8 weeks of age will be studied. These mice will be either irradiated, as discussed below, or left un-irradiated as a control group. The irradiated and un-irradiated mice will be divided into two subsets (cohorts) of animals to allow for evaluation of acute and delayed pulmonary effects. To examine acute pulmonary effects, the

first cohort will undergo necropsy/evaluation at day approximately day 14 to day 30 (2 to 4 weeks) post irradiation exposure; to examine delayed pulmonary effects, the second cohort will undergo necropsy/evaluation at approximately week 12 to 30 weeks. Within these cohorts, there will be three separate treatment groups: 1) untreated; 2) saline (NaCl vehicle) control; and 3) KL4 surfactant-treated. Thus, there will be a total of 12 separate groups, as demonstrated in Table 2.

Table 2: Test groups showing approximate number of mice per group

Mouse Cohorts	ACUTE (3 weeks)		Chronic (18 weeks)	
	No IR	IR	No IR	IR
None	10	15	10	20
Vehicle	10	15	10	20
KL4	10	15	10	20
Group total	**30**	**45**	**30**	**60**
TOTAL	**165 mice total**			

Radiation procedure:

Mice will be irradiated as previously described (Christofidou-Solomidou M, et al. *BMC Cancer* 2011: 11:269; Lee JC, et al. *Radiat Res* 2010;173(5): 590-601; Lee JC, et al. *Cancer Biol Ther* 2009;8(1):47-53; Machtay M, et al. *Radiother Oncol* 2006;81(2): 196-205). Briefly, mice are mildly anesthetized with a combination of ketamine/xylazine (70/7 mg/kg) and irradiated in immobilization chambers that allow exposure of the entire lung (bilateral) with shielding of the head, abdomen, and extremities from radiation. Up to 8 mice can be irradiated simultaneously. A dose of 13.5 Gy (roughly corresponding to LD_{50}) will be delivered as a single fraction to mid-plane using a 250kVp orthovoltage machine with a 2mm copper filter and a tube current of 13mA. Dosimetric analysis, including Thermoluminescent dosimeters (TLDs), will be performed. Irradiated mice tend to lose their fur at the irradiated section of their body; this serves as a superb indicator of the success of the irradiation procedure. This internal control indicates the precise section that has been irradiated. Although the mice are lightly anesthetized for the irradiation procedure, they might occasionally move during the 8-minute exposure procedure, resulting in exposure of their heads or abdomen instead of the required thorax. Mice that have not been optimally irradiated are thus easily detectable and will be eliminated from the study.

Treatment:

KL4 surfactant liquid instillate or normal saline (NaCl) as control will be delivered via the intranasal route twice daily (mice are obligate nasal breathers and effectiveness of delivery method has been previously established) beginning 24 hr post irradiation and lasting for 2 weeks. Methods for intranasal instillations are well described. In brief, mice will be placed in an induction chamber and lightly anesthetized with isoflurane. Once anesthetized, the mouse will be removed from the induction chamber and placed in a supine position and 50-100 µl of instillate delivered drop-wise to the nares using a pipette device. Approximately 60 - 120 mg TPL/kg body weight of KL4 surfactant will be delivered per dose, which corresponds to a dose that has been shown to be effective in the ALI and RDS models in terms of restoring lung function and modulating an immune response. Following treatment, mice will be allowed to recover in a warm environment under observation prior to being returned to their home cage.

Acute pneumonitis:

Acute pneumonitis will be evaluated at approximately day 14 to 30 (2 to 4 weeks) post exposure. Assessment of degree of lung injury will include evaluation of lung function and cardio-respiratory dynamics (breathing rate; arterial oxygenation; heart rate; and pulse distention) non-invasively using mouse-adapted pulse-oximeter (Starr Life Sciences, Oakmont, PA),, and analysis of bronchoalveolar fluid for protein, cytokines/chemokines and cellular content, as well as biochemical analysis (lipid peroxidation of lung tissues, F2α-isoprostanes and 8-oxo-dGuo levels in lung tissue and/or biological fluid as biomarkers of pulmonary oxidative stress) as previously described.

Delayed pulmonary effects:

To investigate delayed pulmonary effects, mouse survival will be recorded twice a week post irradiation and the experiment will be terminated after 12 to 30 weeks (long term). Assessments of degree of lung injury will include evaluation of lung function and cardio-respiratory dynamics non-invasively using mouse-adapted pulse-oximeter as described above., Assessments of altered lung architecture/fibrosis and tissue oxidative stress will include quantitative assessment of lung hydroxyproline content and malondialdelhyde concentration (MDA), respectively; semiquantitative assessment will be based on histopathologic injury score as previously described.

Tissue examination:

All tissues generated will be stored to be homogenized and evaluated for MDA by colorimetrically evaluating MDA levels in lung and plasma using commercially available kits (OXIS International) as a measure of lipid peroxidation as previously described. The results will be expressed as μmol MDA/g wet lung tissue. Liquid chromatography tandem mass spectrometry will be used to measure F2α-isoprostane formation in the tissue.

Anticipated results:

It is expected that pulmonary surfactant, when delivered to the lung pre, peri, or post radiation exposure, will mitigate radiation induced lung damage including acute pneumonitis and/or the delayed subacute/chronic inflammatory response associated with radiation exposure of the lung. The following will be expected to be observed (including but not limited to): improved respiration dynamics (including but not limited to pulmonary resistance, compliance, and arterial oxygenation (measured by arterial oxygen tension (PaO_2; sometimes described as arterial oxygen saturation), and/or peripheral oxygen saturation (SpO_2), and reduced protein, profibrotic and pro-inflammatory cytokines/chemokines (including but not limited to IL-6, TNF, TGF ß, MCP-1, IL-1ß, RANTES), and/or upregulation of antifibrotic and anti-inflammatory cytokines/chemokines, and leak (neutrophil/macrophage migration as an example of white cells, but not limited thereto) in the lung and/or systemic circulation; reduced signs of pulmonary injury on biochemical analysis of bronchoalveolar fluid or lung tissue (reduced lipid peroxidation of lung tissues, blood levels and or tissue and or biological fluid levels of 8-oxo-dGuo, secretory proteins to surfactant protein ratios, and other biomarkers of oxidative stress), reduced alterations in lung architecture, less fibrosis and tissue oxidative stress (quantitatively measure by assessment of lung hydroxyproline content and malondialdelhyde concentration, respectively); and less lung damage based on semi-quantitative and or quantitative histopathologic assessment and histopathologic injury score.

