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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISION DE NUEVAS CREACIONES

E. S. D.

Re: Expediente No. **09.036.247**

Solicitud de patente a nombre de **CHIESI FARMACEUTICI S.p.A.**

Yo, SILVANA MARIA LOPEZ DÍAZ identificada como aparece al pie de mi firma, domiciliada en la Carrera 11 No. 86-53, piso 6, obrando como apoderada de la Sociedad **CHIESI FARMACEUTICI S.p.A.** domiciliada en **Parma, Italia**, solicitante de la patente de la referencia, en respuesta al oficio No. **3086** notificado por fijación en lista el 11 de Marzo de 2014, relacionado con concepto técnico de fondo, me permito manifestar:

1. Reivindicaciones

Con el objeto de atender a las objeciones hechas por su Despacho a las reivindicaciones en cuanto a la extensión del objeto como a la definición de la composición farmacéutica, presento un nuevo capítulo de reivindicaciones en donde se ha limitado el alcance del objeto de manera razonable y se ha definido la composición farmacéutica por sus componentes y proporciones así

- En cuanto a los análogos SP-C, se limitan a la fórmula (Ic) que comprende solamente el péptido SP-C33 y análogos muy similares, tal como el polipéptido (If), indicado como SP-C33(Leu);
- En cuanto a los análogos SP-B, a la formula general (II) limitada convenientemente para comprender solamente el péptido ox Mini-B y análogos muy similares, tal como el polipéptido (IIc), indicado como ox Mini-B(Leu);
- En cuanto a los fosfolípidos, se limita a las mezclas que comprenden las mezclas de dipalmitoilfosfatidilcolina (DPPC) y palmitoil-oleoil-fosfatidilglicerol (POPG).

Creemos que las anteriores limitaciones representan una generalización de los péptidos (Id) y (IIa) razonable y aceptable, y relacionadas con las combinaciones con los fosfolípidos reportados en los Ejemplos (en particular en el Ejemplo 3).

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Respecto al número de reivindicaciones, atentamente nos permitimos decir que no se paga tasa por reivindicaciones adicionales, toda vez que el nuevo capítulo reivindicatorio corresponde en esencia al mismo anteriormente presentado y contiene menor número de cláusulas.

También, con el objeto de complementar los anteriores argumentos se agregan los siguientes para dar más soporte a la similaridad de los péptidos SP-C33 y Mini-B del Ejemplo 3 y los objetos análogos de las dos nuevas fórmulas generales.

En cuanto a los análogos SP-C, las versiones truncadas en el terminal C del SP-C son igualmente eficientes in vitro e in vivo al péptido de longitud completa (Ver, Anexo 1, Almlen).

Así, el segmento en donde se localiza la única diferencia entre el SP-C33 y el SP-C33(Leu) puede dejarse fuera completamente sin consecuencias funcionales.

En cuanto a los análogos SP-B, el único remplazo de residuo entre los péptidos en cuestión, (mini.B vs. Mini-B(Leu)), es Met a Leu. Met y Leu tienen preferencias de estructura secundaria e hidrofobicidad similares (Ver, Anexo 2, Johansson), haciendo improbable que el remplazo vaya a afectar sus estructuras secundarias o sus asociaciones de fosfolípidos, y por lo tanto sus actividades in vitro e in vivo.

(mini.B vs. Mini-B(Leu)), es Met a Leu. Met y Leu tienen preferencias de estructura secundaria e hidrofobicidad similares (Anexo 2), haciendo improbable que el remplazo vaya a afectar sus estructuras secundarias o sus asociaciones de fosfolípidos, y por lo tanto sus actividades in vitro e in vivo.

Anexo también una evidencia posterior a la presentación de la solicitud que muestra que la combinación if+IIc tiene la misma actividad de la combinación Id+IIa luego del ensayo en el mismo modelo del Ejemplo 3 (Ver, Anexo 3).

3. Nivel inventivo

El señor examinador indica que la reivindicación 1 y sus dependientes originales carecen de nivel inventivo frente a la combinación de los documentos **D3 y D2**".

Estamos en total desacuerdo con su opinión.

La enseñanza de D3 está dirigida a tensoactivos reconstituidos que comprenden análogos de SP-C como aquellos de la presente invención, y SP-B o una

polimixina, en particular polimixina B como análogo SP-B (Página 5, líneas 3-4).

El problema técnico solucionado por D3 es suministrar una mezcla de portador lipídico mejorada según lo encontrado por los inventores de que la presencia de ácido palmítico es perjudicial para las propiedades del tensoactivo reconstituido.

D3 no dice nada acerca del efecto sobre el volumen del gas pulmonar y sobre la permeabilidad alveolar al final de la expiración (para las definiciones de volumen tidal y volumen del gas pulmonar véase la página 10 de la presente solicitud).

De otro lado, la solicitante encontró que *‘los tensoactivos reconstituidos [del arte previo] dan lugar a volúmenes de gas pulmonar y grado de permeabilidad alveolar pobres al final de la expiración, y se requiere ventilación con una presión de expiración final positiva (PEEP) para poder lograr una actividad in vivo comparable a aquella lograda con tensoactivos naturales modificados’* (Página 3, líneas 10-14).

La ventilación con PEEP en niños prematuros con el síndrome de distensión respiratoria (RDS), a su vez, ha sido reconocida por contribuir a daños pulmonares que pueden llevar al desarrollo de displasia del neumotórax y/o broncopulmonar y otros efectos colaterales.

Por lo tanto, empezando con D3, el problema técnico objetivo subyacente en la presente invención es el de suministrar “una preparación de tensoactivo reconstituido que sea capaz de garantizar la estabilidad alveolar, y por lo tanto mantener la permeabilidad alveolar al final de la expiración sin requerir ventilación con PEEP” (Página 5, líneas 20-23).

Esta solución es ofrecida por el tensoactivo reconstituido descrito en la nueva reivindicación 1.

A este respecto, la solicitante también observa que, como se mencionó en la descripción de la presente solicitud, el sistema portador proteína/péptido : lípido es una mezcla bastante compleja cuyas propiedades dependen grandemente de la interacción de los componentes y no se puede predecir “*a priori*”.

Por lo tanto, iniciando con las enseñanzas de D3, las cuales guardan silencio acerca del problema de la permeabilidad alveolar, la persona versada en la técnica relevante no obtendría ninguna señal o pista que lo llevara a seleccionar el análogo particular de D2 entre los diversos análogos divulgados en la técnica para hacer un tensoactivo *capaz de garantizar la estabilidad alveolar, y así mantener la permeabilidad o patencia alveolar al final de la expiración sin que se requiera de ventilación con PEEP*.

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En particular, considerando que D3 da una pista no ambigua para usar polimixina B como sustituyente funcional del SP-B, y la polimixina B es más pequeña y mucho más barata que los análogos de SP-B de D2 y también la persona versada en la materia no tiene nada que lo conduzca a remplazar la polimixina B por los análogos de D2.

Adjunto al presente una evidencia posterior a la solicitud que demuestra que el efecto de una combinación del péptido (Id), también referida como SP-C33 de la presente invención, con polimixina B sobre el volumen de gas pulmonar no es significativamente diferente de la del SP-C33 sola (Ver el anexo 4 en la forma de una Declaración para los Estados Unidos).

Por lo tanto, la reivindicación 1 tiene nivel inventivo frente a los documentos D3 y D2.

Habiendo atendido cabalmente las objeciones presentadas por ese despacho, atentamente solicitamos se conceda el privilegio de patente de invención a la solicitud en estudio o si el señor examinador considera que subsisten razones para objetar la presente invención, rogamos expedir otro examen de fondo con los debidos motivos y razones que sustenten su objeción con el fin de que nuestro mandante tenga la posibilidad de exponer sus argumentos con base en la interpretación de los artículos 45 de la Decisión 486 -el cual otorga la facultad al examinador para emitir los exámenes de fondo-, 35 y 59 del Código Contencioso Administrativo que señala que: *“habiéndose dado oportunidad a los interesados para expresar sus opiniones, y con base en las pruebas e informes disponibles, se tomará la decisión que será motivada al menos en forma sumaria si afecta a particulares”* y *“(..) deberá proferirse la decisión definitiva. Esta se motivará en sus aspectos de hecho y de derecho, y en los de conveniencia si es el caso”*, respectivamente (El subrayado es nuestro).

En consecuencia, solicitamos a ese despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el artículo 29 de la Constitución Nacional.

