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(54) **MUCOLYTIC AND ANTI-ELASTASE COMPOUNDS AND METHODS OF USE THEREOF**

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(60) Provisional application No. 60/556,516, filed on Mar. 24, 2004. Provisional application No. 60/650,865, filed on Feb. 7, 2005. Provisional application No. 60/409,960, filed on Sep. 10, 2002. Provisional application No. 60/462,082, filed on Apr. 11, 2003.

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(52) **U.S. Cl.** **424/46**; 514/18; 514/440

(57) **ABSTRACT**

Disclosed are compositions and methods for decreasing the viscosity and/or cohesiveness of and/or increasing the liquefaction of excessively or abnormally viscous or cohesive mucus or sputum. Also disclosed are compositions and methods for inhibiting elastase in a patient. The compositions contains a compound containing a dithiol active-site in reduced state and optionally further contains a reducing system.

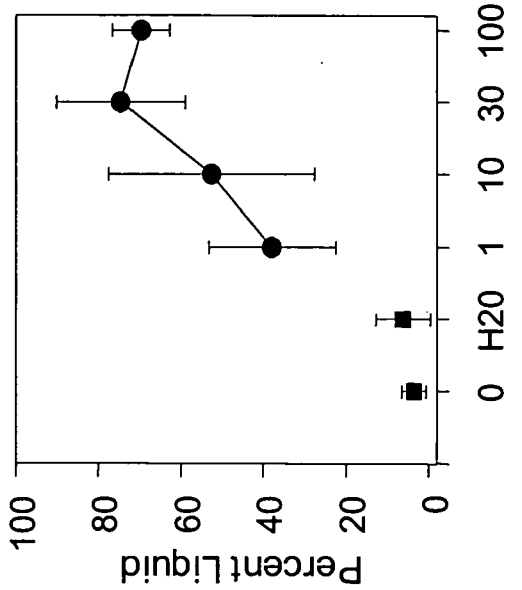

Percent Liquid
100
80
60
40
20
0
0
H20
1
10
30
100

FIG. 1A

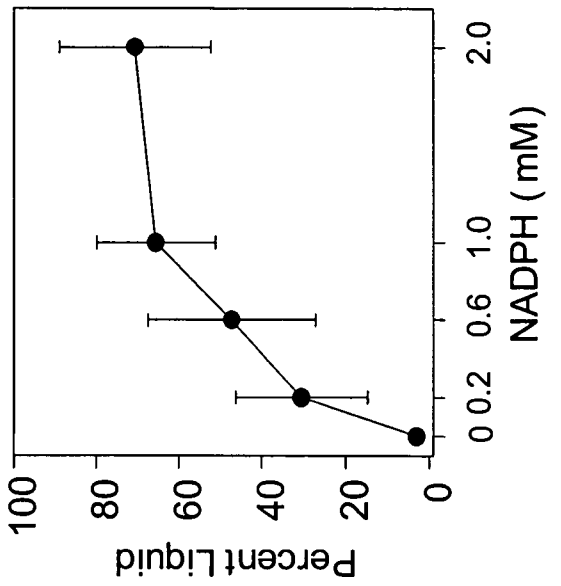

Percent Liquid
100
80
60
40
20
0
Trx (μM)
0
0.2
0.6
1.0
2.0
NADPH (mM)

FIG. 1B

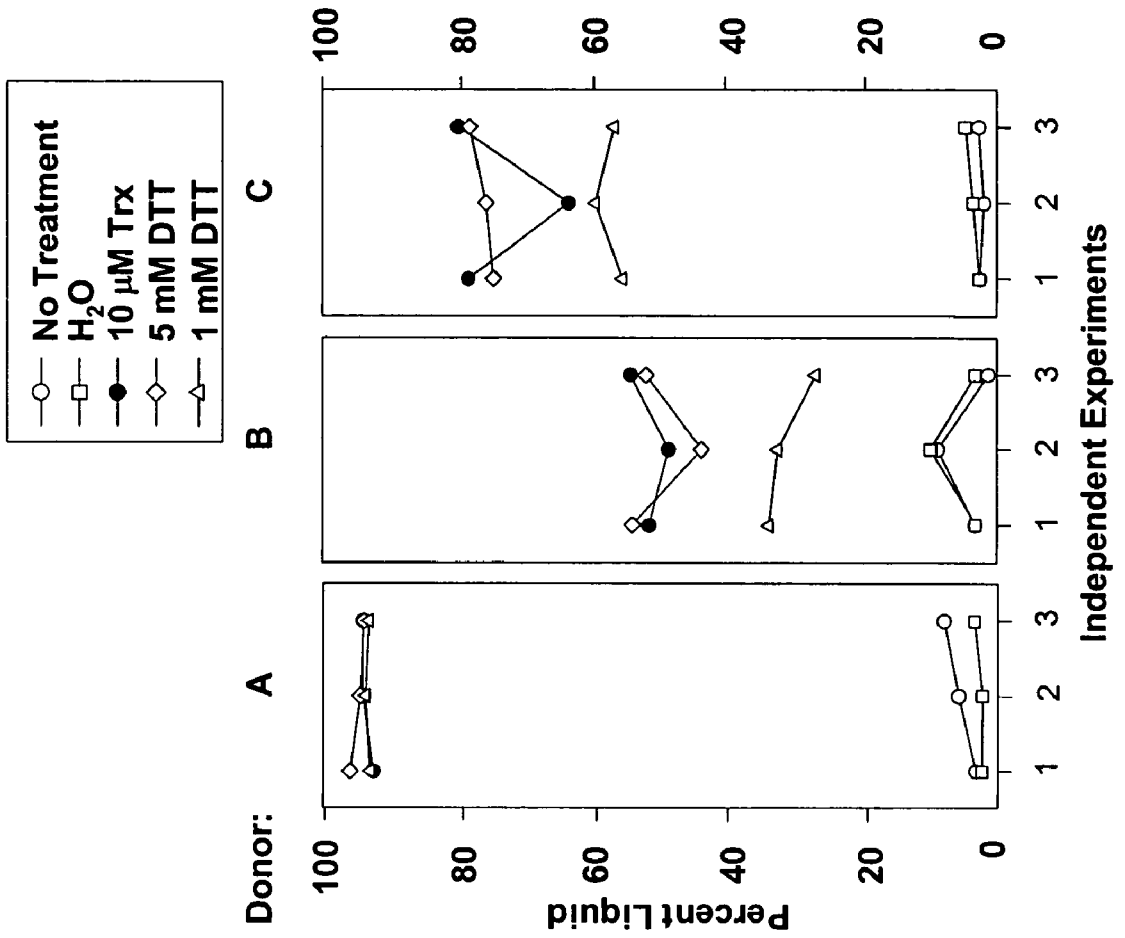

No Treatment
H2O
10 µM Trx
5 mM DTT
1 mM DTT
Donor:
A
B
C
Percent Liquid
0
20
40
60
80
100
1
2
3
Independent Experiments

FIG. 2

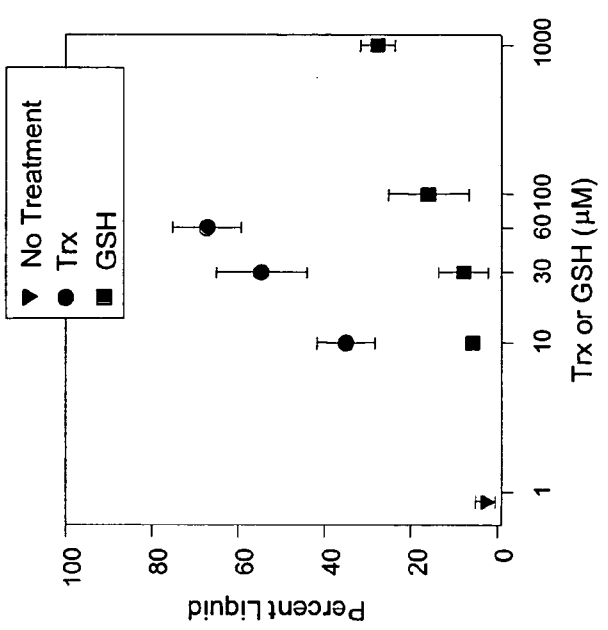

No Treatment
Trx
GSH
Percent Liquid
Trx or GSH (μM)
0
20
40
60
80
100
1
10
30
60
100
1000

FIG. 3A

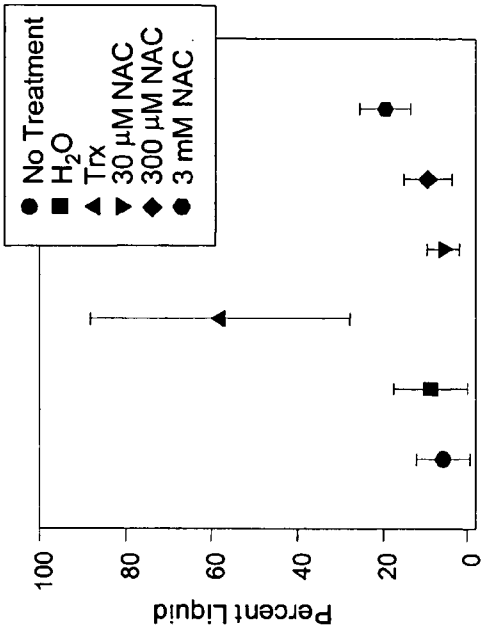

No Treatment
H2O
Trx
30 μM NAC
300 μM NAC
3 mM NAC
Percent Liquid
0
20
40
60
80
100

FIG. 3B

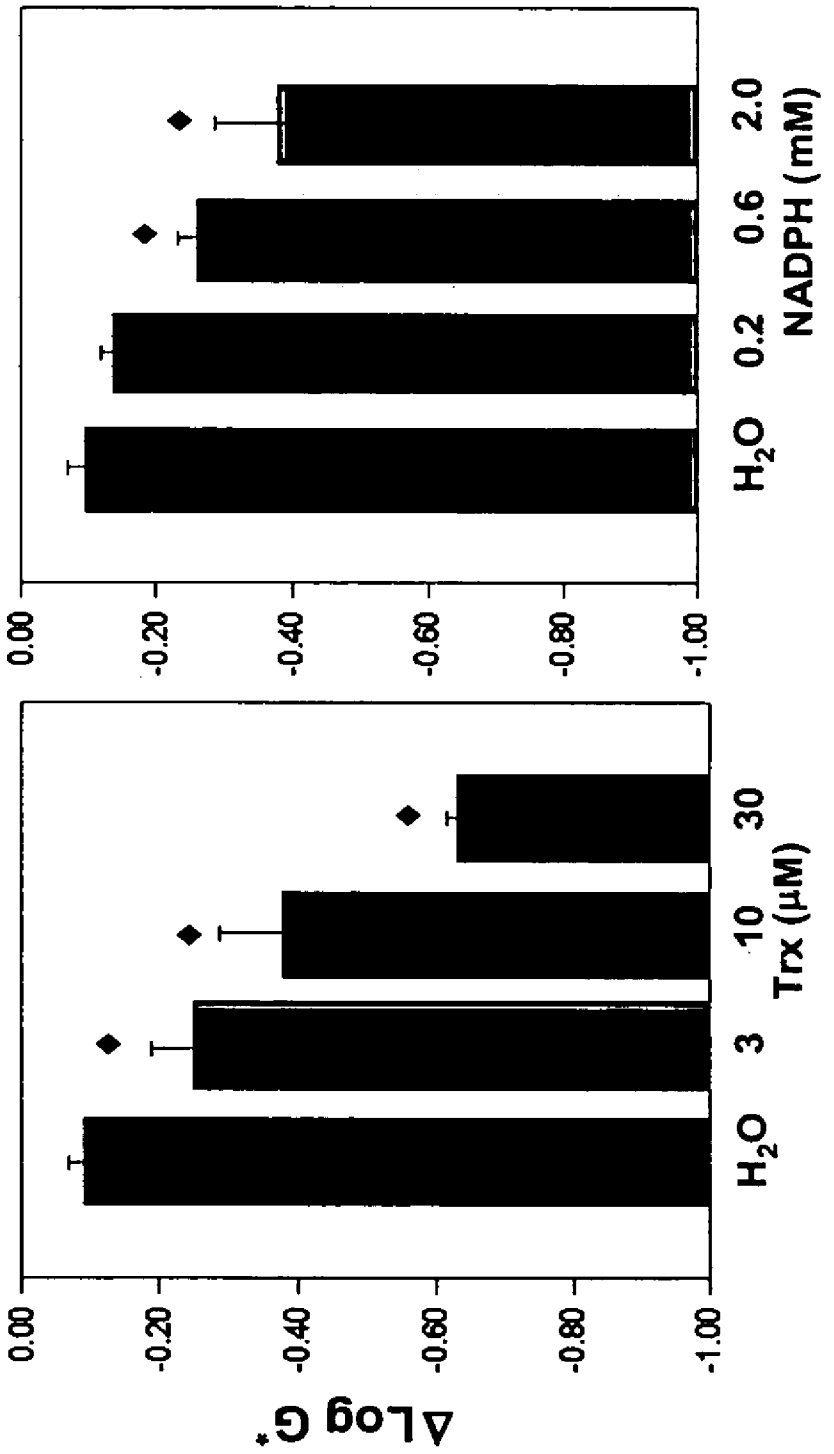

ΔLog G*
0.00
-0.20
-0.40
-0.60
-0.80
-1.00
H2O
3
10
30
Trx (μM)
H2O
0.2
0.6
2.0
NADPH (mM)

FIG. 4A

FIG. 4B

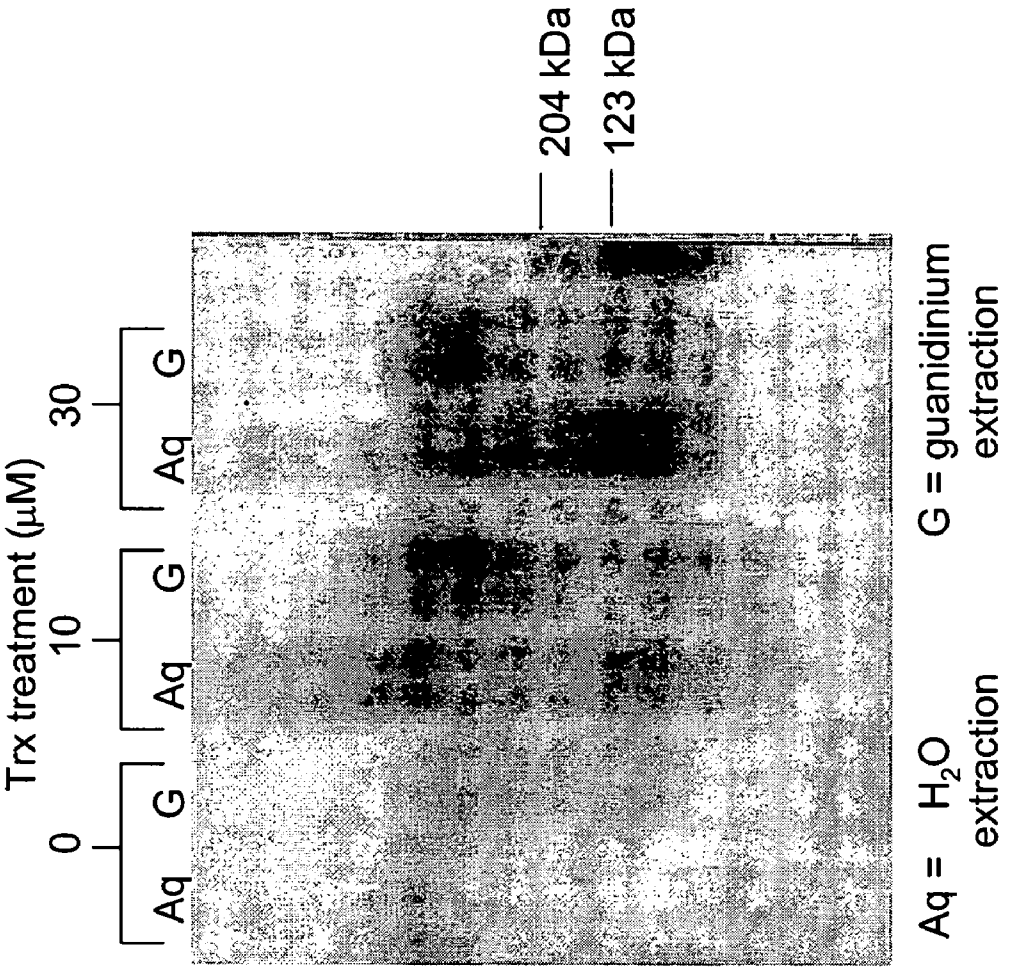

204 kDa
123 kDa
Trx treatment (μM)
0
10
30
Aq
G
Aq = H_2O extraction
G = guanidinium extraction

FIG. 5

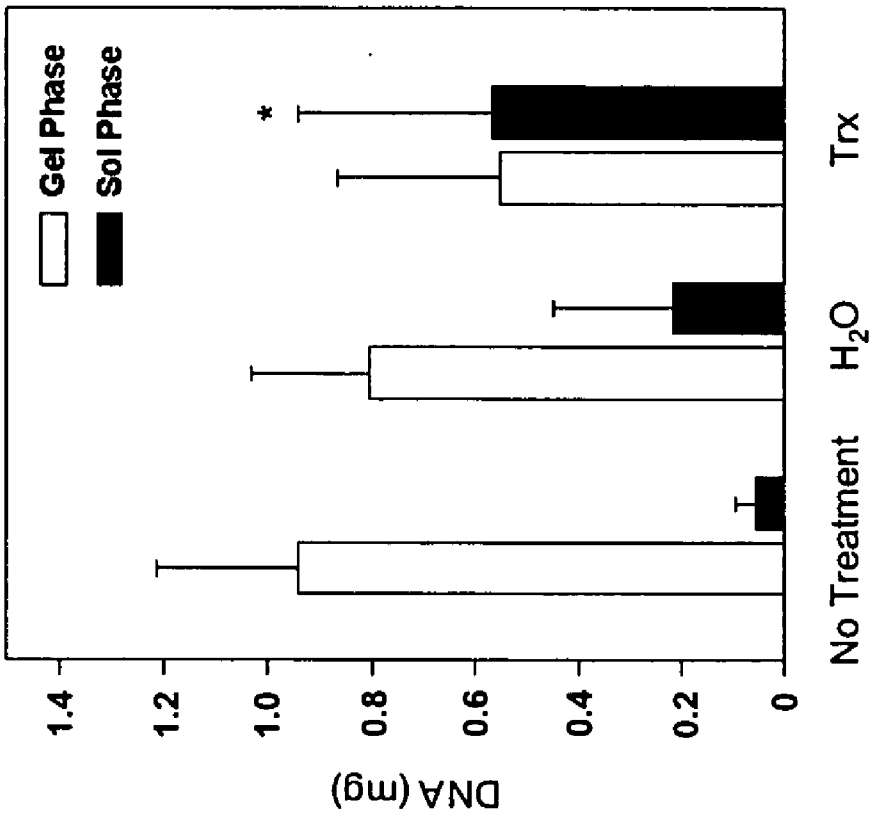

Gel Phase
Sol Phase
*
DNA (mg)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
No Treatment
H2O
Trx

FIG. 6

FIG. 7

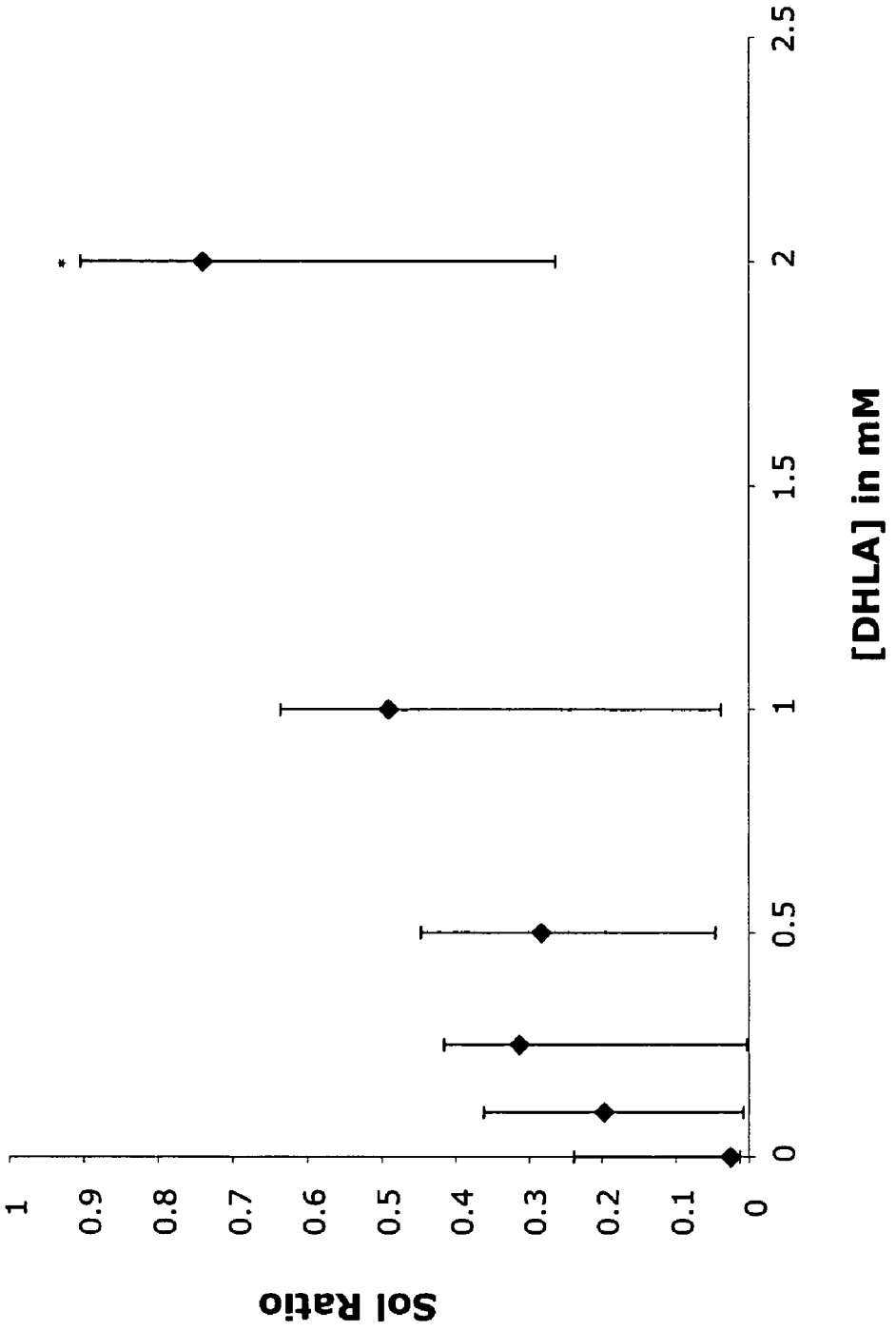


FIG. 8A

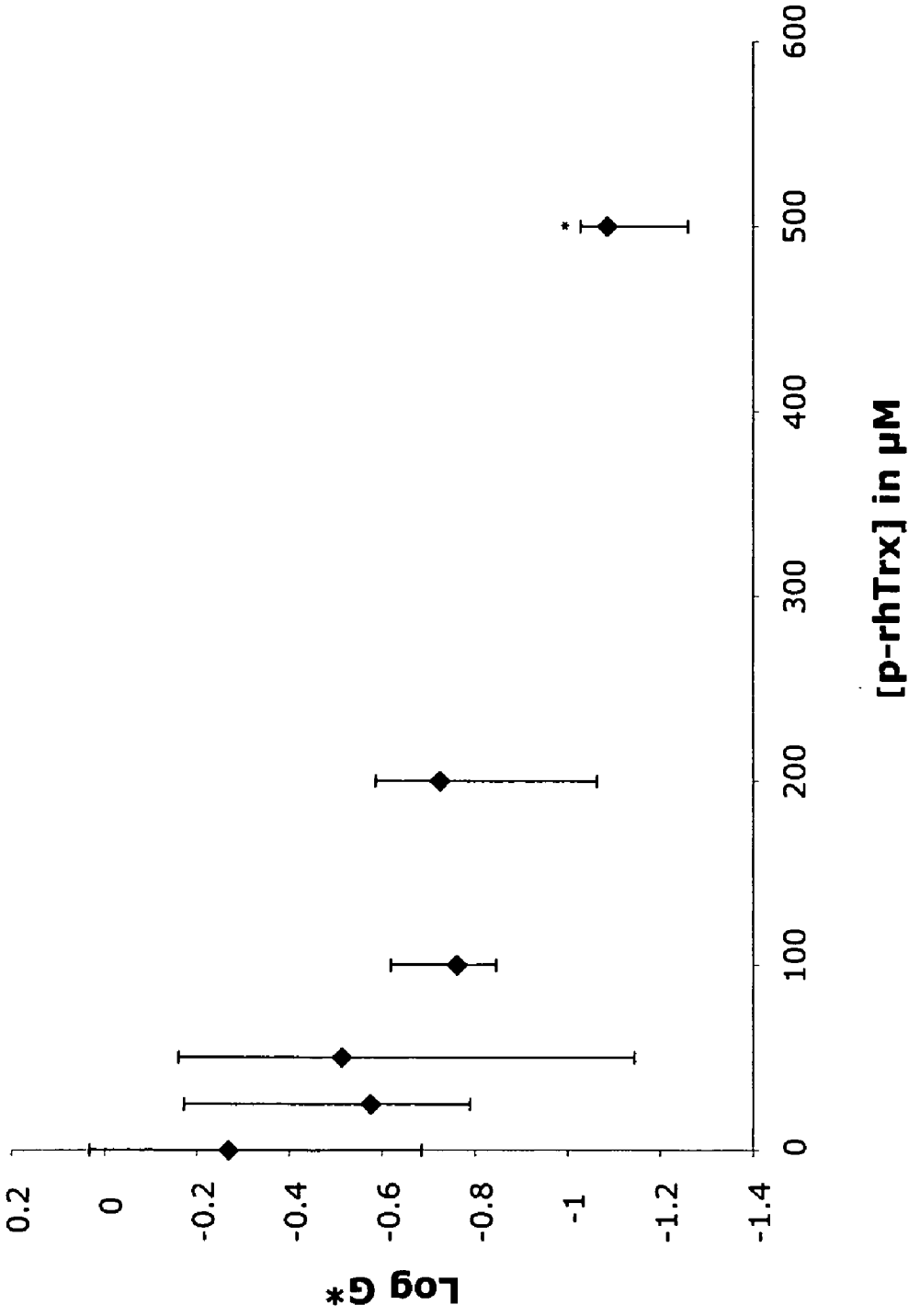


FIG. 8B

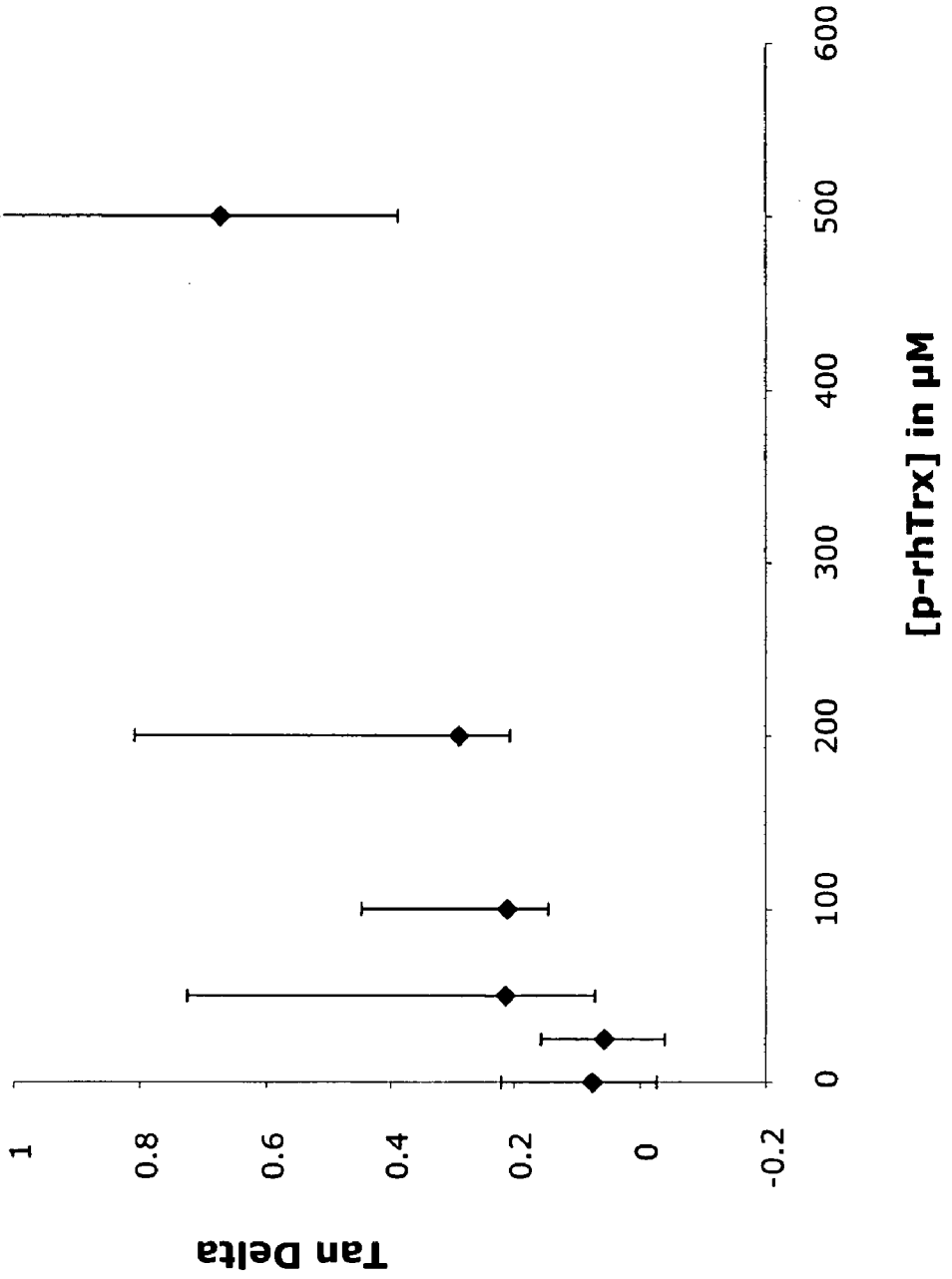


FIG. 9A

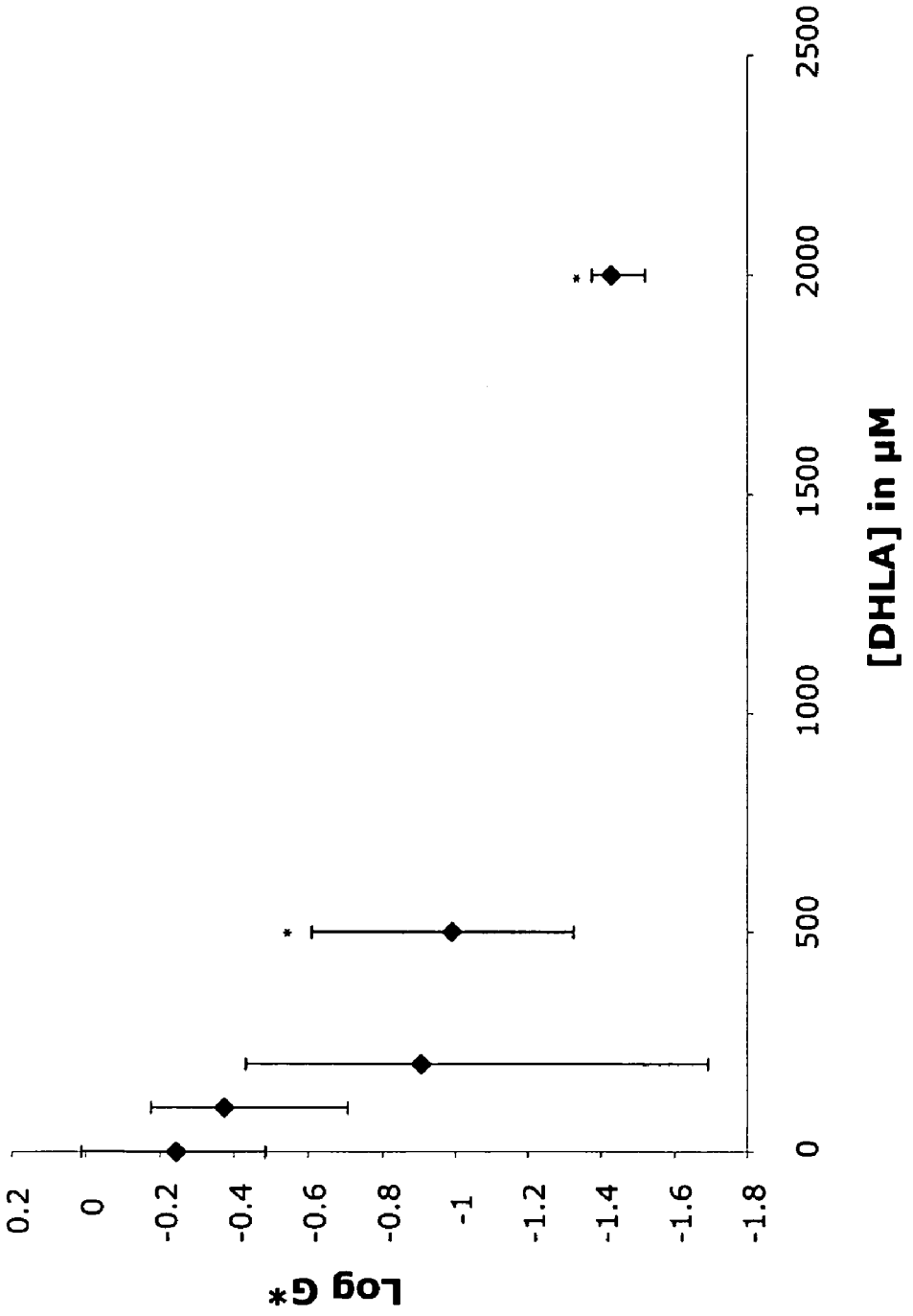

Log G*
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
-1.6
-1.8
0
500
1000
1500
2000
2500
[DHLA] in µM