EXAMPLE 1

As described in Prophetic Example 1 above, mice were irradiated with dose of 13.5 Gy in accordance with Table 2 and treated with KL4 surfactant (as liquid instillate) or normal saline (NaCl) as control, delivered *via* the intranasal route twice daily beginning 24 hr post irradiation

and continuing for 2 weeks to compare the effects of KL4 surfactant treatment with the effects of normal saline as control on lung function and mitigation of radiation-induced lung injury.

Acute pneumonitis was evaluated at day 14 (week 2), 21 (week 3) and 30 (week 4) post irradiation exposure. Assessment of the degree of lung injury (pneumonitis) included evaluation of lung function by measurement of arterial oxygenation, using a mouse-adapted pulse-oximeter (Starr Life Sciences, Oakmont, PA), concentration of total white blood cells and neutrophils, respectively, in bronchoalveolar fluid (cells/ml) measured at day 21 (week 3) post irradiation, as well as oxidative damage, measured as percent (%) change in μmol malondialdehyde (MDA)/g lung. Heart rate, pulse distension and respiratory rate were also measured using the mouse-adapted pulse-oximeter (Starr Life Sciences, Oakmont, PA).

As shown in Figure 1A and Table 3, a potentially clinically meaningful reduction in arterial oxygenation (which was statistically significant) was observed in the irradiated untreated and saline-treated (NaCl) control animals at day 14 (week 2) and day 30 (week 4) versus non-irradiated animals, confirming the presence of acute radiation-induced pneumonitis in the irradiated-untreated and irradiated-saline-treated (NaCl) control animals. That is, the observed decrease in arterial oxygenation indicates that oxygen exchange in the lungs was diminished as a result of radiation-induced lung injury as compared to the non-irradiated animals. In contrast, KL4 surfactant-treated irradiated animals showed no significant reduction in arterial oxygenation from non-irradiated animals; i.e., arterial oxygenation remained unchanged after irradiation. Notably, the differences between arterial oxygenation in KL4 surfactant-treated irradiated animals and untreated irradiated or saline-treated irradiated mice were statistically significantly different.

Also, of note in Figure 1B and Table 3, a potentially clinically meaningful reduction in heart rate, expressed in beats per minute (BPM) was observed in the irradiated untreated and saline-treated (NaCl) control animals at day 14 (week 2) and day 30 (week 4) versus non-irradiated animals (and which was statistically significantly different), suggesting the presence of myocardial injury in the irradiated-untreated and irradiated-saline-treated (NaCl) control animals. In contrast, KL4 surfactant-treated irradiated animals showed no significant reduction in heart rate from non-irradiated animals; i.e., heart rate remained unchanged after irradiation, suggesting that there was no myocardial damage.

Table 3.

(A) Arterial Oxygen saturation in percent (%) and heart rate in beats per minutes (BPM), measured at day 14 (week 2) and day 30 (week 4).

		Arterial O2 Saturation (%) Mean (SEM)	Heart Rate (Beats/min) Mean (SEM)
Week 2	Untreated control	96.8 (0.50)	736.7(17.02)
	XRT	93.8 (1.18)	537.1 (62.04)
	NaCl	96.4(0.51)	756.2 (11.63)
	NaCl + XRT	93.5 (0.90)	657.1 (10.26)
	KL4	96.4 (0.20)	737.3 (8.77)
	KL4 + XRT	97.4 (0.58)	732.1 (4.44)
Week 4	Untreated control	96.2 (0.20)	760.9 (5.43)
	XRT	91.1 (0.60)	495.8 (32.79)
	NaCl	97.5 (0.38)	684.4 (12.70)
	NaCl + XRT	90.9 (0.64)	472.6 (31.48)
	KL4	96.3 (0.26)	648.7 (16.36)
	KL4 + XRT	97.0 (0.42)	720.9 (16.88)

(B) Summary statistics comparing treatment groups (t-test)

		Arterial O2 Saturation p-value	Heart Rate p-value
Week 2	Untreated vs XRT	<0.01	<0.01
	Untreated vs KL4+XRT	0.56	0.91
	NaCl vs NaCl+XRT	<0.05	<0.05
	KL4 vs KL4+XRT	0.35	0.89
	XRT vs NaCl+XRT	0.76	<0.01
	XRT vs KL4+XRT	<0.01	<0.01
	NaCl+XRT vs KL4+XRT	<0.01	0.06
Week 4	Untreated vs XRT	<0.01	,0.01
	Untreated vs KL4+XRT	0.229	0.21
	NaCl vs NaCl+XRT	<0.01	<0.01
	KL4 vs KL4+XRT	0.289	0.03
	XRT vs NaCl+XRT	0.78	0.45
	XRT vs KL4+XRT	<0.01	<0.01
	NaCl+XRT vs KL4+XRT	<0.01	<0.01

Similarly, as shown in Figures 2A and 2B and Table 4, the concentration of white blood cells (WBC) and neutrophils (PMNs) in bronchoalveolar fluid (BAL), expressed in cells/ml of BAL, measured at day 21 (week 3) was lower in the KL4-treated irradiated animals versus the irradiated-untreated and irradiated-saline-treated (NaCl) control animals, and the differences were statistically significant.

Table 4. Concentration of white blood cell counts (WBC) neutrophils (PMNs) in bronchoalveolar fluid (BAL; cells/ml) measured at day 21 (week 3).