Señor Superintendente,


SILVANA MARIA LÓPEZ DÍAZ
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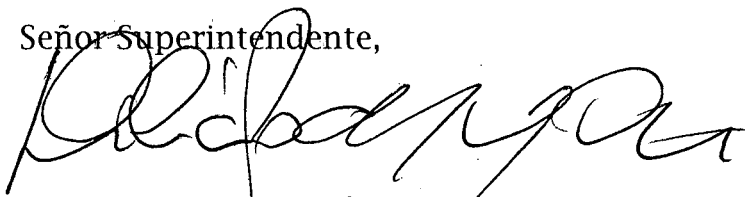
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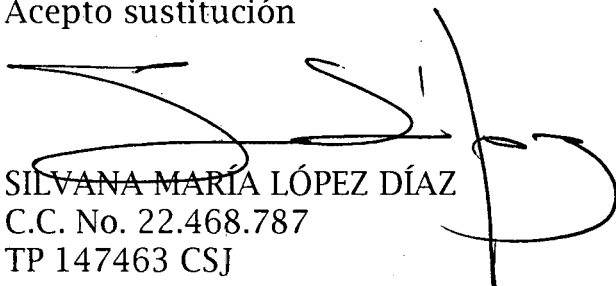
Yo, **DILIA RODRÍGUEZ D' ALEMAN**, identificada como aparece al pie de mi firma, domiciliada en Bogotá, Colombia, atentamente informo a usted que sustituyo el poder con que obro a la doctora **SILVANA MARÍA LÓPEZ DÍAZ**, domiciliada en Bogotá, identificada con la Cédula de Ciudadanía No. 22.468.787 y portadora de la tarjeta profesional No. 147.463 del CSJ, con las mismas facultades con que me fueron conferidas para este caso.

Señor Superintendente,



DILIA RODRIGUEZ D' ALEMAN
C.C. 41.654.882 de Bogotá
TP 20824 CSJ

Acepto sustitución



SILVANA MARÍA LÓPEZ DÍAZ
C.C. No. 22.468.787
TP 147463 CSJ

PRESENTACION PERSONAL

El anterior escrito fue presentado ante el
NOTARIO ONCE DEL CIRCULO DE BOGOTA, D.C.
personalmente por D. ALFONSO RODRIGUEZ

quien exhibió la c.c. 4651002

y Tarjeta Profesional No. 708284

Fecha 31 OCT 2013

Guillermo Chávez Cristancho
NOTARIO



[Large handwritten signature]
Guillermo Chávez Cristancho
NOTARIO ONCE DEL CIRCULO DE BOGOTA, D.C.

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ANEXOS

Nuevo capítulo de reivindicaciones

Anexo 1 – Almlen 2012

Anexo 2 – Johansson 2010

Anexo 3

Anexo 4 – Declaración

1113 PCT Argumentos presentados con la Demanda PCT

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REIVINDICACIONES

1. Tensoactivo reconstituido que comprende:

i) Una mezcla de dipalmitoil fosfatidilcholina (DPPC) y palmitoil oleoil fosfatidilglicerol (POPG);

5 ii) un análogo de polipéptido de la proteína tensoactiva nativa SP-C que tiene la secuencia representada por la fórmula general (Ic)



10 en donde:

Ω es un residuo de aminoácido seleccionado a partir del grupo que consiste de M o su oxidado sobre el átomo de azufre I, L y nL;

p es un entero que tienen un valor de 0 o 1; y

15 iii) un análogo de polipéptido de la proteína tensoactiva nativa SP-B, es un polipéptido de la fórmula general (II):



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en donde:

Ω es un residuo de aminoácido seleccionado independientemente a partir del grupo que consiste de M y L;

f es un número entero que tiene un valor de 0 o 1;

25 y

por lo cual dicho polipéptido de formula (II) está en la forma de molécula unida a disulfuro con un enlace disulfuro intramolecular entre dos residuos Cys en las posiciones 1 y 33 y/o entre dos residuos Cys en las

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posiciones 4 y 27.

2. Tensoactivo reconstituido de conformidad con la reivindicación 1, caracterizado porque el análogo de la proteína tensoactiva nativa SP-C y el análogo de la proteína tensoactiva SP-B, están presentes en una combinación fija.

3. Composición tensoactiva reconstituida de conformidad con cualquiera de las reivindicaciones 1 o 2, caracterizada porque el análogo de la proteína tensoactiva nativa SP-B, es representado por la fórmula general (IIa)

CWLCRALIKRIQAMIPKGGRLPQLVCRLVLRCS (IIa)

4. Composición tensoactiva reconstituida de conformidad con la reivindicación 1, caracterizada porque el análogo de la proteína tensoactiva nativa SP-C se selecciona a partir del grupo que consiste de:

IPSSPVHLKRLKLLLLLLLLLLLLLILGALLMGL	(Id)
IPSSPVHLKRLKLLLLLLLLLLLLLILGALLIGL	(Ie)
IPSSPVHLKRLKLLLLLLLLLLLLLILGALLLGL	(If)
IPSSPVHLKRLKLLLLLLLLLLLLLILGALLnLGL	(Ig)
IPSSPVHLKRLKLLLLLLLLLLLLLILGALL	(Ih)

5. Tensoactivo reconstituido de conformidad con la reivindicación 1, caracterizado porque el análogo de polipéptido de la proteína tensoactiva nativa SP-C es

IPSSPVHLKRLKLLLLLLLLLLLLLILGALLLGL (If)

y el análogo de polipéptido de la proteína tensoactiva nativa SP-B es

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CWLCRALIKRIQAMIPKGGRLPQLVCRLVLRCS (IIa)

6. Tensoactivo reconstituido de conformidad con la reivindicación 1, caracterizado porque el análogo de polipéptido de la proteína tensoactiva nativa SP-C es

IPSSPVHLKRLKLLLLLLLLLILLILGALLGL (If)

y el análogo de polipéptido de la proteína tensoactiva nativa SP-B es

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CWLCRALIKRIQALIPKGGRLLPQLVCRLVLRCS (IIc)

7. Una composición farmacéutica para la profilaxis y/o el tratamiento de una enfermedad respiratoria de un neonato que comprende el tensoactivo reconstituido de acuerdo con una cualquiera de las reivindicaciones 1 a 6.

8. La composición farmacéutica de acuerdo con la reivindicación 7 caracterizada porque está en la forma de una solución, o dispersión, o suspensión o polve seco.

20 9. La composición farmacéutica de acuerdo con la reivindicación 8 caracterizada porque está en la forma de suspensión acuosa.

10. La composición farmacéutica de acuerdo con la reivindicación 9 caracterizada porque comprende el tensoactivo reconstituido en una concentración comprendida entre 2 y 160 mg/ml.

11. La composición farmacéutica de acuerdo con la reivindicación 10 en donde la concentración del tensoactivo reconstituido está comprendida entre 20 y 80 mg/ml.

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Alterations of the C-terminal end do not affect *in vitro* or *in vivo* activity of surfactant protein C analogs

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ABSTRACT

The secondary structure, orientation and hydrogen/deuterium exchange of SP-C33, a surfactant protein C analog, in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine/egg phosphatidylglycerol (8:2, wt./wt.) bilayers, was studied by attenuated total reflection Fourier transform infrared spectroscopy. This showed a transmembrane α -helix, in which about 55% of the amide hydrogens do not exchange for up to 20 h. Moreover, C-terminally modified SP-C33, either truncated after position 30, or having the methionine at position 31 exchanged for either lysine or isoleucine, showed the same secondary structure and orientation. The different peptides, suspended in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol 68:31 (wt./wt.), were tested for surfactant activity *in vitro* in a captive bubble surfactometer and *in vivo* in an animal model of respiratory distress syndrome using premature rabbit fetuses. All preparations showed similar surface activity in the captive bubble surfactometer. Also, in the rabbit model, all preparations performed equally well and significantly better than non-treated controls, both regarding tidal volumes and lung gas volumes. Thus, truncation or residue replacements in the C-terminal part of SP-C33 do not seem to affect membrane association or surfactant activity.

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1. Introduction

The complex lipid/protein mixture of pulmonary surfactant reduces the surface tension at the alveolar air–liquid interface to near-zero values and thereby prevents the alveoli from collapsing at exhalation. Lack of active surfactant is associated with respiratory distress syndrome (RDS) which is common in premature babies. Surfactant consists of several components with different functional properties [1,2]. The main component, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), is responsible for surface tension reduction but is dependent on more fluid phospholipids and the hydrophobic surfactant specific proteins (SP-) SP-B and SP-C to rapidly adsorb to the air/liquid interface [3,4]. Both SP-B and SP-C are required for establishment of alveolar patency at end-expiration [5].