FIG. 9B

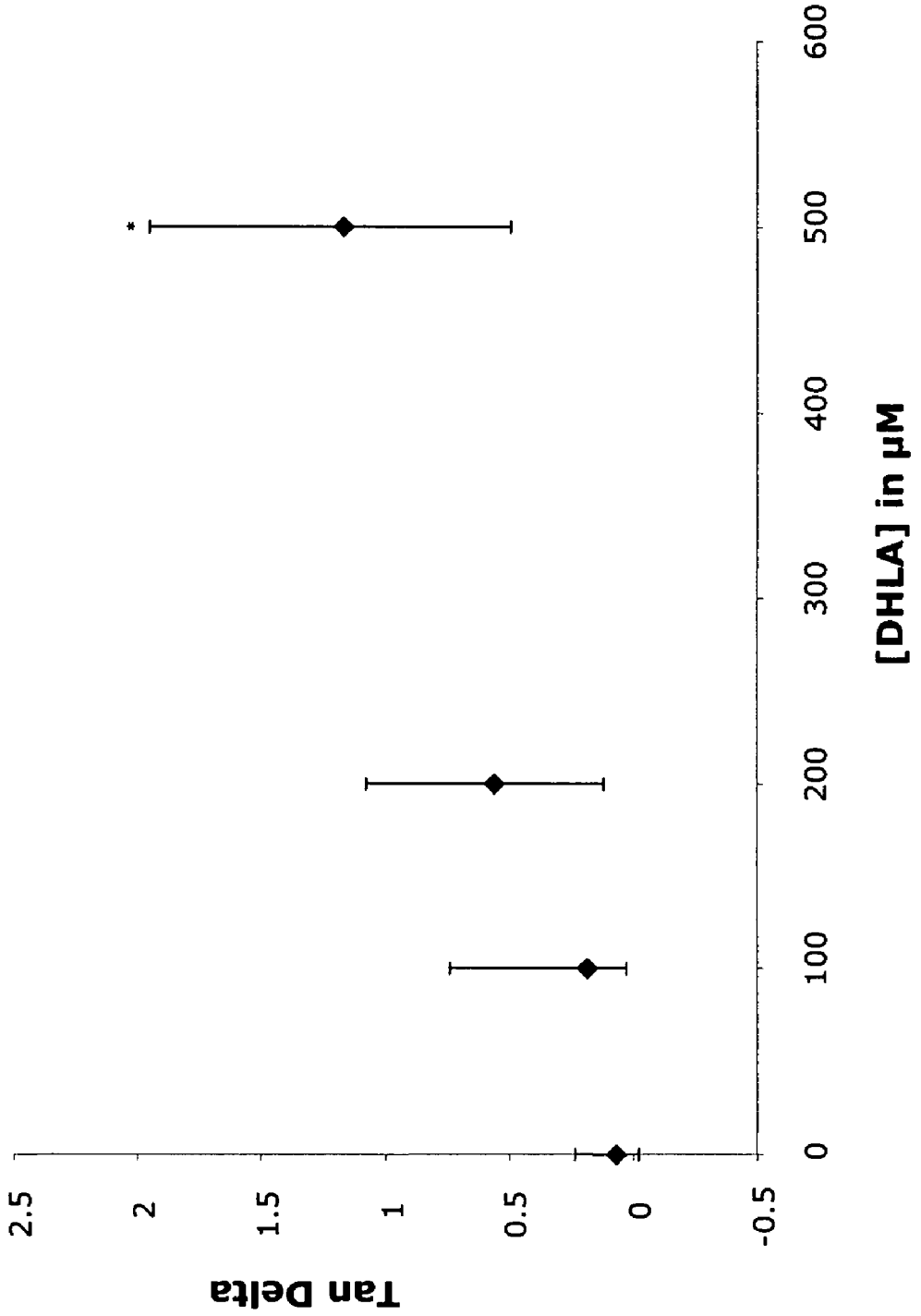


FIG. 10

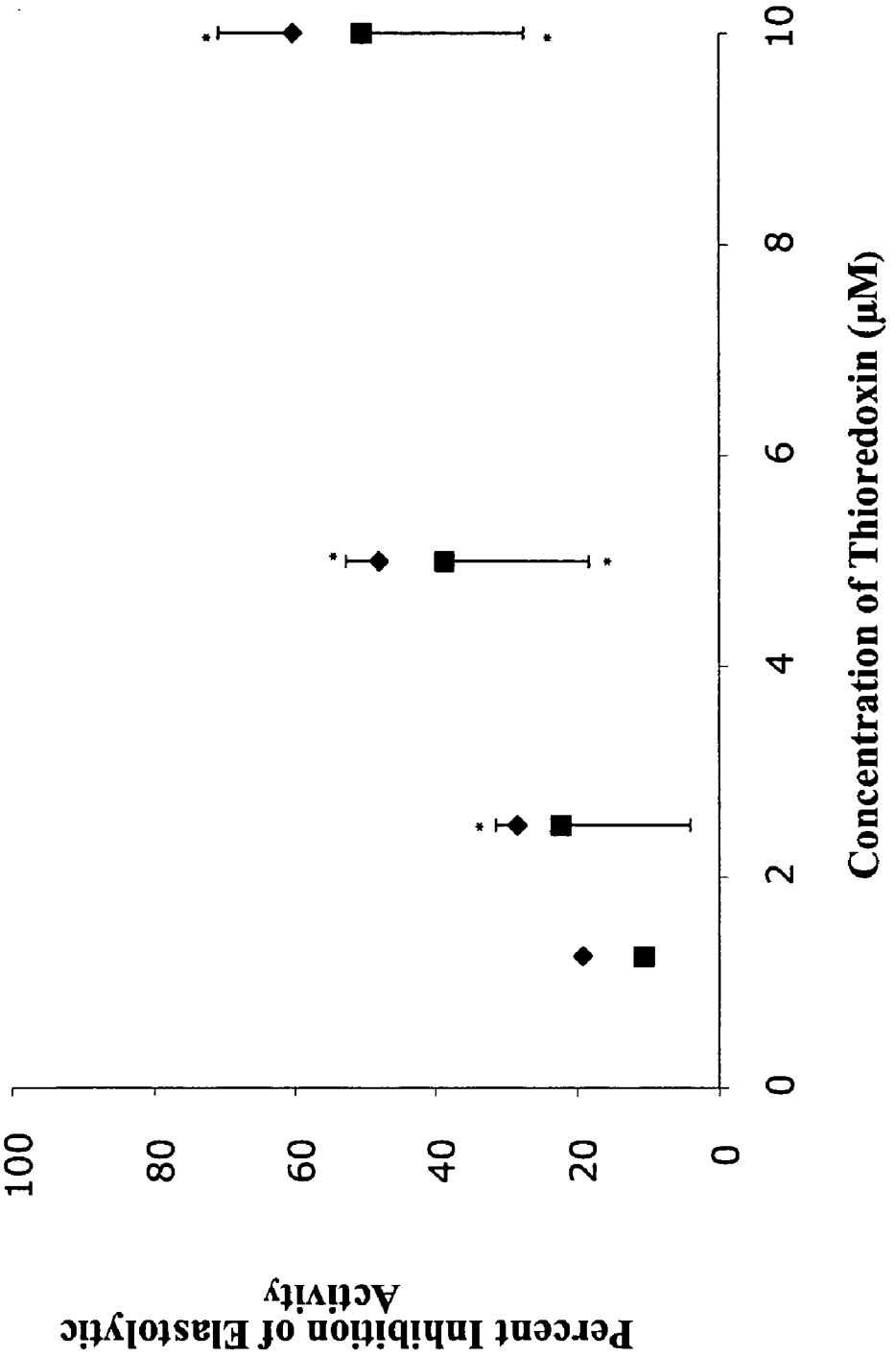

Percent Inhibition of Elastolytic Activity
0
20
40
60
80
100
0
2
4
6
8
10
Concentration of Thioredoxin (μM)

FIG. 11A

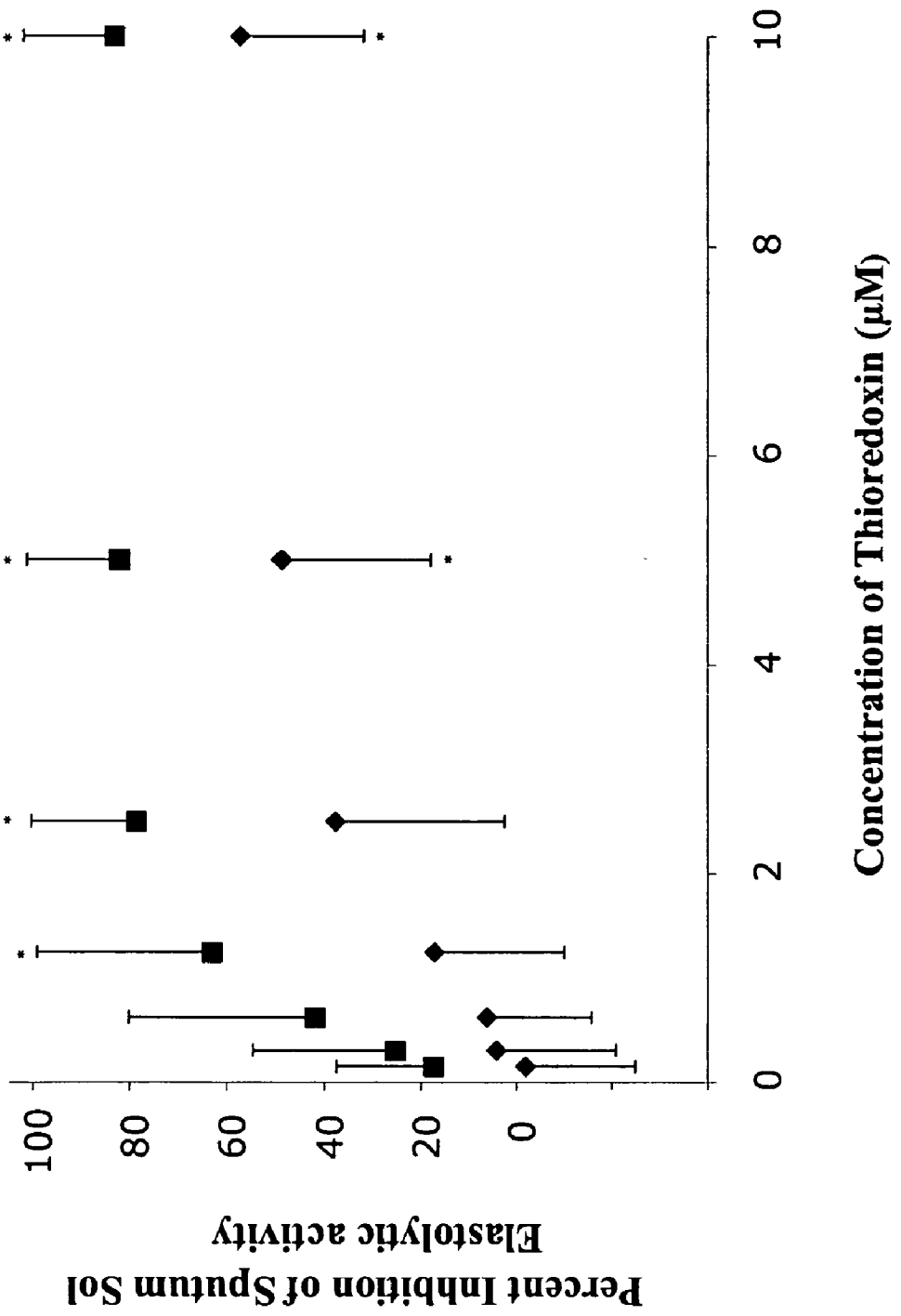

Percent Inhibition of Sputum Sol Elastolytic activity
100
80
60
40
20
0
0
2
4
6
8
10
Concentration of Thioredoxin (μM)

FIG. 11B

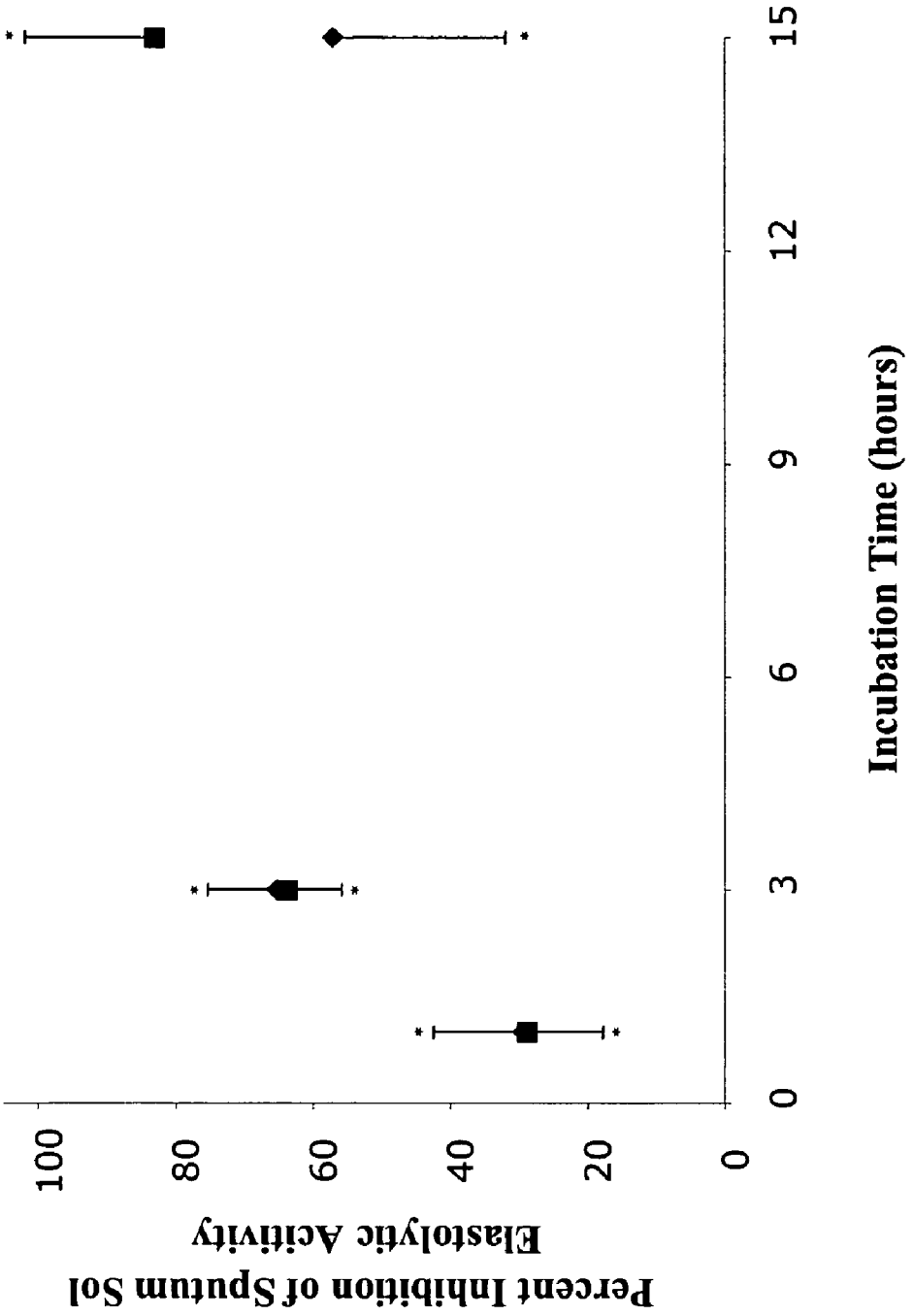

Percent Inhibition of Sputum Sol Elastolytic Activity
100
80
60
40
20
0
0
3
6
9
12
15
Incubation Time (hours)

FIG. 11C

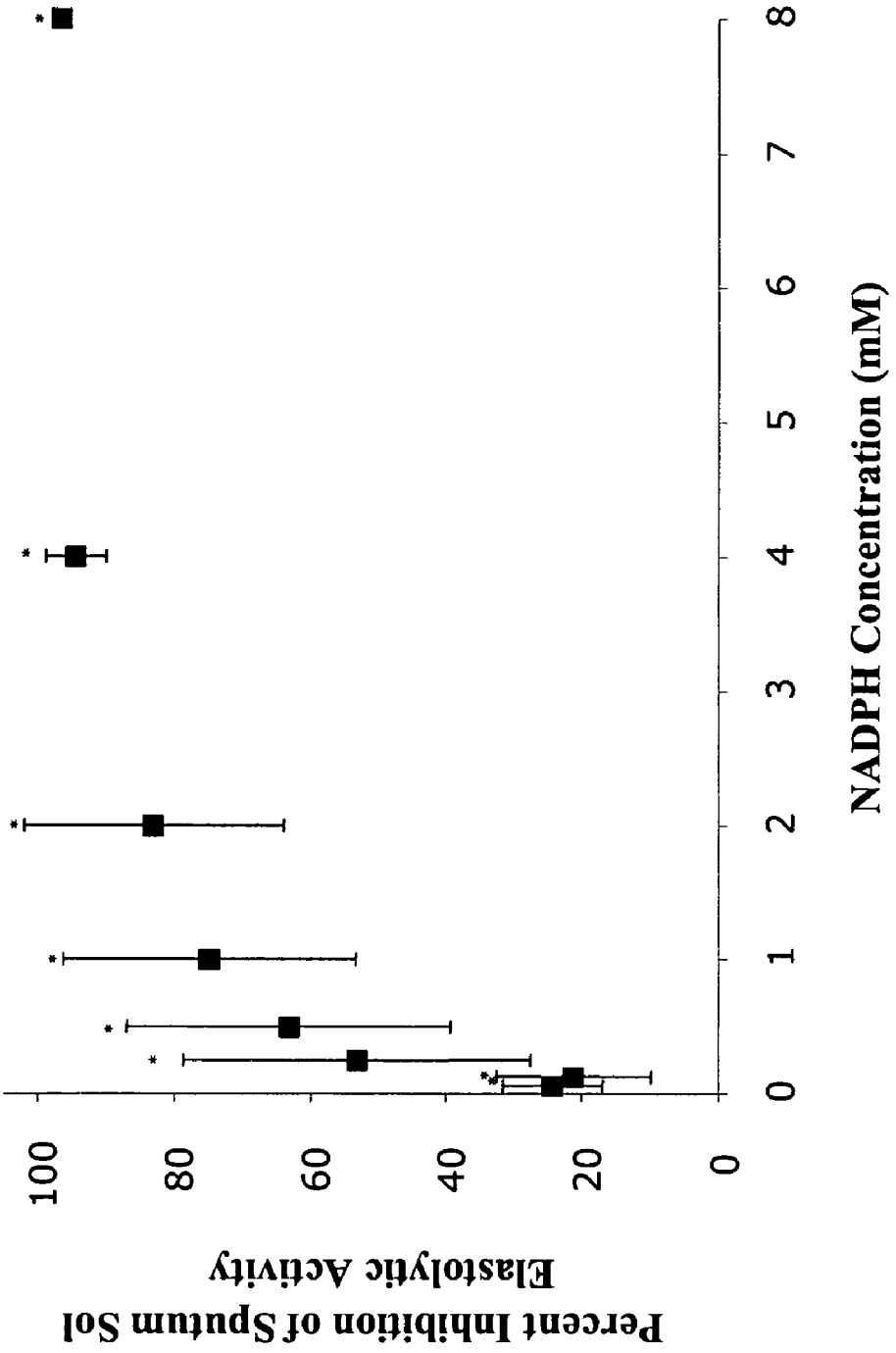

Percent Inhibition of Sputum Sol Elastolytic Activity
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
NADPH Concentration (mM)

FIG. 12

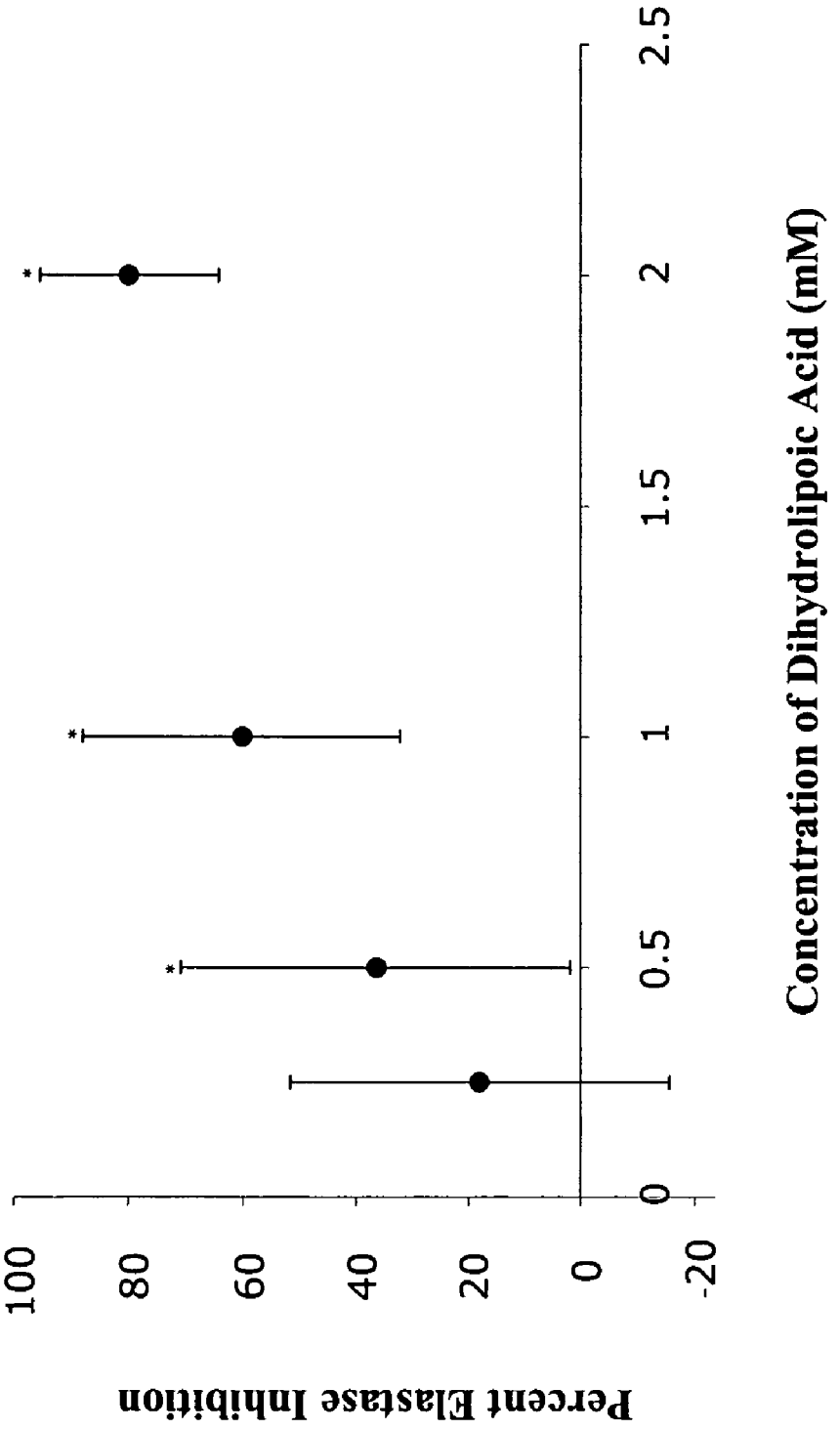

Percent Elastase Inhibition
100
80
60
40
20
0
-20
0
0.5
1
1.5
2
2.5
Concentration of Dihydrolipoic Acid (mM)
*
*
*

FIG. 13

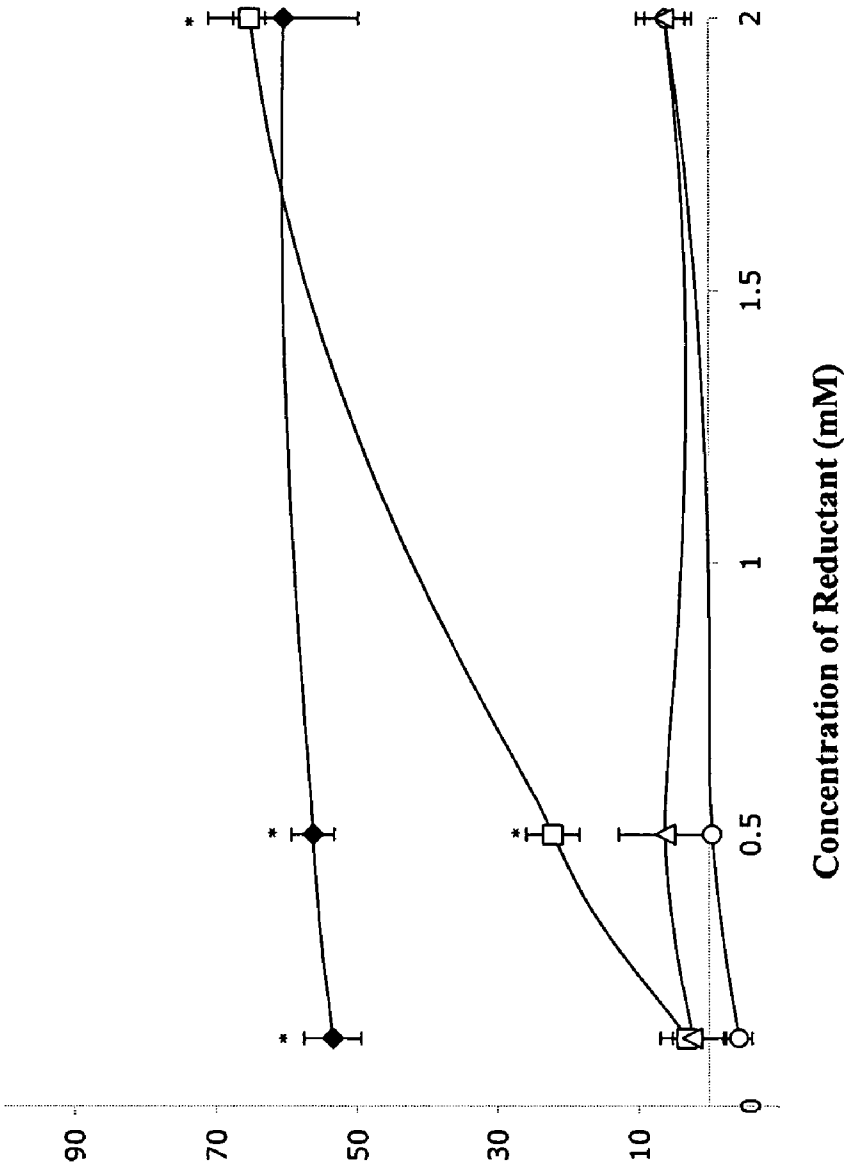


FIG. 14

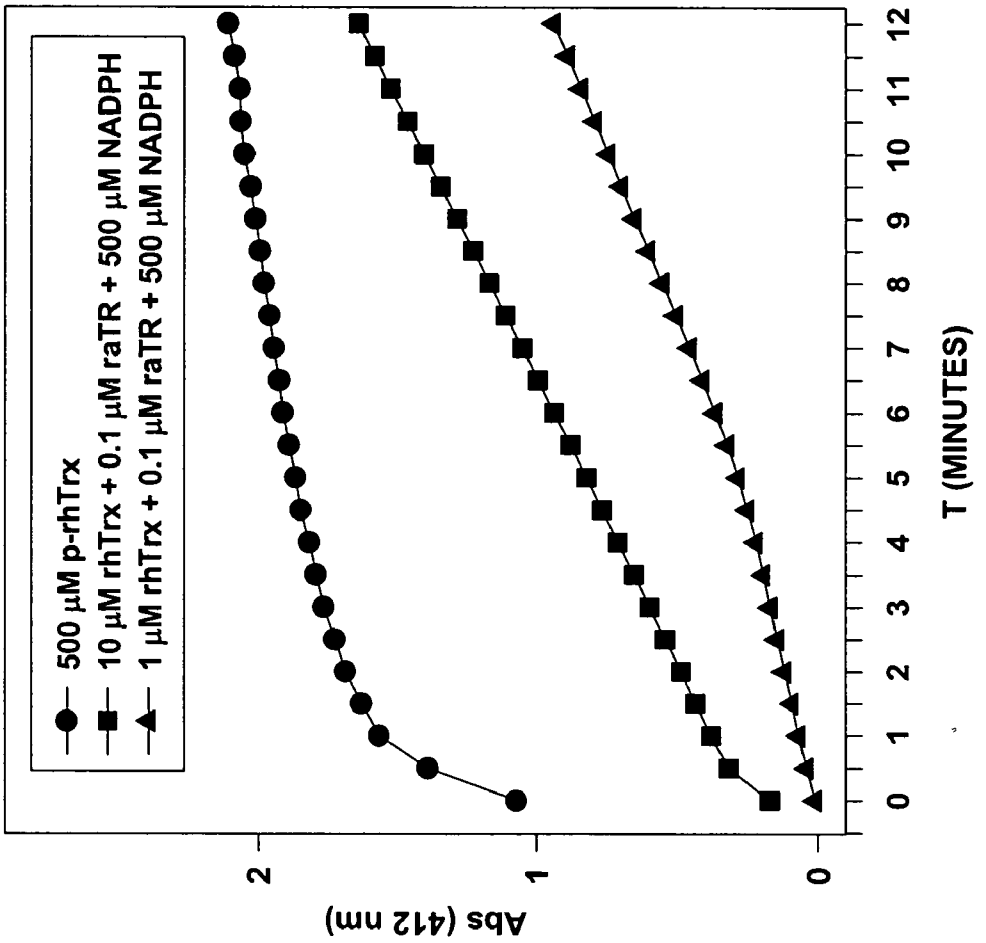

500 µM p-rhTrx
10 µM rhTrx + 0.1 µM raTR + 500 µM NADPH
1 µM rhTrx + 0.1 µM raTR + 500 µM NADPH
Abs (412 nm)
0
1
2
T (MINUTES)
0 1 2 3 4 5 6 7 8 9 10 11 12

FIG. 15A

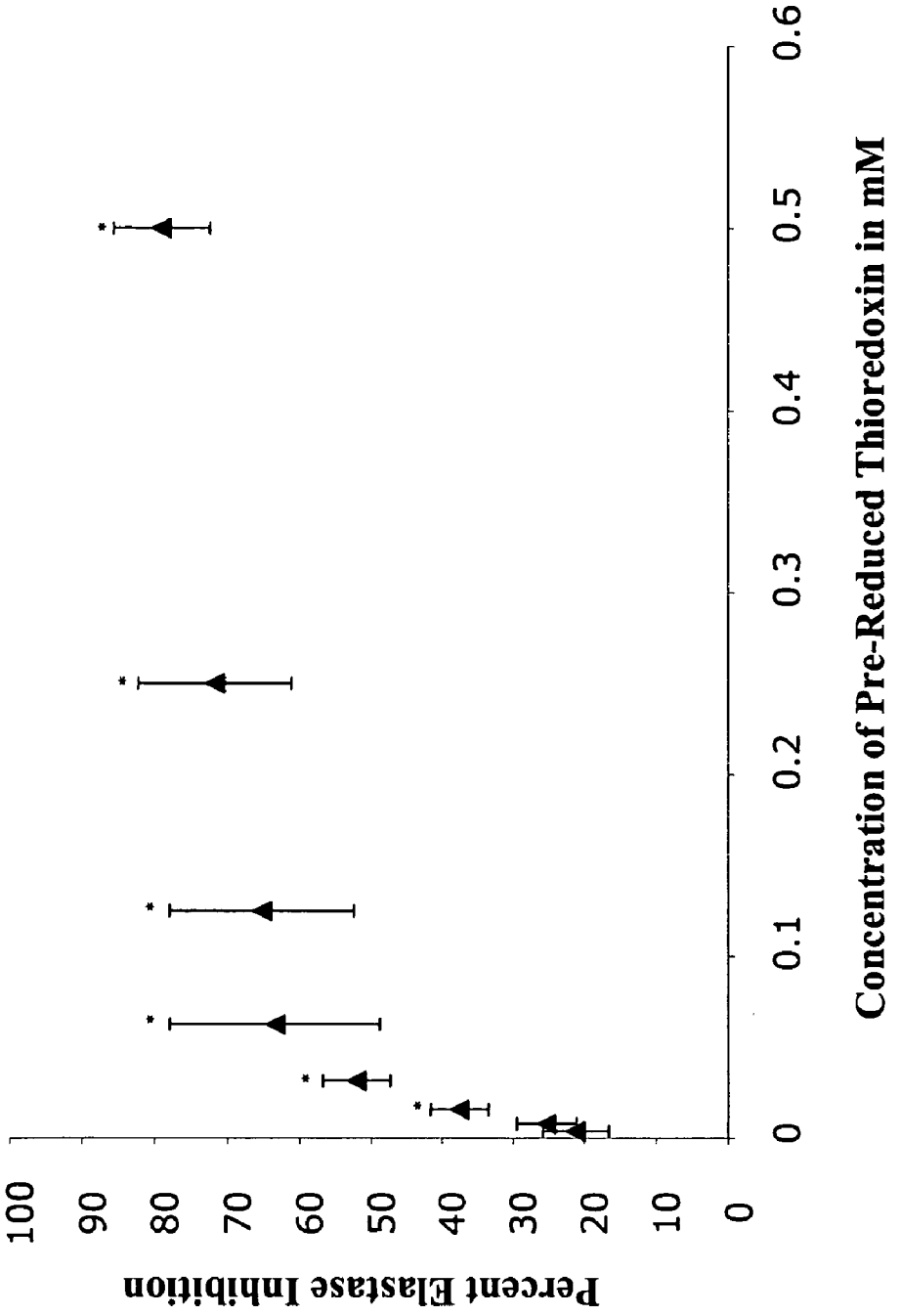

Percent Elastase Inhibition
100
90
80
70
60
50
40
30
20
10
0
0
0.1
0.2
0.3
0.4
0.5
0.6
Concentration of Pre-Reduced Thioredoxin in mM

FIG. 15B

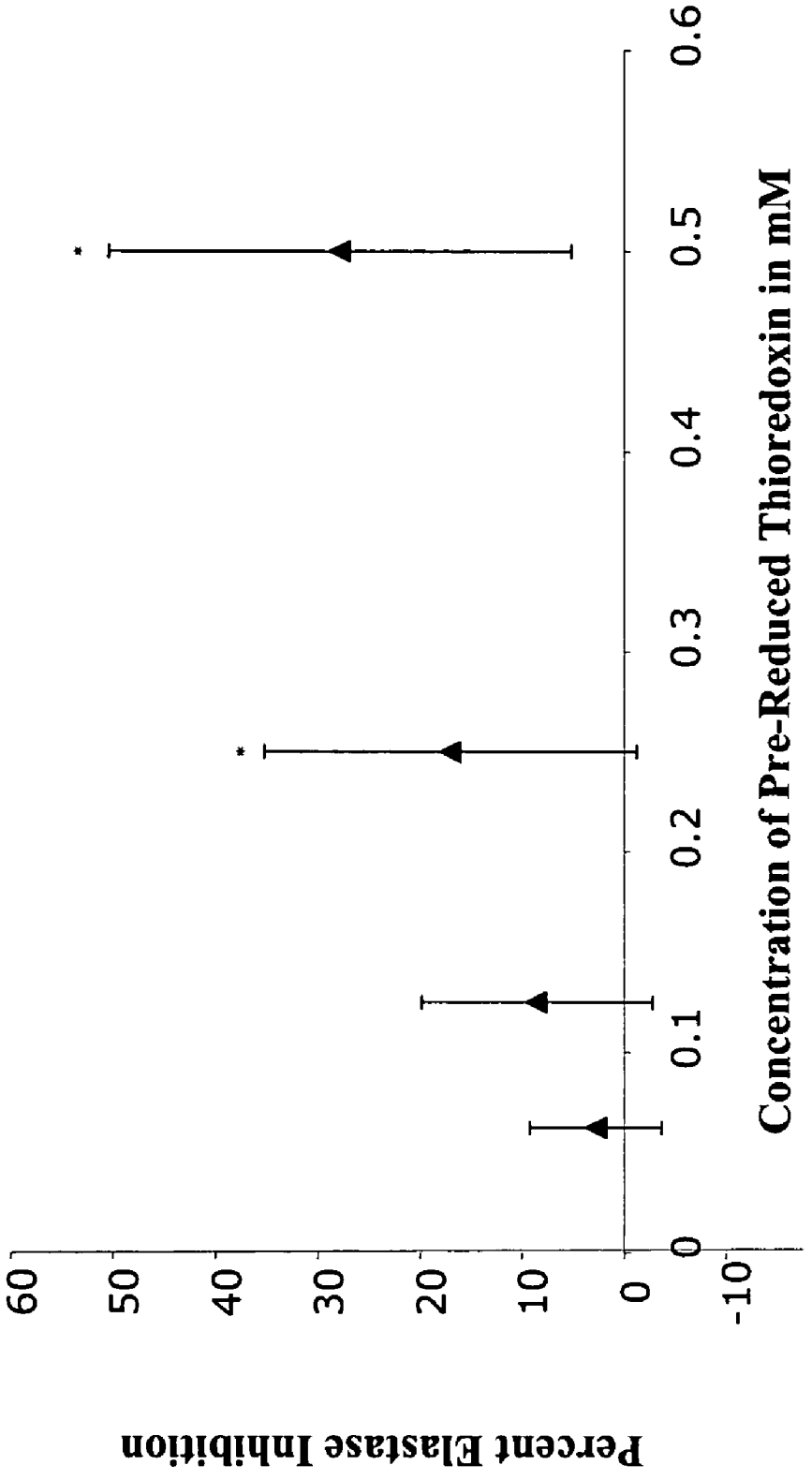

Percent Elastase Inhibition
60
50
40
30
20
10
0
-10
0
0.1
0.2
0.3
0.4
0.5
0.6
Concentration of Pre-Reduced Thioredoxin in mM
*
*