	WBC (cells/ml) Mean (SEM)	PMN (%) Mean (SEM)	PMN (cells/ml BAL) Mean (SEM)
Untreated control	8775 (-)	-	-
XRT	64167 (3005)	31 (4.37)	20067 (2724)
NaCl	47375 (2625)	4 (2.87)	1460 (1140)
NaCl + XRT	74625 (11187)	23 (6.14)	16099 (4120)
KL4	38625 (3793)	1 (0.48)	268 (157)
KL4 + XRT	44875 (4235)	26 (5.51)	11496 (2381)

Figure 3 shows a graph depicting percent (%) change in μmol malondialdehyde (MDA) / g lung measured at day 21 (week 3) post irradiation. KL4 surfactant-treated animals had significantly less oxidative damage versus both NaCl-treated irradiated animals, and their respective, non-irradiated controls (15% increase vs. 1.3%, respectively).

These observation individually and collectively indicate that KL4 surfactant mitigated acute radiation-induced lung injury in mice that were exposed to a dose of radiation (13.5 Gy) that is known to cause radiation pneumonopathy (i.e., the LD_{50} ("Lethal Dose"). LD_{50} is the amount of a material (e.g., irradiation), given all at once, which causes the death of 50% (one half) of a group of test animals). This dose in mice would translate to a dose in humans that would be approximately equivalent (Hopewell et.al, *Int J Radiat Oncol Biol Phys* 1995: 31(2):361-369), and confirmed in published work in rodents (Christofidou-Solomidou M, et al. *BMC Cancer* 2011: 11:269; Lee JC, et al. *Radiat Res* 2010;173(5): 590-601; Lee JC, et al. *Cancer Biol Ther* 2009;8(1):47-53; Machtay M, et al. *Radiother Oncol* 2006;81(2): 196-205.)

Moreover, the therapeutic administration of KL4 surfactant appears to have mitigated lung injury-induced myocardial injury, likely resulting from down regulation/production of proinflammatory cytokines/chemokinesin the lung, since the activation of cytokines/chemokine pathways that play a role in the pathogenesis of radiation pneumonopathy (Rubin and colleagues (*Int J Radiat Oncol Biol Phys* 1992: 24(1):93-101) may also cause multi-organ damage, especially myocardial injury (Braunwald E: *N Engl J Med* 2008;358:2148-59; Weintraub NL et. al: *Circulation.* 2010;122:1975-1996).

PROPHETIC EXAMPLE 2:

Whole body irradiation using a mouse model

Experimental Approach:

Approximately 700 C57BL/6 wild-type mice at 6-8 weeks of age will be studied. These mice will be either irradiated, as discussed below, or left un-irradiated as a control group. The mice will be divided into three subsets (cohorts) of animals to allow for evaluation of acute and delayed pulmonary effects after an acute (24-72 hrs) treatment with KL4 surfactant, or an acute treatment with KL4 surfactant followed by a second round of treatment with KL4 surfactant at approximately 8-12 weeks post injury, or a delayed KL4 surfactant treatment only, beginning approximately around 8-12 weeks post exposure just prior to the onset of subacute pneumonitis (possibly assessed by increased breathing rate, and or decreased arterial oxygenation for example, and/or biochemical evidence of lung injury). The mice will receive either KL4 surfactant or saline as a vehicle control. Groups of 8 mice will be sacrificed at intervals following radiation and surfactant treatment. The first cohort treated 24-72 hrs post radiation will undergo necropsy/evaluation at approximately day 14, 21, 28, and 8, 16, 20, 26, and 30 weeks to assess the effect of early intervention on the development of pneumonitis and fibrosis. The second cohort will, in addition, receive a second round of surfactant beginning at 8-12 weeks just prior to the onset of active pneumonitis (as assessed by increased breathing rate and/or decreased SpO_2 for example, and/or biochemical evidence of lung injury), and will undergo a similar evaluation with sacrifice at 16, 20 and 26 weeks. The third cohort will only receive a KL4 surfactant beginning at 8-12 weeks just prior to the onset of subacute pneumonitis (possibly assessed, but not limited to increased breathing rate, and or decreased arterial oxygenation for

example, and/or biochemical evidence of lung injury), and will undergo a similar evaluation with sacrifice at approximately 16, 20, 26 and 30 weeks. Mice will be irradiated as previously described. Briefly, mice are held in modified jigs and irradiated with 5Gy whole body irradiation (WBI) and an additional 11Gy to the lung only (as discussed for Example 1) for a total of 16Gy to the lung tissue.

Treatment:

KL4 surfactant liquid instillate or normal saline (as control) will be delivered via the intranasal route twice daily (mice are obligate nasal breathers and effectiveness of delivery method has been previously established) beginning 24 to 72 hrs post irradiation and lasting for 2 to 3 weeks. A second treatment phase could be initiated around week 8-12 weeks post radiation exposure, or the initial KL4 surfactant treatment could be initiated at this 8-12 week time-point just prior to the onset of active pneumonitis (possibly assessed by increased breathing rate, and or decreased SpO_2 for example, and/or biochemical evidence of lung injury). Methods for intranasal instillations are well described. In brief, mice will be placed in an induction chamber and lightly anesthetized with isoflurane. Once anesthetized, the mouse will be removed from the induction chamber and placed in a supine position and 50-100 µl of instillate delivered drop-wise to the nares using a pipette device. Approximately 60-120 mg/kg TPL (total phospholipid) of KL4 surfactant will be delivered per dose, which corresponds to dose that has been shown to be effective in the ALI and RDS models in terms of restoring lung function and modulating an immune response in the mouse. Following treatment, mice will be allowed to recover in a warm environment under observation prior to being returned to their home cage.

