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EXPEDIENTE 16-128-893

TÍTULO SOLICITADO: UNA COMPOSICIÓN QUE COMPRENDE UNA SOLUCIÓN NEBULIZADA DE ACTIVADOR DE PLASMINÓGENO DE UROQUINASA DE CADENA SIMPLE (SCUPA) O UN ACTIVADOR DE PLASMINÓGENO TISULAR (TPA) Y UN MÉTODO PARA PRODUCIRLA.

PUBLICACIÓN:

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PRIORIDAD:

US 61/899,739 04/11/2013.

SOLICITANTE:

BOARD OF REGENTS THE UNIVERSITY OF TEXAS SYSTEM

APODERADO:

CAROLINA MERCEDES DAZA MONTALVO

TRÁMITE:

REMISIÓN DE INFORMACIÓN.

JUAN PABLO REYES VILLAMIZAR, mayor de edad, identificado como aparece al pie de mi firma, obrando en calidad de apoderado de la sociedad **LABORATORIOS LEGRAND S.A.**, en el trámite de la referencia y con el propósito de coadyuvar en la decisión sobre la solicitud colombiana 16-128-893, referida a métodos y composición para administrar enzimas al tracto respiratorio de un sujeto, una composición de enzima nebulizada, tal como activadores de plasminógeno y composiciones de perfluorocarbono que comprenden enzimas, tales como activadores de plasminógeno, de manera respetuosa me permito presentar argumentos para demostrar que la solicitud en cuestión incumple los requisitos de patentabilidad aplicables, y no merece por ello, recibir el beneficio patentario.

Superintendencia de Industria y Comercio



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I. REMISIÓN DE INFORMACIÓN.

Con el propósito de demostrar que la solicitud colombiana en trámite carece de los requisitos de patentabilidad establecidos por los artículos 14, 16 y 18 de la Decisión 486, me permito plantear los siguientes argumentos:

En primer lugar, es importante señalar que la modificación en el capítulo reivindicatorio presentada el 10 de noviembre de 2016, corresponde a un cambio formal en las reivindicaciones inicialmente presentadas y que no aportan mayor claridad, concisión o criterios de patentabilidad y por lo tanto no son susceptibles a obtener el privilegio de patente. Veamos:

Falta de claridad, concisión y sustento del capítulo reivindicatorio modificado.

Se observa que aunque la solicitante presentó un nuevo capítulo reivindicatorio, aún persisten problemas de claridad y concisión, como primera medida, la reivindicación 1 reclama una solución enzimática pero no revela la cantidad de los componentes de la misma ni la enzima específica o la sal que puede estar incluida en dicha solución enzimática:

1. Una solución enzimática para administración en las vías respiratorias de un sujeto, que comprende:

- Una enzima que es un activador de plasminógeno, seleccionado del grupo que consiste de un activador de plasminógeno de uroquinasa de cadena simple (scuPA) o un activador de plasminógeno tisular (tPA), y*
- Una sal a una concentración fisiológicamente aceptable.*

De esta reivindicación se entiende que la solución comprende dos componentes, pero dichos componentes son: una enzima definida funcionalmente por ser scuPA o tPA y una sal, lo cual, si bien ya es conocido abarca una cantidad de compuestos posibles que podrían cumplir con dichas restricciones funcionales, no delimitan de forma clara la materia objeto de la solicitud.

Estas mismas falencias son evidentes en las reivindicaciones 2 a 4 que presentan información poco clara y no definen inequívocamente la materia de interés de la solicitante:

2. La solución de la reivindicación 1, en donde el activador de plasminógeno es un scuPA.
3. La solución de la reivindicación 1, que además comprende un amortiguador de pH
4. La solución de la reivindicación 1, en el que la solución enzimática es una solución acuosa.

Los problemas de claridad señalados para la solución, también se hacen presentes en las reivindicaciones 7 a 11, en donde se puede observar que para la composición tampoco se han definido los componentes ni las proporciones de los mismos:

7. Una composición que comprende una solución nebulizada de activador de plasminógeno de uroquinasa de cadena simple (scuPA) o un activador de plasminógeno tisular (tPA) y una sal a una concentración de fisiológicamente aceptable.
8. La composición de la reivindicación 7, que además comprende un agente amortiguador de pH.
9. La composición de la reivindicación 7, en la que la solución es una solución acuosa.
10. La composición de la reivindicación 7, en la que la solución es una solución salina amortiguada con fosfato (PBS).
11. La composición de la reivindicación 7, que comprende scuPA.

De acuerdo con lo anterior, la solicitud falla en cumplir con lo dispuesto por el Artículo 30 de la D. 486 que precisa lo siguiente: *Las reivindicaciones definirán la materia que se desea proteger mediante la patente. Deben ser claras y concisas y estar enteramente sustentadas por la descripción.*

La solicitud además falla en sustentar todas y cada una de las modalidades enseñadas por las reivindicaciones tal como puede observarse en los ejemplos donde su aplicación únicamente se establece para las enzimas tPA y scuPA; dichas enzimas a pesar de estar definidas ampliamente, corresponden a una modalidad entre un grupo indefinido de posibilidades abarcadas por el total de las reivindicaciones, por lo que la extrapolación presentada no es razonable. Así las cosas, las reivindicaciones no encuentran soporte en la descripción por lo que además este criterio se incumple en la presente solicitud.

Resultados a alcanzar

Adicionalmente, la definición tampoco es clara en lo referente al tamaño de partícula de la solución nebulizada ya que las reivindicaciones 12 a 14 se caracterizan por incluir cualquier composición de acuerdo a la reivindicación 7 que tenga dicho tamaño de partícula, lo cual - teniendo en cuenta que la reivindicación 7 tiene un alcance muy amplio - podría afectar la operación de cualquier otra composición para nebulización que pudiera llegar a presentar dicho tamaño de partícula:

12. La composición de la reivindicación 7, en la que la solución nebulizada tiene un tamaño de partícula mediano de entre 2,5 μ m y 10 μ m.

13. La composición de la reivindicación 7, en la que la solución nebulizada tiene un tamaño de partícula mediano de entre 2,5 μ m y 8 μ m.

14. La composición de la reivindicación 7, en la que la solución nebulizada tiene un tamaño de partícula mediano de entre 3,0 μ m y 6 μ m.

Es evidente que el tamaño de partícula depende de la estructura de la composición y sus componentes, no es una característica estructural sino un resultado de dichas características, por lo que estas definiciones no aportan a la claridad del capítulo sino que establecerían un efecto técnico asociado.

Unidad de invención

Adicional a la falta de claridad, el capítulo reivindicatorio no guarda relación estructural o funcional entre sus reivindicaciones ya que se puede identificar que el método de la reivindicación 6 permite obtener cualquier tipo de enzima, no solo tPA o scuPA en una solución. Comprendiendo que la estructura y función de las reivindicaciones independientes es diferente, las reivindicaciones 1 – 5, 6, 7-14 y 15 – 20 se sitúan en materias diferentes dando como resultado al menos cuatro (4) conceptos inventivos diferentes.

Carencia de novedad y nivel inventivo

Pese a las objeciones planteadas en cuanto a claridad, la materia reclamada ya estaba comprendida en el estado de la técnica. Prueba de ello consiste en los

siguientes documentos publicados anteriormente a la fecha de presentación de la solicitud en estudio y que cito a continuación:

La Publicación americana US2003113271 titulada ***"Formulations for pulmonary delivery"*** publicada por Katyama et al., y con fecha 2003-06-19, objeta la novedad de la presente solicitud al referirse a formulaciones para administración pulmonar que incluyen una proteína y un agente tensioactivo no fisiológico en la CMC o por encima de la misma y métodos para prepararla y usarla:

Formulations for pulmonary delivery that include a protein and a non-physiological surfactant at or above the CMC of the surfactant, and methods for preparing and using the same.

Con relación a la reivindicación 1 y sus dependientes de la solicitud en trámite, esta anterioridad incluye formulaciones *enzimáticas para administración en las vías respiratorias de un sujeto, que comprende una proteína terapéutica y un surfactante, que en particular se refiere a un activador de plasminógeno:*

[0002] The present invention relates to surfactant formulations for pulmonary drug delivery and methods for using the same. The formulations include a therapeutic protein and a surfactant. More particularly, the present invention relates to formulations that include a plasminogen activator and a surfactant, which formulations can be used to promote fibrinolysis and/or to reduce inflammation.

(Katyama et al., pág. 1)

Incluso, en dicho documento se encuentran los componentes de modalidades particulares de la solicitud en estudio, tales como la presencia de sales, buffers, producto liofilizado y en general los componentes normales de una formulación para administración por las vías respiratorias:

[0089] The compositions of the invention can be further formulated according to known methods to prepare pharmaceutical compositions. Suitable formulations for administration to a subject include aqueous and non-aqueous sterile injection solutions which can contain anti-oxidants, buffers, bacteriostats, antibacterial and antifungal agents (e.g., parabens, chlorobutanol, phenol, ascorbic acid, an thimerosal), solutes that render the formulation isotonic with the bodily fluids of the intended recipient (e.g., sugars, salts, and polyalcohols), suspending agents and thickening agents. Surfactant formulations can optionally include a spreading agent such as a fatty alcohol (e.g., cetyl alcohol) or a lung surfactant protein in an amount effective to spread the surfactant formulation on the surface of lung alveoli. Suitable solvents include water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol), and mixtures thereof. The formulations can be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and can be stored in a frozen or freeze-dried (lyophilized) condition requiring only the addition of sterile liquid carrier immediately prior to use.

(Katyama et al., pág. 7)

Por lo tanto, la estructura de la formulación, la solución para administración pulmonar y los componentes activos (activadores de plasminógeno) y compuestos asociados ya eran conocidos a partir de Katyama et al.

Tal como se mencionó, la solicitud en estudio busca la protección de moléculas ya conocidas entre las cuales se puede encontrar los activadores de plasminogeno alteplase o reteplase, siendo esto además materia ya reivindicada en el estado de la técnica:

[0042] The term "tissue-type plasminogen activator," which is abbreviated as "PA," refers to a tissue-type plasminogen activator polypeptide, as described herein below, including a tPA pro-peptide (i.e., alteplase or reteplase), and derivatives and structural variants of tPA that contain amino acid substitutions, deletions, additions and/or replacements.

(Katyama et al., pág. 3)

Así entonces, la idea de proveer *una solución enzimática para administración en las vías respiratorias de un sujeto, que comprende una enzima que es un activador de plasminógeno, seleccionado del grupo que consiste de un activador de plasminógeno de uroquinasa de cadena simple (scuPA) o un activador de plasminógeno tisular (tPA), y una sal a una concentración fisiológicamente aceptable*, era conocida a partir del documento de Katyama et al.

Ahora bien, a pesar que este documento por si solo desvirtua la novedad de la solicitud en trámite, la publicación americana US2005036951 "**Methods of treating lung diseases**" publicada por Henderson con fecha 2005-02-17, también objeta la novedad de la solicitud en trámite ya que describe composiciones y métodos para tratar enfermedades del pulmón que implican administrar al sujeto vía pulmonar, orofaríngea, o ruta nasofaríngea un compuesto o composición que contiene un reactivo terapéutico y un elemento objetivo dirigido a un ligando (ver abstract):

Abstract: The present invention discloses compositions and method for treating lung diseases. In preferred embodiments the methods involve administering to the subject via pulmonary, oropharyngeal, or nasopharyngeal route a compound or composition that contains a therapeutic agent and a targeting element directed to a ligand. The ligand is preferably an epitope on plgR receptor.

De la lectura del documento de Henderson se pueden obtener composiciones terapéuticas que comprenden bajos pesos moleculares y que son activas en pulmones:

[0005] Pulmonary administration of therapeutic compositions comprised of low molecular weight drugs has been observed, for example, beta-androgenic antagonists to treat asthma. Other therapeutic agents that are active in the lungs have been administered systemically and targeted via pulmonary absorption. However, not all low molecular weight drugs can be efficaciously administered through the lung. Moreover, pulmonary delivery of higher molecular weight therapeutics, such as polypeptides or proteins, is much more difficult.

(Henderson, pág. 1)

Adicionalmente, ésta anterioridad incluye los mismos componentes de la formulación, siendo parte de la misma el activador plasminogeno tisular tPA y una sal farmacéuticamente aceptable -componentes indispensables de tal desarrollo-. Veamos:

[0444] Expression of sFv- β -IFN in mammalian cells required the use of a suitable signal peptide sequence. The PelB signal peptide is an *E. coli* signal sequence. For mammalian cells we used the tissue plasminogen activator (TPA) signal peptide (GenBank #NM_033011). The TPA signal peptide was fused to the sFV via PCR primer MG TPA-APL10 5' primer (SEQ ID NO: 25) and MG APL10 3' primer (SEQ ID NO: 26).

(Henderson, *pág. 32*)

[0388] The compositions of the present invention provide for delivery of therapeutic agents to a subject in need thereof. The compositions of the invention can further comprise other chemical components, such as diluents and excipients. A "diluent" is a chemical compound diluted in a solvent, preferably an aqueous solvent, that facilitates dissolution of the therapeutic agent in the solvent, and it may also serve to stabilize the biologically active form of the targeting element or one or more of its components. Salts dissolved in buffered solutions are utilized as diluents in the art. For example, preferred diluents are buffered solutions containing one or more different salts. A preferred buffered solution is phosphate buffered saline (particularly in conjunction with compositions intended for pharmaceutical administration), as it mimics the salt conditions of human blood. Since buffer salts can control the pH of a solution at low concentrations, a buffered diluent rarely modifies the biological activity of a biologically active peptide.

(Henderson, *pág. 23*)

Dado que las características técnicas esenciales de la solicitud en trámite corresponden con las determinadas por el documento del estado del arte analizado, no puede atribuirse novedad a la solución enzimática para administración por vías respiratorias reivindicadas.