FIG. 16

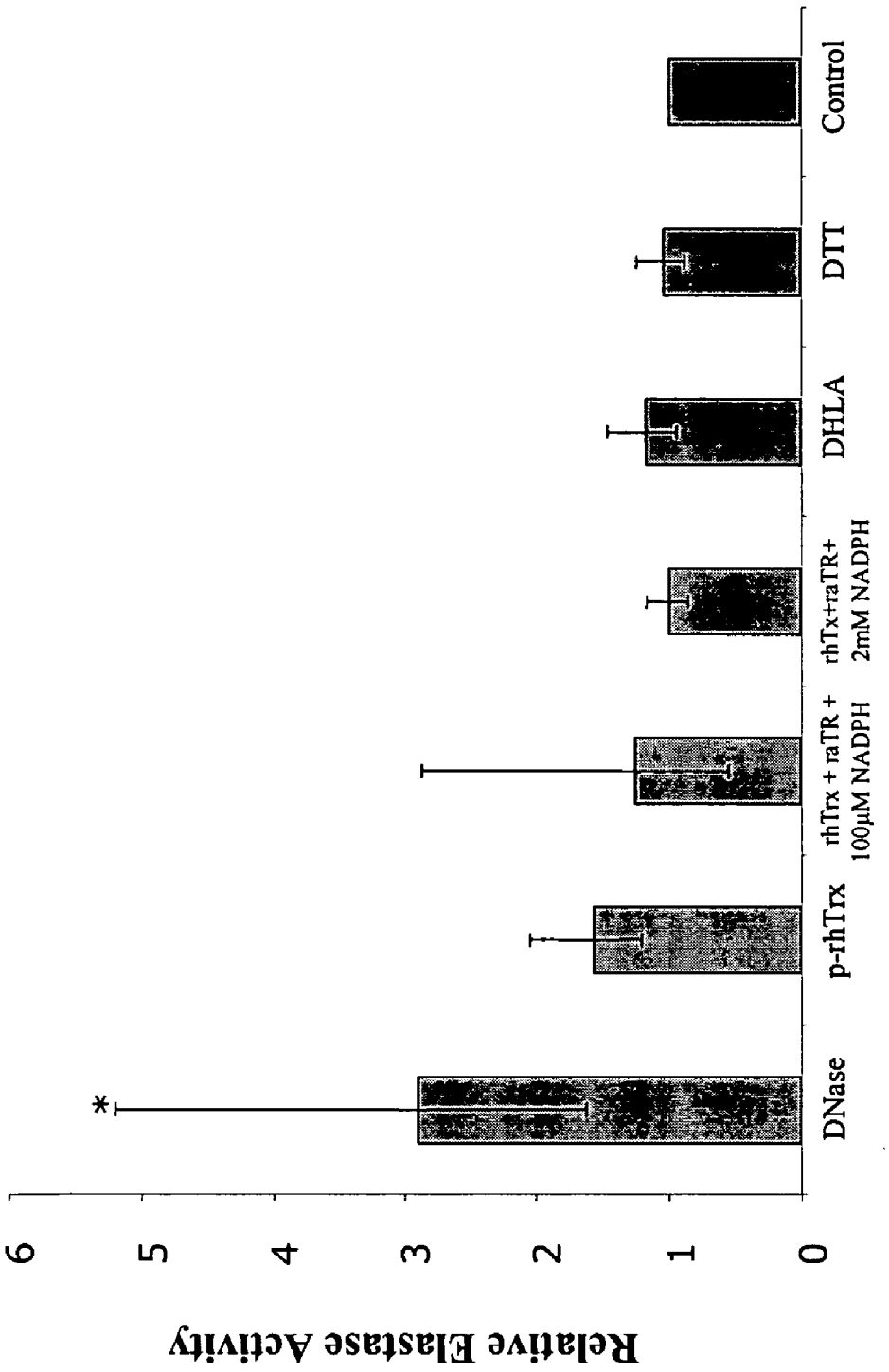

Relative Elastase Activity
0
1
2
3
4
5
6
*
DNase
p-rhTrx
rhTrx + raTR + 100μM NADPH
rhTx+raTR+ 2mM NADPH
DHLA
DTT
Control

FIG. 17

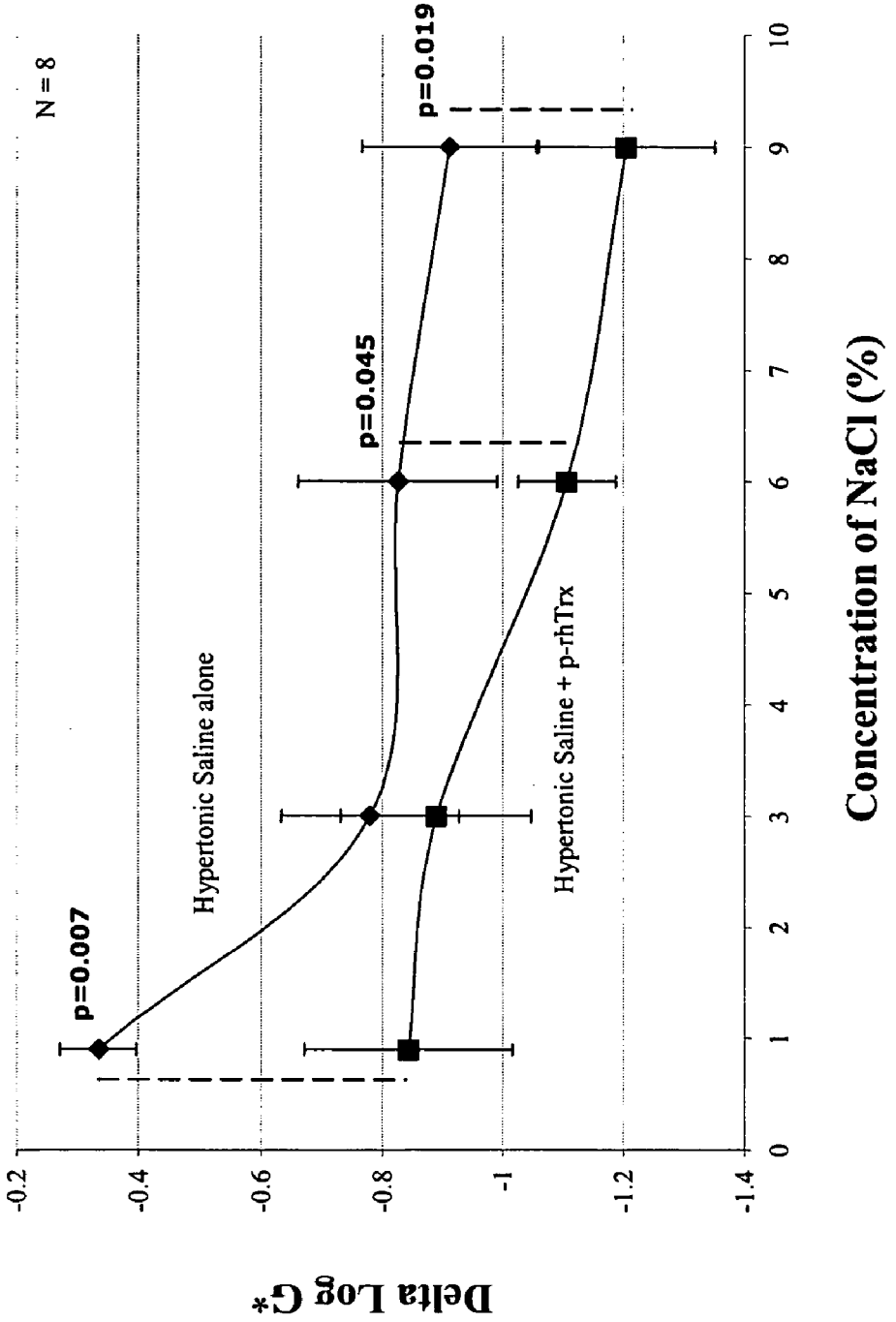

N = 8
p=0.007
p=0.045
p=0.019
Hypertonic Saline alone
Hypertonic Saline + p-rhTrx
Delta Log G*
Concentration of NaCl (%)
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
0
1
2
3
4
5
6
7
8
9
10

FIG. 18

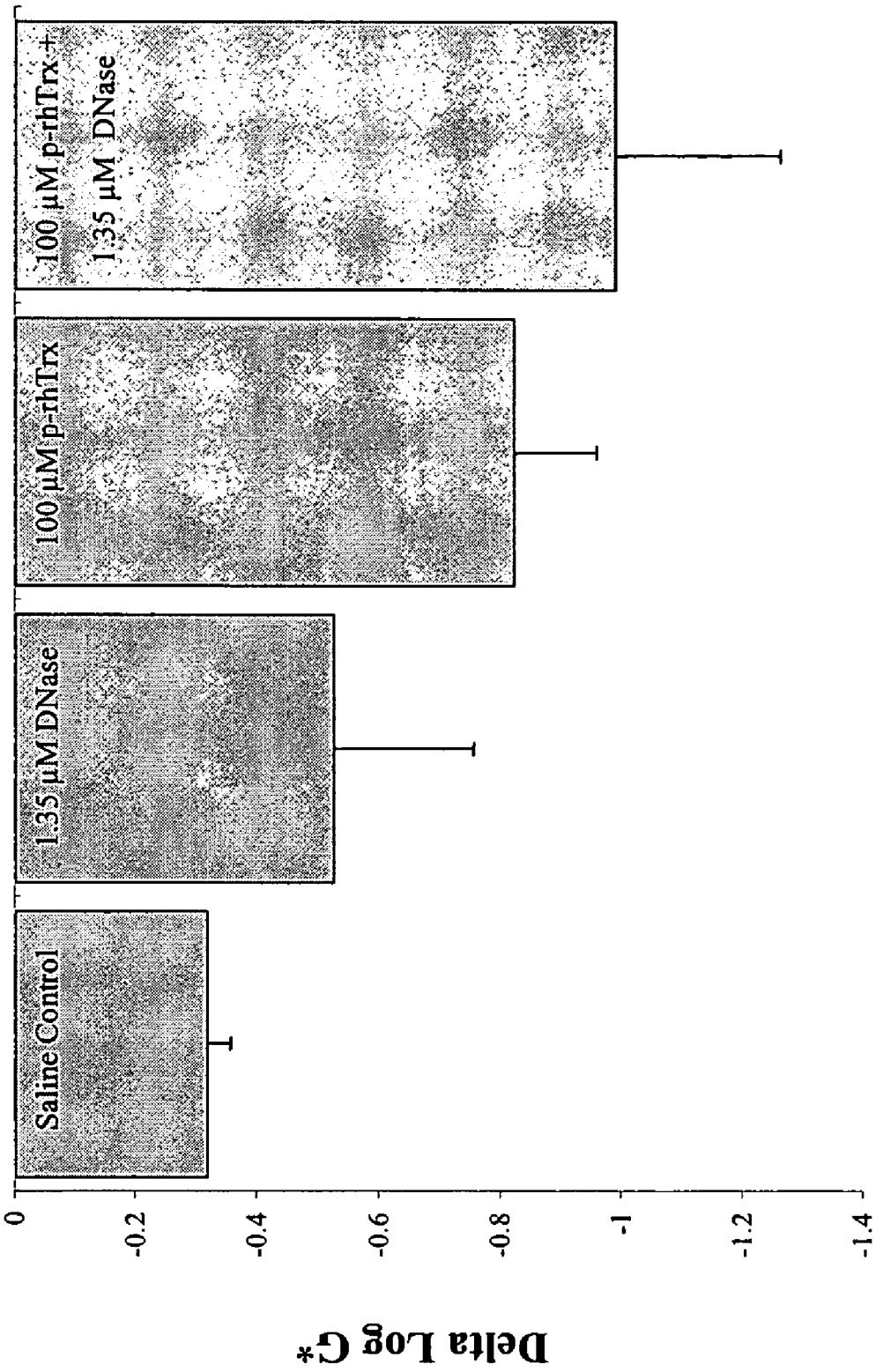

Saline Control
1.35 µM DNase
100 µM p-rhTrx
100 µM p-rhTrx+
1.35 µM DNase
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
Delta Log G*

FIG. 19

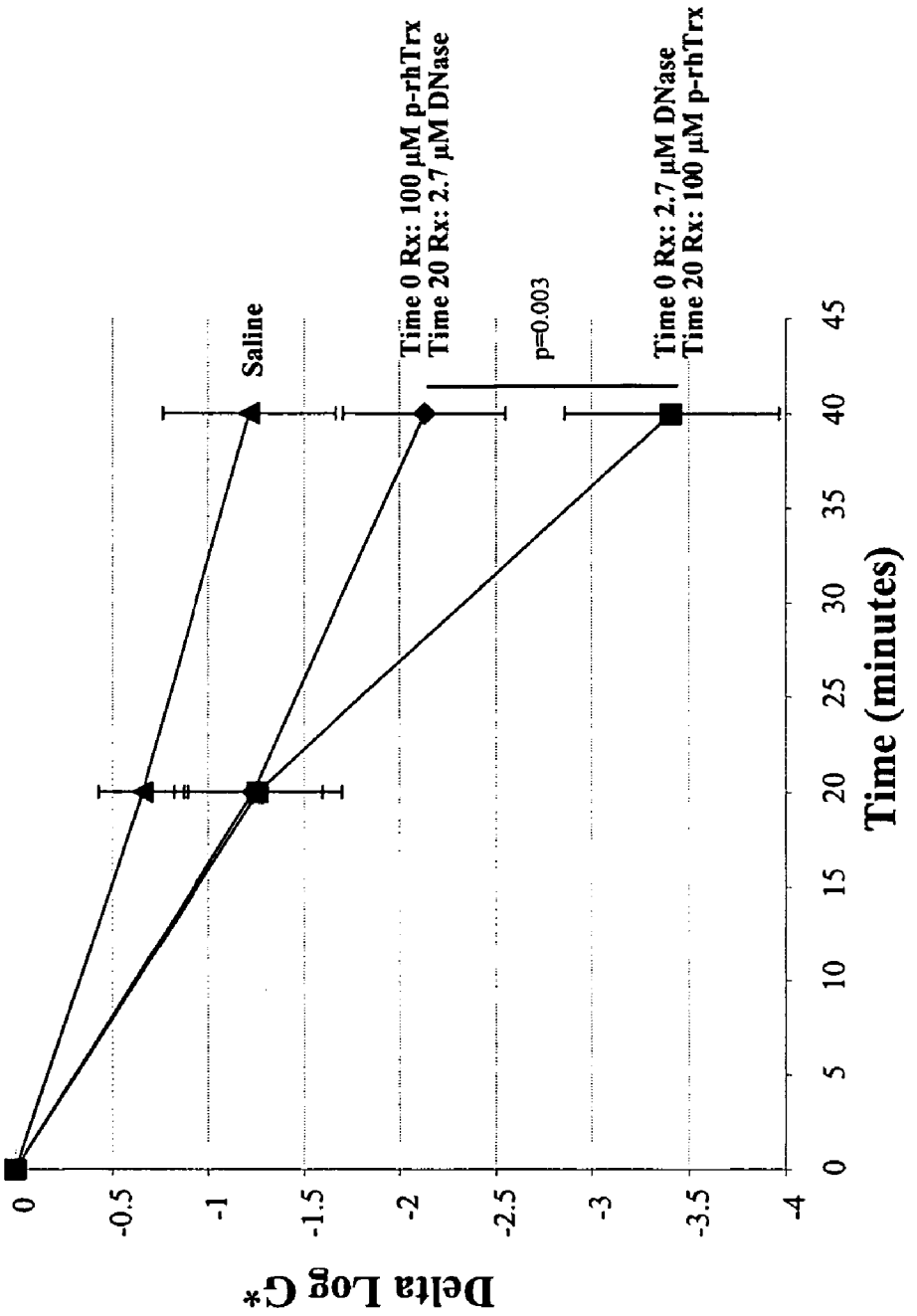

Delta Log G*
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
0
5
10
15
20
25
30
35
40
45
Time (minutes)
Saline
Time 0 Rx: 100 µM p-rhTrx
Time 20 Rx: 2.7 µM DNase
p=0.003
Time 0 Rx: 2.7 µM DNase
Time 20 Rx: 100 µM p-rhTrx

FIG. 20

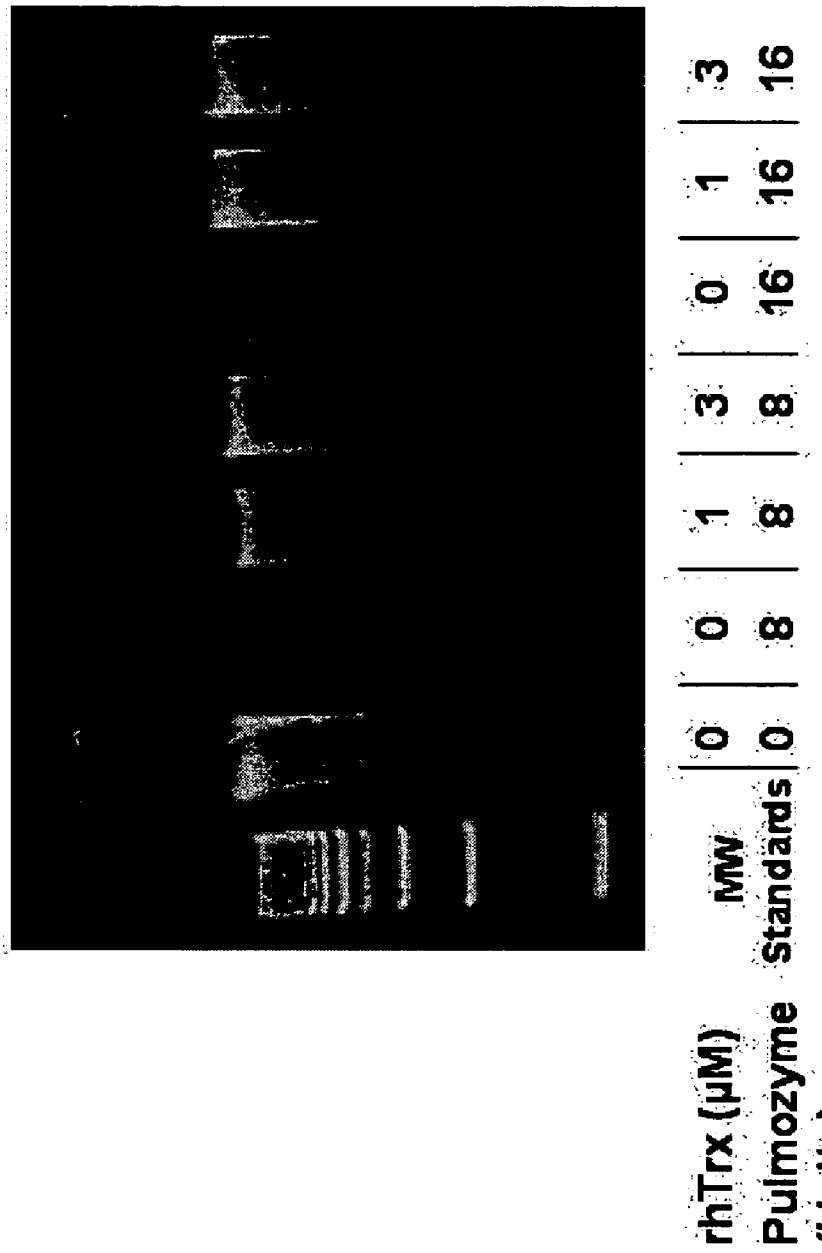
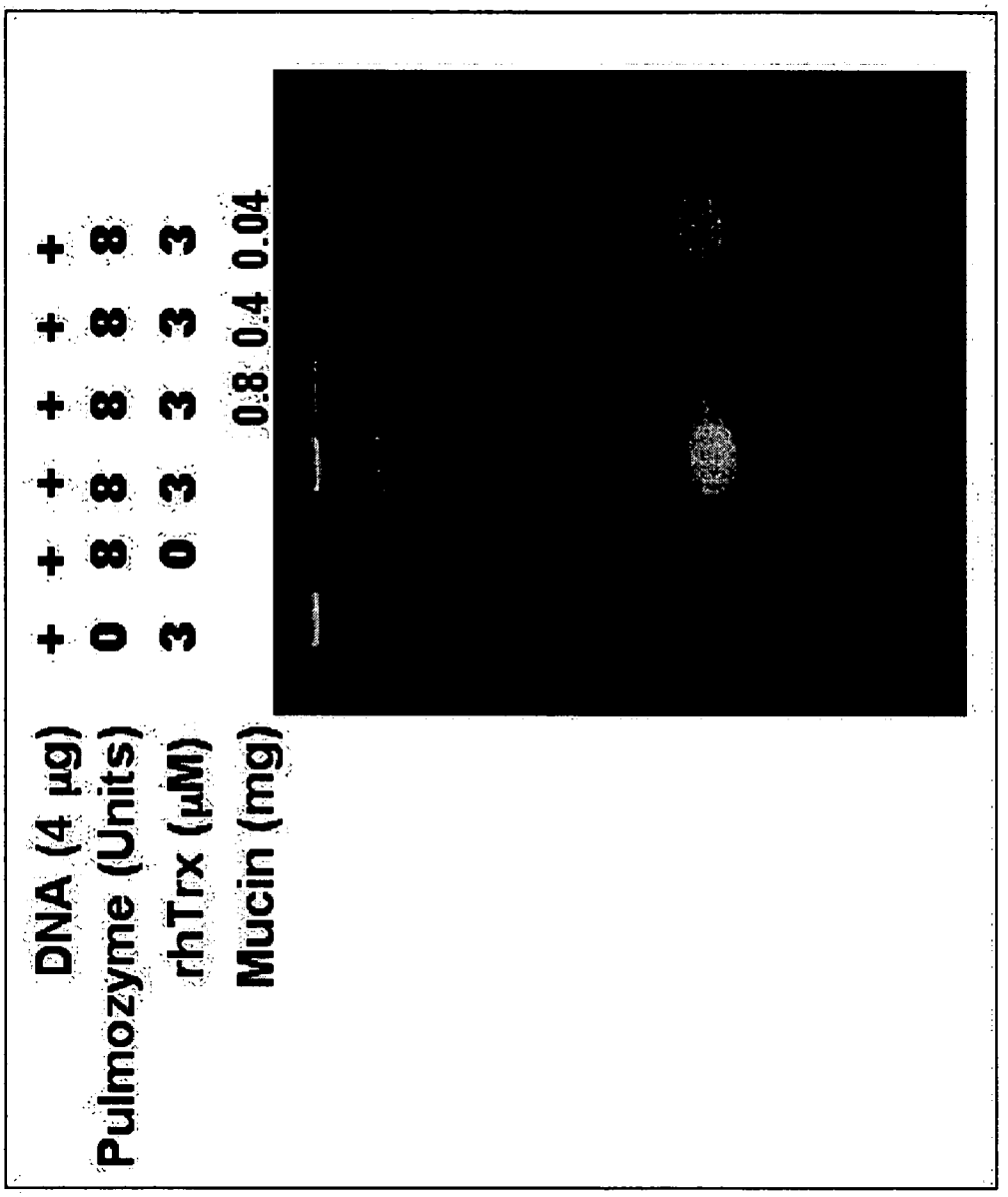


FIG. 21


DNA (4 µg) + + + + + + +
Pulmozyme (Units) 0 8 8 8 8 8 8
rhTrx (µM) 3 0 3 3 3 3 3
Mucin (mg) 0.8 0.4 0.04

MUCOLYTIC AND ANTI-ELASTASE COMPOUNDS AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority under 35 U.S.C. § 119(e) from U.S. Provisional Application Ser. No. 60/556,516, filed Mar. 24, 2004, and from U.S. Provisional Application Ser. No. 60/650,865, filed Feb. 7, 2005. This application is also a continuation-in-part of U.S. patent application Ser. No. 10/660,118, filed Sep. 10, 2003, which claims the benefit of priority under 35 U.S.C. § 119(e) from U.S. Provisional Application Ser. No. 60/409,960, filed Sep. 10, 2002 and from U.S. Provisional Application Ser. No. 60/462,082, filed Apr. 11, 2003. Each of the above-identified applications is incorporated herein by reference in its entirety.

REFERENCE TO SEQUENCE LISTING

[0002] This application contains a Sequence Listing submitted on a compact disc, in duplicate. Each of the two compact discs, which are identical to each other pursuant to 37 CFR § 1.52(e)(4), contains the following file: "Sequence Listing", having a size in bytes of 14 KB, recorded on 24 Mar. 2005. The information contained on the compact disc is hereby incorporated by reference in its entirety pursuant to 37 CFR § 1.77(b)(4).

FIELD OF THE INVENTION

[0003] This invention generally relates to the use of a compound containing a dithiol active site, preferably in reduced state, to induce, enhance and/or increase the liquefaction of mucus or sputum through mucolysis, and/or to inhibit elastase.

BACKGROUND OF THE INVENTION

[0004] Cystic fibrosis (CF) is a common lethal genetic disease that results from a mutation in the gene encoding a chloride channel protein, the CF transmembrane conductance regulator. As a result of this defect, epithelia within the body are impermeable to chloride ion transport (Boucher et al., *Lung* 161:1-17, 1983; Welsh, *Physiol Rev* 67:11443-1184, 1987). Although several organs are affected, including pancreas, intestine, and male genital tract, complications within the lung account for 95% of the morbidity and mortality (Means, M. Cystic Fibrosis: the first 50 years. In: *Cystic Fibrosis—Current Topics* Volume 1, edited by Dodge J A, Brock D J H, and Widdicombe J H. Chichester: Wiley and Sons, 1992, p. 217-250). In lung impaired by the disease, chloride transport into the airway lumen leads to excessive absorption of Na^+ and fluid, reducing the volume of airway surface liquid (Jiang et al., *Science* 262:424-427, 1993). Desiccation of airway surface liquid leads to the concentration of mucin macromolecules, which are the gel forming constituents of mucus (Matsui et al., *Cell* 95:1005-1015, 1998). The viscoelastic properties of normal mucus are dependent on the concentration, molecular weight, and entanglements between mucin polymers (Verdugo et al., *Biorheology* 20:223-230, 1983). Further interaction of mucins with DNA (Potter et al., *Am J Dis Child* 100:493-495, 1960; Lethem et al., *Am Rev Respir Dis* 100:493-495, 1990; Lethem et al., *Eur Respir J* 3:19-23, 1990) and f-actin polymers (Sheils et al., *Am J Path* 148:919-927, 1996; Tomkiewicz et al., DNA and actin filament ultrastructure in cystic fibrosis sputum. In: Cilia, mucus, and mucociliary interactions, edited by Baum G L, Priel Z, Roth Y, Liron N, and Ostfeld E J. New York, N.Y.: Marcel Dekker, 1998) released from dying inflammatory cells is responsible for the dense and viscous nature of CF sputum. The inability to clear such mucus by cough or mucociliary clearance facilitates colonization of the lung with opportunistic pathogens.

[0005] While the etiology of CF lung disease can be attributed to the altered Theological properties of sputum, compromised lung function is rarely evident at birth. Instead, bronchiectasis and airway obstruction progress with age of patient. This chronic lung injury results from a persistent cycle of bacterial infection and inflammatory response. Airway damage results when neutrophils recruited into the lung release matrix degrading enzymes, such as elastase, and harmful reactive oxygen species (reviewed in Konstan and Berger, *Pediatr Pulmonol* 24:137-142, 1997). The elastase and other proteases overwhelm the antiprotease defenses, and this protease/antiprotease imbalance is believed to play an important role in the development of structural lung damage such as bronchiectasis, progressive pulmonary function decline, and early death observed in CF.

[0006] Neutrophil elastase is a member of the chymotrypsin superfamily of serine proteases contained within the neutrophil azurophilic granules. Upon neutrophil activation as part of an inflammatory process, large amounts of elastase are released into the extracellular space with smaller amounts remaining membrane bound. Once released from neutrophils, elastase catalyzes the proteolysis of a variety of extracellular matrix proteins including collagen and elastin. Normally, this proteolytic activity is modulated by a number of antiproteases, including α-1 antiprotease and secretory leukoprotease inhibitor (SLPI). However, in certain pathologic states such as cystic fibrosis, neutrophil elastase escapes the action of these endogenous protease inhibitors due to a protease/antiprotease imbalance.

[0007] Despite some promising advances, correction of CF by gene therapy is not yet attainable. Currently, antibiotic regimens coupled with drugs that facilitate the clearance of purulent airway secretions remain the mainstay treatments for progressive airway disease. Inhalation of purified rhDNase (Pulmozyme; Genentech, USA), which digests extracellular DNA present in the CF airway, is widely used as a respiratory decongestant. Such treatment is clinically effective for diminishing sputum viscosity and stabilizing the forced expiratory volume (FEV) (Fuchs et al., *N Engl J Med* 331:637-642, 1994). Other investigative therapies aimed at breaking down mucin or actin polymers, including N-acetylcysteine, nacystelyn (an N-acetyl-L-cysteine derivative), and gelsolin, can also reduce sputum viscosity experimentally, but have yet to attain clinical approval specifically for treatment of CF in the United States.

[0008] Therefore, there is a need in the art for improved therapeutic approaches for the treatment of cystic fibrosis, as well as other diseases and conditions that are associated with abnormally or excessively viscous or cohesive mucus or sputum and diseases and conditions that are associated with excess, harmful, or unrestrained elastase activity.

SUMMARY OF THE INVENTION

[0009] One embodiment of the present invention relates to a method to inhibit elastase in a patient that has a disease or

condition associated with excessive elastase activity. The method includes administering to the patient a composition comprising a compound containing a dithiol active-site in reduced state effective to reduce elastase levels and/or activity, as compared to prior to the step of contacting. In this method, the composition is administered in the absence of a protease inhibitor (e.g., α1-antitrypsin). In one aspect, the composition is administered in the absence of a requirement for elevated temperature conditions. In one aspect, the step of contacting the patient with the composition is performed by introducing the composition to the patient by a route selected from the group consisting of nasal, intratracheal, bronchial, direct installation into the lung and inhaled.

[0010] Compounds useful in the above-embodiment include compounds that can be catalytically recycled to a reduced state after oxidation of the compound, including a non-protein dithiol that can be catalytically recycled to a reduced state after oxidation of the compound.

[0011] In one aspect, the compound is dihydrolipoic acid or a biologically active derivative thereof. In another aspect, the compound is orally administered α-lipoic acid, or a biologically active derivative thereof.

[0012] In another aspect, the compound is a protein or peptide having a thioredoxin active-site. The thioredoxin active-site can include the amino acid sequence C-X-X-C, wherein C residues are in reduced state, wherein X residues are any amino acid residue; the amino acid sequence X-C-X-X-C-X, wherein C residues are in reduced state, wherein X residues are any amino acid residue; the amino acid sequence X-C-G-P-C-X (SEQ ID NO:2), wherein C residues are in reduced state, wherein X residues are any amino acid residue; and the amino acid sequence W-C-G-P-C-K (SEQ ID NO:3), wherein C residues are in reduced state. In one aspect, the protein is a thioredoxin selected from prokaryotic thioredoxin, yeast thioredoxin, plant thioredoxin, and mammalian thioredoxin. In a preferred aspect, the protein is human thioredoxin. In a particularly preferred aspect, the protein is recombinant human thioredoxin, and even more preferably, recombinant human thioredoxin produced in a substantially reduced, monomeric state. In this aspect of the invention, the composition can further comprise nicotinamide-adenine dinucleotide phosphate (reduced form) (NADPH) for reducing the thioredoxin active site of the protein. The composition can also include thioredoxin reductase. In another aspect, a compound having a thioredoxin active-site in reduced state can be combined with another dithiol, such as dihydrolipoic acid or a biologically active derivative thereof.

[0013] The method of the invention can be used to treat a variety of diseases and conditions including, but not limited to, cystic fibrosis or chronic obstructive pulmonary disease (COPD). Preferably, the composition is administered to the patient in a pharmaceutically acceptable carrier. In one aspect, the compound is administered to the patient in an amount that is between about 1.5 mmoles/kg weight of the patient and about 150 mmoles/kg weight of the patient. In another aspect, the compound has a half-life in the patient of between about 5 minutes and about 24 hours.

[0014] In one aspect, the method further includes administering another compound useful for inhibiting elastase or otherwise treating the patient to the patient. A preferred compound includes, but is not limited to, DNase. The DNase can be administered prior to the dithiol compound.

[0015] Yet another embodiment of the present invention relates to a method to increase the liquefaction of mucus or sputum in a patient that has excessively viscous or cohesive mucus or sputum. The method includes the contacting the mucus or sputum of the patient with a composition comprising a compound containing a dithiol active-site in reduced state effective to increase the liquefaction of the mucus or sputum as compared to prior to the step of contacting. In one aspect, the patient has a lung disease in which abnormal or excessive viscosity or cohesiveness of mucus or sputum is a symptom or cause of the disease. The step of contacting the mucus or sputum of the patient with the composition can be performed, for example, by introducing the composition to the patient by a route selected from oral, nasal, intratracheal, bronchial, direct installation into the lung and inhaled. In one aspect, the mucus or sputum to be contacted is located in the respiratory tract, the gastrointestinal tract or the reproductive tract of the patient. In another aspect, a liquid phase of a total volume of a sample of mucus or sputum from the patient shows a statistically significant increase after administration of the composition.

[0016] Compounds useful in this method include, but are not limited to, a non-protein dithiol that can be catalytically recycled to a reduced state after oxidation of the compound, such as dihydrolipoic acid or a biologically active derivative thereof. In one aspect, the compound is orally administered α-lipoic acid, or a biologically active derivative thereof. In another aspect, the compound is recombinant human thioredoxin, with recombinant human thioredoxin produced in a substantially reduced, monomeric state being particularly preferred. In this embodiment, the thioredoxin can be administered with another dithiol, such as dihydrolipoic acid or a biologically active derivative thereof.

[0017] In one aspect, the method includes the additional step of administering an additional mucolytic compound to the patient, including, but not limited to, a DNase or hypertonic saline. The additional mucolytic compound can, in one aspect, be administered prior to the compound containing a dithiol active-site in reduced state.