SP-C contains 35 residues with a random coil N-terminal segment and a middle/C-terminal transmembrane α -helix which is rich in valines. In spite of its rather simple structure, chemical synthesis of SP-C is complicated by the propensity of valine to form β -sheets instead of α -helices [6]. SP-C therefore is a metastable protein that tends to unfold and turn into β -sheets [7,8]. Different approaches have been taken to obtain SP-C analogs and we have previously designed the SP-C analog, SP-C33, that has been shown to have surfactant properties similar to native SP-C [9]. Differences of this peptide compared to human SP-C includes removal of the two N-terminal amino acid residues, replacement of the palmitoyl-Cys residues at positions 5 and 6 (positions 3 and 4 in SP-C33) with Ser, replacement of Leu 14 with Lys, and replacement of the poly-valine part (positions 15–21 and 23–28) with poly-leucine.

The mechanism of SP-C action is not known, and several different hypotheses have been discussed. It has been shown that SP-C disorders the acyl chains of a phospholipid bilayer while the head groups are stabilized, possibly due to its mobility gradient across the bilayer [10,11]. The mobility gradient is suggested to derive from interactions of a Lys–Arg pair in the N-terminal end of the transmembrane α -helix with phospholipid head-groups and lack of residues in the C-terminal end that are able to interact with surrounding phospholipids. In the “squeeze-out” hypothesis, SP-C is assumed to contribute to the process of removal of non-DPPC lipids from the air/liquid interface upon overcompression [1]. SP-C is also thought to facilitate the reinsertion of surface active

Abbreviations: CBS, captive bubble surfactometer; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; ATR-FTIR, attenuated total reflection Fourier transform infrared spectroscopy; LGV, lung gas volume; PEEP, positive end expiratory pressure; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; RDS, respiratory distress syndrome; SP, surfactant protein

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molecules into the interface from surface-associated reservoirs during alveolar expansion [12].

In this study, we have examined the secondary structure, orientation, and dynamics of SP-C33 and how modifications of the C-terminal end of SP-C33 affect the activity of a synthetic surfactant preparation. Three different SP-C33 variants were studied. SP-C30 is a truncated variant that is predicted to be too short to fully span a phospholipid bilayer and thereby may obtain increased mobility. SP-C33M31K, in which the methionine at position 31 has been exchanged with a lysine, may anchor the C-terminal part of the α -helix in the bilayer and thereby reduce its mobility gradient. SP-C33M31I, where the methionine at position 31 was substituted with an isoleucine, is not expected to affect mobility of the peptide.

2. Materials and methods

2.1. Lipids and peptides

DPPC and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) were obtained from Sigma Chemical (St. Louis, MO, USA).

SP-C33 (IPSSPVHLKRLKLLLLLLLLLILGALLMGL) was provided by Chiesi Farmaceutici S.p.A (Parma, Italy).

SP-C33 M31K (IPSSPVHLKRLKLLLLLLLLLILGALLKGL), SP-C33 M31I (IPSSPVHLKRLKLLLLLLLLLILGALLIGL), and SP-C30 (IPSSPVHLKRLKLLLLLLLLLILGALL) were obtained from Thermo Electron GmbH.

2.2. Surfactant preparations

DPPC/POPG (68:31, wt./wt.) was dissolved in chloroform/methanol (1:1, vol./vol.) and mixed with either of the four SP-C analogs. The peptide content in the preparations was 2% in relation to the phospholipid weight. The solvents were evaporated under nitrogen and the preparations were resuspended in normal saline to a final phospholipid concentration of 80 mg/ml by slow rotation at 37 °C. The preparations were stored at –20 °C. Prior to examination of *in vitro* surface activity, all solutions were diluted with saline to a final concentration of 10 mg phospholipids per milliliter.

For attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy of the SP-C analogs in DPPC/PG (8:2, wt./wt.), lipid/peptide mixtures in chloroform/methanol (lipid/peptide mass ratio, 100:10 or 100:20) were evaporated under nitrogen and dried under vacuum for 3 h. The lipid/peptide films were then suspended in 5 mM HEPES buffer, pH 7.4. Lipid vesicles containing the peptides were separated from unbound constituents by centrifugation in a 5%–40% (wt./vol.) continuous sucrose gradient. The sucrose gradient was fractionated and the proteoliposomes were detected by turbidity measurement at 410 nm. To eliminate sucrose, the vesicles were pelleted twice by ultracentrifugation and resuspended in 5 mM HEPES buffer, pH 7.4.

2.3. Structure determination and hydrogen/deuterium (H/D) exchange measurements

Structure and orientation of SP-C analogs in phospholipid membrane and H/D exchange for SP-C33 were obtained by ATR-FTIR spectroscopy [13]. ATR-FTIR spectra were recorded at 20 °C on a Bruker IF-55 spectrometer (Ettlingen, Germany), equipped with a liquid nitrogen cooled mercury cadmium telluride detector. The internal reflection element was a germanium crystal (50×20×2 mm; ACM, Villiers St. Frédéric, France) with an aperture angle of 45° yielding 25 internal reflections. The samples were spread on the ATR element by slowly evaporating the solvent under nitrogen. The spectra were recorded at a nominal resolution of 2 cm^{–1} with 256 accumulations to improve the signal/noise ratio. The secondary structure was determined from the shape of the deuterated amide I band [14]. Briefly, Fourier self-

deconvolution was applied to detect the different secondary structure components. To quantify the percentage of each resulting band, a least square iterative curve-fitting was performed to fit Lorentzian line shapes to the initial spectrum between 1700 and 1600 cm^{–1}. The percentage of each secondary structure was determined from the Lorentzian band areas in the respective frequency region.

The orientation of SP-C analogs in a lipid bilayer was determined from the orientation of the peptide bond C=O group as described previously [14]. Additional spectra with parallel and perpendicular polarized light with respect to the incidence plane were recorded. To determine the α -helix orientation, a 27° deviation angle between the α -helix axis and the C=O transition dipole moment was taken into account. The mean angle between the helix axis and a normal to the ATR plate was calculated from the dichroic ratio. The dichroic ratio corresponding to the transition dipole moment of the $\gamma_{\text{w}}(\text{CH}_2)$ band at 1200 cm^{–1} was used to characterize the lipid acyl chain orientation.

H/D exchange of accessible amide protons was obtained by flushing the sample on the ATR element with D₂O saturated nitrogen at room temperature. The decay of the NH-associated amide II band area (1520–1580 cm^{–1}) to lipid $\nu(\text{C}=\text{O})$ band area was used to monitor the H/D exchange in the amide group [14].

2.4. Circular dichroism (CD) spectroscopy

SP-C33 was dissolved in either methanol or in sodium dodecyl sulfate (SDS) micelles in sodium phosphate buffer, pH 6.0, to a final concentration of 25 μM . CD spectra from 250 to 190 nm were recorded in 1.0 mm path length quartz cuvettes using a J-810 spectropolarimeter (Jasco, Tokyo, Japan). The scan speed was 50 nm/min, response time was 2 s, and with an acquisition interval of 0.5 nm and band width of 1 nm. The residual molar ellipticity (θ) is expressed in $\text{kdeg} \times \text{cm}^2 \times \text{dmol}^{-1}$. The α -helical content was calculated with the following equations: $(\theta_{208} - 4000 / -37000) \times 100$, $(\theta_{222} - 3000 / -39000) \times 100$ [15].

2.5. In vitro surface activity measurements

Surface tension was measured in triplicates in an *in vitro* simulation of the alveolus by a captive bubble surfactometer (CBS) [16]. In the CBS, surfactant and an air-bubble representing the alveolus are present in an air-tight enclosed chamber. To evaluate surface activity under dynamic circumstances, the chamber is compressed and surface tension can be calculated by studying the shape and height/width ratio of the bubble.

In the experiment, 2 μl of the surfactant preparation (10 mg/ml) was inserted into the sucrose-filled test chamber. After insertion, an air-bubble was created and surface tension was measured during 5 min of adsorption. In the following quasi-static cycling experiments, the bubble was compressed stepwise from the initial volume until a surface tension less than 5 mN/m was reached, alternatively to a maximum area compression of 50%, and then expanded during five cycles. For each run, minimum and maximum surface tensions as well as rate of compression were registered and a median value was calculated.