De acuerdo con los dos documentos analizados, la siguiente tabla permite comparar las características esenciales de la solicitud y el estado de la técnica:

Tabla 1. Comparación de las características esenciales de la solicitud y los documentos citados

Reiv.	Materia reclamada	US2003113271	US2005036951
1	Una solución enzimática para administración en las vías respiratorias de un sujeto, que comprende:	[0002]	[0005]
	Una enzima que es un activador de plasminógeno, seleccionado del grupo que consiste de un activador de plasminógeno de uroquinasa de cadena simple (scuPA) o un activador de plasminógeno tisular (tPA)	[0009]	[0444]
	Una sal a una concentración fisiológicamente aceptable.	[0089]	[0388]
7	Una composición que comprende una solución nebulizada de activador de plasminógeno de uroquinasa de cadena simple (scuPA)	[0041]	[0444]
	o un activador de plasminógeno tisular (tPA)	[0042]	[0444]
	y una sal a una concentración de fisiológicamente aceptable.	[0089]	[0388]

Se concluye entonces que las características esenciales del capítulo reivindicatorio de la solicitud en trámite ya había sido divulgado por los documentos del estado de la técnica y que por tanto la solicitud no cumple con el requisito de novedad.

Por último, tanto las enzimas y las formulaciones reivindicadas por la presente solicitud, no exhiben ninguna propiedad técnica sorprendente o inesperada que pueda ser considerada útil para demostrar el nivel inventivo de la solicitud sobre el estado de la técnica citado, y, en consecuencia, dada la falta de novedad a la materia reivindicada, un técnico medio en la materia habría derivado de forma obvia las modalidades particulares de la materia de la solicitud, bajo una selección de las características técnicas sugeridas por los documentos del estado de la técnica y por los conocimientos y datos propios para llegar al objeto reclamado.

Así las cosas, y con fundamento en los argumentos expuestos, respetuosamente se solicita a ese Despacho declarar la negación de la presente solicitud y ordenar en consecuencia el archivo del expediente respectivo.

II. PRUEBAS.

Ruego que se tengan como pruebas de este escrito, los documentos mencionados en el texto del mismo, y de aquellas que el Despacho considere oficiosamente, las siguientes:

- Publicación americana US2003113271 "**Formulations for pulmonary delivery**". Katyama et al., 2003-06-19. Portada, Pag 1 – 3, 7, 9.
- Publicación americana US2005036951 "**Methods of treating lung diseases**". Henderson 2005-02-17. Portada, pág. 1 – 5, 23 y 32

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(54) **FORMULATIONS FOR PULMONARY DELIVERY**

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(21) Appl. No.: **10/327,476**

(22) Filed: **Dec. 24, 2002**

Related U.S. Application Data

(63) Continuation-in-part of application No. 09/355,522, filed on Oct. 22, 1999, now Pat. No. 6,497,877, filed as 371 of international application No. PCT/US98/01948, filed on Jan. 29, 1998.

(60) Provisional application No. 60/036,566, filed on Jan. 29, 1997.

Publication Classification

(51) **Int. Cl.⁷** **A61L 9/04; A61K 38/48**

(52) **U.S. Cl.** **424/45; 514/2**

(57) **ABSTRACT**

Formulations for pulmonary delivery that include a protein and a non-physiological surfactant at or above the CMC of the surfactant, and methods for preparing and using the same.

FORMULATIONS FOR PULMONARY DELIVERY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to each of U.S. patent application Ser. No. 09/355,522 filed on Oct. 22, 1999 which is the National Phase of PCT/US98/01948 filed Jan. 29, 1998, and claims priority thereto under 35 U.S.C. 371 and further claims priority to U.S. Patent Application Serial No. 60/036,566 filed Jan. 29, 1997, all of which are incorporated by reference in their entirety herein.

FIELD OF THE INVENTION

[0002] The present invention relates to surfactant formulations for pulmonary drug delivery and methods for using the same. The formulations include a therapeutic protein and a surfactant. More particularly, the present invention relates to formulations that include a plasminogen activator and a surfactant, which formulations can be used to promote fibrinolysis and/or to reduce inflammation.

[0005] Presently there is no effective pharmacotherapy for ARDS. Anti-inflammatory therapies (anti-inflammatory drugs or drugs with the capacity of reducing or modifying inflammatory mediators) such as ketoconazole, lisofylline, and steroids have not been beneficial in patients with ARDS (Siegel, Slutsky), and every inflammatory mediator manipulation trial in ARDS to date has been negative (MacIntyre). Antioxidants such as vitamin E, superoxide dismutase, catalase, N-acetylcysteine, and the xanthine oxidase inhibitor, allopurinol, are also not beneficial to patients with ARDS (Siegel; Slutsky).

[0006] Thus, there exists a long-felt need in the art for additional compositions and methods for pulmonary delivery. In particular, there exists a need for preparing formulations for pulmonary drug delivery that preserve the biological activities of therapeutic proteins. To meet this need, the present invention provides formulations suitable for pulmonary delivery, methods for preparing formulations that include a therapeutic protein and a surfactant, and methods for pulmonary delivery of the same.

SUMMARY OF THE INVENTION

[0007] The present invention provides compositions comprising a biologically active protein and a non-physiological surfactant, wherein the non-physiological surfactant is included in an amount greater than the CMC of the surfactant. The compositions are suitable for aerosolization. Optionally, a composition of the invention can further comprise a detectable label.

[0008] Also provided are methods for preparing compositions of the invention. In a representative embodiment the method comprises preparing a composition comprising a biologically active protein and a non-physiological surfactant, wherein the non-physiological surfactant is included in an amount greater than the CMC of the surfactant, and wherein the composition is suitable for pulmonary delivery.

[0009] A composition of the invention comprises a biologically active protein, such as a therapeutic protein or a diagnostic protein. Preferably, the biologically active protein comprises a human protein. In one embodiment of the invention, the biologically active protein comprises a plasminogen activator, and more preferably a tissue-type plasminogen activator.

[0010] Non-physiological surfactants used to prepared compositions of the invention can comprise ionic surfactants or non-ionic surfactants. Preferred surfactants include block copolymer surfactants (e.g., block copolymers of propylene oxide and ethylene oxide, PLURONIC® surfactants, and particularly PLURONIC®-F68 surfactant) and polysorbates (e.g., TWEEN® surfactants, and particularly polysorbate 80 and/or TWEEN®-80 surfactant).

[0011] Surfactants used to prepare a composition of the invention are included in an amount greater than the CMC of the surfactant, typically in an amount from 0.01% (w/w) to 0.5% (w/w), an amount from 0.03% (w/w) to 0.5% (w/w), an amount from 0.05% (w/w) to 0.5% (w/w), an amount from 0.1% (w/w) to 0.5% (w/w), or an amount about 0.1% (w/w).

[0012] In particular embodiments of the invention, a composition comprises fibrinolytic activity, anti-inflammatory activity, or a combination thereof. Representative anti-in-

Table of Abbreviations

ARDS	acute respiratory distress syndrome
CMC	critical micelle concentration
DLS	dynamic light scattering
FTIC	fluorescein isothiocyanate
fMLP	N-formylmethionyl-leucyl-phenylalanine
HPF	high powered field
IL-1	interleukin 1
MDI	metered dose inhaler
mOD	change in optical density
MPO	myeloperoxidase
NO	nitric oxide
PMA	phorbol ester
PPACK	D-Phe-Pro-Arg-chloromethyl ketone HCl
ROS	reactive oxygen species
SK	streptokinase
tPA	tissue plasminogen activator
UK	urokinase (uPA)
uPA	urokinase plasminogen activator
UV	ultraviolet
XO	xanthine oxidase

DESCRIPTION OF THE RELATED ART

[0003] Effective treatment of respiratory diseases and disorders often involves direct delivery of medications to the lungs of the patient. Pulmonary delivery is preferable to oral, intravenous and subcutaneous delivery because it is non-invasive, localized, permits rapid action of medicament, requires a relatively small dosage, is not filtered through the liver of the patient, and produces a low incidence of systemic side effects. However, most medications for the treatment of lung diseases and disorders are not available in formulations suitable for respiratory delivery, in part because lung delivery methods can disrupt the structure of therapeutic proteins.

[0004] As one example, acute respiratory distress syndrome (ARDS) contributes significantly to human morbidity and mortality. It is estimated that tens of thousands of patients develop ARDS annually in the United States and more than 50% of them die (Abraham et al., 2000; Artigas et al., 1998). Importantly, ARDS patients who survive hospitalization have no increased risk of subsequent death (Davidson et al., 1999).

flammatory activities include inhibition of radical oxygen (reactive oxygen species, ROS) production and inhibition of lung leak, as described in the Examples.

[0013] When preparing a composition of the invention, aerosolization can be performed using any suitable device. Representative devices include a jet nebulizer, an ultrasonic nebulizer, a metered dose inhaler, and an aerosolization device based on forced passage through a nozzle.

[0014] The present invention further provides methods for pulmonary delivery of a biologically active protein to a subject, the method comprising administering an effective amount of a composition, wherein the composition comprises a biologically active protein and a non-physiological surfactant, and wherein the non-physiological surfactant is included in an amount greater than the CMC of the surfactant.

[0015] Typically, a composition of the invention is administered to a mammalian subject, preferably a human subject. A subject can display a lung disease or disorder, for example an inflammatory disease or disorder (e.g., acute lung injury, acute respiratory distress syndrome, asthma, bronchitis, or cystic fibrosis), an embolism, or cancer.

[0016] In accordance with the disclosed methods, administration of a composition of the invention can be used to treat a lung disease or disorder via pulmonary delivery of the composition to a subject. Thus, the present invention includes pulmonary administration of an effective amount of a composition, whereby lung inflammation is reduced, whereby embolism is reduced, or whereby cancer growth is inhibited. When an aerosol composition of the invention further includes a detectable label, the disclosed methods can further comprise detecting the detectable label.

[0017] In one embodiment of the present invention, a method is provided for inhibiting pulmonary inflammation in a subject via pulmonary administration of an effective amount of a composition to a subject, wherein the composition comprises a biologically active tissue-type plasminogen activator and a non-physiological surfactant, wherein the non-physiological surfactant is included in an amount greater than the CMC of the surfactant, and whereby pulmonary inflammation is reduced in the subject.

[0018] Accordingly, it is an object of the present invention to provide compositions for pulmonary drug delivery, and methods for preparing and using the same. This object is achieved in whole or in part by the present invention. An object of the invention having been stated above, other objects and advantages of the present invention will become apparent to those skilled in the art after a study of the following description of the invention and non-limiting Examples.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] FIG. 1 shows the effect of tPA on superoxide anion production by human neutrophils stimulated with PMA in vitro. Adding tPA concentrations of 20-100 $\mu\text{g/ml}$ significantly reduced neutrophil O_2^- production compared to values obtained following no additions or addition of 5 $\mu\text{g/ml}$ tPA. Each value is the mean $\pm$ standard error of three or more determinations. Asterisk, $p < 0.05$.

[0020] FIG. 2 shows the effect of L-arginine on O_2^- production by human neutrophils in vitro. Adding 175 or

700 $\mu\text{g/ml}$ of L-arginine, a component of the tPA preparation, did not decrease ($p > 0.05$) O_2^- production by PMA stimulated neutrophils in vitro. By comparison, adding 1400 or 3500 $\mu\text{g/ml}$ of L-arginine increased O_2^- production by PMA stimulated neutrophils in vitro. Each value is the mean $\pm$ standard error of three or more determinations. Asterisk, $p < 0.05$.

[0021] FIG. 3 shows the effect of tPA or PPACK-treated tPA on O_2^- production by neutrophils in vitro. Compared to PMA alone, neutrophils treated with tPA or PPACK treated tPA had comparable ($p > 0.05$) decreases in O_2^- generation. Neutrophils were pretreated with tPA, PPACK, or PPACK:tPA (5:1 mole ratio) and subsequently activated by PMA. The time course of cytochrome C reduction (O_2^- production) was monitored by changes in optical density (mOD). Neither neutrophils alone, tPA alone, PPACK alone, or acetic acid (PPACK vehicle) altered cytochrome C reduction. The V_{max} (mOD/minute) or rate of cytochrome C reduction was significantly less following administration of both tPA+PMA, filled square (0.198 ± 0.09), and PPACK:tPA+PMA, open triangle (0.182 ± 0.01), when compared to administration of PMA alone, filled diamond (0.476 ± 0.15); $p = 0.0004$ and 0.006 , respectively. In addition, there was no significant difference in V_{max} between the tPA+PMA and PPACK:tPA+PMA groups ($p > 0.05$). Data represent the mean of triplicate experiments.

[0022] FIG. 4 shows the effect of tPA on produced by xanthine oxidase in vitro. Adding increasing amounts of tPA did not decrease ($p > 0.05$) generation by xanthine oxidase (XO) in vitro. Each value is the mean $\pm$ standard error of three or more experiments.

[0023] FIG. 5 shows the effect of tissue plasminogen activator (tPA) on carrageenan induced edema in rat footpad. Open square, saline alone; filled square, carrageenan alone; filled triangle, 12 mg/kg tPA+carrageenan; filled circle, 6 mg/kg tPA+carrageenan; open circle, 3 mg/kg tPA+carrageenan. Edema index reflects changes in hind paw volume at different times after plantar carrageenan or saline administration. Data represent the mean ($\pm$ SEM) for ten experiments. Asterisk represents $p \leq 0.05$ tPA when compared to carrageenan alone.

[0024] FIG. 6 shows the effect of streptokinase (SK) on carrageenan induced edema in rat footpad. Open square, saline alone; filled square, carrageenan alone; filled triangle, 40,000 U/kg SK+carrageenan; filled circle, 20,000 U/kg SK+carrageenan; open circle, 10,000 U/kg SK+carrageenan. Edema index reflects changes in hind paw volume at different times after plantar carrageenan or saline administration. Data represent the mean ($\pm$ SEM) for ten experiments. Asterisk represents $p \leq 0.05$ for SK vs. carrageenan alone.

[0025] FIG. 7 shows modulation of IL-1 induced lung leak. Data are presented as the means $\pm$ SEM. Asterisk represents $p < 0.05$ vs. IL-1 control group. Lung leak induced by L-arginine was not significantly different than that induced by saline alone.

[0026] FIG. 8 shows tPA induced inhibition of oxidant production by a rat alveolar macrophage line. Cells were pretreated with tPA (100 $\mu\text{g/ml}$) or vehicle, and subsequently exposed to phorbol ester (PMA, 1.25 $\mu\text{g/ml}$), zymosan (ZMA, 60 $\mu\text{g/ml}$), or opsonized zymosan (opZMA, 60 $\mu\text{g/ml}$). Large open circle, control; large closed circle, tPA;

12

12

open square, PMA; closed square, PMA+tPA; open triangle, ZMA; closed triangle, ZMA+tPA; small open circle, opZMA; and small closed circle, opZMA+tPA. Data represent the mean of triplicate estimations.