[0018] Another embodiment of the present invention relates to a composition for use in the inhibition of elastase in a patient, consisting essentially of a compound comprising a dithiol active-site in reduced state and at least one additional agent for treatment of a disease or condition associated with excessive elastase activity, wherein the compound can be catalytically recycled to a reduced state after oxidation of the compound, and wherein the composition does not comprise a protease inhibitor. In one aspect, the compound is dihydrolipoic acid or a biologically active derivative thereof. In another aspect, the compound is a protein or peptide comprising a thioredoxin active-site in reduced form, including any of such compounds as described above. In this aspect, the composition can further comprise nicotinamide-adenine dinucleotide phosphate (reduced form) (NADPH) and/or thioredoxin reductase. In this aspect, the composition can further comprise dihydrolipoic acid or a biologically active derivative thereof. The composition may also include a compound to catalytically reduce the compound comprising a dithiol active-site after the compound comprising the dithiol active-site has been oxidized. The additional agent, in one aspect, is formulated

separately for administration prior to the administration of the compound comprising a dithiol active-site.

[0019] Yet another embodiment of the present invention relates to a composition for the inhibition of elastase in a patient, comprising α-lipoic acid, wherein the composition is formulated for oral delivery. In one aspect, this composition further comprises at least one additional agent for treatment of a disease or condition associated with excessive elastase activity.

[0020] Another embodiment of the invention relates to a composition for use in the liquefaction of mucus or sputum, comprising a compound comprising a dithiol active-site in reduced state and at least one additional agent for treatment of excessively viscous or cohesive mucus or sputum, wherein the compound can be catalytically recycled to a reduced state after oxidation of the compound. In one aspect, the compound is a non-protein dithiol that can be catalytically recycled to a reduced state after oxidation of the compound, such as dihydrolipoic acid or a biologically active derivative thereof. In another aspect, the protein is recombinant human thioredoxin, and preferably, recombinant human thioredoxin produced in a substantially reduced, monomeric state. In this aspect, the composition can further include dihydrolipoic acid or a biologically active derivative thereof.

[0021] Another embodiment of the present invention relates to a composition for use in the liquefaction of mucus or sputum, comprising α-lipoic acid and at least one additional agent for treatment of excessively viscous or cohesive mucus or sputum, wherein the composition is formulated for oral delivery.

[0022] Yet another embodiment of the present invention relates to a method to increase the liquefaction of mucus or sputum in a patient that has excessively viscous or cohesive mucus or sputum. The method includes contacting the mucus or sputum in the respiratory tract of the patient with a composition comprising dihydrolipoic acid or a biologically active derivative thereof, wherein the contact of composition increases the volume of the liquid phase in a sample of mucus or sputum from the patient as compared to prior to contact with the composition.

BRIEF DESCRIPTION OF THE DRAWINGS OF THE INVENTION

[0023] **FIG. 1A** is a line graph showing that liquefaction of CF sputum by exposure to the Trx reducing system is dose-dependent.

[0024] **FIG. 1B** is a line graph showing that liquefaction of CF sputum by Trx is dependent upon NADPH.

[0025] **FIG. 2** is a line graph showing an assessment of compaction assay reproducibility.

[0026] **FIG. 3A** is a graph showing that the Trx reducing system (Trx+0.1 μM TR+2 mM NADPH) is more potent in sputum liquefaction than a glutathione reducing system (GSH+0.1 μM Gr+2 mM NADPH).

[0027] **FIG. 3B** is a graph showing that the Trx reducing system (Trx+0.1 μM TR+2 mM NADPH) is more potent in sputum liquefaction than N-acetylcysteine (NAC).

[0028] **FIG. 4A** shows the effect of Trx dose on viscoelasticity (log G*) of CF sputum in vitro.

[0029] **FIG. 4B** shows the effect of NADPH dose on viscoelasticity (log G*) of CF sputum in vitro.

[0030] **FIG. 5** is a digitized image showing the glycoprotein mass profile of CF sputum after diluent or Trx exposure.

[0031] **FIG. 6** is a graph showing that Trx exposure increases the solubility of DNA present in CF sputum.

[0032] **FIG. 7** is a graph showing that dihydrolipoic acid exposure increases the liquefaction of sputum in CF patients.

[0033] **FIGS. 8A and 8B** are graphs showing the effect of pre-reduced recombinant human thioredoxin (p-rhTrx) on CF sputum viscoelasticity as measured by change in LogG* (**FIG. 8A**) and Tan Delta (**FIG. 8B**).

[0034] **FIGS. 9A and 9B** are graphs showing the effect of dihydrolipoic acid on CF sputum viscoelasticity as measured by change in LogG* (**FIG. 9A**) and Tan Delta (**FIG. 9B**).

[0035] **FIG. 10** is a graph showing the effect of human and *E. coli* thioredoxins on purified human neutrophil elastase activity.

[0036] **FIGS. 11A, 11B** and 11C are graphs (**FIG. 11A**: dose response; **FIG. 11B**: time course; **FIG. 11C**: NADPH concentration) showing a comparison of in vitro sputum sol elastolytic activity after treatment with thioredoxin.

[0037] **FIG. 12** is a graph showing in vitro CF sputum sol elastolytic activity following treatment with DHLA.

[0038] **FIG. 13** is a graph showing the effect of *E. coli* thioredoxin system (♦), DTT (□), NAC (Δ) and GSH (○) on purified human neutrophil elastase activity.

[0039] **FIG. 14** is a graph showing the reduction of DTNB by rhTrx system and p-rhTrx.

[0040] **FIG. 15A** is a graph showing purified human neutrophil elastase activity following treatment with p-rhTrx (♦) for one hour at 37° C.

[0041] **FIG. 15B** is a graph showing elastase activity (in the sol phase from cystic fibrosis patients (1:14 dilution)) following treatment with p-rhTrx (♦) for one hour at 37° C.

[0042] **FIG. 16** is a graph showing a comparison of elastolytic activity in the sol phase collected from whole CF sputum after treatment with DNase or dithiol reductant systems (p-rhTrx, rhTrx, DHLA or DTT).

[0043] **FIG. 17** is a graph showing the effect of hypertonic saline alone or in combination with pre-reduced recombinant human thioredoxin (p-rhTrx) on sputum viscoelasticity as measured by change in LogG*.

[0044] **FIG. 18** is a graph showing the effect of DNase (Pulmozyme) and pre-reduced recombinant human thioredoxin (p-rhTrx), alone and in combination, on sputum viscoelasticity as measured by change in LogG*.

[0045] **FIG. 19** is a graph showing the effect of administration of DNase prior to administration of pre-reduced recombinant human thioredoxin (p-rhTrx) on sputum viscoelasticity as measured by change in LogG*.

[0046] **FIG. 20** is a digitized image of a gel showing digestion of DNA by DNase alone, or in combination with pre-reduced recombinant human thioredoxin (p-rhTrx).

[0047] **FIG. 21** is a digitized image of a gel showing the effect of mucin on the digestion of DNA by DNase alone, or in combination with pre-reduced recombinant human thioredoxin (p-rhTrx).

DETAILED DESCRIPTION OF THE INVENTION

[0048] The present invention generally relates to the use of a compound containing a dithiol active-site that is preferably in reduced state to induce, enhance and/or increase the liquefaction of mucus or sputum, and/or to inhibit the activity of elastase. Such compounds are useful for the treatment of a variety of diseases and conditions in which excess viscosity or cohesivity of mucus or sputum is problematic, and/or in diseases and conditions in which elastase activity is excessive, harmful, or unrestrained.

[0049] More specifically, in one embodiment of the invention, the present inventor has discovered that compounds (including proteins, peptides and other, non-protein compounds) with dual redox active site cysteines or thiol groups (e.g., thioredoxin, lipoic acid, and derivatives thereof) can decrease the viscosity and/or cohesivity of sputum or mucus and thereby are effective agents for the liquefaction of sputum or mucus (i.e., effective mucolytic agents). Accordingly, any protein, peptide or other compound containing an active-site comprising dual redox active cysteines or thiol groups in a reduced state, can be used alone or in a composition to treat a variety of conditions or diseases associated with undesirable mucus or tenacious and viscous sputum. In one embodiment, native thioredoxin, proteins or peptides containing the active-site of thioredoxin in reduced state, or nucleic acid molecules encoding such proteins, can be used alone or in a composition according to the present invention. In another embodiment, lipoic acid (LA), and particularly dihydrolipoic acid (DHLA), or derivatives thereof, can be used in accordance with the present invention. In a preferred embodiment, the present invention encompasses any protein, peptide or other compound containing an active-site comprising dual redox active cysteines or thiol groups that can be cyclically re-reduced to its effective (reduced) form. Such compounds can be referred to as "recyclable", "recycling" or "re-reducible" dithiol compounds according to the present invention. In another embodiment, the dithiol compounds of the invention, even if they are recyclable, are used in the absence of a recycling system.

[0050] Respiratory diseases such as cystic fibrosis are particularly amenable to treatment using the product and process of the invention, as well as various gastrointestinal or reproductive disorders. The present invention relates to the use of compounds containing the active-site dithiols in reduced state, and preferably recyclable dithiols, for the increased liquefaction of mucus or sputum, particularly mucus or sputum that is abnormally or excessively viscous and/or cohesive. The compounds are administered to a patient that is suffering from or affected by such abnormal or excessive mucus or sputum in a manner and amount effective to increase the liquefaction of the mucus or sputum and preferably, to provide a therapeutic benefit to the patient.

[0051] Another embodiment of the present invention relates to the use of a compound containing a dithiol active-site that is preferably in reduced state to inhibit elastase (i.e., as an anti-elastase agent), and particularly in the absence of any protease inhibitor or elevated temperature conditions. In a preferred embodiment, this embodiment encompasses any protein, peptide or other compound containing an active-site comprising dual redox active cysteines or thiol groups that can be cyclically re-reduced to its effective (reduced) form. Human neutrophil elastase has four disulfide bonds which are important in maintaining its tertiary structure and enzymatic activity. The present inventors have assessed the ability of dithiols, exemplified by thioredoxin, to inhibit elastase activity, in the absence of protease inhibitors or heat sources. The inventors have determined that such a single component form of a dithiol has great potential for therapeutic use, particularly in disease processes with excessive elastolytic activity. The dithiol, dithiothreitol, was able to inhibit the activity of elastase, presumably by disrupting the disulfide linkages. However, dithiothreitol is not used in clinical practice due to poor patient tolerance. The present invention preferably encompasses dithiols that are tolerated by the patient. As such, in one aspect, the invention does not include the use of dithiothreitol as a dithiol in the present compositions and methods. In one aspect, a protease inhibitor can be included with the composition, although as discussed above, such a protease inhibitor is not required and may not be preferred in all embodiments.

[0052] A variety of lung, sinus, nasal, gastrointestinal, or reproductive diseases or conditions can benefit from treatment using this method of the present invention. Any condition or disease that is exacerbated by, and/or is associated at least some of the time with, the secretion or presence of elastase in the tissues can be treated using such method. The present invention relates to the use of compounds containing the active-site dithiols in reduced state, and preferably recyclable dithiols, to inhibit elastase activity in the tissues of a patient. The compounds are administered to a patient that is suffering from or affected by abnormal, excessive or harmful elastase activity in a manner and amount effective to decrease elastase activity and preferably, to provide a therapeutic benefit to the patient.

[0053] Proteins containing a dual redox thiol active site, such as proteins having a thioredoxin active site or other compounds having a recyclable dithiol, such as α-lipoic acid (dihydrolipoic acid in reduced form), have advantages over other reducing agents for use as a mucolytic compound or as an anti-elastase compound, such as in the treatment of conditions such as cystic fibrosis. For example, unlike other reducing agents (e.g., N-acetylcysteine (NAC), Nacystelyn (NAL), dithiothreitol (DTT)), recyclable dithiols such as thioredoxin and α-lipoic acid (also referred to herein as lipoic acid) can be cyclically re-reduced to their effective (reduced) forms. In addition, auto-oxidation (e.g., producing superoxide, hydrogen peroxide, hydroxyl radical and other toxic oxygen metabolites) of compounds such as thioredoxin and lipoic acid occurs at a low level as compared with other reducing agents such as NAC, NAL and DTT. Therefore, in one aspect, the invention excludes the use of dithiols such as NAC, NAL and DTT that can not be recycled to their reduced forms. However, it is not a requirement in all aspects of the present invention that the dithiols used in the present methods be recycled, thus including the use of any dithiol as a mucolytic or as an anti-elastase agent. Furthermore, certain dithiols useful in the present invention, such as thioredoxin, are a naturally occurring compound which

normally exists in the extracellular space, and therefore, introduction of such compounds into the airway should not induce an immune response and should be non-irritating. Proteins such as thioredoxin and other dithiol compounds are also not glycosylated, and as such, administration of the protein or other compound in natural or recombinant form should not induce an innate immune response. Perhaps even more significantly, the recyclable dual redox thiol compounds described herein, in contrast to other reducing agents, maintain the treated mucus or sputum in the liquid state. NAC and DTT, for example, become "spent" or oxidized over time and at this stage, liquified sputum or mucus can revert back to the more viscous "gel" state. In contrast, the liquefaction produced by compounds such as thioredoxin and dihydrolipoic acid appears to endure for hours, most likely due to its cyclic re-reduction by its reducing system. Finally, the dual redox thiol compounds described herein are more potent than other reducing agents and therefore, they can be used at significantly lower doses than other agents to achieve a beneficial effect.

[0054] In addition to the above-described advantages, certain of the dithiol compounds described herein, such as thioredoxin, have other benefits which increase their usefulness in disease conditions. For example, it is known that thioredoxin induces MnSOD (e.g., see U.S. Pat. No. 5,985,261 to White et al., incorporated herein by reference in its entirety) which is predicted to decrease the toxicity of certain bacterial toxins (including, but not limited to, endotoxin from bacterial cell walls of gram-negative bacteria, pyocyanin from *Pseudomonas aeruginosa*, and others) in disease sputum (e.g., cystic fibrosis sputum). In addition, thioredoxin has anti-inflammatory properties which can enhance the overall treatment of a respiratory condition.

[0055] As discussed above, human neutrophil elastase has four disulfide bonds which are important in maintaining its tertiary structure and enzymatic activity. The present inventors have demonstrated herein that the potent reducing agents, thioredoxin and DHLA, both of which contain vicinal thiols, disrupt the disulfide linkages within elastase. Significant reductions in neutrophil elastase activity using micromolar amounts of dithiothreitol (DTT) have been demonstrated in vitro (e.g., Del Val et. al., 2001, *Mol. Immunol.* 38:759-763), although its use in vivo is restricted due to toxicity and poor tolerance issues. Factors influencing the inhibition of elastase included DTT concentration, time and reaction temperature. For example, the effective use of DTT to reduce elastase activity required either increased temperature conditions (e.g., up to 50° C. in the presence of DTT) or high concentrations of DTT, thereby increasing expected toxicity effects. Del Val et. al. also showed that a thioredoxin system, with the requirement of the presence of al-antitrypsin, was able to act as a potent porcine pancreatic elastase inhibitor. However, the data presented herein, in contrast to these prior studies, demonstrates a direct inhibitory effect of thioredoxin (and DHLA) on elastase in the absence of an antiprotease (i.e., protease inhibitor) such as al-antitrypsin and in the absence of increased temperature conditions. This discovery is significant, as it dramatically facilitates and enables the clinical use of dithiols as anti-elastase agents.

[0056] Similar to DTT, both Trx and DHLA have two sulfhydryl moieties at their active sites (thus they are referred to as "dithiols") and are strong reductants with redox potentials of −280 mV and −320 mV, respectively. By contrast, monothiols such as glutathione (GSH) and N-acetyl-cysteine (NAC) have only one sulfhydryl group with redox potentials of −240 mV and −150 mV, respectively. These monothiols require much greater concentrations to inhibit elastase. In the experiments described herein, the inventors determined that in the concentration range at which dithiols inhibited elastase (up to 2 mM), the monothiols had little inhibitory effect. Previous work has also shown that neither GSH nor the monothiol β-mercaptoethanol had a significant effect on elastase activity at low millimolar amounts. Higher concentrations of monothiols can inhibit elastase; however, to achieve a 50% reduction of purified neutrophil elastase activity, approximately 60 mM NAC is required, while even 120 mM of the monothiol mercaptoethane sulphonate (MESNA, Mistabron) does not achieve this level of elastase inhibition. By contrast, p-rhTrx showed a 50% reduction in elastase activity at only 0.03 mM. Therefore, the dithiols used in the present invention are used at substantially lower doses than the doses that would be required to obtain the same or similar effect using monothiols. Again, this is significant for the use of dithiols in clinical applications, where toxicity and patient tolerance are a concern.

[0057] The active site of Trx contains two cysteine sulfhydryl groups in a highly conserved "C-X-X-C" conformation. In mammals, thioredoxin participates in a variety of cell activities including regulation of transcription factors such as NF-κ, AP-1, HIF-1 and 2, nuclear glucocorticoid receptor activation, and regulation of apoptosis. While the majority of thioredoxin is intracellular, a portion is secreted. In human plasma, the thioredoxin concentration is approximately 6 nM, significantly less that the 1 μM of glutathione in plasma. The concentration of thioredoxin in the airway surface liquid is unknown. DHLA is also a naturally occurring dithiol, first identified by Lester Reed in 1951. This small molecule (molecular weight 208.3) has been described as the "universal antioxidant" but has been shown to have both antioxidant and prooxidant activities. In its lipoamide form, it participates as a cofactor of the multienzyme complexes catalyzing the oxidative decarboxylation of α-keto acids. Recent safety data regarding treatment of diabetic polyneuropathy with the oxidized form of DHLA, α-lipoic acid, has been reported. While the dithiols commonly used in laboratory experiments, such as DTT, are toxic and/or irritants to mammals, Trx and DHLA are native intracellular and extracellular mammalian reductants and are believed by the present inventors to hold great promise for medicinal development.

[0058] In addition to elastase inhibition, the experiments described herein confirm that dithiols including Trx and DHLA have potent mucolytic effects. In perhaps the most clinically pertinent experiment, the inventors compared the elastolytic and mucolytic activity in CF sputum after treatment with dithiols and DNase (Pulmozyme). DNase is the most commonly prescribed mucolytic in the United States for CF patients. At concentrations which caused comparable mucolytic effects, most dithiols did not affect elastase activity. A mild increase in elastase activity was noted after p-rhTrx (100 μM) treatment, but this was less than one-third the increase in elastase activity in the DNase treated samples. After DNase treatment, the elastase activity increased almost three-fold that of control sputum. Others have documented acute increases in elastase activity after

DNase treatment both in vitro as well as in patients with CF. Long-term use of DNase by CF patients results in decreased sputum elastase load and activity relative to untreated patients, presumably through enhanced airway clearance. However, sputum elastase activities do not return to the normal range, despite DNase treatment. In fact, attempts to return the protease/antiprotease balance to normal have met with only marginal success. Konstan and Davis noted that "since the neutrophils reach the airway by migrating through the epithelium, much of the release of proteases will occur beneath the mucus blanket and the inhibitors must penetrate this protective layer." In contrast to the DNase treatment, since dithiols act as both potent mucolytics and elastase inhibitors, it is expected that these agents will be able to penetrate beneath the mucus layer. The present inventors therefore propose that airway elastase activity and excessive or abnormal mucus viscosity and/or cohesiveness could be substantially diminished through regular use of dithiols by CF patients, as well as by other patients that have diseases or conditions associated with these symptoms (excessive or abnormal elastase activity and/or excessive or abnormal mucus viscosity and/or cohesiveness).

[0059] According to the present invention, a compound useful in the present invention is a protein, peptide, or other compound (e.g., a natural or synthetic organic or inorganic compound, small molecule, etc.) that comprises dual redox thiol groups. Such a compound can be referred to as a dual redox thiol compound, a dithiol compound, a compound containing dual redox cysteines or thiols, and derivatives thereof. A thiol is any compound containing the monovalent sulfhydryl (—SH) group, which can be attached to a carbon, and can include a hydrosulfide, for example. A dithiol or a compound with dual thiol groups is a compound containing two thiol groups that are in proximity to each other (i.e., are related). The thiols exist as "free" sulfhydryl groups when the compound is in reduced form, and when the compound is in oxidized form, the thiol groups can form a disulfide, for example. Thiol groups that participate in redox reactions (e.g., dual redox thiols) participate in oxidation-reduction reactions that involve the transfer of electrons from one molecule to another. Through the oxidation of the active-site dithiol to a disulfide, dithiols catalyze dithiol-disulfide exchange reactions.

[0060] Preferred dithiols for use in the present invention are any dithiols that have the properties of (1) increasing the liquefaction and/or decreasing viscosity, decreasing elasticity, and/or decreasing cohesiveness, of mucus or sputum, and/or (2) decreasing the activity and/or levels of elastase. Such dithiols include, but are not limited to, any vicinal dithiols. Particularly preferred dithiols for use in the present invention are dithiol compounds that can be recycled, or re-reduced (e.g., catalytically reduced) also referred to as a redox-cycling dithiol, such as by using the compounds in an environment or with a system that reduces the dithiol for reuse (e.g., by combination with other compounds that recycle the compound back to a reduced state). For example, suitable reducing systems useful for thioredoxin are described in detail below. Dithiols are used in the present invention in a reduced form (e.g., pre-reduced dithiols), in a manner and/or in an environment that results in the reduction of the dithiol to a reduced form, and/or with a system that allows for the catalytic reduction of the dithiol to a reduced form. Examples of dithiols that are particularly suitable for use in the present invention include, but are not limited to, dihydrolipoic acid, any protein or peptide with a thioredoxin active site (discussed below) such as thioredoxin, or any other dithiol known in the art, such as any of those described in Lamoureux and Whitesides, 1993, *J. Org. Chem.* 58:633-641, incorporated herein by reference in its entirety, or any derivatives (analogs) of such dithiols. In one embodiment, a dithiol useful in the present invention is one with a reduced tendency to autooxidize or one that is placed in an environment that reduces autooxidation. In another embodiment a dithiol useful in the present invention has a relatively long half-life (e.g. greater than 5 minutes, and more preferably greater than 30 minutes). In another embodiment, a dithiol useful in the present invention is relatively non-toxic to the patient.

[0061] Without being bound by theory, the present inventors believe that the beneficial action of the dithiol compounds in the present invention are through the following two mechanisms, which are separately identifiable and can be utilized to treat different diseases or conditions. First, the dithiol compounds reduce disulfide bonds in mucin polymers, thereby reducing the viscoelasticity of mucus or sputum containing these polymers (discussed in detail below). Second, dithiols tested by the present inventors, including thioredoxin and dihydrolipoic acid, inhibit purified elastase and sputum elastolytic activity (i.e., have an anti-elastase effect), which contributes to the decrease in sputum and mucus viscosity, and also inhibits or decreases excessive or abnormal proteolysis of extracellular matrix proteins that can be harmful to a patient. In addition, the present inventor has shown that thioredoxin, by way of example, can inhibit the actions of purified human neutrophil elastase, including in human cystic fibrosis sputum samples. Furthermore, dithiols used in accordance with the present invention can preserve α1-antitrypsin activity in human mucus, which is a minor, albeit valid mechanism contributing to the anti-elastase effect.

[0062] As discussed above, thioredoxin (Trx) is a protein disulfide reductase that catalyzes numerous thiol-dependent cellular reductive processes. Thioredoxin (Trx) contains two redox-active cysteines which are highly conserved across species. In their oxidized form, these cysteines form a disulfide bridge that protrudes from the three dimensional structure of the protein (Holmgren, *Annu Rev Biochem* 54:237-271, 1985).

[0063] Reduction of this active center by the NADPH-dependent thioredoxin reductase (TR) enzyme allows Trx to function as an electron carrier with dithiol/disulfide exchange capability (Oblong et al., *Biochemistry* 32:7271-7277, 1993). Protein disulfides are a preferred substrate for Trx-mediated reducing action. The persistent and viscous nature of airway secretions in cystic fibrosis disease leads to airway obstruction, opportunistic infection, and deterioration of lung function. Recognizing that respiratory mucins contain several cysteine domains that are believed to play an essential role in polymerization (Bell et al., *Biochem J* 357:203-209, 2001; Asker et al., *Biochem J* 333:381-387, 1998), the present inventors sought to determine whether Trx could serve as an effective mucolytic by reduction of mucin disulfides.

[0064] Dihydrolipoic acid (DHLA; also called dihydrolipoate) and its oxidized form, lipoic acid (LA), is a dithiol discovered in 1951 by Lester Reed that is found in animals

and plants. Lipoic acid (also called α-lipoic acid, lipoate or thioctic acid) is readily converted to and from dihydrolipoic acid (i.e., it is recyclable). Lipoic acid acts as a coenzyme in reactions that occur in the Krebs cycle and plays an essential role in mitochondrial dehydrogenase reactions. Both LA and DHLA are potent lipophilic antioxidants that participate in many enzymatic reactions and are easily absorbed through the gut and transported across cell membranes. LA or DHLA react with reactive oxygen species such as superoxide radicals, hydroxyl radicals, hypochlorous acid, peroxyl radicals, and singlet oxygen. It also protects membranes by interacting with vitamin C and glutathione, which may in turn recycle vitamin E. In addition to its antioxidant activities, dihydrolipoate may exert prooxidant actions through reduction of iron (see Packer et al., *Nutrition* 2001 October; 17 (10):888-95). Being the reduced form of the compound, DHLA is preferred for use in the present invention. However, it is noted that orally administered α-lipoic acid is rapidly absorbed into the bloodstream, taken up by the cells of the body and converted within the cell into dihydrolipoic acid (DHLA). This DHLA synthesized within the cell is then secreted into the extracellular space and in the lung tissue, into the airways. Thus, the present invention encompasses the use of oral α-lipoic acid as an effective "precursor" to the reduced form, DHLA, whereby the result is the delivery of a dithiol in reduced state at the target site of action.

[**0065**] In the experiments discussed herein, the present inventors first examined the effects of the Trx reducing system (thioredoxin, thioredoxin reductase, and NADPH) on the physical and rheologic properties of CF sputum in vitro. Sputum obtained from CF patients was treated with TRX and its reducing system [0.1 μM Thioredoxin reductase (TR)+2 mM NADPH] and liquid phase:gel phase ratio (percent liquid phase) assessed by compaction assay. Exposure to low Trx concentrations (1 μM) caused significant increases in the percentage of liquid phase of sputum. Maximal increases in percent liquid phase occurred with 30 μM Trx. Additional measurements revealed that sputum liquefaction by the Trx reducing system is dependent on NADPH concentration. The relative potency of the Trx reducing system also was compared with other disulfide reducing agents. In contrast with Trx, glutathione and N-acetylcysteine were ineffective in liquefying sputum when used at concentrations below 1 mM. Sputum viscoelasticity, measured by magnetic microrheometry, was also significantly diminished following 20 minute treatment with 3, 10, or 30 μM Trx. Similarly, this reduction in viscoelasticity was also dependent upon NADPH concentration. Further experimentation has indicated that Trx treatment increases the solubility of high molecular weight glycoproteins, and causes redistribution of extracellular DNA into the liquid phase of sputum. The experiments described herein demonstrate that in vitro treatment with catalytic amounts of Trx and its reducing system can liquefy and decrease the viscoelasticity of purulent CF sputum. The increased solubility of high molecular weight glycoproteins present in Trx-treated sputum indicates that mucin macromolecules may be the substrates reduced by Trx during the mucolytic process.

[**0066**] It is important to note that the method of the present invention can result in viscosity changes of about 3-10 fold even in the range where no statistically perceptible change in the percent liquefaction is changing, and such a change in viscosity can be sufficient to provide a therapeutic benefit to a patient. Indeed, sputum liquefaction may change by as little as 1-3% or less, yet a therapeutic benefit in the patient is observed using the methods of the invention.

[**0067**] In subsequent studies, the present inventors have now discovered that dihydrolipoic acid not only reduces thioredoxin (and therefore is clearly a suitable alternative for the NADPH and thioredoxin reductase system that can be used to recycle thioredoxin to a reduced state), but also inhibits elastase and is a mucolytic agent when used alone. Therefore, in addition to being particularly useful in conjunction with compounds containing a thioredoxin active-site according to the present invention, DHLA is a potent dithiol for use as a primary agent in the methods of the invention.

[**0068**] Mucus obstruction of the airways can cause significant morbidity and mortality in patients with CF. The present inventors have demonstrated that the viscoelastic properties facilitating the persistence of these secretions within airways are markedly diminished by Trx and also by DHLA. This conclusion is supported by two lines of experimental evidence. First, compaction assay results indicate that large amounts of liquid are released from the gel matrix of CF sputum during incubation with Trx and also with DHLA. Occurring simultaneously with this release were decreases in the volume of solid matter, indicating that the gel forming constituents of sputum were being solubilized. This liquefaction of CF sputum could often be observed grossly in CF sputum samples during the incubation period, and therefore, is not an artifact of centrifugal disruption. The liberation of liquid by Trx is expected to have important therapeutic implications since restoration of water volume at airway surfaces can restore the mucociliary transport ability of CF epithelium (Jiang et al., *Science* 262:424-427, 1993). Second, magnetic microrheometry measurements provide direct evidence that sputum viscoelasticity declines as a result of reduction of sputum components by Trx.

[**0069**] CF sputum is a non-newtonian fluid exhibiting both liquid and solid characteristics. Polymers when present in solutions at low concentration are able to rotate freely. When polymers become concentrated or cross-linked to such a degree that their rotation is hindered, a solution has reached a transition phase called the percolation threshold (Forgacs, *J Cell Sci* 108:2131-2143, 1995). At the percolation threshold the solution begins to acquire characteristics of a solid, and the elastic moduli continue to increase as more cross-polymer interactions are added, until each filament in the sample is incorporated into the matrix. Biochemical analyses have revealed that mucins MUC5AC and MUC5B, secreted by cells lining the respiratory tract, are the major gel forming polymers components of airway mucus (Hovenberg et al., *Glycoconj J* 13:839-847, 1996; Thornton et al., *Biochem J* 316:967-975, 1996; Thornton et al., *J Biol Chem* 272:9561-9566, 1997). Cysteine domains present on these mucins contribute to polymer formation, and possibly interaction with neighboring mucin chains, by disulfide bond formation (Bell et al., *Biochem J*357:203-209, 2001; Asker et al., *Biochem J* 333:381-387, 1998). Since disulfide bonds on proteins are the preferred substrates for Trx enzymatic activity, it was hypothesized by the inventor that mucin polymers were targets for reduction during the liquefaction of sputum by Trx. This hypothesis was supported by PAS staining which revealed changes in the solubility of high molecular weight glycoforms in Trx treated sputum. Detection of greater concentrations of glycoproteins in the

liquid phase of Trx-exposed sputum was further indicated by a more intense yellow color and had greater opacity than liquid phase derived from diluent-treated samples. The enhanced electrophoretic mobility of PAS-detectable glycoproteins in Trx-exposed sputum also suggests that these macromolecules may decrease in size during enzymatic reduction. Findings from this electrophoretic analysis are in agreement with compaction assay measurements by demonstrating that glycoprotein release into liquid phase coincides with the decrease in mass of the gel matrix during exposure to Trx.

[0070] Neutrophil lysis within the airways of diseased CF lungs results in the deposition of extracellular DNA into airway secretions (Lethem et al., *Eur Respir J* 3:19-23, 1990). By non-covalent interactions, this DNA becomes entangled within mucin glycoproteins, increasing mucus gel viscoelasticity (Sachdev et al., *Chest* 81:41S-43S, 1982). In the experiments described herein, the present inventors found that DNA present in sputum becomes increasingly soluble following Trx treatment. A logical explanation is that Trx activity causes structural changes within the gel matrix which are sufficient to relieve entanglement interactions between DNA and the affected macromolecules. It is uncertain what the relative contribution of this increased DNA solubility has toward viscoelastic changes observed during exposure of CF sputum to Trx. Nonetheless, from a clinical standpoint, removal of DNA from the insoluble gel phase of sputum could render it more susceptible to DNase activity during such treatment in CF. Therefore, the method of the invention has a strong potential for synergy with other existing "state of the art" therapies for CF.

[0071] In studies comparing the sputum liquefying abilities of other thiol reducing agents, Trx demonstrated greater efficacy than the glutathione (GSH; reduced glutathione) reducing system. Since Trx has two redox active cysteine residues (dithiol), whereas GSH contains only one (monothiol), Trx may be more efficient in reduction of disulfide bonds in the gel-forming constituents of CF sputum. With regard to non-recycling mucolytic drugs, DTT was more effective on an equimolar basis than NAC (or Mucomyst®) solutions (**FIGS. 2 and 3**; and data not shown). Efficacy of these compounds may again be dependent on the number of redox active cysteine residues, DTT having two, NAC only one. On the basis of these compaction assay measurements, enzymatic disulfide bond reduction using proteins, peptides or other compounds with dual redox active cysteines or thiol groups are expected to be a potent mucolytic strategy. Indeed, the present inventor has demonstrated that the reduced form of the dithiol, lipoic acid, or DHLA, is a potent mucolytic compound and also has direct anti-elastase properties.

[0072] In summary, dithiols such as thioredoxin and DHLA increase the liquid fraction and are useful for diminishing the viscoelasticity of CF sputum or mucus. Increases in glycoprotein solubility occur during treatment of sputum with Trx, and this may be the mechanism for these rheological changes. The development of mucus reducing systems that stimulate release of liquid, and reduce the viscosity of airway secretions, is expected to have therapeutic potential for CF, as well as for the treatment of excessive or abnormal mucus viscosity and/or cohesiveness that may be associated with other respiratory conditions (e.g., chronic or acute bronchitis; bronchiectasis; acute tracheitis; acute or chronic sinusitis; atelectasis resulting from acute or chronic mucus plugging of the airways; bronchiolitis) or with various gastrointestinal or reproductive disorders associated with or exacerbated by excessive or abnormal mucus viscosity and/or cohesiveness (e.g., acute, subacute or chronic bowel obstruction due to mucus inspissation; infertility due to obstruction of vital reproductive structures). Since Trx is a native protein which lacks glycosylation and post-translational modification, and normally appears at low level within extracellular space, its chronic administration could be tolerated well by the immune system.

[0073] Accordingly, one embodiment of the present invention relates to a method to increase the liquefaction of mucus or sputum in a patient that has excessively viscous or cohesive mucus or sputum. The method includes the step of contacting the mucus or sputum of the patient with a composition comprising a compound (e.g., a protein, peptide, or other compound) containing a dual redox thiol active-site in reduced state (e.g., a dithiol in reduced state). The compound is effective to increase the liquefaction of the mucus or sputum as compared to prior to the step of contacting. Preferably, the dithiol is a recycling dithiol in reduced state. In one embodiment, the dithiol does not include dithiols that do not recycle, such as NAC, NAL or DTT. In one aspect, the compound is a protein or peptide containing a thioredoxin active-site in reduced state, with human thioredoxin, and particularly, recombinant human thioredoxin, being preferred. In another embodiment of the invention, the compound is lipoic acid in reduced state (i.e., dihydrolipoic acid) or a derivative thereof. In yet another embodiment, the method includes the step of contacting the mucus or sputum of the patient with a composition comprising lipoic acid in reduced state (or a derivative thereof) and a protein or peptide containing a thioredoxin active-site in reduced state. In one embodiment, the dithiol does not include a protein or peptide containing a thioredoxin active-site in reduced state. The composition can further include, if desired or necessary, a reducing system for the dithiol (i.e., a system of compound(s) that maintains, recycles or reduces the dithiol active site in the protein or compound to the reduced state, as described elsewhere herein).