Assessment of injury and treatment efficacy:

Mouse survival will be recorded twice per week post irradiation and the experiment will be terminated after 26 weeks. Assessment of acute radiation damage will begin at approximately days 14, 21 and/or 28 post exposure and will include assessment of protein leak, neutrophil/macrophage migration as an example of white cells but not limited to, and cytokines/chemokine production (such as but not limited to IL-6, TNF, TGF ß). A reduction in any or all of these parameters relative to untreated controls of at least 5%, or preferably at least 10%, will be considered as evidence of a treatment effect. Alternatively, a statistical trend ($p<0.1$, unadjusted for multiple comparisons) in reduction in any or all of these parameters

relative to untreated controls will be considered evidence of a treatment effect. Beginning at 6-8 weeks post-irradiation (onset of pneumonitis/alveolitis), animals will be subjected to breathing rate analysis to monitor disease progression. At that time (approximately 8-10 weeks), a group of animals from each group will be sacrificed for detailed histological examination and a more extensive pulmonary function analysis (resistance, compliance) will be performed. Assessment of alveolitis will consist of a qualitative analysis using a histological scoring system, as well as a quantitative analysis of inflammatory cell recruitment measured histologically using cell specific markers in the bronchoalveolar lavage (BAL) and tissue (by image analysis), as well as flow cytometric analysis of cell populations in the lung tissue and blood compartments following enzyme disaggregation. Additional measures will include protein leak, neutrophil/macrophage migration as an example of white cells, but not limited thereto, cytokines/chemokine production (for example but not limited to IL-6, TNF, TGF ß), and the ratio in plasma of secretory proteins to surfactant proteins. A difference of at least 5%, or preferably at least 10%, in terms of reduction in inflammation versus irradiated, untreated controls will be considered meaningful evidence of effectiveness of the treatment, as observed in one or more parameters. Alternatively, a statistical trend ($p<0.1$, unadjusted for multiple comparisons) in reduction in any or all of these parameters relative to untreated controls will be considered evidence of a treatment effect. The delayed subacute inflammatory response and altered lung architecture/fibrosis will be examined at 24-30 weeks post exposure. At the later time points, assessment of fibrosis will utilize a quantitative image analysis technique looking at collagen deposition and the ratio of tissue to airspace as a measure of tissue remodeling. A difference of at least 5%, or preferably, at least 10%, in terms of reduction in collagen deposition or disruption of lung architecture versus irradiated, untreated controls will be considered meaningful. Alternatively, a statistical trend ($p<0.1$, unadjusted for multiple comparisons) in reduction in any or all of these parameters relative to untreated controls will be considered evidence of a treatment effect. Semiquantitative assessments of altered lung architecture/fibrosis and tissue oxidative stress will be based on histopathologic injury score as previously described. Furthermore, lavage fluid, plasma, and tissue homogenate will be assayed for message and protein expression levels including but not limited to profibrotic (TGFß, IL-1 ß) and proinflammatory (TNF, MCP-1, IL-1ß, RANTES) cytokines/chemokines, and/or antifibrotic and anti-inflammatory cytokines/chemokines. A

reduction in any or all of the parameters relative to irradiated untreated controls by at least 5%, or preferably, by at least 10%, will be considered meaningful. Alternatively, a statistical trend ($p<0.1$, unadjusted for multiple comparisons) in reduction in any or all of these parameters relative to untreated controls will be considered evidence of a treatment effect.

EXAMPLE 2

As described in Prophetic Example 2 above, mice were irradiated with a dose 5Gy whole body irradiation (WBI) and an additional 11Gy to the lung only (as discussed for Example 1) (total 16Gy), and treated with KL4 surfactant (as liquid instillate) or normal saline (NaCl) as control, delivered via the intranasal route twice daily beginning 24 hr post irradiation and continuing for 2 weeks to compare the effects of KL4 surfactant treatment with the effects of normal saline as control on mitigation of radiation-induced lung injury.

As shown in Figure 4, neutrophil population density in lung tissue assayed by immunostaining and image analysis was lower in the KL4-treated irradiated animals versus the irradiated untreated and saline-treated (NaCl) control animals.

These observations, consistent with those in Example 1, show that KL4 surfactant treatment mitigated acute radiation-induced lung injury in mice that were irradiated with a dose 5Gy whole body irradiation (WBI) and an additional 11Gy to the lung only (total 16Gy), and treated with KL4 surfactant (as liquid instillate). As in Example 1, the irradiation dose of radiation dose 5Gy whole body irradiation (WBI) and an additional 11Gy to the lung only (total dose of 16 Gy) is known to cause radiation pneumonopathy and approximates the LD_{50} in this model. Similarly, this dose in mice would translate to a dose in humans that would be approximately equivalent (Hopewell et.al: *Int J Radiat Oncol Biol Phys* 1995: 31(2):361-369).

PROPHETIC EXAMPLE 3:

Pulmonary surfactant lung decorporation animal model

The use of pulmonary surfactant to facilitate removal of inhaled insoluble radionuclides (radioactive particles) from the lung will be studied by employing the following techniques:

1. Dilute KL4 surfactant (in a range of 1 - 50 mg/mL TPL; preferably 10 mg/mL TPL) will be administered via BAL. The inclusion of pulmonary surfactant is expected to improve

BAL efficiency in removing inhaled insoluble radioactive particles from the lung. More specifically, pulmonary surfactant is expected to 1) augment the contact efficiency (wetting) of the lavage fluid in the lung parenchyma given its surface lowering properties; 2) decrease the degree of adhesiveness *in vivo* of alveolar macrophages that have phagocytized radioactive particles, making them more amenable to being washed out of the lung by the lavage fluid; and 3) allow more efficient distal lavage of the lung parenchyma given the ability of surfactant to expand terminal airways and alveoli.

2. A modification of the BAL lavage technique using segmental bronchoscopic lavage (BSPL) using dilute pulmonary surfactant (in a range of 1 - 50 mg/mL TPL; preferably 10 mg/mL TPL) will be used. This modified technique is expected to further improve efficiency in removing inhaled insoluble radioactive particles from the lung.

3. Aerosolized pulmonary surfactant will be administered to decorporate inhaled insoluble radioactive particles from the lung, thereby reducing the radioactive body burden, by increasing natural mucociliary clearance of terminal airway and alveolar macrophages that have phagocytized radioactive particles, for example through expectoration and swallowing of cleared material and subsequent elimination in the feces.