[0027] FIG. 9 shows tPA alone significantly reduced the rate of apoptosis and the percent apoptotic cells at 24 hours. Open circle, cells alone; closed circle, tPA alone; open square, PMA alone; closed square, fMLP alone; open triangle, tPA+PMA; and closed triangle, tPA+fMLP.

[0028] FIG. 10 is a bar graph that shows the specific activity of tPA recovered following nebulization performed as described in Example 6. PS-80, TWEEN®-80 surfactant (ICI Americas, Inc. of Bridgewater, N.J.); neb'd, nebulized; F-68, PLURONIC® F68 surfactant (BASF Corporation of Mount Olive, N.J.).

[0029] FIG. 11 is a line graph that depicts inhibition of human neutrophil O_2^- production by nebulized tPA (100 μ g/ml) containing 0.01% TWEEN®-80 surfactant (ICI Americas Inc. of Bridgewater, N.J.). Data are mean ($\pm$ SEM) of two experiments performed in triplicate. *, $p < 0.05$ when compared to PMA alone.

[0030] FIG. 12 is a bar graph that depicts the log of the Aggregation Index for each of the indicated samples. A value < 1 (bold line) indicates substantially no aggregation. Nebulized tPA in the absence of surfactant (Genetech of South San Francisco, Calif.) is substantially aggregated when compared to non-nebulized tPA (native). Co-nebulization of tPA and TWEEN®-80 surfactant reduces nebulization-induced tPA aggregation.

[0031] FIG. 13 is a bar graph that depicts the percent of recovered tPA having fibrinolytic activity, which was assessed as described in Example 7. Co-nebulization of tPA and at least about 0.1% TWEEN®-80 protects tPA in a biologically active form during the nebulization process.

[0032] FIG. 14 is a line graph that depicts the ability of nebulized tPA (neb'd tPA) to inhibit PMA-induced ROS production by neutrophils. The assay was performed as described in Example 7.

DETAILED DESCRIPTION OF THE INVENTION

[0033] I. Definitions

[0034] While the following terms are believed to be well understood by one of ordinary skill in the art, the following definitions are set forth to facilitate explanation of the invention.

[0035] The terms "composition" and "formulation" are used interchangeably to refer to a product which results by combining or mixing more than one element or ingredient.

[0036] The term "aerosolization" refers to a process whereby a liquid formulation is converted to an aerosol. Representative devices for aerosolization include a jet nebulizer, an ultrasonic nebulizer, a metered dose inhaler, and an aerosolization device based on forced passage through a nozzle. The resulting compositions are referred to herein as "aerosol" compositions.

[0037] The phrase "suitable for pulmonary delivery" means that a protein included in the composition remains biologically active following pulmonary delivery.

[0038] The term "surfactant" refers to an agent having surface active, emulsifying, dispersing, solubilizing, and/or wetting activity.

[0039] The term "critical micelle concentration," abbreviated as "CMC," refers to a minimal concentration of monomer surfactant at which the surfactant monomers polymerize to form micelles.

[0040] The term "micelle" refers to a globular polymer of surfactant monomers.

[0041] The term "plasminogen activator" refers to a tissue-type plasminogen activator or to a urokinase plasminogen activator.

[0042] The term "tissue-type plasminogen activator," which is abbreviated as "tPA," refers to a tissue-type plasminogen activator polypeptide, as described herein below, including a tPA pro-peptide (i.e., alteplase or reteplase), and derivatives and structural variants of tPA that contain amino acid substitutions, deletions, additions and/or replacements.

[0043] The term "fibrinolytic" refers to an activity that promotes blood clot dissolution involving digestion of insoluble fibrin by plasmin. For example, fibrinolytic activity can comprise activation of plasminogen to plasmin.

[0044] The term "anti-inflammatory" refers to an activity that reduces or prevents inflammation.

[0045] The term "inflammation" refers to a condition typically characterized by redness, warmth, swelling, and/or pain, which is produced in response to injury or infection. The term "inflammation" encompasses local as well as systemic responses. Local inflammation involves increased blood flow, vasodilation, and/or infiltration leukocytes into tissues, and in some severe cases, intravascular thrombosis, damage to the blood vessels and blood extravasation. Systemic inflammation can involve fever, leukocytosis, and/or release of acute phase reactants into the serum.

[0046] The terms "inflammatory disease" and "inflammatory disorder" refer to conditions characterized by inflammation, as well as to symptoms of inflammation resulting from a separate disease or condition. Thus, as described further herein below, the terms "inflammatory disease" and "inflammatory disorder" encompass inflammation associated with acute lung injury, acute respiratory distress syndrome, arthritis, asthma, bronchitis, cystic fibrosis, reperfusion injury artery occlusion, stroke, ultraviolet light induced injury, vasculitis, autoimmune disease, transplantation, and/or leukocyte dysfunction.

[0047] The term "about", as used herein when referring to a measurable value such as an amount, a temporal duration, etc., is meant to encompass variations of $\pm 20\%$ or $\pm 10\%$, more preferably $\pm 5\%$, even more preferably $\pm 1\%$, and still more preferably $\pm 0.1\%$ from the specified value, as such variations are appropriate to perform the disclosed methods.

[0048] The terms "a," "an," and "the" are used in accordance with convention in the art to refer to one or more.

[0049] II. Aerosolization Methods

[0050] The present invention provides methods for preparing formulations for pulmonary delivery via nebulization or other means, and the formulations produced thereby. A composition of the invention comprises a protein and a

cide gene products, and combinations thereof. See Kirk & Mule (2000) *Hum Gene Ther* 11:797-806; Mackensen et al. (1997) *Cytokine Growth Factor Rev* 8:119-128; Walther & Stein (1999) *Mol Biotechnol* 13:21-28; and references cited therein.

[0087] A protein used to prepare a composition of the invention can also comprise a diagnostic protein. The term "diagnostic protein" refers to a protein whose binding properties are indicative of a particular condition, disease, or disorder. A representative diagnostic protein comprises a protein that specifically binds to, or is indicative of, a lung cancer cell.

[0088] IV. Methods for Pulmonary Administration to a Subject

[0089] The compositions of the invention can be further formulated according to known methods to prepare pharmaceutical compositions. Suitable formulations for administration to a subject include aqueous and non-aqueous sterile injection solutions which can contain anti-oxidants, buffers, bacteriostats, antibacterial and antifungal agents (e.g., parabens, chlorobutanol, phenol, ascorbic acid, an thimerosal), solutes that render the formulation isotonic with the bodily fluids of the intended recipient (e.g., sugars, salts, and polyalcohols), suspending agents and thickening agents. Surfactant formulations can optionally include a spreading agent such as a fatty alcohol (e.g., cetyl alcohol) or a lung surfactant protein in an amount effective to spread the surfactant formulation on the surface of lung alveoli. Suitable solvents include water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol), and mixtures thereof. The formulations can be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and can be stored in a frozen or freeze-dried (lyophilized) condition requiring only the addition of sterile liquid carrier immediately prior to use.

[0090] The formulations according to the invention are buffered to a pH of from about 5 to about 7, preferably about 6. Suitable buffers are those which are physiologically acceptable upon administration by inhalation. Such buffers include citric acid buffers and phosphate buffers, of which phosphate buffers are preferred. Particularly preferred buffers for use in the formulations of the invention are monosodium phosphate dihydrate and dibasic sodium phosphate anhydrous.

[0091] The present invention provides that an effective amount of a non-pathogenic virus is administered to a subject. The term "effective amount" is used herein to describe an amount of a non-pathogenic virus sufficient to elicit a desired biological response. For example, when a formulation comprises a plasminogen activator, an effective amount comprises an amount sufficient to promote fibrinolysis, to reduce inflammation, to reduce oxidative injury, and/or to reduce oxidant production. An effective amount can also comprise an amount sufficient to elicit an anti-cancer activity, including cancer cell cytotoxicity, inhibition of cancer growth, inhibition of cancer metastasis, and/or cancer resistance.

[0092] For diagnostic applications, a detectable amount of a composition of the invention is administered to a subject. A "detectable amount," as used herein to refer to a diagnostic composition, refers to a dose of such a composition that

the presence of the composition can be determined in vivo following pulmonary administration.

[0093] Actual dosage levels of active ingredients in a composition of the invention can be varied so as to administer an amount of the composition that is effective to achieve the desired diagnostic or therapeutic outcome for a particular subject. Administration regimens can also be varied. A single injection or multiple injections can be used. The selected dosage level and regimen will depend upon a variety of factors including the activity of the therapeutic composition, formulation, the route of administration, combination with other drugs or treatments, the disease or disorder to be detected and/or treated, and the physical condition and prior medical history of the subject being treated. Determination and adjustment of an effective amount or dose, as well as evaluation of when and how to make such adjustments, are known to those of ordinary skill in the art of medicine.

[0094] For additional guidance regarding formulation, dose and administration regimen, see Berkow et al. (1997) *The Merck Manual of Medical Information*, Home ed. Merck Research Laboratories, Whitehouse Station, N.J.; Goodman et al. (1996) *Goodman & Gilman's the Pharmacological Basis of Therapeutics*, 9th ed. McGraw-Hill Health Professions Division, New York; Ebadi (1998) *CRC Desk Reference of Clinical Pharmacology*. CRC Press, Boca Raton, Fla.; Katzung (2001) *Basic & Clinical Pharmacology*, 8th ed. Lange Medical Books/McGraw-Hill Medical Pub. Division, New York; Remington et al. (1975) *Remington's Pharmaceutical Sciences*, 15th ed. Mack Pub. Co., Easton, Pa.; Speight et al. (1997) *AverV's Drug Treatment: A Guide to the Properties, Choice, Therapeutic Use and Economic Value of Drugs in Disease Management*, 4th ed. Adis International, Auckland/Philadelphia, Pa.

[0095] Pulmonary administration of a surfactant composition of the present invention can be combined with other techniques for pulmonary delivery, for example carbon dioxide enhancement of inhalation therapy (see e.g., U.S. Pat. No. 6,440,393) and bronchodilation (see e.g., U.S. Pat. No. 5,674,860 and U.S. Published Patent Application No. 20020151597). A treatment regimen can also comprise pulmonary delivery with other delivery routes (e.g., oral and intravascular delivery).

[0096] V. Applications

[0097] The surfactant compositions of the present invention, and methods for pulmonary administration of the compositions to a subject, are useful for the treatment of a disease or disorder of the lung, such as an infection, an immunodeficiency syndrome, an inflammatory disease, an autoimmune disease, a neoplasm, or cancer. In particular, the present invention provides improved methods for formulating therapeutic proteins for pulmonary delivery.

[0098] It is envisioned that the disclosed methods are generally useful in mammalian subjects, including human and non-human subjects. The term "subject" generally refers to mammalian animals, including livestock animals (e.g., ungulates, such as bovines, buffalo, equines, ovines, porcines and caprines), primates (e.g., monkeys, chimpanzees, baboons, and gorillas), as well as rodents (e.g., mice, hamsters, rats and guinea pigs), canines, felines, and rabbits. The term "non-human" is meant to include all mammalian animals, especially mammals and including primates other than human primates.

[0099] In a one embodiment of the invention, surfactant formulations comprising plasminogen activator are prepared. Preferably, such compositions have fibrinolytic activity, anti-inflammatory activity, or a combination thereof. Plasminogen activator compositions of the invention can also have anti-cancer activity and/or can be used to ameliorate unwanted side-effects of anti-cancer therapies.

[0100] As disclosed herein, plasminogen activator can inhibit leukocyte generation of oxygen radicals (e.g., hydroxides, peroxides, superoxides) by a mechanism that is independent of thrombolytic activity and scavenging of oxygen free radicals. By separating the thrombolytic and the anti-inflammatory functions of plasminogen activator, the present invention provides a method of reducing tissue damage due to oxidative injury (e.g., reperfusion injury) while mitigating complications from excessive bleeding, such as stroke and intracerebral hemorrhage. Moreover, because the present invention does not inhibit neutrophil migration and infiltration, use of plasminogen activator as an anti-inflammatory agent does not interfere with processes mediated at least in part by neutrophils such as, for example, wound healing or tissue remodeling, which is a shortcoming of existing steroidal and non-steroidal anti-inflammatory agents.

[0101] Representative therapeutic embodiments of the methods of the present invention are described herein below, including methods for pulmonary administration to modulate fibrinolytic balance, to reduce inflammation, and to inhibit cancer growth. Representative embodiments of the invention for diagnosis and/or imaging or pulmonary diseases and disorders. The present invention also provides that the disclosed therapeutic and diagnostic methods can be used in combination. In addition, the disclosed methods can be used in combination with therapeutic and diagnostic methods known in the art. For example, for the treatment of ARDS, a aerosol composition of the invention can be used in combination with other ARDS treatments, including tPA administration via an alternate administrative route (e.g., parenteral administration, such as intravascular injection).

[0102] V.A. Fibrinolytic Balance

[0103] Plasminogen activators play an important physiological role in the regulation of thrombolysis. This action is exploited therapeutically in conditions such as, for example, acute myocardial infarction, pulmonary embolism, and thrombotic stroke.

[0104] An equilibrium between two opposing reactions, coagulation and fibrinolysis, maintains an intact vascular endothelium. To stop blood loss from a leaking blood vessel, blood clots form a hemostatic plug at the site of a break in the vessel wall. But if the blood clot obstructs flow through a blood vessel, myocardial infarction, pulmonary embolism, or thrombotic stroke can result.

[0105] The interruption of flow through the blood vessel will lead to tissue ischemia. In this condition, the tissue is deprived of oxygen and becomes jeopardized, a state in which the tissue is injured but still potentially viable. If the hypoxic condition is maintained for a period of several hours, the tissue becomes necrotic and cannot recover. Therefore, it is important that reperfusion, the restoration of blood flow, be accomplished as soon as possible to minimize tissue necrosis. See Hansen (1995) *Circulation* 91:1872-85.

Plasminogen activator can be used to induce thrombolysis in patients with acute myocardial infarction. See e.g., Dakik & Nasrallah (2001) *Heart Dis* 3:362-4, Kehl et al. (1996) *Intensive Care Med* 22:968-71, and Munkvad (1993) *Dan Med Bull* 40:383-408. This benefit is due to blood clot fibrinolysis and timely opening of the infarct-related artery. Thrombolysis of brachial arteries has also been reported (Grieg, 1998).