[0074] According to the present invention, the term "mucus" generally refers to a usually clear viscid fluid that is secreted by mucous membranes in various tissues of the body, including by the respiratory, gastrointestinal, and reproductive tracts. Mucus moistens, lubricates and protects the tissues from which it is secreted. It comprises mucin macromolecules, which are the gel forming constituents of mucus. The viscoelastic properties of normal mucus are dependent on the concentration, molecular weight, and entanglements between mucin polymers. The term "sputum" generally refers to a mixture of saliva and discharge from the respiratory passages, including mucus. Sputum is typically an expectorated mixture of saliva and mucus (and other discharge from the respiratory tissues). Therefore, mucus is a primary component of sputum, and as such, the presence of excessively viscous mucus in sputum results in a sputum which is itself excessively viscous. The term "liquefaction" refers to the act of becoming liquid. Therefore, an increase in the liquefaction of mucus or sputum refers to the increase in liquid phase or liquid state of mucus or sputum, as compared to a more solid or viscous phase.

[0075] The general functions of mucus and sputum in the body require that the mucus (and thus the mucus component of the sputum) have viscoelastic properties. In an individual with normal mucus and sputum (i.e., a healthy individual, or more particularly, an individual who does not suffer from symptoms or a condition caused or exacerbated by the viscosity or cohesiveness of mucus or sputum), the viscoelasticity is dependent on the concentration, molecular weight, and entanglements between mucin polymers (Verdugo et al., *Biorheology* 20:223-230, 1983). When mucins in the mucus interact with DNA (Potter et al., *Am J Dis Child* 100:493-495, 1960; Lethem et al., *Am Rev Respir Dis* 100:493-495, 1990; Lethem et al., *Eur Respir J* 3:19-23, 1990) and f-actin polymers (Sheils et al., *Am J Path* 148:919-927, 1996; Tomkiewicz et al., DNA and actin filament ultrastructure in cystic fibrosis sputum. In: Cilia, mucus, and mucociliary interactions, edited by Baum G L, Priel Z, Roth Y, Liron N, and Ostfeld E J. New York, N.Y.: Marcel Dekker, 1998) released from dying inflammatory cells, the mucus (and thus sputum) becomes much more dense and viscous, such as in CF sputum. The inability to clear such mucus by cough or mucociliary clearance facilitates colonization of the lung with opportunistic pathogens. Therefore, abnormally or excessively viscous and/or cohesive mucus is characterized as mucus that is measurably or detectably more viscous or cohesive than mucus from a normal or healthy patient (preferably an age and sex-matched patient), and/or as mucus which, by virtue of its level of viscosity and/or cohesiveness, causes or contributes to at least one symptom in a patient that causes discomfort or pain to the patient, or that causes or exacerbates a condition or disease. In other words, abnormally or excessively viscous and/or cohesive sputum is a deviation from normal mucus or sputum wherein it is desirable to treat the patient to provide some relief from the condition or other therapeutic benefit.

[0076] The method and composition of the present invention can be used to treat any patient in which it is desirable to increase the liquefaction of mucus or sputum. In particular, patients that have certain lung, sinus, nasal, gastrointestinal, or reproductive diseases or conditions can benefit from treatment using the method of the present invention. The present invention is most useful for ameliorating or reducing at least one symptom of a condition or disease that is caused by or exacerbated by abnormal or excessive viscosity and/or cohesiveness of the mucus or sputum, which of course can include the lung-associated disease, cystic fibrosis. Other diseases may, at least some of the time, be associated with abnormal or excessive viscosity and/or cohesiveness of the mucus or sputum, and when such a symptom occurs, the method of the present invention can be used to increase liquefaction of the mucus or sputum and provide at least some relief or therapeutic benefit to the patient. Examples of such diseases include, but are not limited to: cystic fibrosis; chronic or acute bronchitis; bronchiectasis (non-CF and CF bronchiectasis); acute tracheitis (bacterial, viral, mycoplasmal or caused by other organisms); acute or chronic sinusitis; atelectasis (lung or lobar collapse) resulting from acute or chronic mucus plugging of the airways (sometimes seen in a variety of diseases such as asthma); bronchiolitis (viral or other); acute, subacute or chronic bowel obstruction due to mucus inspissation including, but not limited to meconium ileus or meconium ileus equivalent in CF or similar disorders; and infertility due to obstruction of (but not limited to) the cervix, seminal ducts or other vital reproductive structures. In addition, the composition and method of the present invention may be useful for reducing symptoms associated with excessive viscosity and/or cohesiveness of the mucus or sputum in patients with a variety of respiratory infections, including both viral and bacterial infections.

[0077] As such, a therapeutic benefit is not necessarily a cure for a particular disease or condition, but rather, preferably encompasses a result which most typically includes alleviation of the disease or condition, elimination of the disease or condition, reduction or elimination of a symptom associated with the disease or condition, prevention or alleviation of a secondary disease or condition resulting from the occurrence of a primary disease or condition (e.g., infectious disease caused by opportunistic pathogenic microorganisms that take advantage of the excessively viscous mucus in the respiratory tract), and/or prevention of the disease or condition, or a symptom associated with the disease or condition. As used herein, the phrase "protected from a disease" refers to reducing the symptoms of the disease; palliative therapy (relieving or soothing a symptom of the disease without effecting a cure); reducing the occurrence of the disease, and/or reducing the severity of the disease. Protecting a patient can refer to the ability of a composition of the present invention, when administered to a patient, to prevent a disease from occurring and/or to cure or to alleviate disease at least one symptom, sign or cause of the disease or condition. As such, to protect a patient from a disease includes both preventing disease occurrence (prophylactic treatment) and treating a patient that has a disease (therapeutic treatment). In particular, protecting a patient from a disease is accomplished by increasing the liquefaction of mucus or sputum in the patient by contacting the mucus or sputum with a dithiol compound in reduced state such that a beneficial effect is obtained. A beneficial effect can easily be assessed by one of ordinary skill in the art and/or by a trained clinician who is treating the patient. The term "disease" refers to any deviation from the normal health of a patient and includes a state when disease symptoms are present, as well as conditions in which a deviation (e.g., infection, gene mutation, genetic defect, etc.) has occurred, but symptoms are not yet manifested.

[0078] Contact of the mucus and/or sputum of a patient with the dithiol compound (or composition comprising such a compound) is intended to result in increased liquefaction of the mucus or sputum as compared to prior to contact with the composition. According to the present invention, an increase in liquefaction of mucus or sputum can be any measurable or detectable increase in the level of liquefaction of mucus or sputum as compared to a prior level of liquefaction, and is preferably a statistically significant increase (i.e., differences in measured level of liquefaction between the patient sample and a baseline control are statistically significant with a degree of confidence of at least $p<0.05$). Typically, the "baseline control" is a patient sample prior to the administration of the treatment, since normal, healthy individuals generally cannot produce a quantity of sputum sufficient to serve as a control, although sputum from a normal, healthy individual is not excluded as a baseline control. Liquefaction of mucus or sputum can be measured using any suitable technique known in the art, including, but not limited to, compaction assays as described in the Examples section. In such an assay, the amount of mucus or sputum in a solid phase (gel) versus aqueous phase (liquid)

is measured. In other aspects of the invention, the relative viscosity or cohesiveness of mucus or sputum can be measured using other parameters or indicators including, but not limited to, viscoelasticity (measured, for example, by magnetic microrheometry), glycoprotein content, or DNA content. In one aspect of the invention, the level of liquefaction is described as the amount of a given mucus or sputum sample that is in an aqueous (liquid) phase as a percentage of the total volume of the mucus or sputum sample. In a patient with cystic fibrosis, for example, the level of liquefaction of mucus or sputum can be as low as less than 10% or even less than 5% of the total volume. Preferably, contact of a protein, compound or composition of the invention with the mucus or sputum results in a change in the liquefaction of the mucus or sputum of at least about such that at least about 15% of the total volume is in liquid phase, and more preferably, at least about 20% of the total volume is in liquid phase, and more preferably, at least about 25% of the total volume is in liquid phase, and more preferably, at least about 30% of the total volume is in liquid phase, and more preferably, at least about 35% of the total volume is in liquid phase, and more preferably, at least about 40% of the total volume is in liquid phase, and more preferably, at least about 45% of the total volume is in liquid phase, and more preferably, at least about 50% of the total volume is in liquid phase or until the blockage or inhibition of function caused by the mucus has cleared (e.g., until the patient airways are cleared sufficiently to begin expectorating the fluid). In general, it is preferred that the liquefaction of the sputum or mucus in increased in small, gradual increments until the airway or other blocked passage (e.g., in the gastrointestinal or reproductive tract) is cleared, but without excessively liquefying the sputum. Excessive liquefaction of the mucus or sputum is not desired, as it can be detrimental to the patient (e.g., liquefied sputum could flow backward and flood the small airways with an infected thin liquid before the sputum can be cleared by the patient). Preferably, the contact of a protein, peptide, compound or composition of the invention with mucus or sputum produces at least about a 1% increase in the liquefaction of the mucus or sputum by volume as compared to prior to the treatment, more preferably, at least about a 2% increase, and so on, in increments of 1%, until the patient airways or other clogged passages are cleared.

[0079] In one aspect, the therapy is conducted in conjunction with methods to clear the thinned material from the affected tissue (respiratory tract, gastrointestinal tract, reproductive tract) of the patient. For example, in the case of the respiratory system, one can use the method of the present invention in conjunction with postural drainage, huff coughing and other respiratory exercises, or any other suitable method for expectorating the liquefied mucus or sputum.

[0080] Another embodiment of the present invention relates to a method to inhibit elastase levels and/or activity in a patient, and particularly, neutrophil elastase. It is noted that this method, while it may be used in conjunction with the above-described method of treating excessive viscosity or cohesiveness of mucus or sputum, is also separable from the above-described method and indeed, there are diseases and conditions that are associated with excess elastase activity, but wherein excessive viscosity or cohesiveness of mucus or sputum is not problematic. Moreover, by varying dose or administration protocol, one may be able to induce a mucolytic effect without a substantial anti-elastase effect, or vice versa. This method of the invention includes the step of contacting the tissues of a patient (e.g., lung tissue or other tissue where elastase is problematic) with a composition comprising a compound (e.g., a protein, peptide, or other compound) containing a dual redox thiol active-site in reduced state (e.g., a dithiol in reduced state). In this embodiment, the composition does not include the use of a protease inhibitor, such as α1-antitrypsin, and the method does not need to be conducted under elevated temperature conditions. The compound is effective to inhibit (reduce, decrease, destroy, eliminate) the levels and/or the activity of elastase in the patient as compared to prior to the step of contacting. Preferably, the dithiol is a recycling dithiol in reduced state. In one embodiment, the dithiol does not include dithiols that do not recycle, such as NAC, NAL or DTT. In one aspect, the compound is a protein or peptide containing a thioredoxin active-site in reduced state. In another embodiment of the invention, the compound is lipoic acid in reduced state (i.e., dihydrolipoic acid) or a derivative thereof. In yet another embodiment, the method includes the step of contacting the tissues of the patient with a composition comprising lipoic acid in reduced state (or a derivative thereof) and a protein or peptide containing a thioredoxin active-site in reduced state, with human thioredoxin, and particularly, recombinant human thioredoxin, being preferred. In one embodiment, the dithiol does not include a protein or peptide containing a thioredoxin active-site in reduced state. The composition can further include, if desired or necessary, a reducing system for the dithiol (i.e., a system of compound(s) that maintains, recycles or reduces the dithiol active site in the protein or compound to the reduced state, as described elsewhere herein).

[0081] This method and composition of the present invention can be used to treat any patient in which it is desirable to inhibit the levels and/or activity of elastase. In particular, patients that have certain lung, sinus, nasal, gastrointestinal, or reproductive diseases or conditions can benefit from treatment using the method of the present invention. The present invention is most useful for ameliorating or reducing at least one symptom of a condition or disease that is exacerbated by, and/or is associated at least some of the time with, the secretion or presence of elastase in the tissues. For example, elastase catalyzes the proteolysis of a variety of extracellular matrix proteins including collagen and elastin. Normally this proteolytic activity is modulated by a number of antiproteases including α-1 antiprotease and secretory leukoprotease inhibitor (SLPI). However, in certain pathologic states such as cystic fibrosis or chronic obstructive pulmonary disorder (COPD), neutrophil elastase escapes the action of these endogenous protease inhibitors due to a protease/antiprotease imbalance. Examples of such diseases include, but are not limited to: cystic fibrosis; chronic obstructive pulmonary disorder (COPD), pulmonary emphysema, pulmonary fibrosis, bronchial asthma, pulmonary infections (e.g., pneumonia and pulmonary tuberculosis), chronic or acute bronchitis; bronchiectasis (non-CF and CF bronchiectasis); acute tracheitis (bacterial, viral, mycoplasmal or caused by other organisms); acute or chronic sinusitis; atelectasis; bronchiolitis (viral or other); sclerosing cholangitis; Crohn's disease; ulcerative colitis; systemic lupus erythematosus; congenital neutropenia; cyclic neutropenia; pancreatic, intestinal and hepato-biliary diseases; renal insufficiency; cardiovascular disorders (e.g., ischemia/reperfusion injury, pulmonary hypertension, vein graft ath-

erosclerosis, transplant arteriopathy and rejection, coronary artery disease, restenosis, myocardial ischemia, myocarditis); and acute, subacute or chronic inflammatory bowel disease. In addition, the composition and method of the present invention is useful for reducing symptoms associated with elastase production in patients with a variety of respiratory infections, including both viral and bacterial infections.

[0082] Contact of the organs, cells or tissues of a patient with the dithiol compound (or composition comprising such a compound) is intended to result in a decrease (inhibition, reduction, elimination or prevention) of elastase levels and/or activity as compared to prior to contact with the composition. According to the present invention, a decrease or inhibition of elastase levels and/or activity can be any measurable or detectable decrease in the level or activity of elastase as compared to a prior level of elastase production or activity, and is preferably a statistically significant decrease (i.e., differences in measured level of elastase production or activity between the patient sample and a baseline control are statistically significant with a degree of confidence of at least $p<0.05$). Typically, the "baseline control" is a patient sample prior to the administration of the treatment, although elastase levels from a normal, healthy individual is not excluded as a baseline control. Elastase protein levels (e.g., amounts of elastase per sample) or elastase activity (e.g., detection of elastase enzyme activity) can be measured using any suitable technique known in the art, including, but not limited to, the assays as described in the Examples section. Preferably, contact of a cell, organ or tissue where elastase is present or produced with the protein, compound or composition of the invention results in a decrease in the elastase levels (e.g., amount of elastase that can be detected in the patient sample, as determined by protein levels or elastase activity) of at least about 5% as compared to prior to the treatment, and more preferably at least about 10%, and more preferably at least about 20%, and more preferably at least about 30%, and more preferably at least about 40%, and more preferably at least about 50%, and more preferably at least about 60%, and more preferably at least about 70%, and more preferably at least about 80%, and more preferably at least about 90%, and more preferably up to substantially 100% (or any percentage in between 5% and 100% in whole percentage increments), or until the patient displays a detectable improvement in at least one symptom of the disease or condition associated with elastase production.

[0083] According to both methods encompassed by the present invention, the patient to be treated is contacted (e.g., the mucus or sputum is contacted, or another appropriate tissue of the patient, such as lung tissue, is contacted) with a compound (or composition comprising the compound) that contains a dithiol active site in reduced state, and preferably a recyclable dithiol active site in reduced state. Various preferred compounds and those that may, in some aspects, be excluded, have been discussed above. In one embodiment, the compound is effective to reduce the viscosity and cohesivity of sputum or mucus and/or to increase the liquefaction of sputum or mucus as compared to prior to the step of contacting. In another embodiment, the compound is effective to reduce the level of elastase production and/or activity as compared to prior to the step of contacting.

[0084] In one embodiment, the compound is a protein, peptide or other compound comprising a thioredoxin-active site in reduced state. As described previously, thioredoxin is a protein disulfide reductase found in most organisms that participates in many thiol-dependent cellular reductive processes. In humans, thioredoxin is also referred to as adult T cell leukemia-derived factor (ADF). Intracellularly, most of this ubiquitous low molecular weight (11,700) protein remains reduced. Reduced or oxidized thioredoxin may be able to enter intact cells or absorb to the cell membrane, where a small amount is gradually internalized over time. It has two vicinal cysteine residues at the active-site which in the oxidized protein form a disulfide bridge located in a protrusion from the protein's three dimensional structure. The flavoprotein thioredoxin reductase catalyzes the NADPH-dependent reduction of this disulfide. Small increases in thioredoxin can cause profound changes in sulfhydryl-disulfide redox status in proteins.

[0085] In addition to its ability to effect the reduction of cellular proteins, it is recognized that thioredoxin can act directly as an antioxidant (e.g. by preventing oxidation of an oxidizable substrate by scavenging reactive oxygen species) although, unlike other thiols, thioredoxin does not generally contribute to the oxidative stress in a cell by autooxidizing (e.g. generating superoxide radicals through autooxidation). U.S. Pat. No. 5,985,261 to White et al., supra, showed that thioredoxin directly induces the production of MnSOD and that such induction is effected by thioredoxin in reduced state.

[0086] A "thioredoxin active-site" of the present invention comprises the amino acid sequence C-X-X-C. As used herein, amino acid residues denoted "C" are cysteine residues and amino acid residues denoted "X" can be any amino acid residue, and in particular, any of the standard 20 amino acid residues. Such a thioredoxin active-site of the present invention preferably comprises the amino acid sequence C-G-P-C (SEQ ID NO:1). A thioredoxin active-site can further comprise the amino acid sequence X-C-X-X-C-X. Preferably, a thioredoxin active-site of the present invention comprises the amino acid sequence X-C-G-P-C-X (SEQ ID NO:2), wherein such amino acid residue denoted "G" is a glycine residue, and wherein such amino acid residue denoted "P" is a proline residue. More preferably, a thioredoxin active-site of the present invention comprises the amino acid sequence W-C-G-P-C-K (SEQ ID NO:3), wherein such amino acid residue denoted "W" is a tryptophan residue, and wherein such amino acid residue denoted "K" is a lysine residue.

[0087] In one aspect of the invention, the protein containing an thioredoxin active site is a full-length thioredoxin protein or any fragment thereof containing a thioredoxin active site as described structurally and functionally above. Preferred thioredoxin proteins include prokaryotic thioredoxin, yeast thioredoxin, plant thioredoxin, and mammalian thioredoxin, with human thioredoxin being particularly preferred, and with recombinant human thioredoxin being even more preferred. The nucleic acid and amino acid sequences of thioredoxins from a variety of organisms are well known in the art and are intended to be encompassed by the present invention. For example, SEQ ID NOs:4-15 represent the amino acid sequences for thioredoxin from *Pseudomonas syringae* (SEQ ID NO:4), *Porphyromonas gingivalis* (SEQ ID NO:5), *Listeria monocytogenes* (SEQ ID NO:6), *Sac-*

charomyces cerevisiae (SEQ ID NO:7), *Gallus gallus* (SEQ ID NO:8), *Mus musculus* (SEQ ID NO:9), *Rattus norvegicus* (SEQ ID NO:10), *Bos taurus* (SEQ ID NO:11), *Homo sapiens* (SEQ ID NO:12), *Arabidopsis thaliana* (SEQ ID NO:13), *Zea mays* (SEQ ID NO:14), and *Oryza sativa* (SEQ ID NO:15). Referring to each of these sequences, the X-C-G-P-C-X (SEQ ID NO:2) motif (which includes the CGPC motif of SEQ ID NO:1) can be found as follows: SEQ ID NO:4 (positions 33-38), SEQ ID NO:5 (positions 28-33), SEQ ID NO:6 (positions 27-32), SEQ ID NO:7 (positions 29-34), SEQ ID NO:8 (positions 31-36), SEQ ID NO:9 (positions 31-36), SEQ ID NO:10 (positions 31-36), SEQ ID NO:11 (positions 31-36), SEQ ID NO:12 (positions 31-36), SEQ ID NO:13 (positions 59-64), SEQ ID NO:14 (positions 88-93) and SEQ ID NO:15 (positions 94-99). Moreover, the three-dimensional structure of several thioredoxin proteins has been resolved, including human and bacterial thioredoxins. Therefore, the structure and active site of thioredoxins from multiple organisms is well known in the art and one of skill in the art would be able to readily identify and produce fragments or homologues of full-length thioredoxins that can be used in the present invention. In one aspect of the invention recombinant human thioredoxin is used, which can be produced in a substantially reduced, monomeric state. Recombinant human thioredoxin-1 is described in the Examples, as is pre-reduced, recombinant human thioredoxin (i.e., recombinant human thioredoxin produced in a substantially reduced, monomeric state).

[0088] The present invention also encompasses dithiols other than compounds having a thioredoxin active-site, including non-protein compounds, such as dihydrolipoic acid (or its oxidized precursor, α-lipoic acid). Dithiols have been discussed in detail above. It is to be understood that the invention also encompasses any derivatives (e.g., analogs, agonists) of such compounds, including compounds with improved properties as compared to the native compound or protein.

[0089] Dithiol compounds and derivatives thereof to be used in the methods of the invention include known organic compounds such as antibodies, products of peptide libraries, and products of chemical combinatorial libraries. Compounds may also be identified using rational drug design. Such methods are known to those of skill in the art. For example, various methods of drug design, useful to design or select mimetics or other therapeutic compounds useful in the present invention are disclosed in Maulik et al., 1997, *Molecular Biotechnology: Therapeutic Applications and Strategies*, Wiley-Liss, Inc., which is incorporated herein by reference in its entirety.

[0090] In one embodiment, homologues of a protein or peptide comprising a dithiol active site, including peptide and non-peptide homologues of thioredoxin or analogs of other compounds such as DHLA, can be products of drug design or selection and can be produced using various methods known in the art. Such homologues can be referred to as mimetics. A mimetic refers to any peptide or non-peptide compound (analog, agonist, antagonist, derivative) that is able to mimic the biological action of a naturally occurring peptide or compound, often because the mimetic has a basic structure that mimics the basic structure of the naturally occurring peptide or native compound and/or has the salient biological properties of the naturally occurring peptide or native compound. Mimetics can include, but are not limited to: peptides that have substantial modifications from the prototype such as no side chain similarity with the naturally occurring peptide (such modifications, for example, may decrease its susceptibility to degradation); anti-idiotypic and/or catalytic antibodies, or fragments thereof; non-proteinaceous portions of an isolated protein (e.g., carbohydrate structures); or synthetic or natural organic molecules, including nucleic acids and drugs identified through combinatorial chemistry, for example. Such mimetics can be designed, selected and/or otherwise identified using a variety of methods known in the art. Various methods of drug design, useful to design or select mimetics or other therapeutic compounds useful in the present invention are disclosed in Maulik et al., 1997, *Molecular Biotechnology: Therapeutic Applications and Strategies*, Wiley-Liss, Inc., which is incorporated herein by reference in its entirety.

[0091] A mimetic can be obtained, for example, from molecular diversity strategies (a combination of related strategies allowing the rapid construction of large, chemically diverse molecule libraries), libraries of natural or synthetic compounds, in particular from chemical or combinatorial libraries (i.e., libraries of compounds that differ in sequence or size but that have the similar building blocks) or by rational, directed or random drug design. See for example, Maulik et al., supra.

[0092] In a molecular diversity strategy, large compound libraries are synthesized, for example, from peptides, oligonucleotides, carbohydrates and/or synthetic organic molecules, using biological, enzymatic and/or chemical approaches. The critical parameters in developing a molecular diversity strategy include subunit diversity, molecular size, and library diversity. The general goal of screening such libraries is to utilize sequential application of combinatorial selection to obtain high-affinity ligands for a desired target, and then to optimize the lead molecules by either random or directed design strategies. Methods of molecular diversity are described in detail in Maulik, et al., ibid.

[0093] Maulik et al. also disclose, for example, methods of directed design, in which the user directs the process of creating novel molecules from a fragment library of appropriately selected fragments; random design, in which the user uses a genetic or other algorithm to randomly mutate fragments and their combinations while simultaneously applying a selection criterion to evaluate the fitness of candidate ligands; and a grid-based approach in which the user calculates the interaction energy between three dimensional receptor structures and small fragment probes, followed by linking together of favorable probe sites.

[0094] Compounds identified or designed by the art known methods can be synthesized using techniques known in the art, and depending on the type of compound. Synthesis techniques for the production of non-protein compounds, including organic and inorganic compounds are well known in the art. For smaller peptides, chemical synthesis methods are preferred. For example, such methods include well known chemical procedures, such as solution or solid-phase peptide synthesis, or semi-synthesis in solution beginning with protein fragments coupled through conventional solution methods. Such methods are well known in the art and may be found in general texts and articles in the area such as: Merrifield, 1997, *Methods Enzymol.* 289:3-13; Wade et

al., 1993, *Australas Biotechnol.* 3 (6):332-336; Wong et al., 1991, *Experientia* 47 (11-12):1123-1129; Carey et al., 1991, *Ciba Found Symp.* 158:187-203; Plaue et al., 1990, *Biologicals* 18 (3):147-157; Bodanszky, 1985, *Int. J. Pept. Protein Res.* 25 (5):449-474; or H. Dugas and C. Penney, BIOORGANIC CHEMISTRY, (1981) at pages 54-92, all of which are incorporated herein by reference in their entirety. For example, peptides may be synthesized by solid-phase methodology utilizing a commercially available peptide synthesizer and synthesis cycles supplied by the manufacturer. One skilled in the art recognizes that the solid phase synthesis could also be accomplished using the FMOC strategy and a TFA/scavenger cleavage mixture. A compound that is a protein or peptide can also be produced using recombinant DNA technology and methods standard in the art, particularly if larger quantities of a protein are desired. A particularly preferred recombinant protein for use in the present invention is recombinant human thioredoxin (rhTrx) (e.g., see Examples).

[0095] The phrase "in reduced state" specifically describes the state of the cysteine residues in the active-site of a protein or peptide of the present invention, or in the case of a non-protein dithiol compound, the state of the thiol groups on the compound. In reduced state, such cysteine residues or thiol groups form a dithiol (i.e. two free sulfhydryl groups, —SH). In contrast, in oxidized form, such cysteine residues or thiol groups form an intramolecular disulfide bridge; such a molecule can be referred to as cystine in the case of cysteine residues. Such compounds also could be rendered ineffective through formation of intermolecular disulfide(s). In reduced state, a thioredoxin active-site or other dithiol site comprising cysteines, for example, is capable of participating in redox reactions through the reversible oxidation of its active-site dithiol to a disulfide, and catalyzes dithiol-disulfide exchange reactions. Similarly, in non-protein dithiols, the free sulfhydryl groups on a compound participate in redox reactions through the reversible oxidation of the active-site dithiol to a disulfide, that catalyzes dithiol-disulfide exchange reactions.

[0096] The reference to a compound being in a reduced state as a preferred embodiment is primarily intended to encompass the state of the dithiol at the site where it is to be active (e.g., at the target site, such as a cell or tissue). Therefore, a dithiol compound useful in the present invention can be initially administered or provided to a subject in a non-reduced, or oxidized, state, particularly if the dithiol will, in the environment of the target site, become reduced or be converted to the more preferable, reduced active form (e.g., by the natural local environmental conditions or by some other step of reduction, such as inclusion of or administration of a reducing system). For example, the present invention encompasses the use of orally administered α-lipoic acid, which is the oxidized form of dihydrolipoic acid (DHLA). This is due to the fact that the α-lipoic acid will be converted to DHLA intracellularly. Specifically, as previously herein, oral α-lipoic acid is rapidly absorbed into the bloodstream, is taken up by cells, and then converted within the cell to DHLA, where it can then enter the extracellular space and tissues in the target environment. Therefore, administration of oral α-lipoic acid is one means of contacting a patient tissue or fluid with a dithiol in reduced state. Other similar examples will be apparent to those of skill in the art.

[0097] As used herein, a protein of the present invention containing a thioredoxin active site can be a thioredoxin active site per se or a thioredoxin active site joined to other amino acids by glycosidic linkages. The following discussion regarding proteins and peptides also generally applies to any other proteins or peptides having dithiol active sites that are distinguished from a thioredoxin active site, although the thioredoxin active site is used to exemplify the embodiments of the invention. Thus, the minimal size of a protein or peptide of the present invention is from about 4 to about 6 amino acids in length, with preferred sizes depending on whether a full-length, fusion, multivalent, or merely functional portions of such a protein is desired. Preferably, the length of a protein or peptide of the present invention extends from about 4 to about 100 amino acid residues or more, with peptides of any interim length, in whole integers (i.e., 4, 5, 6, 7 . . . 99, 100, 101 . . .), being specifically envisioned. In a further preferred embodiment, a protein of the present invention can be a full-length protein or any homologue of such a protein. As used herein, the term "homologue" is used to refer to a protein or peptide which differs from a naturally occurring protein or peptide (i.e., the "prototype" or "wild-type" protein) by modifications to the naturally occurring protein or peptide, but which maintains the basic protein and side chain structure of the naturally occurring form, and/or which maintains a basic three-dimensional structure of at least a biologically active portion (e.g., the thioredoxin active site) of the native protein. Such changes include, but are not limited to: changes in one or a few amino acid side chains; changes one or a few amino acids, including deletions (e.g., a truncated version of the protein or peptide (fragment)), insertions and/or substitutions; changes in stereochemistry of one or a few atoms; and/or minor derivatizations, including but not limited to: methylation, glycosylation, phosphorylation, acetylation, myristoylation, prenylation, palmitation, amidation and/or addition of glycosylphosphatidyl inositol. According to the present invention, any protein or peptide useful in the present invention, including homologues of natural thioredoxin proteins, have a thioredoxin active-site such that, in reduced state, the protein or peptide is capable of participating in redox reactions through the reversible oxidation of its active-site dithiol to a disulfide, of catalyzing dithiol-disulfide exchange reactions, and/or of decreasing the viscosity or cohesivity of mucus or sputum or increasing the liquefaction of mucus or sputum. As used herein, a protein or peptide containing a thioredoxin active-site can have characteristics similar to thioredoxin, and preferably, is thioredoxin selected from the group of prokaryotic thioredoxin, yeast thioredoxin, plant thioredoxin, or mammalian thioredoxin. In a particularly preferred embodiment, the protein is human thioredoxin.

[0098] Homologues can be the result of natural allelic variation or natural mutation. A naturally occurring allelic variant of a nucleic acid encoding a protein is a gene that occurs at essentially the same locus (or loci) in the genome as the gene which encodes such protein, but which, due to natural variations caused by, for example, mutation or recombination, has a similar but not identical sequence. Allelic variants typically encode proteins having similar activity to that of the protein encoded by the gene to which they are being compared. One class of allelic variants can encode the same protein but have different nucleic acid sequences due to the degeneracy of the genetic code. Allelic

variants can also comprise alterations in the 5' or 3' untranslated regions of the gene (e.g., in regulatory control regions). Allelic variants are well known to those skilled in the art.

[0099] Homologues can be produced using techniques known in the art for the production of proteins including, but not limited to, direct modifications to the isolated, naturally occurring protein, direct protein synthesis, or modifications to the nucleic acid sequence encoding the protein using, for example, classic or recombinant DNA techniques to effect random or targeted mutagenesis.

[0100] Modifications in homologues, as compared to the wild-type protein, or modifications in derivatives of a compound, either agonize, antagonize, or do not substantially change, the basic biological activity of the homologue or derivative as compared to the naturally occurring protein or native or lead compound. In general, the biological activity or biological action of a protein or any non-protein compound refers to any function(s) exhibited or performed by the protein or compound that is ascribed to the naturally occurring or native (lead) form of the protein or compound as measured or observed in vivo (i.e., in the natural physiological environment of the protein or the natural physiological environment for activity of the native compound) or in vitro (i.e., under laboratory conditions).

[0101] Modifications of a protein or compound, such as in a homologue, derivative or mimetic (discussed below), may result in proteins or compounds having the same biological activity as the naturally occurring protein or native compound, or in proteins or compounds having decreased or increased biological activity as compared to the naturally occurring protein or native compound. Modifications which result in a decrease in protein expression or a decrease in the activity of the protein or compound, can be referred to as inactivation (complete or partial), down-regulation, or decreased action of a protein or compound. Similarly, modifications which result in an increase in protein expression or an increase in the activity of the protein or compound, can be referred to as amplification, overproduction, activation, enhancement, up-regulation or increased action of a protein or compound.

[0102] In one embodiment of the present invention, a protein suitable for use in the present invention has an amino acid sequence that comprises, consists essentially of, or consists of a full length sequence of a thioredoxin protein or any fragment thereof that has a thioredoxin active site as described herein, or of another protein or fragment thereof that has a dithiol active site as described herein. For example, any one of SEQ ID NOs:4-12 or a fragment or other homologue thereof that contains a thioredoxin active site as described herein is encompassed by the invention. Such homologues can include proteins having an amino acid sequence that is at least about 10% identical to the amino acid sequence of a full-length thioredoxin protein, or at least 20% identical, or at least 30% identical, or at least 40% identical, or at least 50% identical, or at least 60% identical, or at least 70% identical, or at least 80% identical, or at least 90% identical, or greater than 95% identical to the amino acid sequence of a full-length thioredoxin protein, including any percentage between 10% and 100%, in whole integers (10%, 11%, 12%, . . . 98%, 99%, 100%).

[0103] As used herein, unless otherwise specified, reference to a percent (%) identity refers to an evaluation of homology which is performed using: (1) a BLAST 2.0 Basic BLAST homology search using blastp for amino acid searches and blastn for nucleic acid searches with standard default parameters, wherein the query sequence is filtered for low complexity regions by default (described in Altschul, S. F., Madden, T. L., Schaaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997) "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." Nucleic Acids Res. 25:3389-3402, incorporated herein by reference in its entirety); (2) a BLAST 2 alignment (using the parameters described below); (3) and/or PSI-BLAST with the standard default parameters (Position-Specific Iterated BLAST. It is noted that due to some differences in the standard parameters between BLAST 2.0 Basic BLAST and BLAST 2, two specific sequences might be recognized as having significant homology using the BLAST 2 program, whereas a search performed in BLAST 2.0 Basic BLAST using one of the sequences as the query sequence may not identify the second sequence in the top matches. In addition, PSI-BLAST provides an automated, easy-to-use version of a "profile" search, which is a sensitive way to look for sequence homologues. The program first performs a gapped BLAST database search. The PSI-BLAST program uses the information from any significant alignments returned to construct a position-specific score matrix, which replaces the query sequence for the next round of database searching. Therefore, it is to be understood that percent identity can be determined by using any one of these programs.

[0104] Two specific sequences can be aligned to one another using BLAST 2 sequence as described in Tatusova and Madden, (1999), "Blast 2 sequences—a new tool for comparing protein and nucleotide sequences", *FEMS Microbiol Lett.* 174:247-250, incorporated herein by reference in its entirety. BLAST 2 sequence alignment is performed in blastp or blastn using the BLAST 2.0 algorithm to perform a Gapped BLAST search (BLAST 2.0) between the two sequences allowing for the introduction of gaps (deletions and insertions) in the resulting alignment. For purposes of clarity herein, a BLAST 2 sequence alignment is performed using the standard default parameters as follows.

[0105] For blastn, using 0 BLOSUM62 matrix:

- [0106] Reward for match=1
- [0107] Penalty for mismatch=−2
- [0108] Open gap (5) and extension gap (2) penalties
- [0109] gap x_dropoff (50) expect (10) word size (11) filter (on)

[0110] For blastp, using 0 BLOSUM62 matrix:

- [0111] Open gap (11) and extension gap (1) penalties
- [0112] gap x_dropoff (50) expect (10) word size (3) filter (on).