2.6. In vivo experiments

To assess the surfactant activity of the different preparations *in vivo*, preterm newborn rabbits obtained at a gestational age of 27 days (term, 31 days) were used. The animals, randomly allocated to different treatment groups, were anesthetized directly after birth, tracheotomized, and then received one of the surfactant preparations via a tracheal cannula. Surfactant, 80 mg/ml, was administered at a dose of 2.5 ml/kg and non-treated animals served as negative controls. The animals were kept in pletysmograph boxes at 37 °C and ventilated in parallel with 100% oxygen at a frequency of 40 Hz and an inspiration/expiration ratio of 1:1. The experiments were

performed with a positive end-expiratory pressure (PEEP) of 3–4 cm H₂O. To open up the lungs, peak inspiratory pressure (PIP) was first set at 35 cm H₂O for 1 min. Then, pressure was lowered to 28 cm H₂O for 2 min and further on to 23 cm H₂O for 2 min, 18 cm H₂O for 15 min, 13 cm H₂O for 5 min, and 10 cm H₂O for 5 min. Finally, pressure was raised again to 18 cm H₂O for 5 min, after which the lungs were ventilated for additional 5 min with nitrogen to minimize volume loss due to diffusion. Tidal volumes were recorded every 5 min during the experiment. At the end of the experiment, the tracheal cannula was clamped at end expiration, the trachea was ligated, and the lungs were excised and weighed. Lung gas volumes were determined by water displacement technique [5,17].

2.7. Statistical analysis

Statistical differences were analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison test using GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA).

2.8. Ethical approval

Animal experiments were approved by the local ethical committee for animal research, Stockholms Norra Djurförsöksetiska Nämnd (205/04, 316/06).

3. Results

3.1. SP-C analogs secondary structure and orientation in a phospholipid membrane

The structure of SP-C analogs reconstituted in a DPPC/PG (8:2, wt./wt.) environment was determined by ATR-FTIR. Analysis of the IR-spectrum in the spectral region associated with secondary structure shows that SP-C33 mainly adopts an α -helical structure, characterized by an amide I band (1700–1600 cm⁻¹) maximum at 1657 cm⁻¹ (Fig. 1A). Quantification of the different secondary structure components by deconvolution and curve-fitting of the amide I band demonstrates that more than 90% of the polypeptide chain forms an α -helical structure. Upon reconstitution in a lipid bilayer, the SP-C33M31K, SP-C33M31I, and SP-C30 peptides adopt a similar α -helical structure as illustrated by the superimposition of their respective amide I bands (Fig. 1B).

SP-C33 dissolved in methanol or SDS micelles was analyzed by CD spectroscopy. The resulting spectra (Fig. 2) showed double minima at 208 and 222 nm, typical features for α -helical proteins. Estimation of the α -helix contents from the residual molar ellipticities at 208 and

222 nm yields about 75% helix for SP-C33 in SDS micelles and more than 90% in methanol.

To determine the orientation of SP-C analogs inserted in a lipid bilayer, spectra were recorded with two orthogonal polarizations of the incident light, parallel (//) and perpendicular ($\perp$) with respect to the incident plane as illustrated for SP-C33M31I in Fig. 3 as an example. The difference spectrum (//– $\perp$) reports an important positive deviation in the amide I band region (Fig. 3C). From the dichroic ratios of this band, the calculated maximum tilts of the long helix axis with respect to a normal to the crystal surface were 22°, 18°, 18°, and 17° for SP-C33, SP-C33M31K, SP-C33M31I, and SP-C30, respectively. From the dichroic ratios of the $\gamma_{\text{w}}(\text{CH}_2)$ band at 1200 cm⁻¹, we have determined that the maximum tilt between the lipid hydrocarbon chains and a normal to the germanium surface was 25°. These data demonstrate that the four SP-C analogs adopt a dominantly transmembrane orientation in a lipid bilayer.

3.2. H/D exchange of SP-C33 in DPPC/PG

H/D exchange kinetics of SP-C33 reconstituted in a DPPC/PG (8:2, wt./wt.) lipid bilayer was recorded using ATR-FTIR where the sample was flushed with D₂O at room temperature. After 12 h of deuteration, 55% of the peptide bond protons had not undergone exchange (Fig. 4). This implies that about half of the helical structure of SP-C33 is rigid enough to resist exchange of amide hydrogens. No modification of the α -helical structure was observed during the kinetics, indicating that the secondary structure of SP-C33 is stable.

3.3. In vitro surface activity of SP-C33 and variants thereof

For comparison of the *in vitro* surface activity of the different peptides in DPPC/POPG (68:31, wt./wt.), the surface tension during dynamic conditions was evaluated in the CBS. After the initial adsorption, quasi-static expansion was performed in which all preparations showed similar values with no significant differences regarding maximum and minimum surface tensions after five pulsation cycles (Table 1). The medians of the different synthetic preparations showed γ_{min} around 1–2 mN/m and γ_{max} between 22–27 mN/m. In addition, the degree of compression required to reach 5 mN/m was similar (22%–32%) with no significant differences between tested preparations.

3.4. In vivo activity of SP-C33 and variants thereof

In line with the *in vitro* results, all peptide-containing preparations behaved similarly in a rabbit model of neonatal RDS. The rabbits were

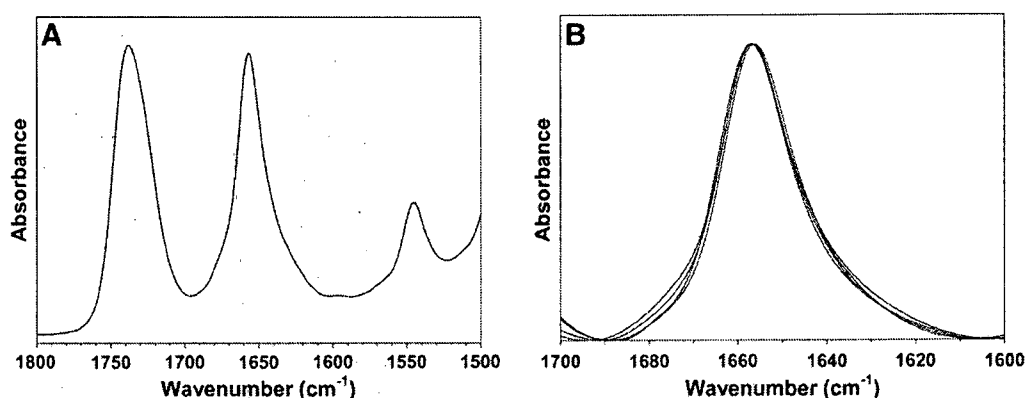


Fig. 1. (A) ATR-FTIR spectrum in the 1800–1500 cm⁻¹ region for SP-C33 in DPPC/PG (8:2, wt./wt.) lipid environment. The amide I band (1700–1600 cm⁻¹) presents the characteristic shape of an α -helical peptide with a maximum at 1657 cm⁻¹. (B) Superimposition of SP-C33, SP-C33M31K, SP-C33M31I, and SP-C30 amide I bands. The four peptides were reconstituted in DPPC/PG (8:2, wt./wt.) lipid environment. The samples in panels A and B were deuterated during 20 min and the contributions of lateral side chains were subtracted.

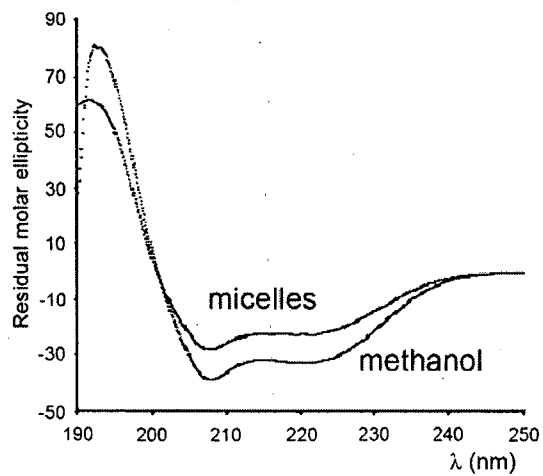


Fig. 2. CD spectra of SP-C33 dissolved in SDS micelles or methanol. The residual molar ellipticity (θ) is expressed in $\text{kdeg} \times \text{cm}^2 \times \text{dmol}^{-1}$.

ventilated with a standardized sequence of insufflations pressure, and throughout experiments, tidal volumes were very similar in all groups that received surfactant. After 35 min of ventilation, tidal volumes around 12–15 ml/kg were observed (Fig. 5). Likewise, the lung gas volumes measured after the experiments were similar for all surfactant-treated groups (Fig. 6). For tidal volumes, throughout the whole experiment, as well as lung gas volumes, all the surfactants containing peptides were significantly better than non-treated controls but with no significant differences between the tested surfactant preparations.