[0106] Surfactant formulations of plasminogen activator of the present invention can be administered to promote fibrinolysis of pulmonary embolisms. As disclosed herein, aerosol compositions retain fibrinolytic activity and are effectively administered to the lung.

[0107] V.B. Inflammation

[0108] Reperfusion is also associated with harmful effects of neutrophil activation and tissue infiltration. The nature of the neutrophil-mediated injury is not fully characterized but is in part due to the production of superoxide anion (O_2^-) and/or related oxidative products. This sequence of events (activation of white blood cells, release of toxic mediators, and resultant pathophysiology in the host) is common to many inflammatory diseases.

[0109] The present invention also provides compositions and methods for treating conditions associated with oxidative injury. For example, aerosol compositions comprising tissue plasminogen activator can be used to reduce cell and/or tissue damage due to oxidative injury, and to inhibit oxidant production by leukocytes. Tissue at risk of oxidative injury may include blood-perfused tissue and inflamed tissue.

[0110] Inflammatory diseases and disorders that can be treated using the disclosed compositions and methods include but are not limited to acute lung injury, acute respiratory distress syndrome, arthritis, asthma, bronchitis, cystic fibrosis, reperfusion injury artery occlusion, stroke, ultraviolet light induced injury, and/or vasculitis. The inflammation can be symptomatic of a separate disease or condition, such as autoimmune disease and transplantation. Inflammatory diseases and disorders also include those conditions characterized by leukocyte dysfunction. The inflammation can be acute, chronic, or temporary inflammation. See e.g., Weissmann et al. (1982) *Ann N Y Acad Sci* 389:11-24, Goldstein et al. (1982) *Ann N Y Acad Sci* 389:368-79, Janoff (1985) *Annu Rev Med* 36:207-16, Hart & Fritzler (1989) *J Rheumatol* 16:1184-91, Doring (1994) *Am J Respir Crit Care Med* 150:S114-7, Demling (1995) *Annu Rev Med* 46:193-202, Hansen (1995) *Circulation* 91:1872-85, Dakik & Nasrallah (2001) *Heart Dis* 3:362-4, Kehl et al. (1996) *Intensive Care Med* 22:968-71, and Munkvad (1993) *Dan Med Bull* 40:383-408.

[0111] In one embodiment of the invention, nebulized tPA formulations are used to treat acute respiratory distress syndrome (ARDS). ARDS is an acute inflammatory disease that involves the sequestration of neutrophils in the lungs (Cooper et al., 1988; Fulkerson et al., 1996). Neutrophils are the primary instigators of lung injury via the generation of ROS (Idell et al., 1989; Idell et al., 1991). Fibrin deposition is also a hallmark of ARDS and may contribute to neutrophil retention in the lung (Cooper et al., 1988; Fulkerson et al., 1996; Idell et al., 1991). This phenomenon may be due to impairment of the intrinsic ability of lung epithelial cells to

produce plasminogen activator with an associated increase in plasminogen activator inhibitor (PAI)-1, which antagonizes tPA (Idell et al., 1989; Idell et al., 1991).

[0112] As disclosed in the Examples, uPA lacks the inhibitory effect on neutrophil ROS production that tPA possesses. Thus, targeted pulmonary delivery of tPA for the treatment of ARDS may be particularly advantageous by providing fibrinolytic and anti-inflammatory activities to the lung while minimizing systemic fibrinolysis.

[0113] In another embodiment of the invention, a surfactant formulation for the treatment of ARDS can include both uPA and tPA. uPA is the predominant plasminogen activator in the lungs and is depressed in ARDS (Bertozzi et al., 1990; Marshall et al., 1990). Thus, treatment can include pulmonary delivery of both tPA and uPA.

[0114] Reduced tissue inflammation can be assayed by detecting proteins induced by inflammation, such as cytokines, monokines, receptors, and proteases. For example, histamine can be measured using a fluorescent assay described by Shore et al. (1959) *J Pharmacol Exp Ther* 127:182-186. Nitric oxide can be measured using a chemiluminescent assay described by Hybertson (1994) *Anal Lett* 127:3081-3093.

[0115] Reduced inflammation can also be assessed by measuring a reduction in oxidant production, including oxidant production by neutrophils, macrophages, monocytes eosinophils, mast cells and/or basophils. Representative methods for assaying the production of oxidants by inflammatory cells are described in the examples. Neutrophil function can also be assayed using techniques known in the art, for example, as described by Bell et al. (1990) *Br Heart J* 63:82-7, Riesenberget al. (1995) *Br Heart J* 73:14-9, Zivkovic et al. (1995) *J Pharmacol Exp Ther* 272:300-9.

[0116] V.C. Lung Cancer

[0117] Reduced levels of tissue-type plasminogen activator are also observed in the core and periphery of non-small cell lung carcinoma (Pavey et al., 1999), suggesting that administration of tPA could be ameliorative in this clinical context as well. Representative methods for detecting and monitoring the progress of non-small cell lung carcinoma are described in U.S. Pat. Nos. 6,242,204; 5,795,871; and 5,756,512; among other places. [000x] Reduced levels of plasminogen activity are observed in patients undergoing conventional cancer therapies, for example, in response to chemotherapy (Ruiz et al., 1989) and radiation treatment (Ts'ao et al., 1983), suggesting that administration of nebulized PA can be used to minimize thromboembolic side-effects associated with these therapies.

[0118] V.D. Pulmonary Imaging

[0119] An aerosol composition of the invention can also comprise a detectable label. Preferably, the detectable label can be detected in vivo, for example by using any one of techniques including but not limited to magnetic resonance imaging, scintigraphic imaging, ultrasound, or fluorescence. Thus, representative detectable labels include fluorophores, epitopes, radioactive labels, and contrast agents.

[0120] In one embodiment of the invention, the detectable label is a protein, e.g., a fluorescent protein. Alternatively, the detectable label is conjugated to a protein to be administered. For example, a composition of the invention can

comprise a diagnostic protein which is conjugated or otherwise bound to a detectable label. Representative detectable labels, labeling methods, and imaging systems suitable for pulmonary imaging and diagnosis are described in Desai (2002) *Clin Radiol* 57:8-17, McLoud (2002) *Clin Chest Med* 23:123-36, and McWilliams et al. (2002) *Oncogene* 21:6949-59, among other places.

EXAMPLES

[0121] The following Examples have been included to illustrate modes of the invention. Certain aspects of the following Examples are described in terms of techniques and procedures found or contemplated by the present co-inventors to work well in the practice of the invention. These Examples illustrate standard laboratory practices of the co-inventors. In light of the present disclosure and the general level of skill in the art, those of skill will appreciate that the following Examples are intended to be exemplary only and that numerous changes, modifications, and alterations can be employed without departing from the scope of the invention.

Example 1

[0122] Plasminogen Activator Inhibits Oxidant Production

[0123] Summary. The following example shows that tissue plasminogen activator inhibits super oxide production by human neutrophils. See Stringer et al. (1997) *Inflammation* 21:27-34. tPA significantly reduced O_2^- production by PMA stimulated human neutrophils in vitro. The inhibitory effect of tPA was not dependent on tPA proteolytic activity, not related to L-arginine in its formulation, and not a consequence of its direct scavenging of O_2^- . These observations show that tPA has another action, inhibition of neutrophil O_2^- production, which may be used to reduce neutrophil O_2^- production and prevent oxidative injury.

[0124] These results indicate that tPA acts directly on the neutrophil to reduce O_2^- production, independent of fibrinolytic activity. These observations could have important clinical implications for optimizing the efficacy of tPA in the management of myocardial infarction as well as other inflammatory processes where a contribution by neutrophil derived O_2^- is likely. Indeed, the possibility that tPA might have anti-inflammatory effects is supported by our related in vivo findings shown below.

[0125] Recovery and Purification of Human Neutrophils. Human neutrophils were isolated from the whole blood of a single, healthy, drug-free donor using a percoll density gradient (POLYMORHPREP™ from Nycomed Pharma of Oslo, Norway) (Ferrante and Thong, 1980). Cells were then suspended in Krebs-Ringers-Phosphate-Dextrose (KRPD) buffer (serum-free), counted, and assessed for viability using trypan blue exclusion. Tissue plasminogen activator (tPA, alteplase from Genentech of South San Francisco, Calif.) was reconstituted following the manufacturer's instructions using sterile water for injection to produce a final concentration of 1 mg/ml. All experiments were performed at 37° C. and pH 7.4, under sterile conditions.

[0126] Measurement of Neutrophil O_2^- Generation by tPA. tPA was added to the neutrophil suspension in sufficient quantities to produce final concentrations of 5, 20, 40, or 100

$\mu\text{g/ml}$. The effect of L-arginine on neutrophil O_2^- generation was also evaluated because L-arginine is a precursor of nitric oxide (NO) and the standard formulation of tPA contains 700 mg L-arginine/20 mg tPA. L-arginine (Sigma Chemical Co. of St. Louis, Mo.) concentrations of 175, 700, 1400, or 3500 $\mu\text{g/ml}$ were evaluated that corresponded to the tPA concentrations used above. Release of O_2^- by neutrophils (5×10^6 cells/ml) stimulated with phorbol myristate acetate (PMA, 1.25 $\mu\text{g/ml}$) was determined during a 30 minute incubation in the absence or presence of each concentration of tPA or L-arginine. O_2^- generation was determined spectrophotometrically by measuring superoxide dismutase (SOD) inhibitable horse heart ferricytochrome C reduction (Babior et al., 1973; Fantone and Kinnes, 1983). Experiments were performed in triplicate.

[0127] PPACK Inhibition of tPA. D-Phe-Pro-Arg-chloromethyl ketone HCl (PPACK, available from Calbiochem of San Diego, Calif.) is an irreversible serine protease inhibitor, that inhibits the proteolytic activity of tPA in vitro (Lijnen et al., 1984). tPA was incubated in the presence of PPACK at varying molar ratios (PPACK:tPA: 5:1, 25:1, 100:1, or 1000:1) for 10 minutes, after which PPACK:tPA complexes or tPA alone (100 $\mu\text{g/ml}$) were incubated with plasminogen (375 $\mu\text{g/ml}$) for 5 hours in a cell incubator (5% CO_2 in air) at 37° C. Subsequently, 50 μl of each of the incubated samples were subjected to 7.5% acrylamide gel electrophoresis along with tPA (100 $\mu\text{g/ml}$), plasminogen (375 $\mu\text{g/ml}$), and plasmin (1 U/ml). Each gel was run at 30V for 16 hours and protein bands were visualized by Coomassie blue stain.

[0128] The effect of the PPACK:tPA complex on human neutrophil O_2^- production was also examined. Briefly, the cell suspension (5×10^6 cells/ml) was divided into four groups: tPA (100 $\mu\text{g/ml}$); PPACK:tPA (5:1); PPACK (140 μM) alone, and PPACK vehicle (10 mM acetic acid). Cells (250 μl of $5 \times 10^6/\text{ml}$) from each group were then plated into a 96-well microtiter plate and incubated for 30 minutes at 37° C. in a cell incubator. Cells were then exposed to PMA (1.25 $\mu\text{g/ml}$) so that the following conditions were met (in triplicate): tPA alone, tPA+PMA, PPACK:tPA alone, PPACK:tPA+PMA, PPACK alone, PPACK+PMA, PMA alone, PPACK vehicle, and cells alone. The plate was then incubated for an additional 30 minutes at 37° C. in a cell incubator after which it was placed in a SPECTRAMAX® plate reader (Molecular Devices of Menlo Park, Calif.) and O_2^- -production was measured as cytochrome C reduction (550 nm OD) every five minutes for 2 hours (Waud et al., 1975). The kinetic disposition of each treatment was compared.

[0129] Measurement O_2^- of Scavenging by tPA. The ability of tPA to scavenge O_2^- was determined by measuring reduction of cytochrome C during a 30 minutes incubation with purified xanthine oxidase (1.6 U/ml) and hypoxanthine in the presence or absence of tPA (concentrations previously mentioned) (Waud et al., 1975). Experiments were performed in triplicate.

[0130] Data Analysis. The mean and standard error of the mean ($\pm\text{SEM}$) for data were determined for each experiment. Treatment groups were compared to each other and to positive and negative controls by analyses of variance and unpaired student's t tests. Concentration dependent effects

were assessed by linear regression followed by an F test for significance. A p value of less than 0.05 was considered significant.

[0131] Effect of tPA on Neutrophil O_2^- Generation In vitro. Adding increasing amounts of tPA significantly ($p < 0.025$) and progressively decreased O_2^- production by human neutrophils stimulated by PMA in vitro (**FIG. 1**). In contrast, adding L-arginine, a component of the tPA formulation, did not decrease ($p > 0.05$) O_2^- production by neutrophils stimulated with PMA (**FIG. 2**). Neither tPA nor L-arginine altered neutrophil O_2^- -production by unstimulated neutrophils.

[0132] Effect of tPA Proteolytic Activity on O_2^- Production. tPA promoted conversion of plasminogen to plasmin. tPA mediated conversion of plasminogen to plasmin was inhibited by PPACK in a concentration dependent fashion. Based on these results, the mole:mole (PPACK:tPA) ratio used in the subsequent experiments was 5:1. In these studies, both tPA and PPACK-treated, proteolytically inactivated, tPA comparably inhibited O_2^- production by neutrophils stimulated with PMA (**FIG. 4**). In addition, analysis of the kinetics of O_2^- production showed that both tPA and proteolytically inactivated tPA decreased the V_{max} of O_2^- production (i.e. rate of cytochrome reduction) similarly (**FIG. 3**).

[0133] Effect of tPA on O_2^- Generation by Xanthine Oxidase In vitro. Adding tPA did not decrease O_2^- concentrations produced by xanthine oxidase (XO) in vitro (**FIG. 4**).