[0113] A protein useful in the present invention can also include proteins having an amino acid sequence comprising at least 10 contiguous amino acid residues of any full-length thioredoxin protein (e.g., SEQ ID NOs:4-12) or other exemplary protein having a dithiol active site (i.e., 10 contiguous amino acid residues having 100% identity with 10 contiguous amino acids of a reference sequence). In other embodiments, a homologue of a thioredoxin protein includes amino

acid sequences comprising at least 15, or at least 20, or at least 25, or at least 30, or at least 35, or at least 40, or at least 45, or at least 50, or at least 55, or at least 60, or at least 65, or at least 70, or at least 75, or at least 80 contiguous amino acid residues of the amino acid sequence of a naturally occurring thioredoxin protein, and so on, up to the full-length of the protein, including any intervening length in whole integers (10, 11, 12, . . .).

[0114] According to the present invention, the term "contiguous" or "consecutive", with regard to sequences described herein, means to be connected in an unbroken sequence. For example, for a first sequence to comprise 30 contiguous (or consecutive) amino acids of a second sequence, means that the first sequence includes an unbroken sequence of 30 amino acid residues that is 100% identical to an unbroken sequence of 30 amino acid residues in the second sequence. Similarly, for a first sequence to have "100% identity" with a second sequence means that the first sequence exactly matches the second sequence with no gaps between nucleotides or amino acids.

[0115] In another embodiment, a protein useful in the present invention includes a protein having an amino acid sequence that is sufficiently similar to a natural thioredoxin amino acid sequence or other protein having a dithiol active site that a nucleic acid sequence encoding the homologue is capable of hybridizing under moderate, high or very high stringency conditions (described below) to (i.e., with) a nucleic acid molecule encoding the natural protein (i.e., to the complement of the nucleic acid strand encoding the natural protein amino acid sequence). Such hybridization conditions are described in detail below.

[0116] A nucleic acid sequence complement of nucleic acid sequence encoding a thioredoxin protein or other dithiol protein of the present invention refers to the nucleic acid sequence of the nucleic acid strand that is complementary to the strand which encodes thioredoxin or other dithiol protein. It will be appreciated that a double stranded DNA which encodes a given amino acid sequence comprises a single strand DNA and its complementary strand having a sequence that is a complement to the single strand DNA. As such, nucleic acid molecules of the present invention can be either double-stranded or single-stranded, and include those nucleic acid molecules that form stable hybrids under stringent hybridization conditions with a nucleic acid sequence that encodes an amino acid sequence of a thioredoxin protein, and/or with the complement of the nucleic acid sequence that encodes such amino acid sequence. Methods to deduce a complementary sequence are known to those skilled in the art.

[0117] As used herein, reference to hybridization conditions refers to standard hybridization conditions under which nucleic acid molecules are used to identify similar nucleic acid molecules. Such standard conditions are disclosed, for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press, 1989. Sambrook et al., ibid., is incorporated by reference herein in its entirety (see specifically, pages 9.31-9.62). In addition, formulae to calculate the appropriate hybridization and wash conditions to achieve hybridization permitting varying degrees of mismatch of nucleotides are disclosed, for example, in Meinkoth et al., 1984, *Anal. Biochem.* 138, 267-284; Meinkoth et al., ibid., is incorporated by reference herein in its entirety.

[0118] More particularly, moderate stringency hybridization and washing conditions, as referred to herein, refer to conditions which permit isolation of nucleic acid molecules having at least about 70% nucleic acid sequence identity with the nucleic acid molecule being used to probe in the hybridization reaction (i.e., conditions permitting about 30% or less mismatch of nucleotides). High stringency hybridization and washing conditions, as referred to herein, refer to conditions which permit isolation of nucleic acid molecules having at least about 80% nucleic acid sequence identity with the nucleic acid molecule being used to probe in the hybridization reaction (i.e., conditions permitting about 20% or less mismatch of nucleotides). Very high stringency hybridization and washing conditions, as referred to herein, refer to conditions which permit isolation of nucleic acid molecules having at least about 90% nucleic acid sequence identity with the nucleic acid molecule being used to probe in the hybridization reaction (i.e., conditions permitting about 10% or less mismatch of nucleotides). As discussed above, one of skill in the art can use the formulae in Meinkoth et al., ibid. to calculate the appropriate hybridization and wash conditions to achieve these particular levels of nucleotide mismatch. Such conditions will vary, depending on whether DNA:RNA or DNA:DNA hybrids are being formed. Calculated melting temperatures for DNA:DNA hybrids are 10° C. less than for DNA:RNA hybrids. In particular embodiments, stringent hybridization conditions for DNA:DNA hybrids include hybridization at an ionic strength of 6×SSC (0.9 M Na^+) at a temperature of between about 20° C. and about 35° C. (lower stringency), more preferably, between about 28° C. and about 40° C. (more stringent), and even more preferably, between about 35° C. and about 45° C. (even more stringent), with appropriate wash conditions. In particular embodiments, stringent hybridization conditions for DNA:RNA hybrids include hybridization at an ionic strength of 6×SSC (0.9 M Na^+) at a temperature of between about 30° C. and about 45° C., more preferably, between about 38° C. and about 50° C., and even more preferably, between about 45° C. and about 55° C., with similarly stringent wash conditions. These values are based on calculations of a melting temperature for molecules larger than about 100 nucleotides, 0% formamide and a G+C content of about 40%. Alternatively, T_m can be calculated empirically as set forth in Sambrook et al., supra, pages 9.31 to 9.62. In general, the wash conditions should be as stringent as possible, and should be appropriate for the chosen hybridization conditions. For example, hybridization conditions can include a combination of salt and temperature conditions that are approximately 20-25° C. below the calculated T_m of a particular hybrid, and wash conditions typically include a combination of salt and temperature conditions that are approximately 12-20° C. below the calculated T_m of the particular hybrid. One example of hybridization conditions suitable for use with DNA:DNA hybrids includes a 2-24 hour hybridization in 6×SSC (50% formamide) at about 42° C., followed by washing steps that include one or more washes at room temperature in about 2×SSC, followed by additional washes at higher temperatures and lower ionic strength (e.g., at least one wash as about 37° C. in about 0.1×-0.5×SSC, followed by at least one wash at about 68° C. in about 0.1×-0.5×SSC).

[0119] A protein of the present invention can also be a fusion protein that includes a segment containing a thioredoxin or other dithiol active-site and a fusion segment that

can have a variety of functions. For example, such a fusion segment can function as a tool to simplify purification of a protein of the present invention, such as to enable purification of the resultant fusion protein using affinity chromatography. A suitable fusion segment can be a domain of any size that has the desired function (e.g., imparts increased stability to a protein, imparts increased immunogenicity to a protein, and/or simplifies purification of a protein). It is within the scope of the present invention to use one or more fusion segments. Fusion segments can be joined to amino and/or carboxyl termini of the segment containing a thioredoxin or other dithiol active-site. Linkages between fusion segments and thioredoxin or other dithiol active-site-containing domains of fusion proteins can be susceptible to cleavage in order to enable straight-forward recovery of the thioredoxin or other dithiol active-site-containing domains of such proteins. Fusion proteins are preferably produced by culturing a recombinant cell transformed with a fusion nucleic acid molecule that encodes a protein including the fusion segment attached to either the carboxyl and/or amino terminal end of a thioredoxin or other dithiol active-site-containing domain.

[0120] In addition, the present invention includes non-protein compounds (e.g., non-protein dithiols) that are complexed (linked, conjugated) with another compound or with a protein to form a chimeric or hybrid compound.

[0121] In one embodiment, a protein or peptide containing a thioredoxin active-site or a protein or peptide containing another dithiol active site suitable for use with the method of the present invention comprises a protein or peptide containing a thioredoxin or other dithiol active-site derived from a substantially similar species of animal as that to which the protein is to be administered. In another embodiment, any protein or peptide containing a thioredoxin or other dithiol active-site, including from diverse sources such as other animal species, microbial and plant species, can be used in a given patient.

[0122] In one embodiment of the present invention, any of the amino acid sequences described herein, such as the amino acid sequence of a naturally occurring thioredoxin protein, can be produced with from at least one, and up to about 20, additional heterologous amino acids flanking each of the C- and/or N-terminal ends of the specified amino acid sequence. The resulting protein or polypeptide can be referred to as "consisting essentially of" the specified amino acid sequence. According to the present invention, the heterologous amino acids are a sequence of amino acids that are not naturally found (i.e., not found in nature, in vivo) flanking the specified amino acid sequence, or that are not related to the function of the specified amino acid sequence, or that would not be encoded by the nucleotides that flank the naturally occurring nucleic acid sequence encoding the specified amino acid sequence as it occurs in the gene, if such nucleotides in the naturally occurring sequence were translated using standard codon usage for the organism from which the given amino acid sequence is derived. Similarly, the phrase "consisting essentially of", when used with reference to a nucleic acid sequence herein, refers to a nucleic acid sequence encoding a specified amino acid sequence that can be flanked by from at least one, and up to as many as about 60, additional heterologous nucleotides at each of the 5' and/or the 3' end of the nucleic acid sequence encoding the specified amino acid sequence. The heterologous nucleotides are not naturally found (i.e., not found in nature, in vivo) flanking the nucleic acid sequence encoding the specified amino acid sequence as it occurs in the natural gene or do not encode a protein that imparts any additional function to the protein or changes the function of the protein having the specified amino acid sequence.

[0123] In another embodiment, a protein or peptide containing a thioredoxin or other dithiol active-site or any other dithiol compound suitable for use with the method of the present invention comprises an isolated, or biologically pure, protein or compound which has been removed from its natural milieu. As such, "isolated" and "biologically pure" do not necessarily reflect the extent to which the protein or compound has been purified. An isolated protein of the present invention can, for example, be obtained from its natural source, be produced using recombinant DNA technology (e.g., polymerase chain reaction (PCR) amplification, cloning), or be synthesized chemically. A non-protein compound is typically synthesized chemically or can be purified from a suitable source material.

[0124] Preferably, the protein or compound containing a thioredoxin or other dithiol active site (e.g., DHLA) to be used in methods of the invention has a half-life in vivo that is sufficient to cause a measurable or detectable increase in liquefaction, or importantly, any decrease in the viscosity or cohesivity, of mucus or sputum in a patient, and/or to cause a measurable, detectable or perceived therapeutic benefit to the patient that is associated with the mucus and sputum in the patient. Such half-life can be affected by the method of delivery of such a protein or other dithiol compound, for example, or by the method of synthesis of the protein or compound (e.g., by linking the protein or compound to a carrier that extends the half-life without compromising the activity of the dithiol compound). A protein or other dithiol compound of the present invention preferably has a half-life of greater than about 5 minutes in an animal, and more preferably greater than about 4 hours in an animal, and even more preferably greater than about 16 hours in an animal. In a preferred embodiment, a protein of the present invention has a half-life of between about 5 minutes and about 24 hours in an animal, and preferably between about 2 hours and about 16 hours in an animal, and more preferably between about 4 hours and about 12 hours in an animal.

[0125] Further embodiments of the present invention include nucleic acid molecules that encode a protein or peptide containing a thioredoxin active site or other protein or peptide comprising a suitable dithiol active site according to the present invention. Such nucleic acid molecules can be used to produce a protein that is useful in the method of the present invention in vitro or in vivo. A nucleic acid molecule of the present invention includes a nucleic acid molecule comprising, consisting essentially of, or consisting of, a nucleic acid sequence encoding any of the proteins described previously herein. In accordance with the present invention, an isolated nucleic acid molecule is a nucleic acid molecule (polynucleotide) that has been removed from its natural milieu (i.e., that has been subject to human manipulation) and can include DNA, RNA, or derivatives of either DNA or RNA, including cDNA. As such, "isolated" does not reflect the extent to which the nucleic acid molecule has been purified. Although the phrase "nucleic acid molecule" primarily refers to the physical nucleic acid molecule and the phrase "nucleic acid sequence" primarily refers to the

sequence of nucleotides on the nucleic acid molecule, the two phrases can be used interchangeably, especially with respect to a nucleic acid molecule, or a nucleic acid sequence, being capable of encoding a protein. An isolated nucleic acid molecule of the present invention can be isolated from its natural source or produced using recombinant DNA technology (e.g., polymerase chain reaction (PCR) amplification, cloning) or chemical synthesis. Isolated nucleic acid molecules can include, for example, genes, natural allelic variants of genes, coding regions or portions thereof, and coding and/or regulatory regions modified by nucleotide insertions, deletions, substitutions, and/or inversions in a manner such that the modifications do not substantially interfere with the nucleic acid molecule's ability to encode the desired protein of the present invention or to form stable hybrids under stringent conditions with natural gene isolates. An isolated nucleic acid molecule can include degeneracies. As used herein, nucleotide degeneracies refers to the phenomenon that one amino acid can be encoded by different nucleotide codons. Thus, the nucleic acid sequence of a nucleic acid molecule that encodes a given protein useful in the present invention can vary due to degeneracies.

[0126] According to the present invention, reference to a gene includes all nucleic acid sequences related to a natural (i.e. wild-type) gene, such as regulatory regions that control production of the protein encoded by that gene (such as, but not limited to, transcription, translation or post-translation control regions) as well as the coding region itself. In another embodiment, a gene can be a naturally occurring allelic variant that includes a similar but not identical sequence to the nucleic acid sequence encoding a given protein. Allelic variants have been previously described above. The phrases "nucleic acid molecule" and "gene" can be used interchangeably when the nucleic acid molecule comprises a gene as described above.

[0127] Preferably, an isolated nucleic acid molecule of the present invention is produced using recombinant DNA technology (e.g., polymerase chain reaction (PCR) amplification, cloning) or chemical synthesis. Isolated nucleic acid molecules include natural nucleic acid molecules and homologues thereof, including, but not limited to, natural allelic variants and modified nucleic acid molecules in which nucleotides have been inserted, deleted, substituted, and/or inverted in such a manner that such modifications provide the desired effect on protein biological activity. Allelic variants and protein homologues (e.g., proteins encoded by nucleic acid homologues) have been discussed in detail above.

[0128] A nucleic acid molecule homologue can be produced using a number of methods known to those skilled in the art (see, for example, Sambrook et al.). For example, nucleic acid molecules can be modified using a variety of techniques including, but not limited to, by classic mutagenesis and recombinant DNA techniques (e.g., site-directed mutagenesis, chemical treatment, restriction enzyme cleavage, ligation of nucleic acid fragments and/or PCR amplification), or synthesis of oligonucleotide mixtures and ligation of mixture groups to "build" a mixture of nucleic acid molecules and combinations thereof. Another method for modifying a recombinant nucleic acid molecule encoding a given protein is gene shuffling (i.e., molecular breeding) (See, for example, U.S. Pat. No. 5,605,793 to Stemmer; Minshull and Stemmer; 1999, *Curr. Opin. Chem. Biol.* 3:284-290; Stemmer, 1994, *P.N.A.S. USA* 91:10747-10751, all of which are incorporated herein by reference in their entirety). This technique can be used to efficiently introduce multiple simultaneous changes in the protein. Nucleic acid molecule homologues can be selected by hybridization with an given gene or by screening the function of a protein encoded by a nucleic acid molecule (i.e., biological activity).

[0129] One embodiment of the present invention relates to a recombinant nucleic acid molecule which comprises the isolated nucleic acid molecule described above which is operatively linked to at least one transcription control sequence. More particularly, according to the present invention, a recombinant nucleic acid molecule typically comprises a recombinant vector and the isolated nucleic acid molecule as described herein. According to the present invention, a recombinant vector is an engineered (i.e., artificially produced) nucleic acid molecule that is used as a tool for manipulating a nucleic acid sequence of choice and/or for introducing such a nucleic acid sequence into a host cell. The recombinant vector is therefore suitable for use in cloning, sequencing, and/or otherwise manipulating the nucleic acid sequence of choice, such as by expressing and/or delivering the nucleic acid sequence of choice into a host cell to form a recombinant cell. Such a vector typically contains heterologous nucleic acid sequences, that is, nucleic acid sequences that are not naturally found adjacent to nucleic acid sequence to be cloned or delivered, although the vector can also contain regulatory nucleic acid sequences (e.g., promoters, untranslated regions) which are naturally found adjacent to nucleic acid sequences of the present invention or which are useful for expression of the nucleic acid molecules of the present invention (discussed in detail below). The vector can be either RNA or DNA, either prokaryotic or eukaryotic, and typically is a plasmid. The vector can be maintained as an extrachromosomal element (e.g., a plasmid) or it can be integrated into the chromosome of a recombinant host cell, although it is preferred if the vector remain separate from the genome for most applications of the invention. The entire vector can remain in place within a host cell, or under certain conditions, the plasmid DNA can be deleted, leaving behind the nucleic acid molecule of the present invention. An integrated nucleic acid molecule can be under chromosomal promoter control, under native or plasmid promoter control, or under a combination of several promoter controls. Single or multiple copies of the nucleic acid molecule can be integrated into the chromosome. A recombinant vector of the present invention can contain at least one selectable marker.

[0130] In one embodiment, a recombinant vector used in a recombinant nucleic acid molecule of the present invention is an expression vector. As used herein, the phrase "expression vector" is used to refer to a vector that is suitable for production of an encoded product (e.g., a protein of interest). In this embodiment, a nucleic acid sequence encoding the product to be produced (e.g., the protein containing a thioredoxin active site) is inserted into the recombinant vector to produce a recombinant nucleic acid molecule. The nucleic acid sequence encoding the protein to be produced is inserted into the vector in a manner that operatively links the nucleic acid sequence to regulatory sequences in the vector which enable the transcription and translation of the nucleic acid sequence within the recombinant host cell.

[0131] In another embodiment of the invention, the recombinant nucleic acid molecule comprises a viral vector. A viral vector includes an isolated nucleic acid molecule of the present invention integrated into a viral genome or portion thereof, in which the nucleic acid molecule is packaged in a viral coat that allows entrance of DNA into a cell. A number of viral vectors can be used, including, but not limited to, those based on alphaviruses, poxviruses, adenoviruses, herpesviruses, lentiviruses, adeno-associated viruses and retroviruses.

[0132] Typically, a recombinant nucleic acid molecule includes at least one nucleic acid molecule of the present invention operatively linked to one or more expression control sequences. As used herein, the phrase "recombinant molecule" or "recombinant nucleic acid molecule" primarily refers to a nucleic acid molecule or nucleic acid sequence operatively linked to an expression control sequence, but can be used interchangeably with the phrase "nucleic acid molecule", when such nucleic acid molecule is a recombinant molecule as discussed herein. According to the present invention, the phrase "operatively linked" refers to linking a nucleic acid molecule to an expression control sequence in a manner such that the molecule is able to be expressed when transfected (i.e., transformed, transduced, transfected, conjugated or conduced) into a host cell. Transcription control sequences are expression control sequences that control the initiation, elongation, or termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include any transcription control sequence that can function in a host cell or organism into which the recombinant nucleic acid molecule is to be introduced. Recombinant nucleic acid molecules of the present invention can also contain additional regulatory sequences, such as translation regulatory sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell. In one embodiment, a recombinant molecule of the present invention, including those which are integrated into the host cell chromosome, also contains secretory signals (i.e., signal segment nucleic acid sequences) to enable an expressed protein to be secreted from the cell that produces the protein. Suitable signal segments include a signal segment that is naturally associated with the protein to be expressed or any heterologous signal segment capable of directing the secretion of the protein according to the present invention. In another embodiment, a recombinant molecule of the present invention comprises a leader sequence to enable an expressed protein to be delivered to and inserted into the membrane of a host cell. Suitable leader sequences include a leader sequence that is naturally associated with the protein, or any heterologous leader sequence capable of directing the delivery and insertion of the protein to the membrane of a cell.

[0133] According to the present invention, the term "transfection" is used to refer to any method by which an exogenous nucleic acid molecule (i.e., a recombinant nucleic acid molecule) can be inserted into a cell. The term "transformation" can be used interchangeably with the term "transfection" when such term is used to refer to the introduction of nucleic acid molecules into microbial cells or plants. In microbial systems, the term "transformation" is used to describe an inherited change due to the acquisition of exogenous nucleic acids by the microorganism and is essentially synonymous with the term "transfection." However, in animal cells, transformation has acquired a second meaning which can refer to changes in the growth properties of cells in culture (described above) after they become cancerous, for example. Therefore, to avoid confusion, the term "transfection" is preferably used with regard to the introduction of exogenous nucleic acids into animal cells, and is used herein to generally encompass transfection of animal cells and transformation of plant cells and microbial cells, to the extent that the terms pertain to the introduction of exogenous nucleic acids into a cell. Therefore, transfection techniques include, but are not limited to, transformation, particle bombardment, electroporation, microinjection, lipofection, adsorption, infection and protoplast fusion.

[0134] One or more recombinant molecules of the present invention can be used to produce an encoded product (e.g., a protein containing a thioredoxin active site, such as recombinant human thioredoxin) of the present invention. In one embodiment, an encoded product is produced by expressing a nucleic acid molecule as described herein under conditions effective to produce the protein. A preferred method to produce an encoded protein is by transfecting a host cell with one or more recombinant molecules to form a recombinant cell.

[0135] In a preferred embodiment, a dithiol compound useful in the present invention is contacted with the mucus or sputum, or the cell, tissue, organ or fluid of a patient to be treated in a composition. The composition comprises the dithiol compound, and may include one or more additional compounds, such as other compounds that can be used to reduce excessively viscous or cohesive mucus or sputum or increase the liquefaction of such mucus or sputum, or to inhibit elastase production and/or activity. In one embodiment, a composition can be used to deliver a nucleic acid molecule encoding a protein or peptide containing a dithiol active site to a cell in the patient to be treated (e.g., an epithelial cell in the lung or airways), such that the cell can become transfected with and express the protein, and so that the protein can contact mucus or sputum in the microenvironment of the cell or so that the protein can contact the elastase in the microenvironment of the cell.

[0136] A composition can also include, for example, a pharmaceutically acceptable carrier, which includes pharmaceutically acceptable excipients and/or delivery vehicles, for delivering a protein, nucleic acid molecule or other regulatory compound to a patient. As used herein, a pharmaceutically acceptable carrier refers to any substance suitable for delivering a therapeutic protein, nucleic acid or other compound useful in the method of the present invention to a suitable in vivo or ex vivo site. Preferred pharmaceutically acceptable carriers are capable of maintaining a protein, nucleic acid molecule or compound in a form that, upon arrival of the protein, nucleic acid molecule or compound at the desired site (e.g., the site where the mucus or sputum to be treated is secreted or drains, or the site where elastase is secreted or is problematic), is capable of contacting the mucus or sputum or tissues of the patient (in the case of a protein or compound), or of entering the cell and being expressed by the cell (in the case of a nucleic acid molecule) so that the expressed protein can contact the mucus or sputum or the tissues of a patient.

[0137] Suitable excipients of the present invention include excipients or formularies that transport or help transport, but

do not specifically target a therapeutic agent (protein, nucleic acid or compound) to a cell, tissue or fluid (mucus or sputum) (also referred to herein as non-targeting carriers). Examples of pharmaceutically acceptable excipients include, but are not limited to water, phosphate buffered saline, Ringer's solution, dextrose solution, serum-containing solutions, Hank's solution, other aqueous physiologically balanced solutions, oils, esters and glycols. Aqueous carriers can contain suitable auxiliary substances required to approximate the physiological conditions of the recipient, for example, by enhancing chemical stability and isotonicity.

[0138] Suitable auxiliary substances include, for example, sodium acetate, sodium chloride, sodium lactate, potassium chloride, calcium chloride, and other substances used to produce phosphate buffer, Tris buffer, and bicarbonate buffer. Auxiliary substances can also include preservatives, such as thimerosal, m- or o-cresol, formalin and benzol alcohol. Compositions of the present invention can be sterilized by conventional methods and/or lyophilized.

[0139] Suitable carriers or excipients include, but are not limited to, preservatives (e.g., EDTA) and other antioxidants that help stabilize the dithiols included in the preparation.

[0140] One type of pharmaceutically acceptable carrier includes a controlled release formulation that is capable of slowly releasing a composition of the present invention into a patient. As used herein, a controlled release formulation comprises one or more therapeutic agents of the present invention in a controlled release vehicle. Suitable controlled release vehicles include, but are not limited to, biocompatible polymers, other polymeric matrices, capsules, microcapsules, microparticles, bolus preparations, osmotic pumps, diffusion devices, liposomes, lipospheres, and transdermal delivery systems. Suitable delivery vehicles for nucleic acids include, but are not limited to liposomes, viral vectors or other delivery vehicles, including ribozymes.

[0141] A liposome delivery vehicle comprises a lipid composition that is capable of delivering a protein, compound or nucleic acid molecule to a suitable cell and/or tissue in a patient. A liposome delivery vehicle can comprise a lipid composition that is capable of fusing with the plasma membrane of a cell to deliver the composition into a cell. A liposome delivery vehicle is preferably capable of remaining stable in a patient for a sufficient amount of time to deliver a composition to a preferred site in the patient. Suitable liposomes for use with the present invention include any liposome.

[0142] A suitable, or effective, amount of a compound containing a dithiol active site to administer to a patient is an amount that is capable of: participating in redox reactions through the reversible oxidation of its active-site dithiol to a disulfide, catalyzing dithiol-disulfide exchange reactions, and particularly, decreasing the viscosity or cohesivity of mucus or sputum and/or increasing the liquefaction of mucus or sputum in a patient, directly inhibiting elastase activity (such as neutrophil elastase activity), inhibiting sputum elastolytic activity, preserving α1-antitrypsin activity, and more particularly, increasing the liquefaction of mucus or sputum in a patient, or decreasing elastase activity in a patient, sufficient to provide a therapeutic benefit to the patient. Decreases in the viscosity or cohesivity or increases in the liquefaction of mucus or sputum can be measured, detected or determined as described previously herein or by any suitable method known to those of skill in the art. Decreases in elastase levels and/or activity can also be measured, detected or determined as described previously herein or by any suitable method known to those of skill in the art.

[0143] In one embodiment, a preferred amount of a protein or peptide containing a thioredoxin or other dithiol active-site to be administered to a patient comprises between about 1 ng×kilogram^{-1} and about less than 10 mg×kilogram^{-1} body weight of a mammal. A more preferred single dose comprises between about 20 ng×kilogram^{-1} and about 5 mg×kilogram^{-1} body weight of the mammal. In one embodiment, a suitable dose is between about 0.1 micromoles×kilogram^{-1} and about 150 micromoles×kilogram^{-1} body weight of a patient. In another embodiment, a preferred amount of a protein or peptide containing a thioredoxin or other dithiol active-site to be administered to a patient comprises between about 1.5 micromoles×kilogram^{-1} and about 150 micromoles×kilogram^{-1} body weight of a patient. A more preferred amount of a protein or peptide containing a thioredoxin or other dithiol active-site to be administered to a patient comprises between about 2 micromoles×kilogram^{-1} and about 25 micromoles×kilogram^{-1} body weight of a patient. An even more preferred amount of a protein or peptide containing a thioredoxin or other dithiol active-site to be administered to a patient comprises between about 3 micromoles×kilogram^{-1} and about 10 micromoles×kilogram^{-1} body weight of a patient.

[0144] In another embodiment, a preferred amount of a compound to deliver to a patient is an amount that results in an airway concentration (e.g., achieved surface concentration of the compound) of between about 0.001 μM and about 1 μM achieved surface concentration of the compound, and more preferably, between about 0.001 μM and about 0.1 μM achieved surface concentration of the compound, including any amount between about 0.0001 μM and 1 μM, in increments of 0.001 μM. In another embodiment, a preferred amount of a compound to deliver to a patient is an amount that results in an airway concentration (e.g., achieved surface concentration of the compound) of between about 0.001 μM and about 1 mM achieved surface concentration, or any increment in between (e.g., 100-500 μM, 100 μM-1 mg), in increments of 0.001 μM.

[0145] It should be noted that the actual delivered dose of a dithiol may be only a small fraction of the nebulized or aerosolized dose, when the compound is delivered by these routes. For example, the actual delivered dose may be only between about 5% and 85% of dose initially administered. One of skill in the art will understand how to adjust the amount of compound administered to achieved the desired delivered dose when using an aerosol or nebulized route of delivery.

[0146] In another embodiment, if the route of delivery is aerosol or a similar route, an amount of a protein or peptide containing a thioredoxin or other dithiol active-site to be administered to a patient comprises at least about 0.25 mg per dosing unit (e.g., a dosing unit for a human is typically about 2-3 ml) and about 25 mg per dosing unit. Preferably, an amount of a protein or peptide containing a thioredoxin active-site to be administered to a patient comprises at least about 0.25 mg per dosing unit, and more preferably at least about 0.3 mg per dosing unit, and more preferably at least

about 0.35 mg per dosing unit, and so on, in increments of 0.05 mg, up to greater than 25 mg per dosing unit. For aerosol delivery, typically, only about 10% of the volume in the aerosol is actually delivered to the airway. Therefore, for other routes of administration when the volume of the composition that will be delivered to the site is greater, it will readily be seen that lower doses of the protein or peptide comprising a thioredoxin active site may be used.

[0147] In one embodiment, an amount of a non-protein dithiol compound to be administered to a patient comprises a dosage amount and interval that can be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the desired effects. Usual patient dosages for systemic administration range from 100-2000 mg/day. Stated in terms of patient body surface areas, usual dosages range from 50-910 mg/m^2/day. Usual average plasma levels should be maintained within 0.1-1000 μM. In cases of local administration or selective uptake, the effective local concentration of the compound can not be related to plasma concentration. Accordingly, when the compound is delivered by aerosolized form, preferred dosages comprise between about 2 ng×$kilogram^{-1}$ and about 100 μg×$kilogram^{-1}$ body weight of the mammal, or between about 2 ng×$kilogram^{-1}$ and about 50 μg×$kilogram^{-1}$, or between about 2 ng×$kilogram^{-1}$ and about 10 μg×$kilogram^{-1}$, or between about 2 ng×$kilogram^{-1}$ and about 5 μg×$kilogram^{-1}$, or between about 2 ng×$kilogram^{-1}$ and about 1 μg×$kilogram^{-1}$, or between about 2 ng×$kilogram^{1}$ and about 0.5 μg×$kilogram^{-1}$, or between about 2 ng×$kilogram^{-1}$ and about 0.25 μg×$kilogram^{-1}$, or between about 2 ng×$kilogram^{-1}$ and about 0.1 μg×$kilogram^{-1}$ body weight of the mammal.

[0148] The optimum amount of a protein or other compound of the present invention to be administered to an animal will vary depending on the route of administration. For instance, if the protein or other compound is administered by an inhaled (aerosol) route, the optimum amount to be administered may be different than the optimum amount to be administered by intratracheal injection. It is within the ability of one skilled in the art to vary the amount depending on such route of administration. It is important to note that a suitable amount of a protein or other compound of the present invention is an amount that has the desired function without being toxic to an animal. In a preferred embodiment, the compounds or the compositions comprising such compounds are formulated in a manner to reduce any noxious or offensive odors or tastes, and to minimize any side effects that may result from administration of the compound.

[0149] In a preferred embodiment of the present invention, a composition of the present invention that contains a dithiol compound is further formulated with one or more agents comprising a reducing system that maintains, recycles or reduces the dithiol active site in the protein or compound to the reduced state. In one embodiment, such an agent includes nicotinamide-adenine dinucleotide phosphate (reduced form) (NADPH) and/or thioredoxin reductase, with formulation with NADPH being particularly preferred. In another embodiment, two cycling dithiols can be combined, such as a protein containing a thioredoxin active site and dihydrolipoic acid, or a protein containing a thioredoxin active site and α-lipoic acid. The present inventors have found that the presence of a reducing system with a protein, peptide or other compound containing a dithiol active site significantly increases the ability of the protein, peptide or other compound containing a dithiol active site to function in the method of the invention (e.g. to increase the liquefaction of mucus or sputum). In addition, for thioredoxin and proteins containing such active site, the present inventors have found that it is sufficient to include NADPH as the reducing system, and therefore, thioredoxin reductase is not a necessary component of the reducing system, but can be included, if desired. Other reducing systems can be used in the present invention and include, but are not limited to, NADH-dependent thioredoxin reductase, lipoic acid, and other biological reductants. In general, the present invention contemplates that an original source of reducing equivalents, most likely NADPH or NADH, will be an important component of a composition of the present invention for optimal therapeutic benefit. However, additional component(s) which serve an intermediary function of transferring the reducing equivalents (H^+, from NADPH or NADH) to the dithiol active site-containing molecule, also can be used.

[0150] When NADPH is included in a composition of the present invention, the composition is preferably formulated with between about 0.1 μM and about 20 mM achieved surface concentration of NADPH, and more preferably, between about 5 μM and about 2 mM achieved surface concentration of NADPH, and even more preferably, between about 50 μM and about 200 μM achieved surface concentration of NADPH. In another embodiment, the composition can be formulated with any suitable amount of NADPH, preferably between about 0.5 μM and about 20 mM achieved surface concentration of NADPH in increments of 0.1 μM (i.e., 0.5 μM, 0.6 μM, . . . 19.9 μM, 20 μM). An "achieved surface concentration" is the concentration of a particular chemical, such as NADPH, that is achieved, or present, at the surface of a cell or tissue, for example, at the surface of lung epithelial lining. Therefore, it may be necessary to actually administer a larger concentration of a particular chemical in order to achieve a certain surface concentration. It is well within the ability of one skilled in the art to determine such concentrations. When thioredoxin reductase in included in a composition of the present invention, it is preferably formulated with between about 0.001 μM and about 1 μM achieved surface concentration of thioredoxin reductase, and more preferably, between about 0.001 μM and about 0.1 μM achieved surface concentration of thioredoxin reductase, including any amount between about 0.0001 μM and 1 μM, in increments of 0.001 μM. In one embodiment, it is not necessary to include any thioredoxin reductase in a composition of the invention. It is within the scope of the present invention that such amounts of thioredoxin reductase and/or NADPH can be modified by one skilled in the art in order to maintain or enhance the reduced state of a thioredoxin active-site or other dithiol active site, as the amount of a protein or other compound containing such active-site or the mode of delivery indicates.

[0151] Another embodiment of the invention relates to the oral administration of a dithiol, and particularly, α-lipoic acid, whereby the α-lipoic acid is naturally converted to dihydrolipoic acid (DHLA) in the cells of the body after administration.

[0152] As discussed above, a composition of the invention can include one or more additional compounds, such as other compounds that can be used to reduce excessively viscous or cohesive mucus or sputum or increase the lique-

faction of such mucus or sputum. Examples of such compounds are known in the art and include, but are not limited to, DNase (e.g., purified rhDNase), hypertonic saline (e.g., between about 0.1% and 12% sodium chloride), N-acetylcysteine, nacystelyn (an N-acetyl-L-cysteine derivative), erdosteine, bromheksin, carbocysteine, guiafenesin, iodinated glycerol, gelsolin and any protease inhibitor (e.g., α1-antitrypsin). Similarly, a composition of the invention can include one or more additional compounds that can be used to reduce elastase levels or activity in the tissues of a patient (e.g., DNase; L-658,758; aerosolized FK706; elafin; α1-antitrypsin). Other useful compounds will be known to those of skill in the art. The use of a dithiol, exemplified by thioredoxin, with the mucolytic agents rhDNase or hypertonic saline to enhance mucolysis of sputum is demonstrated in the Examples.

[0153] In addition, a composition of the present invention may contain additional agents that further enhance the ability of the composition to reduce excessively viscous or cohesive mucus or sputum or increase the liquefaction of such mucus or sputum, or reduce elastase levels or activity in the tissues of a patient. For example, it may be desirable to provide excipients, buffers, or other agents that improve the efficacy of the dithiol compounds and additional mucolytic or anti-elastase agents used in the present invention. An example of such an additional agent is illustrated in the Examples, wherein mucin is provided in a composition of thioredoxin and DNase to reduce the inhibition of DNase by thioredoxin. Other similar agents that can serve as an alternate dithiol target, for example, can be used in compositions to improve the efficacy of the composition.