Experimental Approach:

The proposed research will involve the use of 30 animals (n = 6 per group). The animals will be exposed once to a ^{85}Sr-FAP aerosol followed by treatment as outlined in Table 3. Animals will be placed into metabolism cages approximately 24 hours prior to ^{85}Sr-FAP exposure for acclimation and will remain there throughout the duration of the study. On the morning of exposure, animals will be anesthetized and exposed to ^{85}Sr-FAP by aerosol inhalation exposure and returned to their metabolism cages as described below. 24 hours after ^{85}Sr-FAP exposures, animals will be anesthetized and treatment will be followed as outlined in Table 4.

Table 5. Experimental groups

GROUP	N	INTERVENTION
1	6	Untreated (negative control)
2	6	Bal saline lavage (positive control)
3	6	Bal saline lavage plus KL4 surfactant
4	6	Bspl saline lavage plus KL4 surfactant
5	6	Aerosolized KL4 surfactant

All animals will be followed for 7 days following therapeutic intervention - this observation period will allow comparison of the relative tolerability of treatments, with the expectation that the surfactant-treatment animals (Group 3 and 4) will tolerate the BAL procedure better than those treated only with saline BAL (Group 2). Lavage fluid, urine, feces, and cage rinses will be collected daily and retained for analysis of ^{85}Sr. All animals will be euthanized at day 7 following therapeutic intervention and will undergo necropsy. The lungs and trachea will be removed for measurement of the ^{85}Sr content; the remaining tissues will be stored for possible analyses.

Treatment:

For the standard BAL procedure, one end of the T-tube is connected to the anesthesia circuit, the other to the lavage apparatus, which consists of a container of normal saline at room temperature that is suspended 60-80 cm above the animal. A second T-tube is used in the lavage tubing to extend a latex tube to the floor, in which used lavage fluid will be drained and collected in a suitable vessel. Once the lavage apparatus has been connected, the animal is manually ventilated for approximately 3 minutes to induce apnea, and then placed on a table with the side to be lavaged in the down position. A volume of saline equal to 40% of the estimated residual functional capacity is then instilled into the lung. That fluid is then drained out of the lung into the vessel on the floor. Apnea is repeatedly induced by additional manual ventilation, and the procedure repeated until about 1 L of saline has been used. Isotonic saline without or with therapeutic pulmonary surfactant (KL4 surfactant, 10mg/mL TPL) will be used for the selected experimental groups (Groups 2 and 3, respectively; Table 5).

For the BSPL procedure (Group 4; Table 5) the lung lavage approach is modified in that the animals will require mechanical ventilation during the 20 to 30 minute procedure, and possibly for a short time thereafter. A bronchoscope is introduced down the single lumen endotracheal tube and then serially wedged into each of the lung segments of the animal to allow 2 lavages to be performed with volumes that approximate 10-20 mL. Suctioning of lavagate is performed to remove macrophages, cellular debris and any non-phagocytized radioactive particles. Since the lavagate contains an exogenous functional surfactant (KL4 surfactant), the risk of alveolar collapse is minimized, in contrast to that reported with saline alone (Muggenburg, BA, *et al*. Improvements in Lung Lavage to Increase its Effectiveness in

Removing Inhaled Radionuclides. In Proceedings of the 8th Congress of the International Radiation Protection Association. Montreal, Canada; pp. 920-923; May 17-22, 1992).

For the aerosolized KL4 surfactant therapeutic inhalation exposure (Group 5; Table 3), KL4 surfactant aerosol (30 mg/mL TPL) will be generated with Capillary Aerosol Generating (CAG) device and delivered via interface technology. Lightly sedated dogs will receive aerosolized KL4 surfactant twice daily for 3 days and up to 30 minutes, beginning 24 hours post-exposure, to deliver approximate 60 - 90 mg/kg TPL of KL4 surfactant to the lung per dose.

Seven days after therapeutic administration, animals will be euthanized. Fasted animals will be sedated with acepromazine and butorphanol. After sedation, an intravenous catheter will be placed to accommodate administration of a ketamine/diazepam cocktail. The animals will be euthanized by an overdose of a barbiturate-based sedative. The animals will then undergo necropsy where the lungs and trachea will be collected for measurement of radionuclide content; all remaining tissues will be saved for possible analyses.

Assessment of injury and treatment efficacy

Filter samples, impactor samples, urine, feces, cage rinse, and lung/trachea samples will be analyzed for ^{85}Sr content using *in vivo* and sample counters. This detector system consists of a pair of dual-scintillator crystal detectors that are capable of measuring the entire photon energy range pertinent to measuring ^{85}Sr spectra. The instrument will be calibrated daily with ^{85}Sr NIST-traceable standards. Peak areas will be compared to a generated linear curve to determine the amount of radioactivity present in each sample. It is expected that a minimum 10% decrease in lung content of radioactive particles with KL4 surfactant lavage treatment (Groups 3 and 4) compared to positive saline lavage controls (group 2) and a 10% decrease in lung content of radioactive particles with aerosolized KL4 surfactant (Group 5) will be observed as compared to untreated controls (Group 1).

The present invention is not limited to the embodiments described and exemplified herein, but is capable of variation and modification within the scope of the appended claims.

What is Claimed:

1. Use of an exogenous pulmonary surfactant to treat an individual exposed or at risk of exposure to ionizing radiation, wherein the use comprises the steps of:

(a) identifying the individual who (i) has radiation induced injury resulting from prior exposure to ionizing radiation, or (ii) who is at risk of radiation induced injury resulting from exposure to ionizing radiation, wherein the radiation induced injury includes at least one of lung injury and multi-organ injury; and

(b) administering the exogenous pulmonary surfactant to the individual in an amount and for a time effective to treat the individual's lungs.

2. Use according to claim 1, wherein the exposure to ionizing radiation comprises direct exposure of the individual's lungs to the ionizing radiation.

3. Use according to claim 1, wherein the exposure to ionizing radiation comprises exposure of the individual's whole body to the ionizing radiation.

4. Use according to claim 1, wherein the exposure to ionizing radiation comprises exposure of portions of the individual's body excluding the lungs to the ionizing radiation.

5. Use according to any one of the preceding claims, wherein the exogenous pulmonary surfactant is administered to the lungs of the individual.