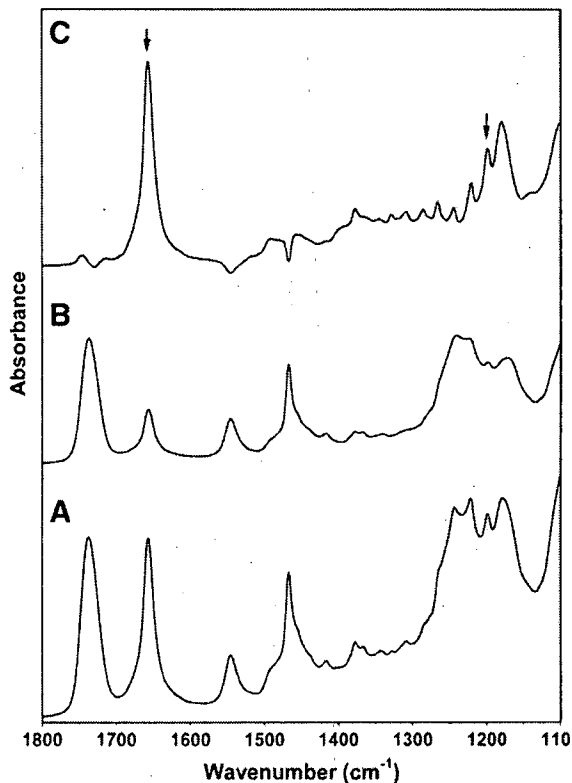


Fig. 3. ATR-FTIR polarized spectra of SP-C33M31I reconstituted in DPPC/PG (8:2, wt./wt.) lipid environment. The spectra were recorded with parallel (A) and perpendicular (B) polarized light with respect to the incident plane. (C) The dichroic spectrum obtained by subtracting (A–B) spectra is expanded twofold in the ordinate direction. The arrows indicate the protein amide I and the phospholipid $\gamma_{\text{w}}(\text{CH}_2)$ bands.

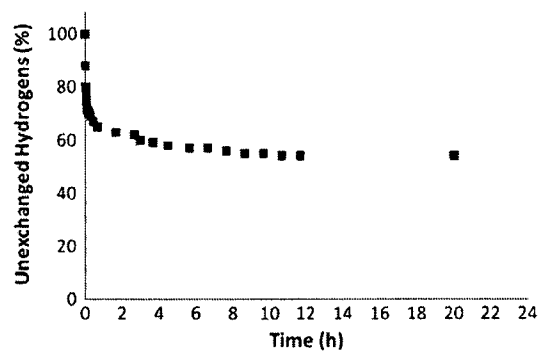


Fig. 4. Hydrogen/deuterium exchange kinetics evaluated using ATR-FTIR for SP-C33 reconstituted in DPPC/PG (8:2, wt./wt.). Evolution of the proportion of unexchanged amide bonds as a function of deuteration time.

4. Discussion

Investigation of the secondary structure of the SP-C analog SP-C33 using FTIR and CD spectroscopy reveals that the peptide adopts an α -helical structure when inserted in a DPPC/PG lipid environment, in SDS micelles and in methanol. The results obtained show that 75%–90% of the peptide adopts helical conformation. Previous FTIR and CD studies of native SP-C indicate an α -helical content of 60%–80% [14,18] while the predecessors to SP-C33, SP-C(Leu) and SP-C(LKS), show an α -helical content of 84% [19] and 75% [20], respectively. This indicates that the overall helical contents of native SP-C and analogs thereof determined by CD and FTIR spectroscopy are similar and in good agreement with the α -helical content (about 75%) deduced from the NMR structure of native SP-C dissolved in chloroform/methanol [21]. We have demonstrated that the three C-terminally modified variants of SP-C33 adopt the same conformation as SP-C33 which is consistent with the fact that all so far analyzed SP-C variants with a poly-leucine sequence or other synthetic analogs SP-C(CC) and SP-C (1–21) have very similar helical contents [22].

The orientation of SP-C in lipid membranes have been studied comprehensively [11,14,18], and in a lipid membrane, native SP-C has been shown to adopt an orientation where the α -helix is inserted parallel to the lipid acyl chains. This transmembrane location is assumed to be an important part of its surfactant activity and is therefore desirable to include in an analog. By recording FTIR-spectra with parallel and perpendicular light with respect to the incidence plane, the transmembrane orientation of SP-C analogs when inserted in DPPC/PG (8:2, wt./wt.) was confirmed.

In contrast to the propensity of native SP-C to transform from α -helix to β -sheet in organic solvents, it largely retains its helical structure when inserted in phospholipids. This is in line with the observation that membrane embedded valine-rich helix domains are protected in a hydrophobic environment. Using H/D exchange, membrane proteins with higher abundance of helix-stabilizing leucines showed less exchange of amide hydrogen atoms than those

Table 1
In vitro surface activity of suspensions (10 mg/ml) containing SP-C analogs (2% wt./wt.), in DPPC/POPG (68:31, wt./wt.) measured in the captive bubble surfactometer.

Peptide in DPPC/POPG (68:31) <i>n</i> = 3 for all preparations	Compression needed to reach 5 mN/m (area, %)	γ_{min} (mN/m)	γ_{max} (mN/m)
SP-C33	32 (25–33)	1.1 (1.0–1.6)	27 (26–27)
SP-C30	26 (25–27)	1.8 (1.0–2.7)	22 (18–27)
SP-C33 M31K	32 (20–33)	2.3 (1.9–4.4)	24 (18–25)
SP-C33 M31I	22 (15–33)	0.9 (0.7–1.1)	26 (25–28)

Compression is measured as the difference in bubble area between maximum surface tension and when surface tension less or equal to 5 mN/m is reached. Presented values for minimum and maximum surface tensions are from the fifth cycle of quasi-static dynamics. Values are given as median (range).

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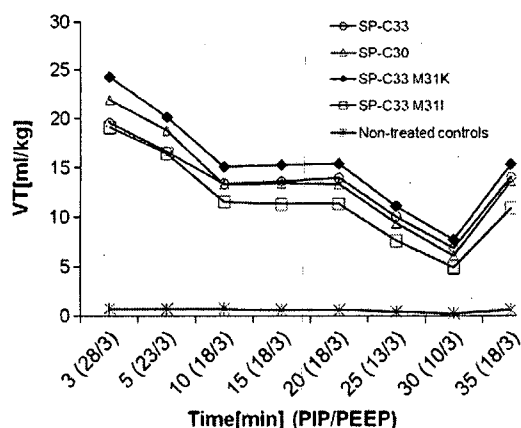


Fig. 5. Tidal volumes (V_T) in preterm newborn rabbits treated with SP-C analogs in DPPC/POPG (68:31, wt./wt.) (80 mg/ml, 2.5 ml/kg, $n=8$ (SP-C33, SP-C30, SP-C33 M31K) or $n=7$ (SP-C33 M31I)) or non-treated controls ($n=7$). Values are given as median. Statistical significance: $p<0.001$ for non-treated controls vs. all other groups at all time points.

containing the helix-destabilizing residues valine and isoleucine [23]. This would suggest that SP-C33, with a poly-leucine helix, is less flexible than native SP-C, with a poly-valine helix. In contrast, our results show a faster H/D exchange with about 60% unexchanged hydrogens for SP-C33 after 4 h compared to 70%–90% for native dipalmitoylated SP-C [14,18]. However, after 20 h, the H/D exchange was rather similar for both SP-C33 and native SP-C with about 55%–60% unexchanged hydrogens. These results suggest that, while the presence of a few helix-destabilizing Val or Ile increases flexibility, a long poly-valine helix like in native SP-C is exceedingly stable. This is in line with the observation that the SP-C helix is stabilized by a high activation barrier to unfolding and that, once helix unfolding occurs, it is not reversible. The presence of peptide bound fatty acyl chains has a stabilizing effect on conformationally dynamic helices in general [24] and the palmitoyl groups in SP-C stabilize the helix [25]. It is possible that the palmitoyl groups linked to SP-C are important for preventing unfolding of the metastable α -helix *in vivo*. The helix of SP-C33, in contrast, is able to unfold and refold and is consequently not dependent on covalently linked fatty acyl chains for stabilization. In line with this supposition, palmitoylation of SP-C33 does not result in alterations of its function [9].

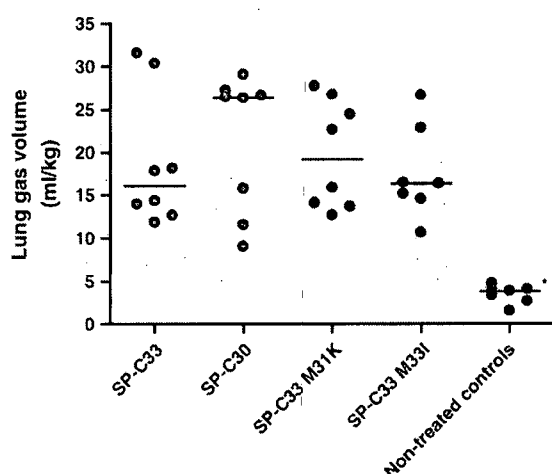


Fig. 6. Lung gas volumes after 35 min of ventilation in preterm newborn rabbits treated with SP-C analogs in DPPC/POPG (68:31, wt./wt.) (80 mg/ml, 2.5 ml/kg) or non-treated controls. Levels of statistical significance: $*p<0.001$ vs. all other groups. The lines indicate median values.