[0154] Agents used in the composition of the invention may be administered together in a single formulation (i.e., substantially at the same time), or the composition may be formulated to deliver the agents in sequential order (e.g., two formulations that are provided together as a single composition for sequential administration to a patient). Such combination therapy may involve the administration of the dithiol used in the invention before, during, and/or after the administration of a second agent, such as an additional mucolytic agent, an additional anti-elastase agent, or another therapeutic or enhancing compound or agent. The administration of the dithiol may be separated in time from the administration of additional agents by up to several hours or days, but more commonly will be separated in time by minutes (e.g., between about 1 minute and 90 minutes, including any 1 minute increment in between). In one embodiment, when a dithiol is administered in combination with a DNase, the DNase is preferably administered prior to the dithiol, as improved mucolysis has been demonstrated by the inventors using such a protocol (see Examples).

[0155] As discussed above, a composition of the present invention is administered to a patient in a manner effective to deliver the composition, and particularly the protein or compound comprising a dithiol active site, a recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a protein or peptide containing a dithiol active site, and/or any other compounds in the composition, to a target site (e.g., mucus or sputum to be treated for proteins and compounds, a target host cell that will be or is in the environment of the mucus or sputum to be treated for recombinant nucleic acid molecules). Suitable administration protocols include any in vivo or ex vivo administration protocol.

[0156] According to the present invention, an effective administration protocol (i.e., administering a composition of the present invention in an effective manner) comprises suitable dose parameters and modes of administration that result in contact of the protein containing a thioredoxin active site and/or other compound in the composition with the mucus or sputum to be treated, or with or near elastase produced by the patient (at the tissues or near cells where elastase is produced or secreted), or in the transfection and expression of a recombinant nucleic acid molecule encoding a protein comprising a dithiol active site in a desired host cell of a patient, preferably so that the patient obtains some measurable, observable or perceived benefit from such administration. In some situations, by sampling the mucus or sputum or a body tissue or cells from the patient, effective dose parameters can be determined using methods as described herein for assessment of mucus or sputum viscosity or liquefaction, and/or elastase levels and/or activity. Alternatively, effective dose parameters can be determined by experimentation using in vitro samples, in vivo animal models, and eventually, clinical trials if the patient is human. Effective dose parameters can be determined using methods standard in the art for a particular disease or condition. Such methods include, for example, determination of survival rates, side effects (i.e., toxicity) and progression or regression of disease.

[0157] According to the present invention, suitable methods of administering a composition of the present invention to a patient include any route of in vivo administration that is suitable for delivering the composition to the desired site into a patient. The preferred routes of administration will be apparent to those of skill in the art, depending on whether the compound is a protein, nucleic acid, or other compound (e.g., a drug), to what part of the body the composition is to be administered, and the disease or condition experienced by the patient. In general, methods of in vivo administration include, but are not limited to, intravenous administration, intraperitoneal administration, intramuscular administration, intracoronary administration, intraarterial administration (e.g., into a carotid artery), subcutaneous administration, transdermal delivery, intratracheal administration, subcutaneous administration, intraarticular administration, intraventricular administration, inhalation (e.g., aerosol), intracerebral, nasal, oral, pulmonary administration, impregnation of a catheter, and direct injection into a tissue. Aural delivery can include ear drops, intranasal delivery can include nose drops or intranasal injection, and intraocular delivery can include eye drops. Aerosol (inhalation) delivery can also be performed using methods standard in the art (see, for example, Stribling et al., *Proc. Natl. Acad. Sci. USA* 189:11277-11281, 1992, which is incorporated herein by reference in its entirety). Oral delivery can include solids and liquids that can be taken through the mouth, for example, as tablets or capsules, as well as being formulated into food and beverage products. When the dithiol to be administered is α-lipoic acid, oral delivery is particularly preferred. Other routes of administration that are useful for mucosal tissues include bronchial, intradermal, intramuscular, intranasal, other inhalatory, rectal, subcutaneous, topical, transdermal, vaginal, transcervical, pericervical and urethral routes. In addition, administration protocols can include

pretreatment devices, such as application of the protein, peptide, compound or composition in a diaphragm (e.g., to the cervix) for use in applications such as infertility. In a preferred embodiment of the present invention, when the protein, other compound or composition of the invention is administered to treat excessively or abnormally viscous or cohesive sputum or mucus in the respiratory tract (airways), a protein or peptide or other compound (or composition) containing a dithiol active-site or other compound is administered by a route including, but not limited to, inhalation (i.e. by inhaling an aerosol, e.g., in or with surfactants); direct installation into the lung via a bronchoscope, endotracheal tube and/or via any artificial ventilation device; nasal administration (intranasal or transnasal), bronchial, or intratracheally (i.e. by injection directly into the trachea or tracheostomy), either directly or via lipid-encapsulation or surfactant. Any conceivable method of introducing the composition or protein into the airways so that it can contact the mucus or sputum therein is encompassed by the invention.

[0158] Various methods of administration and delivery vehicles disclosed herein have been shown to be effective for delivery of a nucleic acid molecule to a target cell, whereby the nucleic acid molecule transfected the cell and was expressed. In many studies, successful delivery and expression of a heterologous gene was achieved in preferred cell types and/or using preferred delivery vehicles and routes of administration of the present invention.

[0159] For example, using liposome delivery, U.S. Pat. No. 5,705,151, issued Jan. 6, 1998, to Dow et al. demonstrated the successful in vivo intravenous delivery of a nucleic acid molecule encoding a superantigen and a nucleic acid molecule encoding a cytokine in a cationic liposome delivery vehicle, whereby the encoded proteins were expressed in tissues of the animal, and particularly in pulmonary tissues. In addition, Liu et al., *Nature Biotechnology* 15:167, 1997, demonstrated that intravenous delivery of cholesterol-containing cationic liposomes containing genes preferentially targets pulmonary tissues and effectively mediates transfer and expression of the genes in vivo. Several publications by Dzau and collaborators demonstrate the successful in vivo delivery and expression of a gene into cells of the heart, including cardiac myocytes and fibroblasts and vascular smooth muscle cells using both naked DNA and Hemagglutinating virus of Japan-liposome delivery, administered by both incubation within the pericardium and infusion into a coronary artery (intracoronary delivery) (See, for example, Aoki et al., 1997, *J. Mol. Cell, Cardiol.* 29:949-959; Kaneda et al., 1997, *Ann N.Y. Acad. Sci.* 811:299-308; and von der Leyen et al., 1995, *Proc Natl Acad Sci USA* 92:1137-1141).

[0160] Delivery of numerous nucleic acid sequences has been accomplished by administration of viral vectors encoding the nucleic acid sequences. Using such vectors, successful delivery and expression has been achieved using ex vivo delivery (See, of many examples, retroviral vector; Blaese et al., 1995, *Science* 270:475-480; Bordignon et al., 1995, *Science* 270:470-475), nasal administration (CFTR-adenovirus-associated vector), intracoronary administration (adenoviral vector and Hemagglutinating virus of Japan, see above), intravenous administration (adeno-associated viral vector; Koeberl et al., 1997, *Proc Natl Acad Sci USA* 94:1426-1431). A publication by Maurice et al. (1999, *J. Clin. Invest.* 104:21-29) demonstrated that an adenoviral vector encoding a β2-adrenergic receptor, administered by intracoronary delivery, resulted in diffuse multichamber myocardial expression of the gene in vivo, and subsequent significant increases in hemodynamic function and other improved physiological parameters. Levine et al. describe in vitro, ex vivo and in vivo delivery and expression of a gene to human adipocytes and rabbit adipocytes using an adenoviral vector and direct injection of the constructs into adipose tissue (Levine et al., 1998, *J. Nutr. Sci. Vitaminol.* 44:569-572).

[0161] In the area of neuronal gene delivery, multiple successful in vivo gene transfers have been reported. Millecamps et al. reported the targeting of adenoviral vectors to neurons using neuron restrictive enhancer elements placed upstream of the promoter for the transgene (phosphoglycerate promoter). Such vectors were administered to mice and rats intramuscularly and intracerebrally, respectively, resulting in successful neuronal-specific transfection and expression of the transgene in vivo (Millecamps et al., 1999, *Nat. Biotechnol.* 17:865-869). As discussed above, Bennett et al. reported the use of adeno-associated viral vector to deliver and express a gene by subretinal injection in the neural retina in vivo for greater than 1 year (Bennett, 1999, ibid.).

[0162] Gene delivery to synovial lining cells and articular joints has had similar successes. Oligino and colleagues report the use of a herpes simplex viral vector which is deficient for the immediate early genes, ICP4, 22 and 27, to deliver and express two different receptors in synovial lining cells in vivo (Oligino et al., 1999, *Gene Ther.* 6:1713-1720). The herpes vectors were administered by intraarticular injection. Kuboki et al. used adenoviral vector-mediated gene transfer and intraarticular injection to successfully and specifically express a gene in the temporomandibular joints of guinea pigs in vivo (Kuboki et al., 1999, *Arch. Oral. Biol.* 44:701-709). Apparailly and colleagues systemically administered adenoviral vectors encoding IL-10 to mice and demonstrated successful expression of the gene product and profound therapeutic effects in the treatment of experimentally induced arthritis (Apparailly et al., 1998, *J. Immunol.* 160:5213-5220). In another study, murine leukemia virus-based retroviral vector was used to deliver (by intraarticular injection) and express a human growth hormone gene both ex vivo and in vivo (Ghivizzani et al., 1997, *Gene Ther.* 4:977-982). This study showed that expression by in vivo gene transfer was at least equivalent to that of the ex vivo gene transfer. As discussed above, Sawchuk et al. has reported successful in vivo adenoviral vector delivery of a gene by intraarticular injection, and prolonged expression of the gene in the synovium by pretreatment of the joint with anti-T cell receptor monoclonal antibody (Sawchuk et al., 1996, ibid. Finally, it is noted that ex vivo gene transfer of human interleukin-1 receptor antagonist using a retrovirus has produced high level intraarticular expression and therapeutic efficacy in treatment of arthritis, and is now entering FDA approved human gene therapy trials (Evans and Robbins, 1996, *Curr. Opin. Rheumatol.* 8:230-234). Therefore, the state of the art in gene therapy has led the FDA to consider human gene therapy an appropriate strategy for the treatment of at least arthritis. Taken together, all of the above studies in gene therapy indicate that delivery and expression of a recombinant nucleic acid molecule according to the present invention is feasible.

[0163] Another method of delivery of recombinant molecules is in a non-targeting carrier (e.g., as "naked" DNA molecules, such as is taught, for example in Wolff et al., 1990, *Science* 247, 1465-1468). Such recombinant nucleic acid molecules are typically injected by direct or intramuscular administration. Recombinant nucleic acid molecules to be administered by naked DNA administration include an isolated nucleic acid molecule of the present invention, and preferably includes a recombinant molecule of the present invention that preferably is replication, or otherwise amplification, competent. A naked nucleic acid reagent of the present invention can comprise one or more nucleic acid molecules of the present invention including a dicistronic recombinant molecule. Naked nucleic acid delivery can include intramuscular, subcutaneous, intradermal, transdermal, intranasal and oral routes of administration, with direct injection into the target tissue being most preferred. A preferred single dose of a naked nucleic acid vaccine ranges from about 1 nanogram (ng) to about 100 μg, depending on the route of administration and/or method of delivery, as can be determined by those skilled in the art. Suitable delivery methods include, for example, by injection, as drops, aerosolized and/or topically. In one embodiment, pure DNA constructs cover the surface of gold particles (1 to 3 μm in diameter) and are propelled into skin cells or muscle with a "gene gun."

[0164] In the method of the present invention, therapeutic compositions can be administered to any member of the Vertebrate class, Mammalia, including, without limitation, primates, rodents, livestock and domestic pets. Preferred patients to protect are humans.

[0165] The following examples are provided for the purpose of illustration and are not intended to limit the scope of the present invention.

EXAMPLES

Examples 1-6

[0166] The following Materials and Methods were used in Examples 1-6 below.

[0167] Reagents and materials. Lyophilized recombinant *E. coli* Trx was obtained from Promega (Madison, Wis.). *E. coli* TR was from American Diagnostica, (Greenwich, Conn.). β-Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), reduced GSH, glutathione reductase, dithiothreitol, disopropylflurophosphate, aprotinin, N-ethyl maleimide, schiff reagent, salmon testes DNA, and Hoechst dye were all obtained from Sigma Chemical (St. Louis, Mo.). N-acetylcysteine was from Fisher Scientific (Pittsburgh, Pa.). All other chemicals were of the highest possible grade.

[0168] Sputum collection. Sputum was obtained from patients with CF at the Children's Hospital (Denver, Colo.). Patients were diagnosed with CF if they had sweat chloride values in excess of 60 mM in two separate pilocarpine iontophoresis sweat tests, and exhibited two allelic CF-producing mutations in subsequent genetic analysis. All samples were donated by either spontaneous expectoration or hypertonic saline induction. Sputum samples containing visibly detectable saliva were discarded. After expectoration, samples were stored at −80° C. until their time of use. Sputum collection protocol, data collection, and consent/assent forms were approved by the Institutional Review Board of the University of Colorado Health Science Center (COMIRB) and affiliated hospitals.

[0169] Compaction assay. CF sputum stored at −80° C. was thawed at room temperature and aliquoted into 1.5 ml Eppendorf centrifuge tubes at volumes of 275 μl using a positive displacement pipette (Rainin, Emeryville, Calif.). Sputum samples were subjected to either diluent (H_2O), Trx+TR+NADPH, GSH+glutathione reductase+NADPH, dithiothreitol (DTT) or N-acetylcysteine treatment by the addition of 25 μl of H_2O containing the appropriate molar concentration of each agent. After brief vortexing (~1 second), sample tubes were loaded onto a microtube rotisserie (Barnstead, Dubuque, Iowa) and incubated at 37° C. for 20 minutes. Samples were then processed for compaction assay according to methodology originated by Daugherty et al. (Daugherty et al., *Biomaterials* 16:553-558, 1995). To perform the assay, the contents of each sample were loaded into 100 μl glass micro-capillary tubes (Fisher Scientific) that had been previously welded to 200 μl pipette tips in order to achieve a tight fit. Three modified capillary tubes were used to draw up >90% of each sputum sample. Capillary tubes were then removed from their pipette tip, sealed with clay, and centrifuged for 10 minutes in a hematocrit centrifuge (IEC, Needham Heights, Mass.), followed by measurement of the length in millimeters of the gel (solid) and aqueous (liquid) phases in each tube. The percent liquid fraction of each capillary tube was calculated by dividing aqueous phase length by total length (gel+aqueous)×100. The three measurements of the liquid fraction (%) derived from each sample were then averaged to generate a single value for each treatment condition.

[0170] Magnetic microrheometry. Viscoelastic change in response to treatment was measured by means of a magnetic microrheometer as developed by King (King M., Magnetic microrheometer. In: *Methods in Bronchial Mucology*, edited by Braga P C, and Allegra L. New York: Raven Press, 1988, p. 73-83). An 80-120 μm steel sphere was placed in a 10 mg sputum sample. An electromagnet was used to oscillate this sphere, whose image was projected onto a pair of photocells via a microscope. The mucus retarded the motion of the sphere and this effect was revealed by plotting the motion of the sphere against the driving force of the magnet on an oscilloscope, from which G* was measured. G* was the mechanical impedance or vector sum of viscosity and elasticity. For Trx and NADPH dose response experiments, log G* at 10 rad/s was measured before any treatment (baseline), and then after 20 minute incubation with no treatment, diluent (H_2O), or Trx with reducing system. All treatments were administered to the sample in a volume of H_2O equal to 10% of total sample volume. One measurement was performed per aliquot of sample.

[0171] Glycoprotein extraction from sputum. Extraction of soluble glycoproteins from sputum was performed according to methodology outlined by Davies et al. (Davies and Carlstedt, *Methods Mol Biol* 125:3-13, 2000). 275 μl of CF sputum was treated for 20 minutes at 37° C. with 25 μl of H_2O alone or H_2O containing Trx (10 or 30 μM)+NADPH (2 mM) and TR (0.1 μM). After treatment, 100 μl of H_2O containing 1 mM disopropylflurophosphate and 10 μg ml aprotinin, was added to each sample, followed by 15 minute centrifugation at 22,000 g at 4° C. The resulting supernatant (aqueous phase) of each sample was transferred to a new

microcentrifuge tube and stored at −20° C. The remaining solid gel portion of each sample was carefully unseated from the tube bottom in the presence of 250 μl of guanidinium extraction buffer (6 M guanidinium chloride; 5 mM EDTA; 10 mM sodium phosphate buffer, pH 6.5; 1 mM N-ethyl maleimide; 100 μM disopropylflurophosphate; and 1 μg/ml aprotinin) using a pipette tip and rotated for 14 hours at 4° C. After centrifugation, the resulting supernatant from this gel phase extraction was then transferred to a clean tube and frozen at −20° C. until time of electrophoresis.

[0172] Analysis of glycoprotein content. The glycoprotein content of aqueous and gel phase samples were evaluated by staining with periodic acid Schiff reagent (PAS) according to methodology outlined by Thornton et al. (Thornton et al., *Methods Mol Biol* 125:77-85, 2000). Aqueous and gel samples were thawed and 80 μl aliquots of each were loaded onto a 1.0% agarose gel (150 mm×125 mm) housed within a Biomax horizontal electrophoresis apparatus (Kodak, Rochester, N.Y.). Electrophoresis reagents were as follows: electrophoresis buffer: 40 mM Tris-acetate, 1 mM EDTA, pH 8.0, 0.1% SDS; sample loading buffer: 60% electrophoresis buffer, 40% glycerol (v/v) and 0.005% (w/v) bromophenol blue. Gel contents were transferred to polyvinylidene (PVDF) membrane by vacuum blotter (Boeckel Scientific, Feasterville, Pa.) using 0.6 M NaCl, 60 mM sodium citrate as a transfer solution. After transfer, membranes were washed in three changes of water and transferred to 200 ml of a 1% periodic acid (v/v) 3% acetic acid (v/v) solution for 30 minutes at room temperature. The membrane was then rinsed twice with 0.1% sodium metabsulfite in 1 mM HCl and placed in Schiff reagent for 6 minutes.

[0173] Measurement of total DNA content. 275 μL of CF sputum was incubated with no treatment, 25 μl of H_2O, or 25 μl of H_2O containing Trx (30 μM)+TR (0.1 μM) and NADPH (2 mM). After 20 minute incubation at 37° C., 100 μl of H_2O was added to each sample, followed by centrifugation (22,000×g) for 10 minutes. Resulting gel and liquid phases were separated and incubated with an equal volume of digestion solution consisting of 100 mM Tris Cl, 5 mM EDTA, 200 mM NaCl, 0.5% Tween 20, and 1 mg/ml proteinase K for 4 hours at 50° C. DNA was purified from liquid and gel phases by phenol/chloroform extraction and resuspended in 100 μl of TE buffer, pH 8.0. DNA concentrations were determined by Hoechst assay (Labarca and Paigen, *Anal Biochem* 102:344-352, 1980) using an F-2000 fluorometer (Hitachi, Schaumburg, Ill.) with an excitation wavelength of 575 nM and an emission wavelength of 555 nm. Salmon testis DNA, dissolved in TE buffer, was used to establish the standard curve.

[0174] Statistics. Data in the figures are presented as mean±standard deviations, except **FIGS. 4A and 4B** which displays standard error. A linear mixed-effects modeling approach was used to analyze the effect of treatments on the liquefaction, viscoelasticity, and DNA solubility of sputum samples. Dunnet's correction was applied when comparing several treatments against a single control. Reproducibility of the compaction assay was assessed via intraclass correlation coefficients calculated in the linear model. All analyses were performed using SAS version 8.2 (SAS Institute Inc, Cary, N.C.). Significance was defined as $p<0.05$.

Example 1

[0175] The following example describes the effect of the Trx reducing system on release of liquid from CF sputum.

[0176] Due to abnormal ion transport caused by defects in the CFTR gene, airway secretions in CF patients are often desiccated. As a consequence, purulent CF sputum is comprised largely of a rigid and nonflowing biopolymer matrix, often referred to as gel phase, and lesser amounts of soluble, liquid phase. To assess the effect of Trx on the ratios of these two phases in sputum, compaction assay measurements were performed. In a first experiment, equal volumes of sputum samples were treated with 25 μl H_2O containing 0, 1, 10, or 30 μM Trx; 0.1 μM TR; and 2 mM NADPH (final concentration). After 20 minute incubation, the liquid fraction of each sample was determined by compaction assay.

[0177] The mean (±SD) percentage of CF sputum present in the liquid phase was 3.5±2.9% prior to Trx exposure (**FIG. 1A**; values are the mean from 5 independent experiments. * $P<0.05$ versus H_2O exposed samples). Aliquots treated with diluent (H_2O) equal to 10% of the sputum demonstrated a small, nonsignificant increase (6.2±6.6%) in the proportion of sputum present in the liquid phase. In contrast, the liquid phase of CF sputum was significantly increased after treatment with the Trx reducing system (Trx+0.1 μM TR, and 2 mM NADPH). Treatment of sputum with Trx (1 μM) increased the liquid fraction of sputum to 37.8±15.4%. Maximal increases in liquid fraction occurred in samples incubated with a higher Trx (30 μM) concentration (74.5±15.6%).

[0178] To examine the effect of NADPH, additional samples from different donors were treated with 30 μM Trx, 0.1 μM TR and either 0, 0.2, 0.6, 1.0 or 2 mM NADPH for 20 minutes. Samples treated with Trx and TR without NADPH had a low percentage of sputum present in the liquid phase (2.9%±1.3%). Aliquots treated with NADPH demonstrated a dose-dependent increase in the liquid fraction, with maximal increase occurring at 2 mM (70.55±18.13%) (**FIG. 1B**). Treatment of sputum with NADPH in the absence of Trx did not cause any increase in the proportion of sputum present in the liquid fraction (not shown).

Example 2

[0179] The following example demonstrates the reproducibility of compaction assay measurements from Example 1.

[0180] To assess the reproducibility of compaction assay measurements, sputum samples from three different CF donors were separated into aliquots and frozen. Specifically, freshly isolated CF sputum from three different donors (A, B, C) was separated into 275 μl aliquots and frozen. After thaw, aliquots were incubated without treatment, or with H_2O, 10 μM Trx (+0.1 μM TR and 2 mM NADPH), or dithiothreitol (DTT, 1 or 5 mM) for 20 minutes and the percent liquid measured by compaction assay. Results obtained from three independent experiments performed on each donor sample were used to evaluate assay reproducibility. On three consecutive days, aliquots were thawed, and treated with water, Trx and its reducing system, dithiothreitol (DTT), or no treatment.

[0181] As shown in **FIG. 2**, aliquots from donor A that had been treated with no additions or diluent (H_2O) had a liquid

phase fraction of less than 10% of their total volume. Treatment of sputum aliquots from donor A with DTT (1 mM or 5 mM), or Trx (30 μM) with reducing system, increased the liquid fraction of these samples to greater than 90% of the total sample volume. These percent liquid fraction values of sputum samples from donor A did not fluctuate to any appreciable degree with identical treatment upon the second and third determinations in subsequent independent experiments. The extent of changes occurring in donor B and C samples in response to Trx or DTT exposure were less extensive than those occurring in sputum samples from donor A, but still differed significantly from the controls. For the sample from donor B, the 3 day range of variation in percent of sputum present in the liquid state after drug treatment was: 1 mM DTT-24-31%; 5 mM DTT-42-57%, 30 μM Trx-46-51%. For sputum from donor C, the range of percent liquid values was: 1 mM DTT-57-61%; 5 mM DTT-77-79%, 30 μM Trx-62-81%. These results show that the compaction assay has sufficient intra-sample reproducibility to validate its use as a method for measuring drug-induced liquefaction in a heterogeneous group of sputum samples.

Example 3

[0182] The following example demonstrates that Trx is a more potent sputum liquefaction agent than glutathione or N-acetylcysteine.

[0183] The effectiveness of Trx in liquefying sputum was compared with other monothiol and dithiol reducing agents. Sputum samples were aliquoted and treated with Trx or GSH for 20 minutes and percent liquid determined by compaction assay. Initial compaction assay experiments compared the potencies of the Trx and GSH reducing systems in liquefaction of sputum in the presence of equimolar concentrations of NADPH. Compared to control (no additions), a progressive and significant increase in percent liquid fraction was observed in sputum treated with 10, 30, or 60 μM Trx (**FIG. 3A**). In contrast, a significant increase in the liquid fraction of sputum was not observed after exposure to GSH at comparable or higher concentrations up to 1 mM. In separate studies, the use of N-acetylcysteine across a range of concentrations (**FIG. 3B**) also was observed to be less effective than Trx in causing liquefaction of sputum. Referring to **FIGS. 3A and 3B**, the actual percentages shown are as follows: No treatment=2.7±2.3%; H_2O=4.5±2.7%; 10 μM Trx=34.8±6.6%; 30 μM Trx=54.6±10.4%; 60 μM Trx=67.0±8.0%; 30 μM GSH=7.8±5.7%; 100 μM GSH=15.9±9.3%; 1 mM GSH=27.6±3.9%. The analysis of Trx and NAC efficacy also was determined after 20 minute incubation, but on a different set of sputum samples. Values are the mean from 5 (GSH) or 4 (NAC) experiments (* P<0.05 versus no treatment).

Example 4

[0184] The following example shows the effect of Trx reducing system on sputum viscoelasticity.

[0185] Magnetic microrheometry was performed to determine the effect of Trx and its reducing system on sputum viscosity. Measurements were performed on sputum samples before and after incubation with the Trx reducing system (Table 1) to determine the change in log G* (viscoelasticity).

TABLE 1

Viscoelasticity data (log G*) of untreated, H_2O, or thioredoxin-exposed CF sputum

Treatment	None	H_2O	3 μM Trx	10 μM Trx	30 μM Trx
Before	3.31 ± 0.05	3.30 ± 0.06	3.37 ± 0.06	3.33 ± 0.06	3.28 ± 0.04
After	3.22 ± 0.04	3.20 ± 0.03	3.11 ± 0.04	2.94 ± 0.05	2.63 ± 0.04

Aliquots of CF sputum were incubated, without treatment, with H_2O, or Trx + reducing system (0.1 μM TR and 2 μM NADPH), at 37° C. for 20 minutes. All data are presented as log G* (mean ± standard error) for 4 samples.

[0186] In the experiment shown in **FIG. 4A**, sputum samples were incubated with H_{20} or 3, 10, or 30 μM Trx+0.1 μM TR and 2 mM NADPH for 20 minutes and log G* was determined by magnetic microrheometry. Data is presented as the difference in log G* measurements recorded before and after exposure (displayed on the Y-axis). Each column represents the mean±standard error for 4 samples (♦ Statistically significant from H_2O diluent value). Incubation of sputum with diluent (H_2O) for 20 minutes, resulted in a modest decline in log G (0.11 log units) compared with pretreatment values. Exposure to Trx (3 μM), TR (0.1 μM) and NADPH (2 mM) resulted in a significant decrease in the log G* (0.26 log units) compared to diluent treatment alone (**FIG. 4A**). More substantial declines were evident in samples exposed to higher concentrations of Trx (0.39 log units decreased at 10 μM Trx and 0.65 log units decreased at 30 μM Trx, respectively).

[0187] The effect of varying concentrations of NADPH was also examined (**FIG. 4B**; change in log G after incubation with H_2O or 10 μM Trx, 0.1 μM TR, and 0.2, 0.6 or 2 mM NADPH). Sputum exposed to Trx (30 μM), TR (0.1 μM) and a low concentration of NADPH (0.2 mM) exhibited a modest decline in viscoelasticity (0.16 log units). A further decline in viscoelasticity occurred with exposure to higher NADPH concentrations, 0.6 mM (0.26 log units) and 2 mM (0.39 log units), demonstrating the importance of provision of reducing equivalents to allow Trx-mediated reduction in sputum viscoelasticity.

Example 5

[0188] The following example describes the effect of Trx on the solubility of sputum glycoproteins.

[0189] Disulfide bonds on mucin glycoprotein polymers are potential targets for reduction by Trx. To examine the effect of Trx on glycoproteins present in sputum, aliquots of sputum were incubated with H_2O, 10 or 30 μM Trx with its reducing system for 20 minutes and separated into aqueous (Aq) and gel fractions by centrifugation. Each insoluble gel

fraction was further treated for 14 hours with guanidinium (G). After treatment, the resulting soluble and insoluble phases of each sample were separated and analyzed for glycoprotein content by periodic acid/Schiff reagent (PAS) staining. Specifically, fractions were loaded and electrophoresed in a 1% agarose (w/v) gel, transferred to PVDF membrane, and stained with periodic acid/Schiff reagent as described in materials and methods. Referring to **FIG. 5**, molecular weight standards are shown in far right lane. Results are representative of three independent experiments.

[**0190**] As shown in **FIG. 5**, a discrete population of high molecular weight glycoproteins was detected in both the soluble and gel fractions derived from sputum treated with diluent. In contrast, greater amounts of PAS-reactive glycoproteins were evident in both phases derived from sputum treated with 10 or 30 μM Trx. During the processing of these samples, it was observed that the gel phase matrix from diluent treated samples retained a high degree of insolubility, despite overnight guanidinium treatment. This insolubility is the likely reason for the quantitative difference in amounts of glycoprotein observed in gel phase lanes from diluent and Trx-treated sputum. In addition to being more abundant, a substantial proportion of the glycoforms in Trx-exposed samples also exhibited greater electrophoretic mobility than those moieties present in diluent-treated samples. These findings indicate that Trx increases the solubility, and reduces the size of glycoprotein polymers in sputum.

Example 6

[**0191**] The following example demonstrates the effect of Trx on the solubility of DNA in sputum.

[**0192**] The presence of high amounts of extracellular DNA in CF airway secretions contributes to the excessive viscoelasticity of CF sputum. To evaluate what effect Trx has on the solubility of DNA in sputum, sputum samples (2751 μl) were incubated with either no additions, diluent (H_2O), or Trx (30 μM)+reducing system for 20 minutes, and then separated into gel (insoluble) and liquid (soluble) phases. Measurement of DNA content by Hoechst assay revealed that most of the DNA present in the untreated samples was retained in the gel phase (gel=0.94±0.26 mg; liquid=0.05±0.03 mg) (**FIG. 6**). Diluent-treated samples demonstrated a modest increase in mean DNA content in their liquid phase (gel=0.80±0.24 mg; liquid=0.21±0.23 mg). With Trx treatment, a further shift in DNA from gel to liquid phase (gel=0.55±0.31 mg; liquid=0.57±0.37 mg) was observed. Referring to **FIG. 6**, shown are mean DNA content±S. D. from each fraction (n=5 experiments) (*P<0.05 versus no treatment soluble phase).

Example 7

[**0193**] The following example describes the treatment of a patient that has excessively viscous or cohesive mucus or sputum with the therapeutic composition of the invention.

[**0194**] A 4 month old female patient presents with poor weight; frequent, bulky, foul-smelling, oily stools; a protruding abdomen and recurrent coughing and wheezing. The patient undergoes a quantitative pilocarpine iontophoresis sweat test and is diagnosed with cystic fibrosis. Pulmonary function tests and a chest X-ray confirm the diagnosis. The patient undergoes periodic evaluation and therapy including prevention and treatment of lung problems as they occur, good nutrition, and physical activity. By age 13, the patient displays slowed growth, delayed puberty, and declining physical endurance, and frequently suffers from lung infections, labored breathing and gastrointestinal discomfort. The patient presents at the physician's office with a severe cough, wheezing and impaired lung function.

[**0195**] A compaction assay is performed on a sample of sputum collected from the airway of the patient and it is determined based on this assay and the prior diagnosis of cystic fibrosis that the patient is suffering from respiratory dysfunction due to excessively viscous and cohesive mucus in the airways. To treat this symptom in the lung, the patient is administered a composition comprising about 2.5 mg per dosing unit of human thioredoxin and a dose of NADPH sufficient to achieve about 5 μM achieved surface concentration in a surfactant by aerosol delivery. The patient is monitored subsequently by additional compaction assays for increased liquefaction of the sputum and by lung function testing for clearing of the airways. Subsequent doses of the composition as described above are administered by aerosol on a daily basis until the patient airways show a significant clearance and the patient symptoms and general health have improved.

Example 8

[**0196**] The following example shows that reduced dithiol compounds can be used as a single component system to liquefy mucus and decrease sputum viscoelasticity. For this example, pre-reduced recombinant human thioredoxin (p-rhTrx) and dihydrolipoic acid (DHLA), both dithiol compounds, were shown to act as potent mucolytic compounds by liquefying mucus and reducing sputum viscoelasticity. DHLA is the reduced form of alpha-lipoic acid (also called thioctic acid) and obtained from a commercial source (Sigma, St. Louis, Mo.). p-rhTrx was synthesized as detailed in Materials and Methods section for Examples 9-13.

[**0197**] In the first experiment, 30 μl of p-rhTrx or DHLA in phosphate-buffered saline (PBS) was added to 270 μl of sputum from cystic fibrosis (CF) patients. The following concentrations of DHLA were tested: 0, 0.125 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM. The concentrations of p-rhTrx tested were: 0, 0.025 mM, 0.05 mM, 0.1 mM, 0.2 mM and 0.5 mM. The mucus was incubated for 20 minutes at 37° C. and then two separate assessments of mucus rheology were performed.

[**0198**] The "compaction assay" was used to assess sputum liquefaction. In this assay, approximately 80 μl of treated sputum is aspirated into a hematocrit tube and centrifuged for 7 minutes in a hematocrit tube centrifuge. After centrifugation, the mucus in the hematocrit tube separates into a solid fraction (gel) and a liquid fraction (sol). The ratio of sol relative to the entire mucus column (sol+gel) was recorded. As shown in **FIG. 7**, the ratio of sol in treated mucus increases proportionally with the concentration of DHLA. DHLA liquefies sputum in a concentration dependent manner. Similarly, thioredoxin also liquefied sputum in a dose dependent manner as measured by the compaction assay (**FIG. 1A**). As described in Example 1, NADPH and thioredoxin reductase were used as a reducing system for thioredoxin.

[**0199**] The second assessment was performed with an AR-1000 cone and plate rheometer (TA Instruments, New

Brunswick, Del.) with a 1 degree cone at an angular velocity of 1 rad/sec. Measurements of viscoelasticity were preformed before treatment and at the end of the 20 minute incubation period. Data is expressed as the change in viscoelasticity. The log of the complex modulus (Log G*) and the tan delta (tan 6) are presented here. Decreases in Log G* are indicative of decreased viscoelasticity while increases in tan 6 represent greater liquefaction of mucus. As shown in **FIGS. 8A and 9A**, both p-rhTrx and DHLA decreased sputum viscoelasticity (Log G*) in a concentration-dependent manner. Similarly, the liquefaction of sputum was supported by the increase in tan 6 as presented in **FIGS. 8B and 9B**.

[**0200**] The inventors have shown in this Example that the reduced forms of dithiol compounds, represented here by p-rhTrx and DHLA, can act in a single component system as potent mucolytics by decreasing sputum viscoelasticity and increasing mucus liquefaction. The effects occur in a concentration-dependent manner.

Examples 9-13

[**0201**] The following Materials and Methods were used in Examples 9-13 below, which show that, in addition to having potent mucolytic effects, dithiols such as thioredoxin and DHLA inhibit neutrophil elastase.