6. Use according to any one of the preceding claims, further comprising, after the administering step, a step of detecting or monitoring one or more signs or symptoms of lung injury, wherein a favorable result of treatment, as compared with an equivalent untreated individual, is indicated by any one or more of: improved respiration dynamics, pulmonary resistance, or pulmonary compliance; maintaining oxygenation in a normal physiological range; reduced leak of protein or profibrotic and pro-inflammatory cytokine, including IL-6, TNF, TGF ß, MCP-1, IL-1ß, or RANTES, in the alveolar space or lung tissue; reduced neutrophil or macrophage migration into the alveolar space or lung tissue; reduced signs of pulmonary injury on biochemical analysis of bronchoalveolar fluid or lung tissue; reduced lipid peroxidation of lung tissues; reduced blood levels and or tissue and or biological fluid levels of 8-oxo-dGuo; reduced ratio of secretory proteins to surfactant proteins; reduced levels of biomarkers of oxidative stress; reduced alterations in lung architecture; reduced fibrosis and tissue oxidative

stress; and reduced lung damage based on semiquantitative and or quantitative histopathologic assessment and histopathologic injury score.

7. Use according to claim 6, wherein a reduction of at least five percent of any of the following criteria, as compared to an equivalent untreated individual, indicates a favorable treatment result: protein leak; neutrophil/macrophage migration; IL-6, TNF, IL-1 ß, MCP-1, RANTES, or TGFß mRNA or protein production; the plasma ratio of secretory proteins to surfactant proteins; assessment of fibrosis utilizing a quantitative image analysis technique looking at collagen deposition or the ratio of tissue to airspace.

8. Use according to claim 7, wherein an improvement of at least ten percent of any of the criteria, as compared to an equivalent untreated individual, indicates a favorable treatment result.

9. Use according to any one of the preceding claims, wherein the exogenous pulmonary surfactant is administered in combination with additional components including therapeutic agents or radio-protectants, wherein said components are administered simultaneously or in series.

10. Use according to any one of the preceding claims, wherein the exogenous pulmonary surfactant is administered as one or more of: a liquid instillate, a dry powder or an aerosol.

11. Use according to any one of the preceding claims, wherein the exogenous pulmonary surfactant is administered at a dosage of 5 to 1000 mg total phospholipid (TPL) per kilogram body weight.

12. Use according to claim 11, wherein the exogenous pulmonary surfactant is administered at a dosage of 5 to 200 mg TPL/kg body weight.

13. Use according to any one of the preceding claims, wherein the individual has been exposed to the ionizing radiation and has lung injury.

14. Use according to claim 13, wherein the lung injury comprises at least one of acute radiation pneumonitis (ARP) or delayed effects of acute radiation exposure (DEARE).

15. Use according to claim 13 or 14, wherein the administration of exogenous pulmonary surfactant begins not more than about 24-48 hours after the exposure to ionizing radiation.

16. Use according to claim 13 or 14, wherein the administration of exogenous pulmonary surfactant begins not more than about 24 hours to about 2 weeks after onset of clinical symptoms of DEARE.

17. Use according to any one of claims 1-12, wherein the individual identified is at risk of future exposure to ionizing radiation.

18. Use according to claim 17, wherein the administration of exogenous pulmonary surfactant begins at least about 2 weeks prior to the anticipated future exposure to ionizing radiation.

19. Use according to claim 17, wherein the administration of exogenous pulmonary surfactant begins at least about 12 hours prior to the anticipated future exposure to ionizing radiation.

20. Use according to any one of claims 1-12, wherein the individual identified has been previously exposed to ionizing radiation but does not yet exhibit symptoms of lung injury.

21. Use according to any one of the preceding claims, wherein the exposure to ionizing radiation comprises inhalation of radionuclides.

22. Use according to claim 21, wherein the individual identified has been exposed to ionizing radiation and the exogenous pulmonary surfactant is administered by bronchoalveolar lavage, by segmental bronchoscopic pulmonary lavage, or by aerosol.

23. Use according to claim 22, wherein the exogenous pulmonary surfactant comprises KL4 (SEQ ID NO:1) and is administered at a concentration of about 1mg/mL to about 50 mg/mL TPL.

24. Use of an exogenous pulmonary surfactant for treating a subject who is exposed to ionizing irradiation of at least 1 Gy and who exhibits lung dysfunction measured by a decrease of arterial oxygenation of at least two percent (2 %), wherein the use comprises administering the exogenous pulmonary surfactant in an amount of 0.5 mg to 2000 mg TPL per kilogram of body weight to the subject at the time of exposure or within 30 minutes to six months after exposure to the irradiation.

25. Use according to claim 24, wherein the exposure to ionizing irradiation comprises direct exposure of the individual's lungs to the irradiation.

26. Use according to claim 24, wherein the exposure to ionizing irradiation comprises exposure of the individual's whole body to the irradiation.

27. Use according to claim 24, wherein the exposure to ionizing irradiation comprises exposure of portions of the individual's body excluding the lungs to the irradiation.

28. Use of an exogenous pulmonary surfactant to treat an individual exposed to ionizing radiation, wherein ionizing radiation has caused or could cause lung injury, and wherein the lung injury can cause lung injury-induced multi-organ injury, wherein the use comprises the steps of:

(a) identifying an individual who (i) has lung injury resulting from prior exposure to ionizing radiation, or (ii) who is at risk of lung injury resulting from exposure of the individual to ionizing radiation; and

(b) administering the exogenous pulmonary surfactant to the individual's lungs in an amount and for a time effective to treat the individual's lungs, resulting in treatment of the multi-organ injury.

29. Use according to claim 28, wherein the multi-organ injury includes one or more of myocardial injury, renal injury and liver injury.

30. Use according to claim 29, wherein the individual exhibits decreased heart rate as a symptom of the myocardial injury.

31. Use according to any one of the preceding claims, wherein the exogenous pulmonary surfactant comprises KL4 (SEQ ID NO:1).