The effects of modifications of the C-terminal end of SP-C on surface activity were investigated using variants of SP-C33. From the CBS *in vitro* study, no significant differences in surface activity due to the alterations were noticed. All preparations were equally active with surface tension shifting from ~25 mN/m to ~1 mN/m at a compression rate of 25%–30%. There were also no significant differences found *in vivo*; tidal volumes and lung gas volumes, reflecting the functional residual capacities, were all approximately the same, despite the exact nature of the C-terminal part. These findings can be related to reports on other surfactant protein analogs. It seems that the presence of an α -helical structure is of greater importance than the primary structure since exchange of the whole helical sequence of SP-C with that from bacteriorhodopsin does not influence the surface activity *in vitro* [22]. In the same study, it was shown that the length of the helix was of importance since truncation of the native SP-C sequence at residues 12, 17, or 21 noticeably decreased surface activities, while in our study, SP-C30 showed no variance compared to the longer peptides. This implies that an α -helical peptide that is able to span at least the nonpolar parts of a phospholipid membrane is necessary for activity of a synthetic surfactant. The importance of the helical length for surface activity has also been shown in a study using peptoid SP-C mimics. Here, it was shown that a helical length of approximately 28 Å was superior in comparison with peptoids containing shorter helices [26].

Earlier studies show surfactant activity of SP-C33 to be similar to the activity of native SP-C. Both *in vivo* and *in vitro* data of the modified peptides in this study, in comparison to SP-C33, thus suggest that all the studied analogs are similarly efficient regarding surfactant activity as native SP-C and could therefore be used as a functional analog in a synthetic surfactant.

Acknowledgments

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Conformational preferences of non-polar amino acid residues: An additional factor in amyloid formation

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ABSTRACT

Amyloid consists of β -sheet polymers and is associated with disease and with functional assemblies. Amyloid-forming proteins differ widely in native structures and sequences. We describe here how conformational preferences of non-polar amino acid residues can affect amyloid formation. The most non-polar residues promote either β -strands (Val, Ile, Phe, and Cys, VIFC) or α -helices (Leu, Ala, and Met, LAM), while the most polar residues promote only α -helices. For 12 proteins associated with disease, the localizations of the amyloid core regions are known. Eleven of these contain segments that are biased for VIFC, but essentially lack segments that are biased for LAM. For the amyloid β -peptide associated with Alzheimer's disease and an amyloidogenic fragment of the prion protein, observed effects of mutations support that VIFC bias favors formation of β -sheet aggregates and amyloid, while LAM bias prevents it. VIFC and LAM profiles combine information on secondary structure propensities and polarity, and add a simple criterion to the prediction of amyloidogenic regions.

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1. Introduction

Amyloid consists of polypeptides that have turned into insoluble β -sheet fibrils and is associated with about 30 human diseases, e.g. Alzheimer's disease (amyloid β -peptide, A β), Creutzfeldt–Jakob disease (prion protein, PrP), and systemic amyloidosis (lysozyme) [1,2]. The most common human amyloid is formed from a fragment of lactadherin, medin, which is found in the aortic wall of virtually all above age sixty [3]. Amyloid can be formed from wildtype, non-processed protein, but in some cases mutations or proteolytic cleavages are required to make the protein amyloidogenic [4]. Amyloid is not only associated with disease but so-called functional amyloid can generate, for example, bacterial pili and human pigment binding templates, or regulate translation in yeast [5,6]. Amyloid fibrils derived from the yeast prion protein Sup35 are formed by self-complementary side-chain interactions in steric zippers, showing that fibril formation is sequence-dependent [7]. Similar types of steric zippers have been found in fibrils derived from A β , PrP and other amyloidogenic regions [8].

The human proteome harbors many proteins that are prone to aggregate and to form self-complementary steric zippers, the “amylome” [9,10], but apparently only a small fraction of these actually forms amyloid. Regions prone to aggregate or form steric zippers are often buried, flanked by charged residues or twisted, making them less likely to form intermolecular β -sheets [10,11].

However, it is not clear what distinguishes truly amyloidogenic proteins from other proteins in the amylome. This has motivated the search for factors that determine amyloid formation, and several algorithms that predict amyloidogenic regions are available. For example, the Waltz algorithm combines polarities, α -helix and β -strand propensities with charge of amino acid residues, and takes into account sequence dependency to identify regions prone to form amyloid fibrils [12]. In an attempt to further elucidate determinants of amyloid formation we have here investigated the conformational preferences of non-polar amino acid residues and their distribution in amyloid-forming proteins.

2. Materials and Methods

Human proteins associated with amyloid disease [13], except immunoglobulins (since their sequences are unique to each individual with light or heavy chain amyloidosis) and keratin (since it exists in several isoforms and it is unknown what isoform is associated with amyloidosis) were studied. The percentages of Val + Ile + Phe + Cys (VIFC) and Leu + Ala + Met (LAM) were calculated for overlapping pentapeptide segments and plotted for the first residue of each segment along the respective amino acid sequence. VIFC bias is defined as a segment in which $\geq 60\%$ are VIFC residues and $\leq 20\%$ are LAM residues. The VIFC and LAM profiles for five functional amyloid proteins, in which the amyloid core regions have been localized were analyzed in the same manner. The average total contents of VIFC and LAM in globular proteins are about 17% and 20%, respectively.

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3. Results

Plotting hydropathy against secondary structure propensities showed that non-polarity of amino acid residues correlates positively with β -strand propensity, but there is no correlation between α -helix propensity and polarity (suppl. Fig. S1). These findings agree well with the fact that β -sheets overall are more non-polar and less surface exposed than α -helices [14] and that β -aggregation regions are generally buried in non-polar protein cores [11]. Plotting the ratios of β -strand and α -helix propensities versus polarities (Fig. 1) reveals that there is a striking positive correlation between the strand/helix ratios and non-polarity for 17 of the 20 amino acid residues. Interestingly, the non-polar residues Leu, Ala and Met (LAM), in sharp contrast to the non-polar Val, Ile, Phe, Cys (VIFC), do not follow this correlation, but favor α -helix over β -strand formation to the same extent as the strongly polar residues do. A striking example of the result of VIFC $\rightarrow$ LAM replacements is offered by lung surfactant protein C (SP-C). SP-C and its precursor proSP-C can form amyloid in association with alveolar proteinosis and lung fibrosis [15,16]. The transmembrane (TM) region of proSP-C consists essentially only of Val, the non-polar residue with highest β -strand propensity (suppl. Fig. S1). The proSP-C TM region is predicted by Waltz [12] (<http://waltz.switchlab.org/>) to be amyloidogenic. Replacement of the polyVal segment of (pro)SP-C with a polyLeu segment, however, abolishes amyloid formation *in vitro* [16,17], but this polypeptide is still predicted to form amyloid with equal probability as SP-C.

The importance of the nature of non-polar residues on amyloid fibril formation has previously been studied for other peptides. For example, the tetra-peptides KVVE and KFFE form β -sheet structure and amyloid-like fibrils *in vitro*, but KLLLE and KAAE do not [18]. Likewise, A β_{12-28} with the central segment K₁₆LVFF₂₀ changed to K₁₆LAAA₂₀ does not form amyloid-like fibrils [19]. The data in Fig. 1 show that the different amyloidogenic behaviors of these otherwise similar peptides can be rationalized in a straightforward