[**0202**] Reagents. Lyophilized recombinant *E. coli* thioredoxin-1 (*E. coli* Trx), *E. coli* thioredoxin reductase (*E. coli* TR), dihydrolipoic acid (DHLA), reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), dithiothreitol (DTT), reduced glutathione (GSH), N-acetyl-cysteine (NAC), human neutrophil elastase, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and N-Methoxysuccinyl-ala-ala-pro-val 4-nitroanilide were obtained from Sigma (St. Louis, Mo.). Recombinant human thioredoxin-1 (rhTrx) and a plant recombinant NADP-thioredoxin reductase (raTR) from *Arabidopsis* were prepared as detailed below. All other chemicals were of the highest possible grade. Throughout this paper, the term "*E. coli* Trx system" refers to *E. coli* Trx+*E. coli* TR+NADPH and the term "rhTrx system" refers to rhTrx+raTR+NADPH.

[**0203**] Cloning of rhTrx and raTR genes: The rhTrx gene was amplified by PCR out of human single strand cDNA from breast tissue and HELA cells (Stratagene; La Jolla, Calif.). The raTR gene was obtained in the same manner from leaf *Arabidopsis* single strand cDNA (Torrey Mesa Research Institute; San Diego, Calif.). In both cases, two primers were designed containing a Nde I restriction site at the 5' end and Xho I restriction site at the 3' end. The PCR fragments were then cloned into the TOPO-TA vector (Invitrogen; Carlsbad, Calif.) and sequenced for gene accuracy. The genes were subcloned Nde I/Xho I in the pET-22b vector for overexpression in bacteria or site directed mutagenesis.

[**0204**] Microbial protein overexpression: B121 DE3 (rhTrx) or B121 DE3 pLys (raTR) bacteria were transformed and plated in LB agar plates containing carbenicillin. Five colonies of freshly transformed bacteria were used to inoculate 100 ml of LB-carbenicillin and were grown in a shaker overnight at 37° C. The 100 ml preculture was added to 1 liter of LB-carbenicillin and grown to an $OD_{600\ nm}$ of 0.6 to 0.8. The bacteria were induced with 1 mM isopropyl-p-D-thiogalactopyranoside (IPTG) and grown until the end of their exponential phase. rhTrx was released by osmotic shock without cell disruption (29). raTR was extracted by disrupting the cells in either 0.1 mg/ml lysozyme or by 5 freeze/thaw cycles. This cell lysate was incubated in the presence of 10 μg/ml DNase 1 and 10 μg/ml RNase I in 50 mM Tris-HCl (pH 8.0) supplemented by 10 mM MgCl2 and 100 μM phenylmethylsulfonyl fluoride (PMSF).

[**0205**] rhTrx and raTR purification: A two-step ammonium sulfate (AS) fractionation was used to precipitate the rhTrx or raTR. AS was added to the protein mixture to yield a 40% AS solution. Precipitated material was removed by centrifugation (17000 g for 15 minutes). Additional AS was added to the supernatant to yield a 90% solution. Following centrifugation, the pellet was resuspended in less than 10 ml of 20 mM K-phosphate 200 mM NaCl buffer (pH 7.0). The 40% AS pellet contained contaminant proteins and the 90% AS pellet contained either rhTrx or raTR. The resulting protein extracts were clarified by centrifugation (20000 rpm, 4° C.) and the supernatant retained for further purification steps. Both protein solutions were separated by size exclusion chromatography in a 450 ml Superdex 75 HP equilibrated in 20 mM K-phosphate 200 mM NaCl buffer (pH 7.0). The raTR was retrieved close to the void volume and was identified either with a bright yellow color absorbing at 460 nm or with the DTNB assay as described by Florencio, et al (10). rhTrx was retrieved in the 10 kDa size range and was identified with the corn NADP-malate dehydrogenase assay as described by Jacquot, et al (14). The active fractions were combined and purified to homogeneity as described below.

[**0206**] rhTrx. The protein sample was supplemented with 2 M AS and separated close to homogeneity on a 6 ml Resource Phenyl custom column using a 120 ml gradient ranging from 2.2 to 0 M AS in 100 mM K-phosphate buffer (pH 7.0). rhTrx was retrieved at ~1.1 M AS and the active fractions were combined and dialyzed against 20 mM Na-acetate (pH 4.7) overnight at 4° C. in 8000 MWCO dialysis bags. Proteins were then separated on a 6 ml Resource Q column in a 120 ml gradient of 0-300 mM NaCl 20 mM Na-Acetate (pH 4.7) and rhTrx was recovered at ~100 mM NaCl. The protein was dialyzed in 8000 MWCO dialysis bags against 20 mM K-phosphate buffer (pH 7.0) and stored at −80° C. raTR. The protein sample was supplemented with 1.4 M AS and then separated on a 6 ml Resource phenyl custom column in a 120 ml gradient of 1.4 to 0 M AS in 200 mM K-phosphate buffer (pH 7.0). The raTR was eluted at ~0.5 M AS. The raTR positive fractions were retrieved by UV monitoring at 460 nm. Active fractions were dialyzed against 20 mM Tris-HCl (pH 8.0) overnight at 4° C. in 50000 MWCO dialysis bags. The proteins were purified to homogeneity on a 6 ml Resource Q column in a 140 ml gradient of 0 to 600 mM NaCl in 20 mM Tris-HCl (pH 8.0). The raTR was recovered at ~180 mM NaCl and stored at −80° C.

[**0207**] Pre-reduced recombinant human Trx (p-rhTrx): Purified recombinant human Trx was incubated in excess DTT (mM concentration) for 1 hour at 37° C. DTT was removed from the protein solution using a PD-10 desalting column (Amersham Bioscience; Uppsala, Sweden). Reduced protein was stored at −80° C.

[**0208**] Measurement of reducing activity: The reducing activity of different Trx formulations was tested through use

of the DTNB reduction assay. For the NADPH dependent Trx system, 50 μl of assay buffer (100 mM potassium phosphate, pH 7.0, 10 mM EDTA, and 0.05 mg/ml bovine serum albumin) containing 5 or 50 mM rhTrx and 0.5 mM raTR was added to a 96 well flat bottom plate (Costar; Corning, N.Y.). Next, 175 μl sample buffer containing 720 mM NADPH was added followed by 25 μl of 6 mM DTNB in sample buffer. To measure the reducing ability of p-rhTrx, 50 μl of 2.5 mM p-rhTrx was added to a 96 well plate followed by 175 μl of sample buffer and 25 μl of 6 mM DTNB. After initiating all reactions by the simultaneous addition of the DTNB, change in kinetic absorbance at 412 nm due to DTNB reduction was monitored by use of a Spectra Max 340 microplate reader (Molecular Devices; Sunnyvale, Calif.). Temperature was maintained at 30° C. and the duration of the assay was set to 12 minutes.

[0209] Purified neutrophil elastase studies: In order to assess direct effects on elastase activity, studies were conducted by exposing purified human neutrophil elastase to the various reductant systems. The reductant to be tested (Trx, DHLA, GSH or NAC) was diluted to the desired concentration in phosphate buffered saline (PBS, pH 7.2). The mixture was added to purified human neutrophil elastase (100 μg/ml in PBS pH 7.2, 0.01% Triton X-100) and incubated at 37° C. for one hour. The final volume during incubation was 210 μl and the concentration of neutrophil elastase was 1.6 μg/ml. Following incubation, 60 μl aliquots where tested in triplicate for elastolytic activity.

[0210] Sputum collection: Sputum was obtained from adult and pediatric patients with cystic fibrosis (CF) at the Children's Hospital (Denver, Colo.). Patients were diagnosed with CF if they had sweat chloride values in excess of 60 mM in two separate pilocarpine iontophoresis sweat tests and exhibited two allelic CF-producing mutations in subsequent genetic analysis. All samples were donated by either spontaneous expectoration or hypertonic saline induction. Sputum samples containing visibly detectable saliva were discarded. Sputum collection protocol, data collection, and consent/assent forms were approved by the Institutional Review Board of the University of Colorado Health Science Center (COMIRB). Sputum was kept on ice until delivered to the laboratory and then kept at −80° C.

[0211] Sputum sol experiments: For experiments with sputum sol, sputum samples were centrifuged at 50,000 g for 90 minutes at 4° C. The supernatant (sol phase) was removed and stored at −80° C. until needed. On the day of the experiment, the stored sol was thawed in a 37° C. water bath. The reductant system to be tested (Trx or DHLA) was diluted to the desired concentration in PBS (pH 7.2), added to the sol and incubated at 37° C. for the designated period of time (1 to 15 hours). The final volume tested was 210 μl with a final sol dilution of 1:14 (sputum sol:PBS). After incubation, three 60 μl aliquots were removed for determination of elastase activity.

[0212] Whole sputum experiments: Trx system or DHLA was mixed to the desired concentration in PBS (pH 7.2). DNase was used as formulated in the medication Pulmozyme. Aliquots of 30 μl were added to 270 μl of sputum from CF patients. This mixture was vortexed briefly (less than 1 second) and incubated for 20 minutes. A 20 minute timeframe was selected as this was a sufficient period to allow measurable mucolysis. The concentrations of DNase and p-rhTrx were chosen so as to give a similar mucolytic effect as assessed by an AR-1000 rheometer (TA Instruments; New Castle, Del.) in oscillation mode with an angular velocity of 1 rad/sec at 37° C. Following incubation, the mixture was centrifuged at 22,000 g for 15 minutes. The supernatant was removed and 5 μl aliquots were tested for elastase activity.

[0213] Assay of Elastase Activity: Elastase activity was determined by adding 120 μl of the substrate N-methoxysuccinyl-ala-ala-pro-val 4-nitroanilide and monitoring kinetic absorbance at 405 nm at 37° C. over 4 minutes (6). The concentration of elastase used in these experiments was determined to be within the linear range. The percent elastase inhibition was determined as follows:

$$\%\ \text{Inhibition} = \left(1 - \frac{\Delta OD}{\Delta OD_0}\right) \cdot 100$$

[0214] where ΔOD and ΔOD_0 are the change in absorbance for the experimental group and PBS control group, respectively.

[0215] Statistics: The data was analyzed with a two-tailed Student's t-test with the null hypothesis that the percent elastase inhibition would be zero. For data with a non-normal distribution, a logarithmic transformation was performed prior to statistical analysis. $\alpha=0.05$ for all analyses. Data variability is expressed as either standard deviation or 95% confidence interval.

Example 9

[0216] This example shows the effect of Thioredoxin on purified elastase and sputum sol elastase activities.

[0217] To determine whether thioredoxin (Trx) can inhibit elastase activity, purified neutrophil elastase solutions were incubated in the presence of *E. coli* Trx system or the recombinant human thioredoxin (rhTrx) system. The *E. Coli* Trx system consisted of *E. Coli* Trx+*E. Coli* Trx reductase+NADPH and the rhTrx system consisted of rhTrx+recombinant *Arabidopsis* NADP-thioredoxin reductase-1 (raTR)+NADPH. raTR was found to be an efficient transporter of reducing equivalents from NADPH to rhTrx (G. del Val, unpublished results). Referring to **FIG. 10**, *E. coli* Trx or rhTrx was incubated with NADPH (2 mM) and Trx reductase (0.1 μM) for one hour at 37° C. Elastase activity is expressed as the percentage inhibition relative to PBS control ($N \geqq 4$ determinations per condition. Data presented as mean±SD, $^*p<0.05$).

[0218] Both *E. coli* Trx and rhTrx systems inhibited purified human neutrophil elastase in a concentration-dependent manner. Keeping the concentrations of Trx reductase (0.1 μM) and NADPH (2 mM) constant during a one hour incubation at 37° C., both *E. Coli* Trx and rhTrx had a concentration-dependent inhibitory effect on purified neutrophil elastase activity (**FIG. 10**). At the highest Trx concentration tested (10 μM), elastase activity was inhibited 50% (SD 23, $p=0.0011$) after rhTrx treatment and 60% (SD 11, $p<0.0001$) after *E. coli* Trx treatment. Sputum sol from cystic fibrosis (CF) patients treated overnight (15 hours) with the same concentrations of the Trx systems showed a similar inhibitory pattern up to a maximal inhibition of 83%

(SD 19, p<0.0001) with rhTrx and 57% (SD 25, p=0.001) with *E. Coli* Trx (**FIG. 11A**; ETrx (♦); rhTrx (●); elastase activity is expressed as percentage inhibition relative to PBS control; N≧3 determinations per condition; data presented as mean±SD, *p<0.05).

[**0219**] The inhibition of purified neutrophil elastase was relatively rapid as shown by the robust response after an one hour incubation (**FIG. 10**). By contrast, the inhibition of sputum sol elastase activity was somewhat delayed. At one hour, most elastase in sputum sol remains in an active state. Only with longer treatment is there greater inhibition. The time-course of sputum sol elastase activity indicated that Trx inhibition was near maximal by three hours for both *E. coli* Trx and rhTrx (**FIG. 11B**). Incubation for longer periods did not cause further inhibition.

Example 10

[**0220**] The following example demonstrates that NADPH is the source of reducing equivalents for thioredoxin systems.

[**0221**] Varying the concentration of NADPH used in the Trx systems altered the inhibition of elastase in a concentration-dependent pattern (**FIG. 11C**). Sputum sol elastase inhibition reached a maximum at 4 mM NADPH (percent elastase inhibition: 94%, SD 4.4, p<0.0001) but statistically significant inhibitory effects on elastase activity were noted with NADPH concentrations as low as 0.0625 mM (percent elastase inhibition: 24%, SD 7.3, p=0.0005).

[**0222**] Each of the three components of the Trx system was essential for inhibition of elastase. The absence of any of the three Trx system components resulted in the elimination of the inhibitory effect (percent inhibition with no Trx: −5.5%, SD 4.3; no Trx reductase: −15.6%, SD 12.1; no NADPH: −11.6%, SD 12.2).

Example 11

[**0223**] The following example demonstrates that comparison of elastase inhibition after treatment with dithiols and monothiols.

[**0224**] Thioredoxin's conserved active site has two cysteine groups in a C-G-P-C conformation. Dihydrolipoic acid (DHLA) is a small molecule with two closely positioned free thiol groups in a similar orientation. A concentration-response curve was developed by incubating increasing concentrations of DHLA overnight with CF sputum sol. DHLA inhibits sol elastase activity in a concentration-dependent manner (**FIG. 12**; elastase activity is expressed as percentage inhibition relative to PBS control; N≧6 determinations per condition; data presented as mean±SD, *p<0.05). At the highest concentration tested (2 mM), DHLA inhibited 80% (SD 18, p<0.0001) of sol elastase activity. Using a similar concentration of reducing equivalents on a molar basis in the rhTrx system (2 mM NADPH), the extent of elastolytic activity inhibition by rhTrx was almost identical (**FIG. 13C**; percent elastase inhibition: 83%, SD 19).

[**0225**] In contrast to the dithiols *E. coli* Trx, rhTrx and DHLA, the monothiols N-acetyl-cysteine (NAC) and reduced glutathione (GSH) did not show the same ability to inhibit elastase, particularly at low concentrations. For this comparison, the concentration of NADPH within the *E. Coli* Trx system was varied since NADPH is the primary source of reducing equivalents. At concentrations between 0.125 mM and 2 mM, neither NAC nor GSH significantly inhibited purified human neutrophil elastase (**FIG. 13**; elastase activity is expressed as percentage inhibition relative to PBS control; N=3 determinations per condition; data presented as the mean±SD, *p<0.05). By contrast, the *E. Coli* Trx system caused potent inhibition throughout this concentration range. Dithiothreitol (DTT) showed significant elastase inhibition at low millimolar concentrations (2 mM) but was less effective at lower micromolar concentrations (**FIG. 13**).

Example 12

[**0226**] The following example demonstrates the use of pre-reduced Thioredoxin.

[**0227**] In the previous experiments, NADPH provided reducing equivalents to thioredoxin via thioredoxin reductase. In order to confirm that the reduced form of thioredoxin was alone sufficient to inhibit elastase activity, a pre-reduced form of human recombinant thioredoxin (p-rhTrx) was obtained. The reducing activity of p-rhTrx was verified by spectrophotometer with 5,5'-dithiobis (2-nitrobenzoic acid-)(DTNB) (**FIG. 14**). 10 or 1 μM rhTrx+0.1 μM raTR+500 μM NADPH; or 500 μM p-rhTrx, were tested for ability to reduce DTNB. DTNB was added to each experimental sample to achieve a final concentration of 600 μM. The reaction was performed at 30° C. over 12 minutes. Using similar amounts of reductant activity (500 μM p-rhTrx or 500 μM NADPH), p-rhTrx demonstrated very rapid reduction of DTNB with over 50% of the DTNB being reduced in the time taken for the spectrophotometer to take the first measurement. By contrast, the reduction kinetics of the rhTrx system was not nearly as rapid and was dependent of rhTrx concentration.

[**0228**] p-rhTrx was a potent inhibitor of purified neutrophil elastase (**FIG. 15A**; N≧3 determinations per data point). Progressive inhibition of elastase activity was noted up to a maximum of 79% (SD 6.6, p=0.0002) at 0.5 mM p-rhTrx after a one hour incubation at 37° C. Treatment of CF sputum sol for the same time period with p-rhTrx also caused a concentration-dependent inhibition (**FIG. 15B**; N=6 determinations per condition; elastase activity is expressed as percentage inhibition relative to PBS control; data presented as mean±SD, *p<0.05), but the absolute inhibition was less (elastase percent inhibition: 27.8%, SD 22.6, p=0.03).

Example 13

[**0229**] The following example demonstrates the treatment of whole sputum from patients with cystic fibrosis.

[**0230**] Thioredoxin has been shown to have potent mucolytic effects in vitro. In vitro treatment of whole sputum with the mucolytic DNase has been shown previously to increase elastolytic activity. In order to investigate the possibility that thioredoxin or other dithiol agents may show a similar effect, whole mucus from cystic fibrosis patients was incubated for 20 minutes with 2.7 μM DNase, 100 μM p-rhTrx, rhTrx system (10 μM rhTrx+0.1 μM raTR) with 100 μM or 2 mM NADPH, 2 mM DHLA, or 2 mM DTT. The concentration of DNase selected was equivalent to that in the medication Pulmozyme. The sol phase was then collected by centrifugation. Based on oscillation rheometry, the concentrations of

DNase and p-rhTrx tested yielded similar mucolysis (100 μM p-rhTrx log ΔG*=−0.70, SD 0.15; 2.7 μM DNase logG*=−0.61, SD 0.37; N=4). DNase and p-rhTrx showed significant increases in sol elastase activity (relative increase in elastase activity versus control: DNase: 2.9, CI: 1.8-4.6, p=0.0001; p-rhTrx: 1.6, CI: 1.3-1.9, p=0.0003). The increase after DNase treatment was much more pronounced than after any dithiol, including p-rhTrx (**FIG. 16**; elastase activity is expressed as a ratio relative to PBS control; N≧5 determinations per condition; data presented as mean±95% CI, *p<0.05 versus control). Sputum sol treated with DNase had 2.6 times the elastase activity relative to p-rhTrx treated sputum (Wilcoxon Rank Sum, p=0.006, N=10).

[**0231**] Discussion Related to Examples 9-13

[**0232**] The studies in Examples 9-13 provide evidence that thioredoxin (Trx) and dihydrolipoic acid (DHLA) can directly inhibit human neutrophil elastase activity. Both recombinant human and *E. coli* Trx systems caused significant inhibition of elastase. *E. coli* Trx has approximately 29% homology with its human counterpart. The *E. coli* Trx contains only two active site cysteine residues, whereas human Trx has these as well as three additional cysteines. The Trx system used in this consists of Trx, thioredoxin reductase (TR) and NADPH. TR serves to transfer reducing equivalents (electrons) from NADPH to Trx. Trx required both TR and NADPH to inhibit elastase. Decreasing NADPH caused a concentration-dependent impairment of elastase inhibition, and removal of either NADPH or TR eliminated inhibition altogether. However, if pre-reduced recombinant human thioredoxin (p-rhTrx) was used, it was effective in inhibiting elastase activity without the presence of TR or NADPH. This demonstrates that reduced Trx is the active constituent of the Trx system.

[**0233**] To explore the possible clinical significance of these findings, the inventors examined sputum from cystic fibrosis (CF) patients. A major component of airway inflammation in CF is an influx of neutrophils, which release large quantities of elastase. Excess elastase overwhelms the anti-elastase protective mechanisms (α-1 antiprotease, secretory leukoprotease inhibitor, α-2 macroglobulin and eglin), resulting in dramatically elevated free elastase in sputum of CF patients. Even patients with mild CF disease have large amounts of free neutrophil elastase present in bronchoalveolar lavage fluid. This free elastase has numerous effects including direct structural damage to airways, increased mucus secretion, impaired opsonophagocytosis as a result of complement and immunoglobulin cleavage, and increased cytokine expression especially IL-8 which acts as a neutrophil chemoattractant. Elastase can also impair clearance of apoptotic neutrophils through cleavage of phosphotidyl serine receptors on macrophages. More recently, elastase has been shown to inactivate the pulmonary surfactant proteins A and D, which also play a role in the clearance of apoptotic neutrophils. Inhibition of elastase could help mitigate the adverse consequences of this uncontrolled inflammation. The present experiments with CF sputum demonstrated significant decreases in the elastolytic activity of the liquid (sol) phase after treatment with the complete Trx system, p-rhTrx or DHLA. This inhibitory effect was concentration-dependent with micromolar concentrations of the reductants showing efficacy.

Example 14

[**0234**] The following example demonstrates the simultaneous use of a dithiol and other mucolytic agents to enhance sputum liquefaction.

[**0235**] These experiments demonstrate that dithiol compounds will not only result in sputum liquefaction and mucolysis when used alone, but will also enhance the mucolytic effect of other mucolytic compounds. These compounds include, but are not limited to, compounds which are currently being used as mucolytics, compounds currently being developed as mucolytics or future compounds that will be developed as mucolytics.

[**0236**] Hypertonic saline is currently being used as a mucolytic for cystic fibrosis patients. Hypertonic saline consists of water with high sodium chloride (NaCl) concentrations. Sodium chloride concentrations between 3% and 9% are commonly used in cystic fibrosis. Occasionally, concentrations as high as 12% can be used if tolerated by the patient. The mucolytic effect of hypertonic saline is likely due to disruption of ionic and hydrogen bonding between and within the mucin polymers. As an example of the additive mucolytic effect of dithiol compounds when used in combination with other mucolytics, the mucolytic effect of hypertonic saline was assessed in the presence and absence of pre-reduced recombinant human thioredoxin (p-rhTrx).

[**0237**] Thirty microliters of various sodium chloride concentrations (0.9%, 3%, 6% or 9%) with or without 100 μM p-rhTrx was added to 270 μl of mucus from eight CF patients and incubated for 20 minutes at 37° C. Using a TA Instruments AR-1000 cone and plate rheometer in oscillation mode with a one degree cone and angular velocity of 1 rad/sec, the viscoelasticity of the sputum sample was determined before and after the mucolytic treatment. Changes in viscoelasticity were expressed as a change in the log of the complex modulus (Delta Log G*). Measurements were performed in triplicate for each sample.

[**0238**] Hypertonic saline was able to decrease the viscoelasticity of CF sputum as shown by the decrease in Log G* (**FIG. 17**). With the addition of 100 μM p-rhTrx in combination with the hypertonic saline, a significantly enhanced mucolytic effect was demonstrated (**FIG. 19**). Using 9% hypertonic saline together with 100 μM p-rhTrx produced a 25% greater mucolytic effect than using the hypertonic saline alone (p=0.019).

[**0239**] In another example, p-rhTrx was used in combination with DNase (Pulmozyme). DNase cleaves the DNA strands that are intertwined with the mucin polymers within mucus. Using 1.35 μM DNase, a modest decrease in viscoelasticity is noted (**FIG. 18**). Adding 100 μM p-rhTrx simultaneously with the DNase resulted in substantially greater mucolysis which exceeded that of p-rhTrx alone.

[**0240**] In the case of DNase, the inventors found that the method of administration could influence the mucolytic effect. In particular, the mucolytic effect was dependent on the order of dithiol (p-rhTrx) and DNase administration. Mucus treated for 20 minutes with 100 μM p-rhTrx followed by an additional 20 minutes of treatment with 2.7 μM DNase showed a decrease in sputum viscoelasticity which exceeded the saline control. However, if the order of mucolytic administration was reversed such that DNase was given first followed by p-rhTrx, the mucolytic effect was dramatically

enhanced (**FIG. 19**). The cause of this enhanced effect is not completely clear but may be the result of a direct interaction between DNase and thioredoxin. The inventors have demonstrated that thioredoxin can inhibit DNase activity, which may affect its mucolytic ability. Purified DNA was incubated with 1 or 3 μM thioredoxin and 8 or 16 units of DNase (Pulmozyme) and then analyzed by polyacrylamide gel electrophoresis. DNase alone resulted in complete digestion of the DNA strands (lanes 3 and 6 of **FIG. 20**). By contrast, DNase incubated with p-rhTrx was inhibited as shown by the continued presence of long strands of DNA on the polyacrylamide gel (lanes 4, 5, 7 and 8 of **FIG. 20**).

[**0241**] This inhibitory effect of thioredoxin on DNase activity could be mitigated by the presence of mucin. Lane 2 of **FIG. 21** again demonstrates the ability of DNase to break down DNA strands as evidenced by the absence of DNA in that polyacrylamide gel lane. In the presence of thioredoxin, undigested DNA remains in the lane (lane 3 of **FIG. 21**), suggesting that DNase activity has been inhibited. With the addition of increasing concentrations of mucin in lanes 4, 5 and 6 (**FIG. 21**), DNase activity appears to be partially restored despite the continued presence of thioredoxin. This continued activity of DNase is suggested by the presence of increasing lower molecular weight DNA strands with increasing concentrations of mucin. Mucin polymers have numerous disulfide bonds and other potential targets for reduction by thioredoxin. Thus the mucin may act to competitively inhibit thioredoxin from interfering with the activity of Dnase.

[**0242**] In summary, the inventors have demonstrated that dithiol compounds, using thioredoxin as an exemplary, but non-limiting compound, are useful in concert with other mucolytics such as hypertonic saline and DNase for an enhanced effect. In the case of DNase, this enhanced mucolytic effect can be made more pronounced by staggering the administration such that DNase is given first followed by thioredoxin. Such a treatment schedule as well as the presence of mucins will prevent DNase inactivation and maximize the overall decrease in sputum viscoelasticity.

[**0243**] Each of the publications and other references discussed or cited herein is incorporated herein by reference in its entirety.

[**0244**] While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention, as set forth in the following claims.

What is claimed is:

1. A method to inhibit elastase in a patient that has a disease or condition associated with excessive elastase activity, comprising administering to the patient a composition comprising a compound containing a dithiol active-site in reduced state effective to reduce elastase levels and/or activity, as compared to prior to the step of contacting;

wherein the composition is administered in the absence of a protease inhibitor.

2. The method of claim 1, wherein the composition is administered in the absence of a 1-antitrypsin.

3. The method of claim 1, wherein the composition is administered in the absence of a requirement for elevated temperature conditions.

4. The method of claim 1, wherein the step of contacting the patient with the composition is performed by introducing the composition to the patient by a route selected from the group consisting of nasal, intratracheal, bronchial, direct installation into the lung and inhaled.

5. The method of claim 1, wherein the compound can be catalytically recycled to a reduced state after oxidation of the compound.

6. The method of claim 1, wherein the compound is a non-protein dithiol that can be catalytically recycled to a reduced state after oxidation of the compound.

7. The method of claim 1, wherein the compound is dihydrolipoic acid or a biologically active derivative thereof.

8. The method of claim 1, wherein the compound is orally administered α-lipoic acid, or a biologically active derivative thereof.

9. The method of claim 1, wherein the compound is a protein or peptide having a thioredoxin active-site.

10. The method of claim 9, wherein the thioredoxin active-site comprises the amino acid sequence C-X-X-C, wherein C residues are in reduced state, and wherein X residues are any amino acid residue.

11. The method of claim 9, wherein the thioredoxin active-site comprises the amino acid sequence X-C-X-X-C-X, wherein C residues are in reduced state, and wherein X residues are any amino acid residue.

12. The method of claim 9, wherein the thioredoxin active-site comprises the amino acid sequence X-C-G-P-C-X (SEQ ID NO:2), wherein C residues are in reduced state, and wherein X residues are any amino acid residue.

13. The method of claim 9, wherein the thioredoxin active-site comprises the amino acid sequence W-C-G-P-C-K (SEQ ID NO:3), wherein C residues are in reduced state.

14. The method of claim 9, wherein the protein is a thioredoxin selected from the group consisting of prokaryotic thioredoxin, yeast thioredoxin, plant thioredoxin, and mammalian thioredoxin.

15. The method of claim 9, wherein the protein is human thioredoxin.

16. The method of claim 9, wherein the protein is recombinant human thioredoxin.

17. The method of claim 16, wherein the recombinant human thioredoxin is produced in a substantially reduced, monomeric state.

18. The method of claim 9, wherein the composition further comprises nicotinamide-adenine dinucleotide phosphate (reduced form) (NADPH) for reducing the thioredoxin active site of the protein.

19. The method of claim 18, wherein the composition further comprises thioredoxin reductase.

20. The method of claim 9, wherein the composition further comprises dihydrolipoic acid or a biologically active derivative thereof.

21. The method of claim 1, wherein the patient has cystic fibrosis.

22. The method of claim 1, wherein the patient has chronic obstructive pulmonary disease (COPD).

23. The method of claim 1, wherein the composition is administered to the patient in a pharmaceutically acceptable carrier.

24. The method of claim 1, wherein the compound is administered to the patient in an amount that is between about 1.5 mmoles/kg weight of the patient and about 150 mmoles/kg weight of the patient.

25. The method of claim 1, wherein the compound has a half-life in the patient of between about 5 minutes and about 24 hours.

26. The method of claim 1, further comprising administering DNase to the patient.

27. The method of claim 26, wherein the DNase is administered prior to the compound.

28. A method to increase the liquefaction of mucus or sputum in a patient that has excessively viscous or cohesive mucus or sputum, comprising contacting the mucus or sputum of the patient with a composition comprising a compound containing a dithiol active-site in reduced state effective to increase the liquefaction of the mucus or sputum as compared to prior to the step of contacting.

29. The method of claim 28, wherein the patient has a lung disease in which abnormal or excessive viscosity or cohesiveness of mucus or sputum is a symptom or cause of the disease.

30. The method of claim 28, wherein the step of contacting the mucus or sputum of the patient with the composition is performed by introducing the composition to the patient by a route selected from the group consisting of oral, nasal, intratracheal, bronchial, direct installation into the lung and inhaled.

31. The method of claim 28, wherein the mucus or sputum to be contacted is located in the respiratory tract, the gastrointestinal tract or the reproductive tract of the patient.

32. The method of claim 28, wherein a liquid phase of a total volume of a sample of mucus or sputum from the patient shows a statistically significant increase after administration of the composition.

33. The method of claim 28, wherein the compound is a non-protein dithiol that can be catalytically recycled to a reduced state after oxidation of the compound.

34. The method of claim 28, wherein the compound is dihydrolipoic acid or a biologically active derivative thereof.

35. The method of claim 28, wherein the compound is orally administered α-lipoic acid, or a biologically active derivative thereof.

36. The method of claim 28, wherein the protein is recombinant human thioredoxin.

37. The method of claim 36, wherein the recombinant human thioredoxin is produced in a substantially reduced, monomeric state.

38. The method of claim 36, wherein the composition further comprises dihydrolipoic acid or a biologically active derivative thereof.

39. The method of claim 28, further comprising administering an additional mucolytic compound to the patient.

40. The method of claim 39, wherein the additional mucolytic compound is a DNase.

41. The method of claim 39, wherein the additional mucolytic compound is administered prior to the compound containing a dithiol active-site in reduced state.

42. A composition for use in the inhibition of elastase in a patient, consisting essentially of a compound comprising a dithiol active-site in reduced state and at least one additional agent for treatment of a disease or condition associated with excessive elastase activity, wherein the compound can be catalytically recycled to a reduced state after oxidation of the compound, and wherein the composition does not comprise a protease inhibitor.

43. The composition of claim 42, wherein the compound is dihydrolipoic acid or a biologically active derivative thereof.

44. The composition of claim 42, wherein the compound is a protein or peptide comprising a thioredoxin active-site in reduced form.

45. The composition of claim 44, wherein the thioredoxin active-site comprises the amino acid sequence X-C-X-X-C-X, wherein C residues are in reduced state, and wherein the X residues are any amino acid residue.

46. The composition of claim 44, wherein the thioredoxin active-site comprises the amino acid sequence X-C-G-P-C-X (SEQ ID NO:2), wherein C residues are in reduced state, and wherein the X residues are any amino acid residue.

47. The composition of claim 44, wherein the thioredoxin active-site comprises the amino acid sequence W-C-G-P-C-K (SEQ ID NO:3), wherein C residues are in reduced state.

48. The composition of claim 44, wherein the protein is thioredoxin selected from a group consisting of prokaryotic thioredoxin, yeast thioredoxin, plant thioredoxin, and mammalian thioredoxin.

49. The composition of claim 44, wherein the protein comprises human thioredoxin.

50. The composition of claim 44, wherein the protein is recombinant human thioredoxin.

51. The composition of claim 50, wherein the recombinant human thioredoxin is produced in a substantially reduced, monomeric state.

52. The composition of claim 44, wherein the composition further comprises nicotinamide-adenine dinucleotide phosphate (reduced form) (NADPH).

53. The composition of claim 52, wherein the composition further comprises thioredoxin reductase.

54. The composition of claim 44, wherein the composition further comprises dihydrolipoic acid or a biologically active derivative thereof.

55. The composition of claim 42, wherein the composition further comprises a compound to catalytically reduce the compound comprising a dithiol active-site after the compound comprising the dithiol active-site has been oxidized.

56. The composition of claim 42, wherein the additional agent is formulated separately for administration prior to the administration of the compound comprising a dithiol active-site.

57. A composition for the inhibition of elastase in a patient, comprising α-lipoic acid, wherein the composition is formulated for oral delivery.

58. The composition of claim 57, wherein the composition further comprises at least one additional agent for treatment of a disease or condition associated with excessive elastase activity.

59. A composition for use in the liquefaction of mucus or sputum, comprising a compound comprising a dithiol active-site in reduced state and at least one additional agent for treatment of excessively viscous or cohesive mucus or sputum, wherein the compound can be catalytically recycled to a reduced state after oxidation of the compound.

60. The composition of claim 59, wherein the compound is a non-protein dithiol that can be catalytically recycled to a reduced state after oxidation of the compound.

61. The composition of claim 59, wherein the compound is dihydrolipoic acid or a biologically active derivative thereof.

62. The composition of claim 59, wherein the protein is recombinant human thioredoxin.

63. The composition of claim 62, wherein the recombinant human thioredoxin is produced in a substantially reduced, monomeric state.

64. The composition of claim 62, wherein the composition further comprises dihydrolipoic acid or a biologically active derivative thereof.

65. A composition for use in the liquefaction of mucus or sputum, comprising α-lipoic acid and at least one additional agent for treatment of excessively viscous or cohesive mucus or sputum, wherein the composition is formulated for oral delivery.

66. A method to increase the liquefaction of mucus or sputum in a patient that has excessively viscous or cohesive mucus or sputum, comprising contacting the mucus or sputum in the respiratory tract of the patient with a composition comprising dihydrolipoic acid or a biologically active derivative thereof, wherein the contact of composition increases the volume of the liquid phase in a sample of mucus or sputum from the patient as compared to prior to contact with the composition.

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