Example 2

[0134] Tissue Type Plasminogen Activator Reduces Inflammation in the Carrageenan-Induced Rat Footpad Model

[0135] Summary. This example shows that tPA, but not streptokinase (SK), can reduce inflammation in an in vivo model, the carrageenan-induced rat footpad model. See Stringer et al. (1997a) *Free Radic Biol Med* 22:985-8. Carrageenan, a mucopolysaccharide derived from Irish sea moss, is a phlogistic agent that provokes a local antigenic inflammatory response which is primarily attributed to neutrophil mediated injury and is highly reproducible (Vinegar et al., 1969; Vinegar et al., 1976; Vinegar et al., 1987). This model has been used extensively to evaluate the anti-inflammatory effects of such drugs as the non-steroidal anti-inflammatory drugs, corticosteroids, and more recently superoxide dismutase (Ando et al., 1991; Vinegar et al., 1987; Winter and Flataker, 1965).

[0136] Mechanisms by which tPA could influence carrageenan-induced footpad inflammation and edema include inhibition of neutrophil infiltration into the footpad, inflammatory mediator release, including neutrophil-generated O_2^- , and/or vascular permeability. The first possibility is unlikely since there was no difference between the plasminogen activators in regard to the magnitude of neutrophil infiltration into the footpad. Generation of O_2^- exerts important pro-inflammatory effects, including deesterification of phospholipids resulting in increased vascular permeability like that observed in ischemia-reperfusion injury (Deby and Goutier, 1990). While not wishing to be bound by any particular mode of operation, the anti-inflammatory activity of tPA likely involves its capacity to reduce O_2^- production by neutrophils.

METHODS OF TREATING LUNG DISEASES

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 60/439,373, filed Jan. 9, 2003, 60/480,047 filed Jun. 20, 2003, and 60/494,841 filed Aug. 12, 2003, the contents of each of which are incorporated herein in their entirety.

FIELD OF THE INVENTION

[0002] The present invention relates to the field of compositions and methods for treating lung diseases.

BACKGROUND OF THE INVENTION

[0003] The following description of the background of the invention is provided simply as an aid in understanding the invention and is not admitted to describe or constitute prior art to the invention.

[0004] Lung diseases comprise a spectrum of manifestations and etiologies, and may be particularly difficult to treat with systemic administration of potential therapeutics. Broad categories of disease classifications exemplify this spectrum of lung diseases. Over 150 diseases of the interstitium are recognized, including many types of fibrosis. Another category includes disorders of gas exchange and blood circulation. Disorders of the airways and disorders of the pleura constitute two additional categories. Lung cancers include both primary lung cancers and metastases from primary cancers of various other organs or tissues. Infectious diseases include viral, bacterial, and fungal infectious agents.

[0005] Pulmonary administration of therapeutic compositions comprised of low molecular weight drugs has been observed, for example, beta-androgenic antagonists to treat asthma. Other therapeutic agents that are active in the lungs have been administered systemically and targeted via pulmonary absorption. However, not all low molecular weight drugs can be efficaciously administered through the lung. Moreover, pulmonary delivery of higher molecular weight therapeutics, such as polypeptides or proteins, is much more difficult.

[0006] The anatomy and physiology of the lung presents several barriers to pulmonary administration. Initially, after passing through the nose or mouth, inhaled air (and any particles contained therein) moves into the respiratory tree, which is composed of numerous dichotomous branches between the trachea and the alveoli. Bronchi, bronchioles, and terminal bronchioles comprise the conducting zone. The epithelium of these conducting airways is pseudo stratified and largely ciliated. The more distal levels of branching form the transitional and respiratory zones, comprised of respiratory bronchioles, alveolar ducts, and alveoli, is where gas exchange and pulmonary absorption occur. The respiratory zone, in contrast to the conducting zone, is non-ciliated and comprised of a single cell layer.

[0007] The air-blood barrier is comprised of the alveolar epithelium, the capillary endothelium, and the lymph-filled interstitial space separating these two cell layers. In the alveolar epithelium, adjacent cells overlap and are bound by non-leaky tight junctions, which, in conjunction with the non-leaky single cell layer comprising the capillary endo-

thelium, limits the movement of fluids, cells, salts, proteins, and numerous other macromolecules from the blood and intercellular spaces into the lumen of the alveoli. Most molecules, including proteins and polypeptides, must be actively or passively transported across this barrier in the absence of lung injury. Also, mucosal secretions from epithelial cells and cilia provide additional physical barriers to the delivery of a potential therapeutic.

[0008] Other cell types present in the alveolar lumen and in the interstitial space separating the alveolar epithelium from the capillary endothelium may also serve as barriers for delivery. Alveolar macrophages migrate from the blood across the air-blood barrier. Additionally, other cell types, such as neutrophils and lymphocytes, can move into the alveoli from the blood in response to infection.

[0009] Immunotherapy directed to tumor-associated or tumor-specific antigens has long been considered an attractive method for safe, nontoxic treatment of tumors. Translating such methods into clinical benefit, however, has been somewhat less successful than might have been hoped. While many tumors express antigens that could be used to generate an in vitro or in vivo immune response, direct targeting of such antigens may not be the most effective mode of providing immunotherapy. Cytokines, such as interleukin-2 ("IL-2"), have also been employed to stimulate immune response to tumors. Such therapies, either alone or with conventional therapies, may provide a more attractive means for achieving clinical benefit in malignant and non-malignant diseases. See, e.g., Xu et al., *Cancer Res.* 60: 4475-84 (2000); Christ et al., *Clinical Cancer Res.* 7: 1385-97 (2001); Steven A. Rosenberg, *The Transformed Cell: Unlocking the Mysteries of Cancer*, Putnam Group, 1992.

[0010] Experimental treatment of certain tumors with cytokines has been performed by various artisans. Cytokines, such as IL-2, have been administered systemically (e.g., by intravenous infusion and/or subcutaneous administration), with the demonstration of some antitumor response. However, serious side effects have also been observed in such treatments, including fever, pulmonary vascular leakage, weight gain, malaise, rigor, anemia, and thrombocytopenia. See, e.g., Heinzer et al., *J. Clin. Oncol.* 17: 3612-20 (1999). More recently, aerosol delivery of cytokines such as IL-2 have been shown to provide reduced toxicity coupled with modest therapeutic benefit. See, e.g., Lorenz et al., *Clin. Cancer Res.* 2: 1115-22 (1996); Zissel et al., *Cancer Immunol. Immunother.* 42: 122-26 (1996); Khanna et al., *J. Pharm. Pharmacol.* 49: 960-71 (1997).

[0011] Acute respiratory infections can affect both the upper or lower respiratory systems. An upper respiratory infection typically involves the ears, nose, throat or sinuses. Examples of upper respiratory tract infections include the common cold (typically viral); the flu (influenza virus); otitis media, pharyngitis, acute sinusitis or chronic sinusitis, and tonsillitis, which involve inflammation of the middle ear, throat, sinuses, and tonsils, respectively. Lower respiratory infections typically involve the trachea, bronchial tubes and the lungs themselves. Examples of lower respiratory tract infections include bronchitis and pneumonia. In a single infection, one or both of the upper and lower respiratory systems can be affected.

[0012] Respiratory tract infections are primarily of bacterial, viral, or fungal origin; although there are also rarer

types, such as parasitic infections. Pulmonary tuberculosis (TB) is an example of a contagious bacterial infection caused by *Mycobacterium tuberculosis*. The lungs are primarily involved, but the infection can spread to other organs. TB is one of the most clinically significant infections worldwide, with an incidence of 3 million deaths and 10 million new cases each year. With improved sanitary conditions and the advent of antimicrobial drugs, the incidence of mortality had been steadily declining. However, in most developed countries, there has been a resurgence of TB infection, in part due to immunocompromised individuals (e.g., HIV-positive) and the emergence of multidrug-resistant (MDR) strains of *M. tuberculosis*.

[0013] Severe acute respiratory syndrome (SARS) is a newly recognized viral respiratory tract infection, first detected in China in late 2002. The viral agent has been identified as a previously unrecognized human coronavirus, called SARS-associated coronavirus (SARS-CoV). SARS is also an example of both upper and lower respiratory tract involvement caused by infection with a single organism. Early symptoms include runny nose and sore throat, which are then followed by dyspnea and dry cough, and may develop into adult respiratory distress syndrome requiring intervention with mechanical ventilation.

[0014] Pneumonia is an example of a respiratory tract that may be caused by either bacteria, viruses, or parasites. It is generally defined as an inflammation of the lung tissue, whereby white cells in the lungs prevent the alveoli from functioning properly. This condition is potentially life-threatening.

[0015] *Candida* and *Aspergillus* are the most common fungal respiratory tract infections, tending to appear in immunocompromised subjects, such as transplant recipients. While *Candida* mainly infests the upper tracheobronchial tree with only an occasional chance of dissemination, *Aspergillus* has the potential to involve the deeper parenchyma. Other potential fungal pathogens include *Cryptococcus*, *Pseudallerschiera* and *Coccidioides*.

[0016] Experimental treatment of certain infections with cytokines has also been performed by various artisans. Cytokines have been used to treat serious bacterial and viral infections (particularly, those caused by drug resistant organisms), either alone or in combination therapies with known treatments or vaccines. For a review of immune modulation in the treatment of respiratory infection, the reader is referred to Kolls and Nelson, *Resp. Res.* 1:9-11, 2000. For example, tuberculosis, the seventh leading cause of morbidity and mortality in the world, has been successfully treated with recombinant interferon- γ in aerosol form (Condos et al., *Lancet* 349:1513-5, 1997). As another example, intranasal interferon- α 2b has been shown to prevent rhinovirus infection, and to lessen symptoms associated with parainfluenza infections (Monto et al., *J. Infect. Dis.* 154:128-133, 1986). Other examples of therapeutic molecules for the treatment of infections include chemokines such as gamma-interferon-inducible protein 10 (IP-10), interferon-inducible T cell alpha chemoattractant (I-TAC) and MIG (monokine induced by interferon-gamma). Antibodies directed against a variety of epitopes of infectious agents causing infection are also known in the art, for both treatment and prevention (e.g., vaccines) of infection.

[0017] To achieve maximum therapeutic impact in the treatment of any lung disease, potential therapeutic agents

should be optimally directly delivered to the respiratory tract. A number of general methods have been described for delivering medically important molecules, including small molecules, nucleic acids, and/or protein or peptide compositions, in an effort to improve bioavailability and/or to target delivery to particular locations within the body. Such methods include the use of prodrugs, encapsulation into liposomes or other particles, co-administration in uptake enhancing formulations, and targeting to specific tissues. For review see, e.g., *Critical Reviews in Therapeutic Drug Carrier Systems*, Stephen D. Bruck, ed., CRC Press, 1991. In the case of cytokines such as IL-2, pulmonary delivery has relied upon both inhalation of free cytokine (either alone or in combination with intravenous delivery of additional cytokine), and inhalation of liposomal formulations. See, e.g., Enk et al., *Cancer* 88: 2042-46 (2000); Khanna et al., *J. Pharm. Pharmacol.* 49: 960-71 (1997). Such delivery modes can provide high cytokine levels within the lung, but relatively modest systemic cytokine levels.

[0018] Certain modes for delivering medically important molecules (e.g., oral, nasopharyngeal, oropharyngeal, pulmonary, buccal, sublingual, mucosal, vaginal, or rectal delivery modes) require that the molecule(s) of interest be delivered across "polarized" cells (e.g., epithelial cells) that have two distinct surfaces. In the case of pulmonary epithelium, these surfaces are referred to as the apical surface, which is exposed to the aqueous or gaseous medium in which the molecule(s) of interest is delivered to the subject; and the opposing basolateral (also known as basal lateral) side that rests upon and is supported by an underlying basement membrane, and that can provide access to the interstitial spaces and the general circulation. Tight junctions between adjacent epithelial cells separate the apical and basolateral sides of an individual epithelial cell. The biological methods that provide and maintain such cellular polarity can also act to limit bioavailability of molecules delivered by these modes.

[0019] Molecules are trafficked into, out of, and within a cell by various means, and it is typically these means that are believed to confer bioavailability to a molecule delivered by oral, nasopharyngeal, oropharyngeal, pulmonary, buccal, sublingual, mucosal, vaginal, or rectal delivery modes. "Active transport" is a general term for the energy-dependent carriage of substances across a cell membrane. "Endocytosis" is a general term for the process of cellular internalization of molecules, i.e., processes in which cells take in molecules from their environment, either passively or actively. "Exocytosis" is a general term for processes in which molecules are passively or actively moved from the interior of a cell into the medium surrounding the cell. "Transcytosis" is a general term for processes in which molecules are transported from one surface of a cell to another. "Paracytosis" is a general term for processes in which molecules are transferred through the interstices between cells, often past tight junctions. "Receptor mediated endocytosis" refers to a particular type of trafficking event by which cells internalize molecules, viruses, bacteria, etc. As its name implies, it depends on the interaction of that molecule with a specific binding protein in the cell membrane called a "receptor." "Forward transport" refers to transport in a basolateral to apical direction, while "reverse transport" refers to transport in an apical to basolateral direction.

[0020] Each publication and patent application in the foregoing Background section is hereby incorporated by reference in its entirety, including all tables, figures, and claims.

SUMMARY OF THE INVENTION

[0021] The present invention discloses methods of treating lung diseases. The methods involve administering to a subject via a pulmonary, oropharyngeal, or nasopharyngeal route a compound or composition that contains a therapeutic agent and a targeting element directed to a ligand present on the surface of cells lining the pulmonary or nasopharyngeal system. The ligand preferably confers transcytosis of the compound or composition across polarized epithelial layers, either in vitro or in vivo. The therapeutic agent is preferably a cytokine or a chemokine, more preferably an interleukin or an interferon, IP-10, I-TAC, or MIG. The therapeutic agent may also be an antibody, for example, an antibody directed against an infectious agent. The invention is described herein in detail with regard to targeting elements that target an epitope on pIgR receptor. In particularly preferred embodiments, the targeting element confers apical to basolateral transcytosis to the therapeutic agent in an in vitro transcytotic assay. The subject is preferably a human that is, for example, diagnosed with a lung disease and in need of treatment, or predisposed to a lung disease and in need of prophylaxis.