FIG. 1A

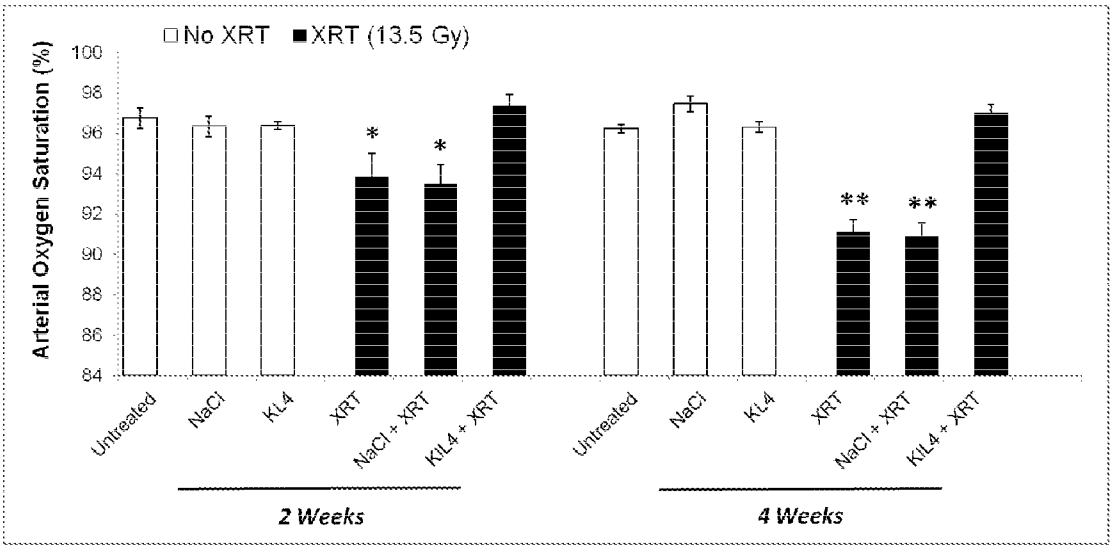

No XRT
XRT (13.5 Gy)
Arterial Oxygen Saturation (%)
100
98
96
94
92
90
88
86
84
*
*
**
**
Untreated
NaCl
KL4
XRT
NaCl + XRT
KL4 + XRT
Untreated
NaCl
KL4
XRT
NaCl + XRT
KL4 + XRT
2 Weeks
4 Weeks

FIG. 1B

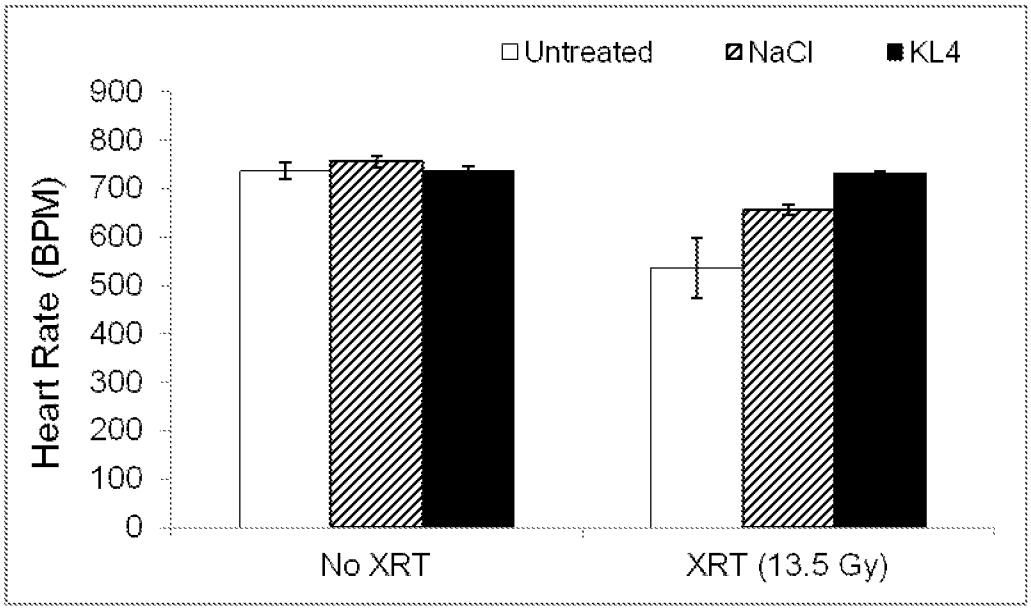

Untreated
NaCl
KL4
Heart Rate (BPM)
900
800
700
600
500
400
300
200
100
0
No XRT
XRT (13.5 Gy)

FIG. 2A

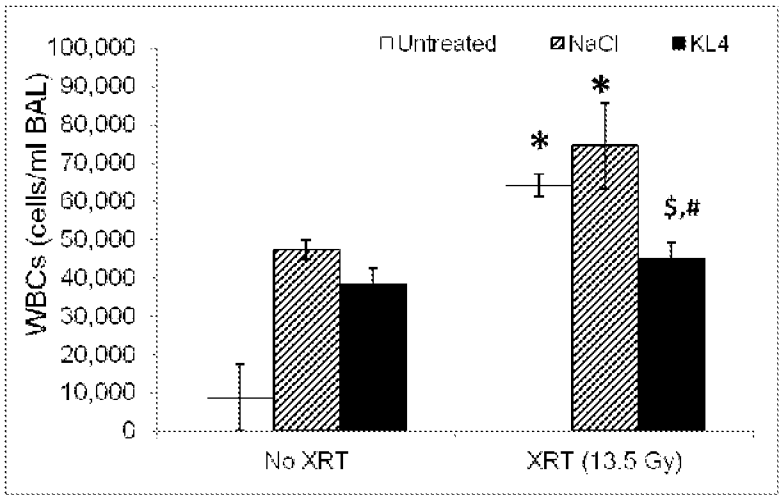

Untreated
NaCl
KL4
WBCs (cells/ml BAL)
100,000
90,000
80,000
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
*
*
$,#
No XRT
XRT (13.5 Gy)