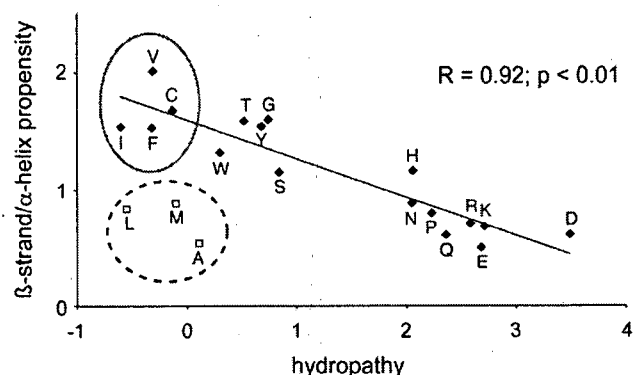


Fig. 1. Correlation between polarities and β -strand/ α -helix propensity ratios. The ratio of β -strand and α -helix propensity values for each amino acid residue is plotted vs. its hydropathy. The linear trend line shows a statistical significant correlation between strand/helix propensity ratios and non-polarity for the 17 residues marked with black diamonds. Leu, Ala, and Met (LAM, marked with open squares and circled with a dashed line) combine non-polarity with low strand/helix ratios. The non-polar β -preferring residues Val, Ile, Phe, and Cys (VIFC) are circled with a red line. The hydropathy values are the biological hydrophobicity values, determined from the ability of the respective residue to insert into the ER membrane [33], residues with values <0 promote membrane insertion. A correlation is likewise obtained when water to lipid partitioning [34], or Kyte–Doolittle hydropathy [35] scales are used to represent hydropathy (Supplementary Fig. S2). The β -strand and α -helix propensities are derived from relative occurrences of amino acid residues in different secondary structure elements [17], and they correlate with intrinsic β -strand propensities derived from physico-chemical properties [36] (correlation coefficient $R = 0.76$), and with experimental α -helix propensities [37] ($R = 0.91$), respectively.

manner from the β -strand/ α -helix propensity ratios of their non-polar residues (high for V and F, low for L and A). This suggests that amyloidogenic behavior in general can be influenced by local distribution of the β -favoring VIFC relative to the α -favoring LAM. We therefore investigated the VIFC and LAM density along the amino acid sequences of 26 proteins that are known to form amyloid in association with human disease [13].

For 12 of the 26 amyloid proteins, the localizations of the amyloid core regions along the polypeptide chain have been determined. Fig. 2 shows the VIFC and LAM profiles for A β , PrP, lysozyme and serum amyloid protein A, and the profiles for the remaining eight proteins with identified amyloid core regions are shown in supplementary Fig. S3. In 11 of these 12 cases (A β , PrP, lysozyme, transthyretin, α -synuclein, cystatin C, serum amyloid protein A, lactadherin, tau, gelsolin, and odontoblast amyloidogenic protein), the respective amyloid core contains one or two VIFC biased segments. However, only one amyloid core region (in α -synuclein) contains a LAM biased segment. Apparently VIFC biased segments are overrepresented and LAM biased segments underrepresented in known disease amyloid cores. The remaining 14 proteins associated with human disease lack yet identified amyloid core regions. Out of these 14, nine proteins (ABri, ADan, insulin, apolipoproteins A-II and A-IV, semenogelin I, keratopithelin, fibrinogen α -chain, and lactotransferrin) contain VIFC biased segments.

For A β_{1-42} , two regions, corresponding to residues 16–22 and 36–42, respectively, are VIFC biased, but no LAM biased segments are found (Fig. 2). In A β_{1-42} fibrils the β -strands are composed of residues 17–26 and 31–42 [20], i.e. they correspond to the VIFC biased regions. The mutations Ala21Gly and Leu34Val in A β give rise to early-onset Alzheimer's disease [21]. Among factors that are known to collectively influence the ability to form β -sheet aggregates [22], these mutations strongly affect the β -strand/ α -helix preferences (i.e. the VIFC and/or LAM profiles), without significantly affecting polarity or charge (see Fig. 1). Moreover, an unbiased search for mutations that reduce the aggregation propensity of A β_{1-42} resulted in 36 A β variants containing in total 96 amino acid replacements [23]. Of these aggregation-reducing replacements, 17 were VIFC $\rightarrow$ LAM, but only one was LAM $\rightarrow$ VIFC. These data suggest that distributions of VIFC and LAM affect the tendency of A β to form amyloid *in vivo* and *in vitro*, where local VIFC bias promotes amyloid formation, while LAM bias prevents it.

The amyloid core of human PrP consists of residues 160–220 [24]. This region contains one VIFC biased segment, corresponding to residues 175–184, but no LAM biased segment (Fig. 2). Most known mutations linked to Creutzfeldt–Jakob disease are found within residues 171–211 [25], i.e. in or close to the VIFC biased region. The VIFC biased region constitutes part of PrP helix 2. This helix region can form amyloid-like fibrils on its own as well as seed fibril formation of full-length PrP, suggesting that it is critical in the initiation of fibril formation [26]. A synthetic 23-residue peptide corresponding to PrP helix 2 (residues 172–194 of human PrP), but not a variant thereof in which seven VIFC residues had been replaced with Ala, formed amyloid-like fibrils after incubation in neutral buffer, as judged by transmission electron microscopy (not shown). Although this result is anticipated from the secondary structure propensities of these residues, it suggests that simple VIFC and LAM profiles can translate the different strand/helix preferences of these two groups of non-polar residues into amyloid propensities.

We also analyzed the VIFC and LAM profiles for five functional amyloid proteins, in which the amyloid core regions have been localized (Suppl. Fig. S4). This showed that Ure2p from *S. cerevisiae* and human Pmel17 have VIFC biased regions in the amyloid cores, and thereby resemble 11 of 12 disease-associated proteins with localized cores (Fig. 2 and suppl. Fig. S3). CsgA from *E. coli*, Sup35

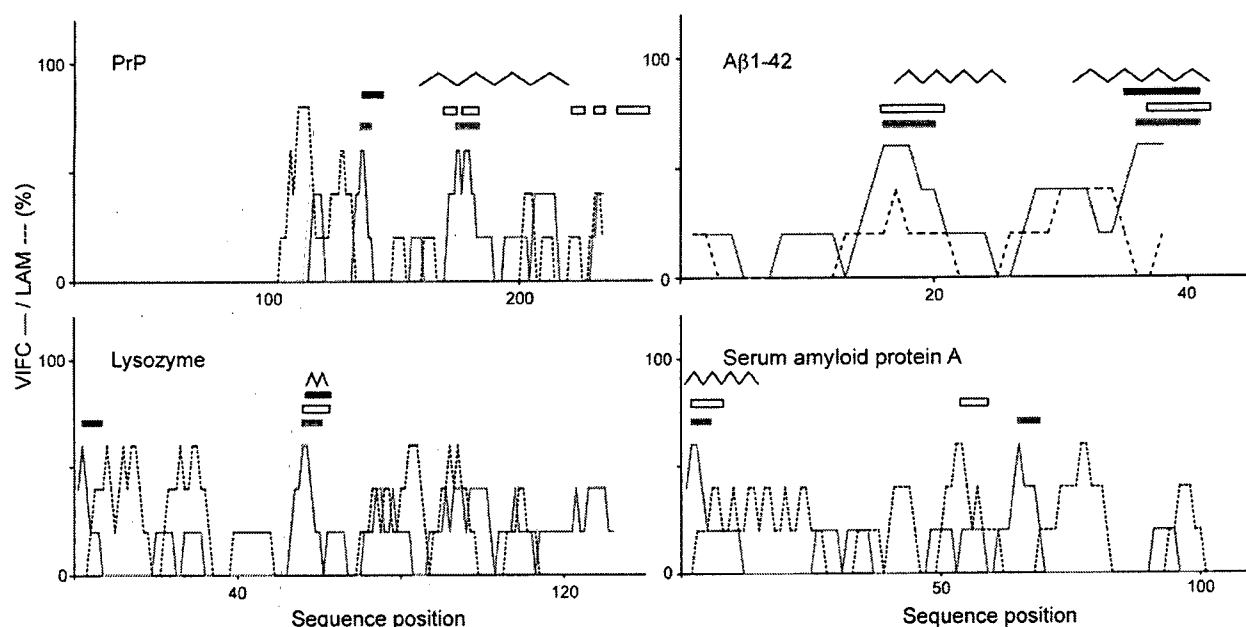


Fig. 2. Non-polar residue composition in amyloid disease proteins. The percentages of Val + Ile + Phe + Cys (VIFC, solid red lines) and Leu + Ala + Met (LAM, dotted black lines) are plotted along the respective amino acid sequence. Localizations of amyloid core regions for Aβ [1], PrP [24], lysozyme [38], and serum amyloid protein A [39] are marked with horizontal wavy lines. VIFC biased segments are indicated with horizontal red lines. Segments that are predicted to be amyloidogenic by the Waltz algorithm [12] are indicated by horizontal open boxes, and segments that form steric zipper fibrils [8] are marked with horizontal black lines.

from *S. cerevisiae* and HET-s from *P. anserine*, in contrast, lack VIFC bias in their amyloid cores. These proteins resemble the only disease-associated protein with a localized core lacking VIFC bias (prolactin, Suppl. Fig. S3), and five disease-associated proteins that completely lack VIFC biased regions (islet amyloid polypeptide (IAPP), calcitonin, apolipoprotein A-I, β2-microglobulin, and atrial natriuretic factor). VIFC bias thus appears to be more common in disease-associated amyloid cores than in functional amyloid counterparts, although the small number of proteins makes comparisons uncertain. It was recently found that several peptide hormones, including prolactin and calcitonin which both lack VIFC bias, can be stored in an amyloid state in secretory granules, and this might be regarded as a type of functional amyloid [27].