[0022] In various embodiments, exemplary ligands include one or more of the following: pIgR, pIgR stalk, transferrin receptor, apo-transferrin, holo-transferrin, vitamin B12 receptor, FeRn, an integrin, Flt-1, Flk-1, Flt-4, a GPI-linked protein, a scavenger receptor, folate receptor, and low density lipoprotein receptor. In the most preferred embodiment, the ligand is pIgR or the pIgR stalk. In preferred embodiments, the targeting element binds a non-secretory component region of pIgR. In additional embodiments, the therapeutic agent is a polypeptide, preferably an enzyme, a cytokine or a chemokine. In various embodiments, the therapeutic agent is one or more of the following: an enzyme, an interleukin, an interferon, a cytokine, a chemokine or an antibody. The following list of interleukins is not inclusive and is provided by way of example only. Other interleukins, those existing and those yet to be discovered, are also contemplated for use in the invention. However, an exemplary list of interleukins includes any of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, IL-18, IL-21, and functional derivatives of any of these foregoing exemplary interleukins. Likewise, the following list of interferons is not inclusive and is provided by way of example only. An exemplary list of interferons include interferon α (including interferon α -2a and -2b), interferon β , and interferon γ . In the most preferred embodiments, the interleukin is IL-2, or a functional derivative thereof, and the interferon is interferon α or interferon β , or a functional derivative thereof of either. Preferred chemokines include IP-10, I-TAC and MIG. Combinations of any two or more cytokines, chemokines, or other therapeutic agents are also provided herein.

[0023] The term "functional derivative" as used herein refers to a chemically modified version, an analog, or a homolog of a compound that retains a biological function of interest of that compound for any given application. In the case of polypeptides, chemical modification may include, by

way of non-limiting example, adding chemical groups to a compound (e.g., glycosylation, phosphorylation, thiolation, pegylation, acetylation, amidation, glycosylphosphoinositolization, etc.), eliminating parts of a compound that do not impact the function of interest (preparing a truncated form of a protein that retains an activity of interest, e.g., Klenow fragment), extending a compound with sequences that add domains or functions to the compound (e.g., preparing fusion proteins); changing sets of one or more amino acids in the polypeptide (preparing muteins). In preferred embodiments, functional derivatives of therapeutic compounds described herein extend the residence time of the therapeutic compound in the lungs, for example, by slowing their release or metabolism.

[0024] Analogs are exemplified by peptidomimetics; and homologs are polypeptides from other species of animals that retain biological activity (e.g., human and porcine insulin, human and salmon calcitonin, etc.) or intraspecies isomers of a polypeptide (protein "families" such as the cytochrome P450 family). Muteins and pegylated functional derivatives of IL-2, for example, are well known to those of skill in the art. See, e.g., Chapes et al., *J. Appl. Physiol.* 86: 2065-76 (1999); Shanafelt et al., *Nature Biotechnol.* 18: 1197-202 (2000). IL-2 biological activity of the functional derivatives are preferably tested by evaluating the ability to sustain proliferation of the IL-2-dependent murine cytotoxic T cell line, CTLL-2. See, e.g., Melani et al., *Cancer Res.* 58:4146-54 (1998). Likewise, functional derivatives of IL-2 linked to Fc or human serum albumin are well known in the art. See, e.g., Zheng et al., *J. Immunol.* 163: 4041-48 (1999); Melder et al., Modulation of anti-infective responses in mice by Albuleukin, an Interleukin-2/human serum albumin fusion protein, Society for Biological Therapy Meeting, November 2001.

[0025] By "pulmonary route" is meant administration of a compound or composition to a subject through the airways leading to the lungs. The pulmonary route includes, but is not limited to, all passageways including the trachea, larynx, bronchioles, bronchus, and alveoli.

[0026] The "nasopharynx" refers to any of the nasal passages, pharynx, trachea, and larynx. By a "nasopharyngeal route" is meant that the compound enters the subject through the nasopharynx. Similarly, the "oropharynx" refers to the oral cavity, and includes the back of the tongue (base of tongue), soft palate, tonsils and its pillars, and the back wall of the throat (posterior pharyngeal wall), through the pharynx, trachea, and larynx. Thus, by an "oropharyngeal route" is meant that the compound enters the subject through any one or more of the membranes of the oropharynx. In various embodiments the mode of administration is instillation, nebulization, aerosolization, atomization, misting, or inhalation, and most preferably inhalation.

[0027] The pharynx stretches from the back of the nose, down the neck to the larynx. The trachea connects the larynx to the bronchial tubes. The larynx is a structure of muscle and cartilage in the upper neck that contains the vocal cords. Air passes through the larynx into the windpipe and then into the lungs.

[0028] Preferred delivery methodologies of the present invention include instillation, or inhalation of a material generated by nebulization, aerosolization, atomization, and misting. "Instillation" refers to direct delivery of liquid in

liquid drops to a pulmonary passageway. "Inhalation" is the most preferably form of administration and refers to inhaling gas (preferably air) that contains the compound into the lungs and/or naso-pharynx of the subject, preferably by force of the subject's own respiration. "Nebulization" refers to creating a fine spray or mist of particles from liquid. "Aerosolization" refers to creating a suspension of fine solid or liquid particles in gas. "Atomization" refers to reducing the composition to fine particles or spray.

[0029] An "anti-tumor agent" is an agent that destroys, shrinks, or arrests the growth of tumors or cancers in a subject, or that extends the life of a subject receiving the agent. The skilled artisan will understand that anti-tumor agents do not necessarily produce an anti-tumor effect in each subject receiving the agent. Rather, whether or not an agent destroys, shrinks, or arrests the growth of tumors or cancers in a subject, or that extends the life of a subject is a statistical question measured in a population receiving the treatment, which is compared to a like population not receiving the treatment. Preferably, an anti-tumor agent extends the average life span of a subject by 3 months, 6 months, 9 months, 1 year, 2 years, 3 years, 5 years, or more, relative to a subject not receiving the treatment. In particularly preferred embodiments, an anti-tumor agent reduces the average incidence or average time to appearance of metastatic disease in a subject, most preferably lung metastases, relative to a subject not receiving the treatment.

[0030] In certain embodiments, the anti-tumor agent may be an anti-angiogenesis agent. An "anti-angiogenesis agent" is a compound that blocks or prevents the function of an angiogenic factor that normally promotes the development of a tumor's blood supply. Tumor angiogenesis is the specific development of an adequate blood supply for a solid tumor mass; and the growth of a tumor depends upon the existence, maintenance, and continued development of sufficient and functional blood vasculature in the tumor mass. Tumor angiogenesis thus involves endothelial cell penetration of the vascular basement membrane in a preexisting blood vessel; followed by endothelial cell proliferation; and then by an invasion of the extracellular matrix surrounding the blood vessel to form a newly created vascular spout (see, e.g., Vernon and E. H. Sage, *Am. J. Pathol.* 147: 873-883 (1995)).

[0031] An "angiogenic factor" as used herein, refers to a compound that promotes angiogenesis. Such factors include, for example, vascular endothelial growth factors (VEGFs) and VEGF receptors, fibroblast growth factors (FGFs), transforming growth factor (TGF) α and β , platelet-derived endothelial cell growth factor (PD-ECGF), tumor necrosis factor- α (TNF- α), matrix metalloproteinases (MMPs), angiopoietin-2 and Tie-2 receptor, scatter factor (hepatocytes growth factor, IL-8, angiogenin, adhesion molecules (e.g., integrins, selectins, cadherins), prostaglandin E1 and E2, angiogenin transforming growth factors, angiotropin, granulocyte-colony stimulating factor, placental growth factor, and proliferin.

[0032] Anti-angiogenesis agents may thus block the normal function of one of these angiogenesis agents, for example, an antibody directed against VEGF. Alternatively, there are natural anti-angiogenesis agents, or anti-angiogenic factors, which normally balance the angiogenesis agents in vivo. Anti-angiogenic factors include angiosta-

tin, endostatin, IFN- α and IFN- β , IFN- γ inducible protein 10, IL-1, IL-6, IL-12, platelet factor 4, thrombospondin-1, 2-methoxyestradiol, tissue inhibitors of metalloproteinases, retinoic acid, prolactin, basic fibroblast growth factor soluble receptor, transforming growth factor- β (TGF- β), placental proliferin-related protein, TNF- α , I-TAC and MIG. The therapeutic agents of the invention may comprise such anti-angiogenesis agents, or may be administered in combination with such anti-angiogenesis agents as a second therapeutic agent.

[0033] In certain embodiments, the therapeutic agent may be an apoptosis inducer. Apoptosis, which is also referred to as programmed cell death, is a form of cell death characterized by membrane blebbing and nuclear DNA fragmentation. Dysregulation of apoptosis has been implicated in a number of human diseases, including cancer. Although apoptotic cell death is initially triggered by a specific death signal received, for example, by ligation of the Fas cell surface molecule, execution of the apoptotic pathway occurs only upon the activation of members of the Ced-3/ICE (caspase) family of cysteine proteases. There are at least 10 known members of the caspase family whose activities lead to site-specific cleavage and consequent activation/inactivation of various target molecules. FLICE and related caspases may initiate apoptosis by activating a downstream caspase cascade, including CPP32 (caspase-3). The decision to engage the apoptotic execution pathway in response to specific death signals depends on the status of various cellular regulators of apoptosis, including p53 and the Bcl-2/Bax set point. The latter set point arises through heterodimerization between the Bcl-2/Bcl-X_L family of suppressors and promoters, respectively, in which the ratio of the heterodimerizing partners determines the outcome, cell death or cell survival, in response to various death signals. Bad, a more distantly related family member, is a direct regulator of the set point, by a mechanism that is governed by phosphorylation. The phosphorylation may, in turn, be affected by Bcl-2-dependent recruitment of Raf-1 kinase. Thus, an "apoptosis inducer" as used herein, is a molecule that interacts with an apoptotic pathway to trigger cell death, or blocks the function of another molecule that prevents apoptosis. The therapeutic agents of the invention may comprise such apoptosis inducers, or may be administered in combination with such apoptosis inducers as a second therapeutic agent.

[0034] An "anti-infective agent" is an agent that prevents infection by an infectious agent, decreases the severity of infection by an infectious agent, interferes with normal infection pathways, arrests infection by an infectious agent, impairs the function of growth of an infectious agent, or kills an infectious agent. The skilled artisan will understand that anti-infective agents do not necessarily produce an anti-infective effect in each subject receiving the agent. Rather, whether or not an agent is effective is a statistical question measured in a population receiving the treatment, which is compared to a like population not receiving the treatment.

[0035] A "ligand," "target molecule" or "molecular target" is a compound, a molecular complex of two or more compounds, a moiety (a portion of a compound), or an interface formed between two or more compounds, that are associated with a cell surface and to which a targeting element specifically binds. Preferred ligands are membrane proteins, most preferably pIgR, pIgR stalk, transferrin recep-

tor, apo-transferrin, holo-transferrin, vitamin B 12 receptor, FcRn, an integrin, Flt-1, Flk-1, Flt-4, a GPI-linked protein, a scavenger receptor, folate receptor, and/or low density lipoprotein receptor.

[0036] The term "targeting element" encompasses any type of composition or compound that is capable of specifically binding to a molecular target. The term "specifically binds" is not intended to indicate that the targeting element binds exclusively to its intended target. Rather, a targeting element specifically binds if its affinity for its intended target is about 2-fold greater when compared to its affinity for a non-target molecule. Preferably the affinity of the targeting element will be at least about 5-fold, preferably 10-fold, more preferably 25-fold, even more preferably 50-fold, and most preferably 100-fold or more, greater for a target molecule than its affinity for a non-target molecule. A compound or composition comprising such a targeting element would be referred to as being "adapted to specifically bind" to the target molecule. Preferred targeting elements can be selected from the group consisting of a polypeptide, a recombinant polypeptide, an antibody, an antibody fragment, a single-chain variable region fragment, a small molecule, an oligonucleotide, an oligosaccharide, a polysaccharide, a carbohydrate, a cyclic polypeptide, a peptidomimetic, and an aptamer, as these terms are defined herein.

[0037] A cell surface component is said to "promote" transport, active transport, endocytosis, or transcytosis if a compound or composition comprising a targeting element that specifically binds to the cell surface component is transported into, around, or through a cell (depending on the type of transport involved) at a higher rate or to a higher absolute amount compared to a similar composition lacking the targeting element. Preferably, a 2-fold, 5-fold, 10-fold, 100-fold, or 1000-fold increase in rate or amount is obtained.

[0038] The term "compound" as used herein refers to a single covalently linked molecule. Preferably, a compound comprises one or more therapeutic agents covalently linked to one or more targeting elements.

[0039] The term "composition" as used herein refers to a plurality of compounds associated by non-covalent means. A composition may include a compound comprising one or more therapeutic agents covalently linked to one or more targeting elements, associated with pharmaceutically acceptable excipients. Alternatively, a composition may refer to one or more therapeutic agents and one or more targeting elements associated with a particle or capsule as described in the entirety of Provisional U.S. Patent Application No. 60/402,029, filed Aug. 7, 2002, which is hereby incorporated by reference.

[0040] As used herein, the term "small molecule" refers to compounds having molecular mass of less than 3000 Daltons, preferably less than 2000 or 1500, still more preferably less than 1000, and most preferably less than 600 Daltons. Preferably but not necessarily, a small molecule is not an oligopeptide.

[0041] As used herein, the term "polypeptide" refers to a covalent assembly comprising at least two monomeric amino acid units linked to adjacent amino acid units by amide bonds. An "oligopeptide" is a polypeptide comprising a short amino acid sequence (i.e., 2 to 10 amino acids). An

oligopeptide is generally prepared by chemical synthesis or by fragmenting a larger polypeptide. Examples of polypeptide drugs include, but are not limited to, therapeutic antibodies, insulin, parathyroid hormone, polypeptide vaccines, and antibiotics such as vancomycin. Novel polypeptide drugs may be identified by, e.g., phage display methods.

[0042] As used herein, the term "antibody" refers to a molecule comprising at least one antigen binding domain formed by two binding regions referred to by those of skill in the art as an immunoglobulin or immunoglobulin-like heavy chain, and an immunoglobulin or immunoglobulin-like light chain. When obtained by in vitro or in vivo generation of an immunogenic response, the heavy and light chains are expressed as separate polypeptides, and are joined by disulfide bonds. In this case, the heavy and light chains may be separated under reducing conditions. Such antibodies include both polyclonal, monospecific and monoclonal antibodies, and antigen binding fragments thereof (e.g., Fab fragments, Fab' fragments, etc.). An "immunogenic response" is one that results in the production of antibodies directed to one or more proteins after the appropriate cells have been contacted with such proteins, or polypeptide derivatives thereof, in a manner such that one or more portions of the protein function as epitopes.