FIG. 2B

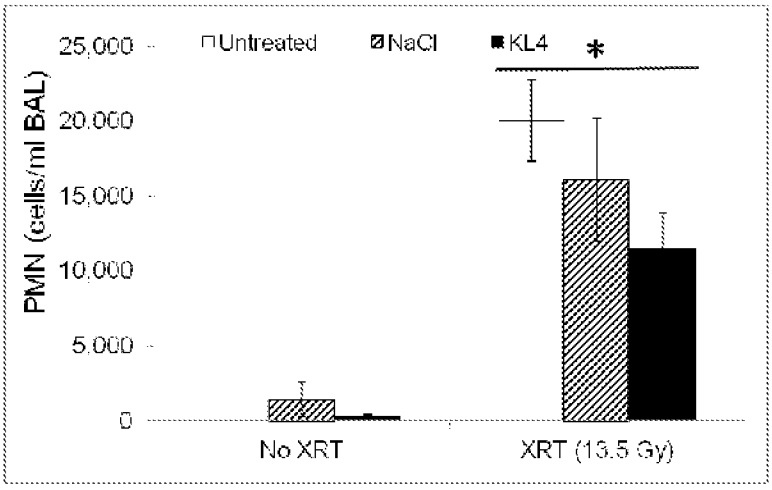

Untreated
NaCl
KL4
PMN (cells/ml BAL)
25,000
20,000
15,000
10,000
5,000
0
*
No XRT
XRT (13.5 Gy)

FIG. 3

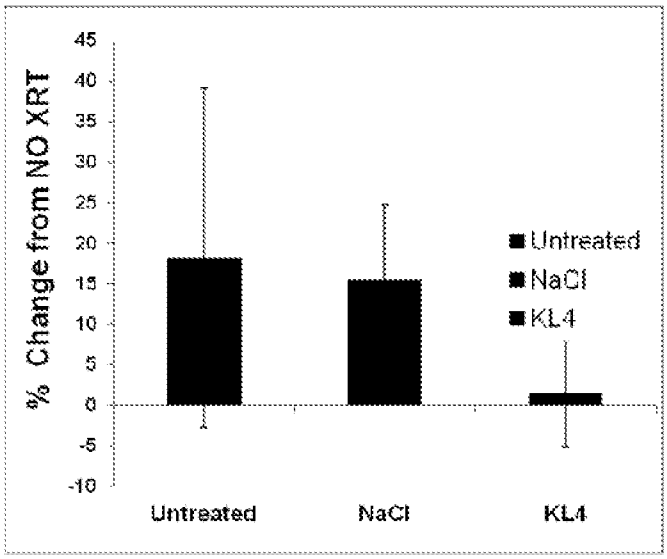
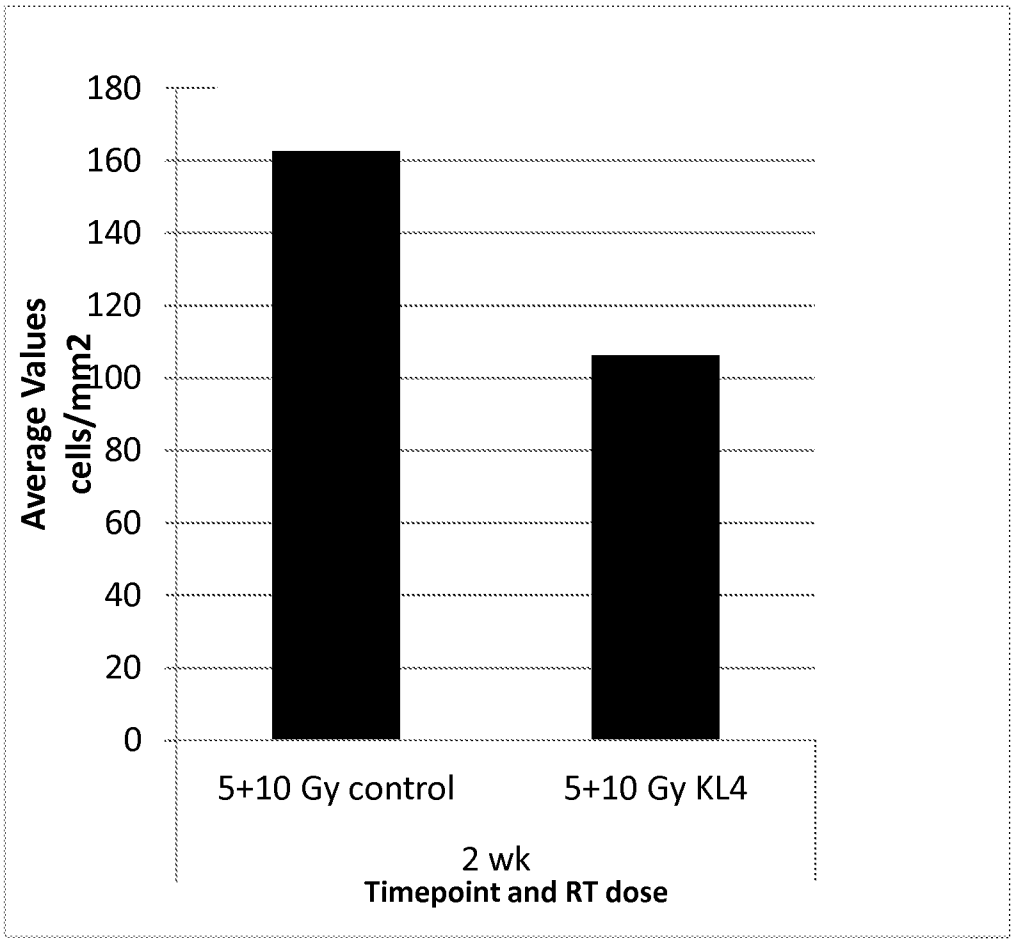

% Change from NO XRT
45
40
35
30
25
20
15
10
5
0
-5
-10
Untreated
NaCl
KL4
Untreated
NaCl
KL4

FIG. 4


Average Values cells/mm2
180
160
140
120
100
80
60
40
20
0
5+10 Gy control
5+10 Gy KL4
2 wk
Timepoint and RT dose