[0043] When a molecule comprising at least one antigen binding domain is formed recombinantly, the heavy and light chains may be linked by disulfide bonds as in the foregoing discussion. However, in various embodiments, the heavy and light chains are linked by non-reducible covalent linkers. As used herein, the term "single-chain variable region fragment" or "sFv" refers to a variable, antigen-binding determinative region of a single antibody light chain and antibody heavy chain linked together by a covalent linkage having a length sufficient to allow the light and heavy chain portions to form an antigen binding site. Such a linker may be as short as a covalent bond; preferred linkers are from 2 to 50 amino acids, and more preferably from 5 to 25 amino acids. The antigen binding site need not be formed from intramolecular association of light and heavy chain portions; rather, two separate sFvs may form multimeric antigen binding molecules (e.g. diabodies) as described hereinafter.

[0044] As used herein, the term "polynucleotide" refers to molecule comprising a covalent assembly of nucleotides linked typically by phosphodiester bonds through the 3' and 5' hydroxyls of adjacent ribose units. An "oligonucleotide" is a polynucleotide comprising a short base sequence (i.e., 2 to 10 nucleotides). Polynucleotides include both RNA and DNA, may assume three-dimensional shapes such as hammerheads, hairpins, dumbbells, etc., and may be single or double stranded. Polynucleotide drugs can include ribozymes, and polynucleotide vaccines.

[0045] As used herein, the term "oligonucleotide analog" refers to a molecule that mimics the structure and function of an oligonucleotide, but which is not a covalent assembly of nucleotides linked by phosphodiester bonds. Peptide nucleic acids, comprising purine and pyrimidine bases linked via a backbone linkage of N-(2-aminoethyl)-glycine units, is an example of an oligonucleotide analog.

[0046] As used herein, a "carbohydrate" is any form of saccharide. Examples of carbohydrates include, but are not limited to, simple sugars or oligosaccharides (such as

a polypeptide, an antibody, an antibody fragment, a single-chain variable region fragment, a small molecule, an oligonucleotide, an oligosaccharide, a polysaccharide, a cyclic polypeptide, a peptidomimetic, and an aptamer, a poly(ethylene oxide), a dextran, etc.) may be chemically cross-linked by a linker having chemistry compatible with a site on each component. Crosslinkers are well known to those of skill in the art, and may be obtained commercially (see, e.g., Pierce Chemical Company Catalog and Handbook 1994-95, pages O-90 through O-110, which is hereby incorporated by reference) or synthesized as needed.

[0384] Alternatively, in cases where both components are peptides, the components may be coupled "genetically"; that is, the first and second elements may be expressed as a chimeric protein or fusion protein. For example, U.S. Pat. No. 6,072,041 to Davis et al. is drawn to fusion proteins that are directed to the secretory component of pIgR. Ferkol et al., *Am. J. Respir. Crit. Care Med.* 161:944-951, 2000, discloses a fusion protein consisting of a single-chain variable region fragment directed to the secretory component (SC) of human pIgR and a human alpha (1)-antitrypsin. U.S. Pat. No. 6,042,833 to Mostov et al. discloses "genetic fusions" and "fusion proteins" that include ricin A, poly-(L)-Lys, or a phage surface protein.

[0385] In a similar manner, molecular biology may be used to introduce domains into a component that can combine with a complementary domain on a second component. For example, a coiled-coil domain sequence may be attached to a first targeting element and a second targeting element to provide the complementarity necessary to achieve binding between the two elements. Alternatively, cysteine residues may be introduced into the two targeting elements for the formation of a disulfide-bonded complex.

[0386] In an alternative approach, the various components of the compositions described herein can be associated with a particle or capsule. Methods for producing particulate administration systems for delivery of biologically-relevant molecules are well known to those of skill in the art. Such particles are preferably porous and/or biodegradable so that molecules (e.g., drugs, vaccines, vitamins, polypeptides, antibodies, etc.) contained within the particle may be released once delivered into the circulation; however, non-porous and/or nonbiodegradable particles (e.g., liposomes) are also known to those of skill in the art. Preferred particles and capsules, including microparticles, nanoparticles, microcapsules, and nanocapsules are disclosed in, e.g., U.S. Pat. No. 5,702,727; U.S. Pat. No. 5,620,708; U.S. Pat. No. 5,607,691; U.S. Pat. No. 4,610,896; U.S. Pat. No. 5,149,794; U.S. Pat. No. 6,197,349; U.S. Pat. No. 6,159,502; U.S. Pat. No. 5,785,976; Chiu et al., *Biomaterials* 23: 1103-12 (2002); Andrianov et al., *Biomaterials* 19: 109-115 (1998); Soppi-math et al., *J. Controlled Release* 70: 1-20 (2001); McPhail et al., *Intl. J. Pharmaceutics* 200: 73-86 (2000); Müller et al., *Eur. J. Pharmaceut. Biopharmaceut.* 50: 161-177 (2000); Franssen et al., *J. Controlled Release* 60: 211-21 (1999); Prokop et al., *Biotechnol. and Bioeng.* 75: 228-232 (2001); Allémann et al., *Adv. Drug Deliv. Rev.* 34: 171-89 (1998); Vinogradov et al., *Adv. Drug Deliv. Rev.* 54: 135-47 (2002); Jung et al., *Eur. J. Pharmaceut. Biopharmaceut.* 50: 147-60 (2000); Martin et al., *Biomaterials* 19: 69-76 (1998); Vervoort et al., *Intl. J. Pharmaceutics* 172: 137-45 (1998); J. Controlled Release 65: 49-54 (2000); Davda and Labhaset-war, *Intl. J. Pharmaceutics* 223: 51-9 (2002); Düzgünes and

Nir, *Adv. Drug Deliv. Rev.* 40: 3-18 (1999); Nagayasu et al., *Adv. Drug Deliv. Rev.* 40: 75-87 (1999); Leroueil-Le Verger et al., *Eur. J. Pharmaceut. Biopharmaceut.* 46: 137-143 (1998); Breton et al., *Biomaterials* 19: 271-81 (1998); Konan et al., *Intl. J. Pharmaceutics* 233: 239-52 (2002); Duncan et al., *Eur. Polymer J.* 37: 1821-6 (2001); and Stenekes et al., *Biomaterials* 22: 1891-8 (2001), each of which is hereby incorporated by reference in its entirety.

[0387] Pharmaceutical Compositions

[0388] The compositions of the present invention provide for delivery of therapeutic agents to a subject in need thereof. The compositions of the invention can further comprise other chemical components, such as diluents and excipients. A "diluent" is a chemical compound diluted in a solvent, preferably an aqueous solvent, that facilitates dissolution of the therapeutic agent in the solvent, and it may also serve to stabilize the biologically active form of the targeting element or one or more of its components. Salts dissolved in buffered solutions are utilized as diluents in the art. For example, preferred diluents are buffered solutions containing one or more different salts. A preferred buffered solution is phosphate buffered saline (particularly in conjunction with compositions intended for pharmaceutical administration), as it mimics the salt conditions of human blood. Since buffer salts can control the pH of a solution at low concentrations, a buffered diluent rarely modifies the biological activity of a biologically active peptide.

[0389] An "excipient" is any more or less inert substance that can be added to a composition in order to confer a suitable property, for example, a suitable consistency or to form a drug. Suitable excipients and carriers include, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol cellulose preparations such as, for example, maize starch, wheat starch, rice starch, agar, pectin, xanthan gum, guar gum, locust bean gum, hyaluronic acid, casein potato starch, gelatin, gum tragacanth, polyacrylate, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents can also be included, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Other suitable excipients and carriers include hydrogels, gellable hydrocolloids, and chitosan. Chitosan microspheres and microcapsules can be used as carriers. See WO 98/52547 (which describes microsphere formulations for targeting compounds to the stomach, the formulations comprising an inner core (optionally including a gelled hydrocolloid) containing one or more active ingredients, a membrane comprised of a water insoluble polymer (e.g., ethylcellulose) to control the release rate of the active ingredient(s), and an outer layer comprised of a bioadhesive cationic polymer, for example, a cationic polysaccharide, a cationic protein, and/or a synthetic cationic polymer; U.S. Pat. No. 4,895,724. Typically, chitosan is cross-linked using a suitable agent, for example, glutaraldehyde, glyoxal, epichlorohydrin, and succinaldehyde. Compositions employing chitosan as a carrier can be formulated into a variety of dosage forms, including pills, tablets, microparticles, and microspheres, including those providing for controlled release of the active ingredient(s). Other suitable bioadhesive cationic polymers include acidic gelatin, polygalactosamine, polyamino acids such as polylysine, polyhistidine, polyomithine, polyquaternary compounds,

prolamine, polyimine, diethylaminoethylidextran (DEAE), DEAE-imine, DEAE-methacrylate, DEAE-acrylamide, DEAE-dextran, DEAE-cellulose, poly-p-aminostyrene, polyoxethane, copolymethacrylates, polyamidoamines, cationic starches, polyvinylpyridine, and polythiodiethylaminomethylethylene.

[0390] The compositions of the invention can be formulated in any suitable manner. Suitable formulations include dry particulate and liquid formulations. Dry formulations include freeze dried and lyophilized powders, which are particularly well suited for aerosol delivery to the sinuses or lung, or for long term storage followed by reconstitution in a suitable diluent prior to administration. The particular amount of biologically active component to be delivered will depend on many factors, including the effect to be achieved, the type of organism to which the composition is delivered, delivery route, dosage regimen, and the age, health, and sex of the organism. As such, the particular dosage is left to the ordinarily skilled artisan's discretion. Additionally, particle size may be controlled to achieve optimal delivery to a specific region of the organ (e.g., the lung). Preferred particle sizes are between about 1 μm and about 20 μm , preferably between about 1 μm and about 10 μm , even more preferably between about 2 μm and about 7 μm , and most preferably between about 3 μm and about 5 μm . The term "about" in this context refers to $\pm 10\%$ of a given measurement.

[0391] It will be readily apparent to those skilled in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

EXAMPLE 1

Administration

[0392] The compounds administered according to the invention can be administered according to various methods, such as instillation, inhalation, exposure to the nasal and/or oral membranes (e.g., sniffing or nasal drops), intravenous administration, or intraperitoneal administration, depending on the particular application. Instillation and inhalation are especially effective methods of administration. The composition can also be nebulized, aerosolized, atomized, or made as a mist, and administered through inhalation or instillation. The most desirable mode of administration will be determined in any particular application, but the most preferable mode of administration is inhalation of the compound, so that administration can occur without surgical intervention or the presence of medical personnel, and the methods can be self-administered by the subject.

EXAMPLE 2

Multimeric sFvs

[0393] In vitro genetic manipulation has been used to alter the reading frame of sFvs so as to create derivatives that have substitutions or insertions of amino acids with reactive sites. See, e.g., U.S. patent application Ser. No. 09/969,748,

Example 6, and International Publication No. WO02/28408, Example 6, each of which is hereby incorporated by reference in this regard. The two variable regions of a sFv that combine to form a ligand binding site are known as V(H) and V(L). In a monomeric sFvs, the V(H) and V(L) of each molecule are associated with each other. In one type of dimeric sFv, the V(H) of one monomer [V(H)1] is associated with the V(L) of another monomer [V(L)2], and vice versa [i.e., V(H)2 is associated with V(L)1].

[0394] The length and composition of the linker between the V(H) and V(L) regions in an sFv is one factor that influences the tendency of an sFv to form monomers or multimers (Todorovska et al., Design and application of diabodies, triabodies and tetrabodies for cancer targeting, *J. Immunol. Meth.* 248:47-66 (2001); Arndt et al., *Biochemistry* 37 12918-12926 (1993). For example, a sFv molecule in which there is a relatively short linker between the V(H) and V(L) regions may be less likely to fold back upon itself and form a monomer. Thus, "short linker" sFv derivatives are often more likely to form dimers, as their V(H) and V(L) regions must pair with, respectively, the V(L) and V(H) regions of a second sFv molecule. Often, sFv derivatives with relatively long linkers between the V(H) and V(L) regions may fold back upon themselves, and therefore may have a greater tendency to form monomers. However, some sFv derivatives with long linkers between V(H) and V(L) may have some tendency to form multimers.

[0395] Various amino acid sequences are known that may serve as suitable spacers in the compounds of the invention (for a review, see Simons, Spacers, probability, and yields, *Bioconjug. Chem.* 1999 Jan.-Feb.; 10(1):3-8). Some non-limiting examples of sequences that have been used in sFvs include EGKSSSGSGSESKEF (SEQ ID NO: 10), one or more copies of GGGGS [also known as (G₄S)_x] (Newton et al., Angiogenin single-chain immunofusions: influence of peptide linkers and spacers between fusion protein domains, *Biochemistry* 1996 Jan. 16;35(2):545-53), GSGS [also known as (GSGS)_x] and GSSG [also known as (GSSG)_x].

[0396] APL10 is an exemplary sFv coding sequence. To facilitate affinity purification, protein A interacts with the VH chain of APL10.

EXAMPLE 3

IL-2-sFv Conjugates

[0397] Human IL-2 is synthesized as a precursor protein of 153 amino acids, which includes a 20 amino acid hydrophobic leader sequence. The IL-2 molecule has a molecular weight of about 15.4 kD and a slightly basic pI. The protein comprises a single intramolecular disulfide bond (Cys58-Cys105) that is necessary for the biological activity of IL-2 (Yamada et al., Importance of disulfide linkage for constructing the biologically active human interleukin-2, *Arch Biochem Biophys* 257:194-199, 1987).

[0398] Some forms of IL-2 comprise chemical modifications. It has been reported that O-glycosylation occurs at Thr3 of bovine IL-2, and that variants with different masses due to glycosylation exist. However, non-glycosylated IL-2 remains biologically active (Kuhnle et al., Bovine interleukins 2 and 4 expressed in recombinant bovine herpesvirus 1 are biologically active secreted glycoproteins, *J Gen Virol* 77(Pt 9):2231-2240, 1996).

19

[0441] The 100 μ l PCR reaction was purified using a QLAquick PCR purification column (cat.# 28104, Qiagen, Valencia, Calif.). 2 μ l of purified product was ligated into the pCR II Blunt TOPO vector (Invitrogen, Carlsbad, Calif.). Colonies were picked and miniprep DNA was prepared (Qiagen miniprep kit #27106). Positive clones were confirmed by DNA sequencing. pCR II Blunt TOPO Hum- β -IFN (pCRIIBT HIFN β) contained the human β -IFN gene and the wild-type N-terminal signal peptide as follows:

β -IFN gene sequence: (SEQ ID NO:21):					
ATGACCAACA	AGTGTCTCCT	CCAAATTGCT	CTCCTGTGTG	GCTTCTCCAC	TACAGCTCTT
					60
TCCATGAGCT	ACAACTTGCT	TGGATTCCTA	CAAAGAAGCA	GCAATTTTCA	GTGTCAGAAG
					120
CTCCTGTGGC	AATTGAATGG	GAGGCTTGAA	TACTGCCTCA	AGGACAGGAT	GAACTTTGAC
					180
ATCCCTGAGG	AGATTAAGCA	GCTGCAGCAG	TTCCAGAAGG	AGGACGCCGC	ATTGACCATC
					240
TATGAGATGC	TCCAGAACAT	CTTTGCTATT	TTCAGACAAG	ATTCATCTAG	CACTGGCTGG
					300
AATGAGACTA	TTGTTGAGAA	CCTCCTGGCT	AATGTCTATC	ATCAGATAAA	CCATCTGAAG
					360
ACAGTCCTGG	AAGAAAAACT	GGAGAAAGAA	GATTTACCCA	GGGAAAAACT	CATGAGCAGT
					420
CTGCACCTGA	AAAGATATTA	TGGGAGGATT	CTGCATTACC	TGAAGGCCAA	GGAGTACAGT
					480
CACTGTGCCT	GGACCATAGT	CAGAGTGGAA	ATCCTAAGGA	ACTTTTACTT	CATTAACAGA
					540
CTTACAGGTT	ACCTCCGAAA	CTGA			564

[0442] The β -IFN gene was fused to the N-terminus of APL10 via the NheI site to make pDIZ HIFN β -APL10. To construct the sFv- β -IFN chimera, 100 ng of pDIZHIFN β -APL10 was used as the template for PCR amplification using Vent DNA polymerase and the primers '122602-1TPF AvrII-G4S-IFN Beta Forward' (SEQ ID NO:22) and '122602-2TPR IFN Beta NheI-SalI-XhoI Reverse' (SEQ ID NO:23):

122602-1TPF AvrII-G4S-IFN Beta	
Forward	
5'-ACCGTCCCTAGGTGGTGGCGAGGGTCAATGAGCTACAACCTGCTTGGATTCCCTA-3': (SEQ ID NO:22)	
122602-2TPR IFN Beta NheI-SalI-XhoI	
Reverse	
5'-TCCTCGAGGTCGACGCTAGCTTATTAGTTTCGGAGGTAACCTGTAAGTCTGTTA-3': (SEQ ID NO:23)	

[0443] The forward primer used to generate the partial APL-10- β -IFN 551 bp PCR product was designed to include sequences encoding a synthetic linker encoding 5 amino acids (Gly-Gly-Gly-Gly-Ser) that can be inserted in frame to the C-terminus of sFv polypeptide APL-10. The 3-step PCR amplification reaction included 5 cycles with annealing temperature at 55° C. followed by 30 cycles at 60° C. The 551 bp PCR product was QIAquick column purified and cloned into the pCR Blunt II TOPO intermediate vector. Miniprep DNA was made and positives clones verified by

DNA sequencing. Following sequence confirmation, the PCR product was inserted into the AvrII/SalI sites of APL-10E vector (pELK vector derivative), or AvrII/XhoI digested APL-2005S vector (pSyn vector derivative) DNAs. Miniprep DNA was prepared and positive clones confirmed by DNA sequencing. Positive vector clones are illustrated in FIG. 2 and contain the chimeric DNA sequence (SEQ ID NO: 24) which encode for a chimeric protein containing the following domains and oriented from the N-terminus: (NH₂)-pel-B leader-sFv-Gly₄Ser linker- β -IFN-(COOH).

sFv-β-IFN chimera DNA sequence (SEQ ID NO: 24):					
ATGAAATACC	TATTGCCTAC	GGCAGCCGCT	GGATTGTTAT	TACTCGCGGC	CCAGCCGGCC 60
ATGGCC ~~CAGG~~	TGCAGCTGCA	GCAATCAGGG	GGAGGCGTGG	TCCAGCCTGG	GAGGTCCCTG 120
AGACTCTCCT	GTGCAGCCTC	TGGATTACCC	TTCAGTAGCT	ATGCTATGCA	CTGGGTCCGC 180
CAGGCTCCAG	GGAAGGGGCT	GGAGTGGGTC	TCAGCTATTA	GTGCTAGTGG	TGGTAGCACA 240
TACTACGCAG	ACTCCGTGAA	GGGCCGGTTC	ACCATCTCCA	GAGACAACGC	CAAGAACTCA 300
CTGTATCTGC	AAATGAACAG	CCTGAGAGCC	GAGGACACGG	CTGTGTATTA	CTGTGCGAGA 360
GATACCCGAG	GGTACTTCGA	TCTCTGGGGC	CGTGGCACCC	TGGTCACCGT	CTCCTCAGGT 420
GGCGGAGGGT	CATCTGAGCT	GACTCAGGAC	CCTGCTATGT	CTGTGGCCTT	GGGACAGACA 480
GTCAGAATCA	CATGTCAAGG	GGACAGTCTC	AGAAAGTATC	ATGCAAGCTG	GTATCAGCAG 540
AAGCCAGGGC	AGGCCCTGT	TCTTGTATC	TATGGTAAGA	ATGAACGTCC	CTCAGGGATC 600
CCAGAGCGAT	TCTCTGGGTC	CACCTCAGGA	GACACAGCTT	CCTTGACCAT	CAGTGGGCTC 660
CAGGCGGAAG	ATGAGGCTGA	CTATTACTGT	CACTCCCGAG	ACTCTAATGC	TGATCTTGTG 720
GTGTTCCGCG	GAGGGACCAA	GGTCACCGTC	CTAGGTGGTG	GCGGAGGGTC	AATGAGCTAC 780
AACTTGCTTG	GATTCTTACA	AAGAAGCAGC	AATTTTCAGT	GTCAGAAGCT	CCTGTGGCAA 840
TTGAATGGGA	GGCTTGAATA	CTGCCTCAAG	GACAGGATGA	ACTTTGACAT	CCCTGAGGAG 900
ATTAAGCAGC	TGCAGCAGTT	CCAGAAGGAG	GACGCCGCAT	TGACCATCTA	TGAGATGCTC 960
CAGAATCTCT	TTGCTATTTT	CAGACAAGAT	TCATCTAGCA	CTGGCTGGAA	TGAGACTATT 1020
GTTGAGAACC	TCCTGGCTAA	TGTCTATCAT	CAGATAAACC	ATCTGAAGAC	AGTCCTGGAA 1080
GAAAAACTGG	AGAAAGAAGA	TTTACCAGG	GGAAAACTCA	TGAGCAGTCT	GCACCTGAAA 1140
AGATATTATG	GGAGGATTCT	GCATTACCTG	AAGGCCAAGG	AGTACAGTCA	CTGTGCCTGG 1200
ACCATAGTCA	GAGTGGAAAT	CCTAAGGAAC	TTTTACTTCA	TTAACAGACT	TACAGGTTAC 1260
CTCCGAAACT	AA				

[0444] Expression of sFv-β-IFN in mammalian cells required the use of a suitable signal peptide sequence. The PelB signal peptide is an *E. coli* signal sequence. For mammalian cells we used the tissue plasminogen activator (TPA) signal peptide (GenBank #NM_033011). The TPA signal peptide was fused to the sFV via PCR primer MG TPA-APL10 5' primer (SEQ ID NO: 25) and MG APL10 3' primer (SEQ ID NO: 26).

APL10 was inserted into pgWIZ cut with the same enzymes. Resulting clones of pgWIZtpaSigP-APL10 were screened and one was chosen and sequence verified.

[0446] The IFNβ region was amplified by PCR using primers MG sigP(-)HIFNβ5' (SEQ ID NO:27) and MG HIFNβ3' (SEQ ID NO:28) and pDIZ HIFNβ-APL10 as a template. The wild-type signal peptide was removed and

MG TPA-APL10 5' primer
5'-GGATATCGCCACCATTGGATGCAATGAA-
GAGAGGGCTCTGTGTGTGTGTGTGTGTGTGGAGCAGTCTTCTGTTTCGCCAGCCAGGTACAGCTGCAGCA-3': (SEQ ID NO:25)

MG APL10 3' primer
5'-CGCGGCCGCTCAACCTAGGACGGTGACCTTGGTCCCTCCGCCGAACACCA-3': (SEQ ID NO:26)

[0445] The resulting TPA signal peptide (tpa SigP)-APL10 per product was digested with EcoRV and NotI and isolated via agarose gel electrophoresis. The digested tpa-SigP-

replaced with a (Gly-Gly-Gly-Ser)×2 linker. The signal peptide minus HIFNβ per product was digested with AvrII and NotI and inserted into pgWIZtpaSigP-APL10 cut with

20
20

the same enzymes to make pgWIZtpaSigP-APL10-HIFN β . The resulting products were screened by miniprep and verified by sequencing. To subclone the tpaSigP-APL10-HIFN β into pDIZ, pgWIZtpaSigP-APL10-HIFN β was cut with EcoRV and NotI and the tpaSigP-APL10-HIFN β fragment was gel purified.

with Lipofectamine 2000 (Invitrogen) and AZ-IFBC protein expression and secretion was examined after 3 days by western blot. We used the anti-human IFN- β , monoclonal antibody (R&D systems, cat.#MAB814). The protein was applied to a 1 ml protein A sepharose column to examine purification potential. The purified AZ-IFBC was assayed

MG sigP(-)HIFN β 5'
5'-GTCCTAGGTGGCGCGGAAGCGGAGGCTCCATGAGCTACAACCTGCTTGGATTCCCTACAAGAAGCAGCA-3': (SEQ ID NO:27)
MG HIFN β 3'
5'-TGC GGCCGCTTAGCTAGCTTATTAGTTTCGGAGGTAACCTGTAAGTCTGTTAATGAAGTAAAGTTCCT-3': (SEQ ID NO:28)

[0447] The tpaSigP-APL10-HIFN β fragment was inserted into pDIZ cut with EcoRV and NotI to make pDIZtpaSigP-APL10-HIFN β . The full-length insert was sequenced and verified to be correct.

for binding to Rat D6 as described above for functionality of the APL10 domain. The IFN β domain was examined by inhibition of virus-induced (vesicular stomatitis virus, VSV) cytopathic effect (cpe) as described below.

TPA SigP-APL10-IFN β (SEQ ID NO:29):					
ATGGATCAA	TGAAGAGAGG	GCTCTGCTGT	GTGCTGCTGC	TGTGTGGAGC	AGTCTTCGTT 50
TCGCCAGCC	AGGTACAGCT	GCAGCAATCA	GGGGGAGGCG	TGGTCCAGCC	TGGGAGGTCC 100
CTGAGACTCT	CCTGTGCAGC	CTCTGGATTG	ACCTTCAGTA	GCTATGCTAT	GCACTGGGTC 150
CGCCAGGCTC	CAGGGAAGGG	GCTGGAGTGG	GTCTCAGCTA	TTAGTGGTAG	TGGTGGTAGC 200
ACATACTACG	CAGACTCCGT	GAAGGGCCGG	TTCACCATCT	CCAGAGACAA	CGCCAAGAAC 250
TCACTGTATC	TGCAAAAGAA	CAGCCTGAGA	GCCGAGGACA	CGGCTGTGTA	TTACTGTGCG 300
AGAGATACCC	GAGGGTACTT	CGATCTCTGG	GGCCGTGGCA	CCCTGGTCAC	CGTCTCCTCA 350
GGTGGCGGAG	GGTCATCTGA	GCTGACTCAG	GACCCTGCTA	TGCTCTGTGC	CTTGGGACAG 400
ACAGTCAGAA	TCACATGTCA	AGGGGACAGT	CTCAGAAAGT	ATCATGCAAG	CTGGTATCAG 450
CAGAAGCCAG	GGCAGGCCCC	TGTTCTTGTC	ATCTATGGTA	AGAATGAACG	TCCCTCAGGG 500
ATCCCAGAGC	GATTCTCTGG	GTCCACCTCA	GGAGACACAG	CTTCCTTGAC	CATCAGTGGG 550
CTCCAGCGCG	AAGATGAGGC	TGACTATTAC	TGCTACTCCC	GAGACTCTAA	TGCTGATCTT 600
GTGGTGTTTG	GCGGAGGGAC	CAAGGTCACC	GTCTTAGGTG	GCGGCGGAAG	CGGCGGAGGC 650
TCCATGAGCT	ACAACCTTGCT	TGGATTCCCTA	CAAAGAAGCA	GCAATTTTCA	GTGTCAGAAG 700
CTCCTGTGGC	AATTGAATGG	GAGGCTTGAA	TACTGCCTCA	AGGACAGGAT	GAACCTTGAC 750
ATCCCTGAGG	AGATTAAGCA	GCTGCAGCAG	TTCAGAAAGG	AGGACGCCGC	ATTGACCATC 800
TATGAGATGC	TCCAGAACAT	CTTTGCTATT	TTCAGACAAG	ATTATCTAG	CACTGGCTGG 850
AATGAGACTA	TTGTTGAGAA	CCTCCTGGCT	AATGTCTATC	ATCAGATAAA	CCATCTGAAG 900
ACAGTCCTGG	AAGAAAAACT	GGAGAAAGAA	GATTTCACCA	GGGAAAAACT	CATGAGCAGT 950
CTGCACCTGA	AAAGATATTA	TGGGAGGATT	CTGCATTACC	TGAAGGCCAA	GGAGTACAGT 1000
CACTGTGCCT	GGACCATAGT	CAGAGTGGAA	ATCCTAAGGA	ACTTTTACTT	CATTAAACAGA 1050
CTTACAGGTT	ACCTCCGAAA	CTAA			1074

[0448] One correct clone was chosen and plasmid DNA (pDNA) obtained by Qiagen Maxiprep. The DNA (pDIZ-tpa SigP-APL10-IFN β) was transfected into CHO dhfr(-) cells

[0449] While the foregoing example describes the preparation of sFv- β -IFN conjugates as fusion proteins, the skilled artisan will understand that additional methods (e.g., chemi-