Proteins not associated with disease but kept under denaturing conditions can form amyloid fibrils *in vitro*, suggesting that the ability to form β-sheet fibrils is a generic property of the polypeptide chain [28]. Myoglobin is not found in amyloid disease, but under denaturing conditions it forms amyloid-like fibrils [29]. Myoglobin contains one VIFC biased region (residues 98–113) and two LAM biased regions (suppl. Fig. S5). A fragment corresponding to the VIFC biased segment is prone to form amyloid-like fibrils *in vitro* [30], again pointing to the relevance of this group of non-polar amino acid residues.

4. Discussion

The β-strand/α-helix propensity ratio (Fig. 1) reflects the preferred secondary structure for each amino acid residue. This preference should be particularly relevant in less polar environments where hydrogen bonding between the polypeptide backbone and solvent becomes unfavorable compared to hydrogen bonding within or between polypeptide backbones. VIFC biased segments will then promote formation of intra- or intermolecular β-sheets, while LAM biased counterparts will promote intramolecular α-helix formation. We note that VIFC biased segments in disease amyloid cores are in most cases isolated, while VIFC biased segments localized outside amyloid core regions are often surrounded by

LAM biased segments, see e.g. PrP, serum amyloid protein A, lactadherin and gelsolin (Fig. 2 and suppl. Fig. S3). It seems likely that isolated VIFC biased segments can promote amyloid formation, while the presence of neighboring LAM biased segments lowers the risk of inadvertent intermolecular β-sheet formation. In an analogous manner, the potential risk of forming β-sheet rather than α-helix during biosynthesis of TM segments may be reduced by their strong over-representation of Leu and Ala [31], which will prevent local VIFC bias without affecting the degree of non-polarity, see Fig. 1. We speculate that the occurrence of LAM in non-polar segments, in general, reduces the risk of inadvertent formation of β-sheets. Another naturally occurring strategy for dealing with potentially amyloidogenic regions can be illustrated by proSP-C, which may have developed a specific way to handle a strongly VIFC biased segment. A chaperone domain, BRICHOS, localized in the C-terminal, ER luminal part of proSP-C can promote folding of the proSP-C polyVal TM segment into α-helix and prevent β-sheet amyloid formation [16,32]. The observation that mutations in proSP-C BRICHOS lead to amyloid formation [16] supports the possibility that this is a physiological way to handle extreme VIFC bias.

Eleven of 18 (61%) VIFC biased segments within amyloid cores overlap with amyloidogenic regions predicted with the Waltz algorithm [12], while only 13 of 44 (30%) VIFC biased segments outside amyloid cores do so (Fig. 2 and suppl. Figs. S3 and S4). Moreover, peptide segments that can form steric zipper fibrils and are derived from the amyloidogenic proteins PrP, Aβ, tau, lysozyme, and α-synuclein [8] coincide almost exactly with VIFC biased segments (Fig. 2 and suppl. Fig. S3). These observations suggest that a combination of VIFC bias and ability to form self-complementary steric zippers in one and the same peptide segment results in a strong determinant for amyloid formation. Moreover, out of 37 amyloidogenic regions predicted by the Waltz program to be localized outside known amyloid cores, only 11 overlap with VIFC biased regions. Adding a VIFC bias criterion, similar to the one used herein, may improve the specificity in the prediction of amyloid core regions. In the nine proteins that contain VIFC biased segments but lack identified amyloid core regions (see above), VIFC bias

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and Waltz predictions coincide in five regions. It will be interesting to find out if these constitute amyloid core regions.

Acknowledgments

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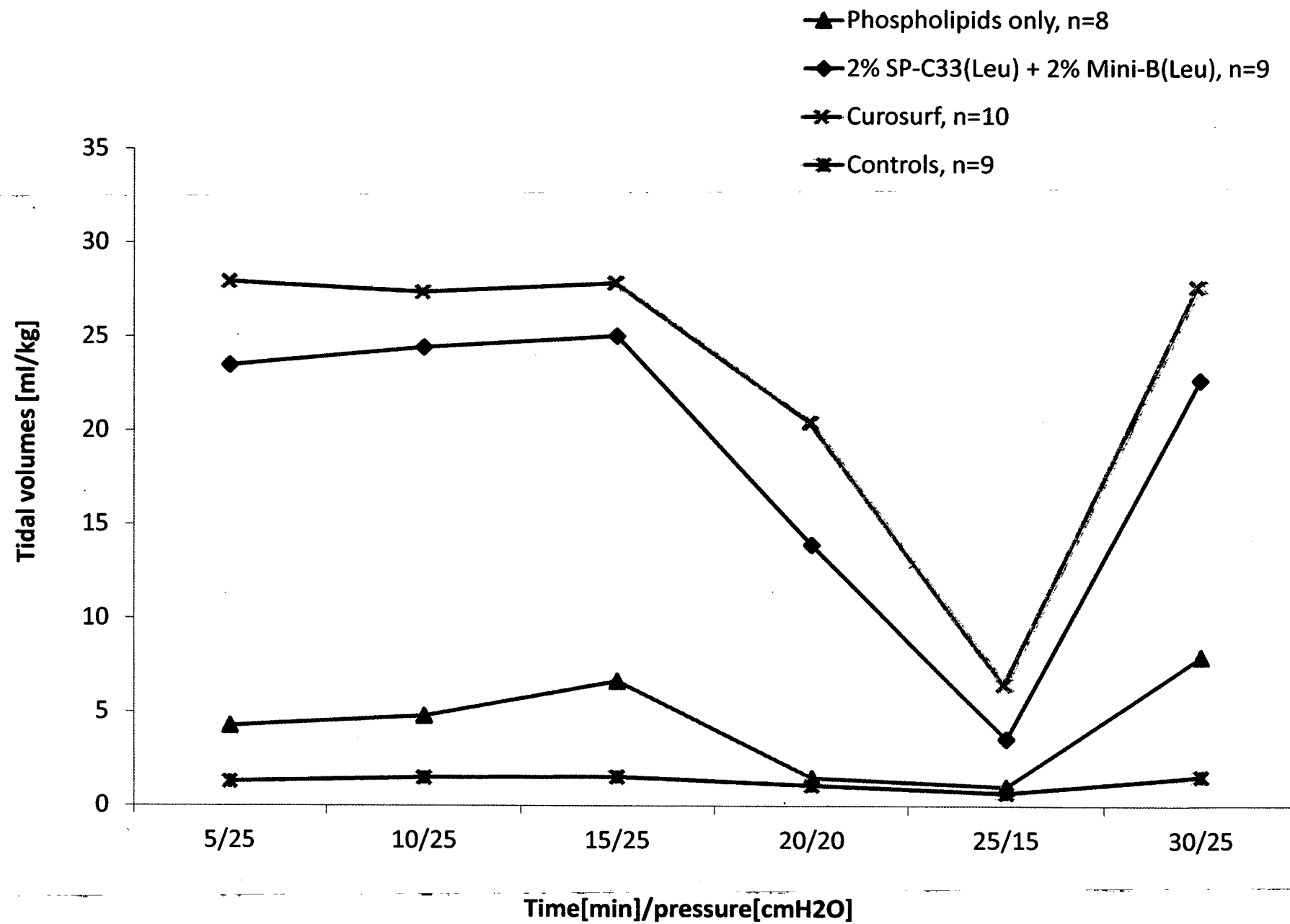
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.10.062.

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DOCKET NO: 395431US68CONT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :
JAN JOHANSSON ET AL : EXAMINER: TELLER, R. R.
SERIAL NO: 13/443,164 :
FILED: APRIL 10, 2012 : GROUP ART UNIT: 1675
FOR: RECONSTITUTED SURFACTANTS :
HAVING IMPROVED PROPERTIES

DECLARATION UNDER 37 C.F.R. § 1.132

COMMISSIONER FOR PATENTS
ALEXANDRIA, VIRGINIA 22313

SIR:

Now, comes Jan Johansson, Ph.D., who deposes and states that:

1. I am a named inventor of the above-identified application.
2. I receive a research grant from Chiesi Farmaceutici S.p.A,
3. A copy of my *curriculum vitae* is attached hereto and is part of this declaration.
4. I am a named inventor and am familiar with the disclosure of WO 00/47623

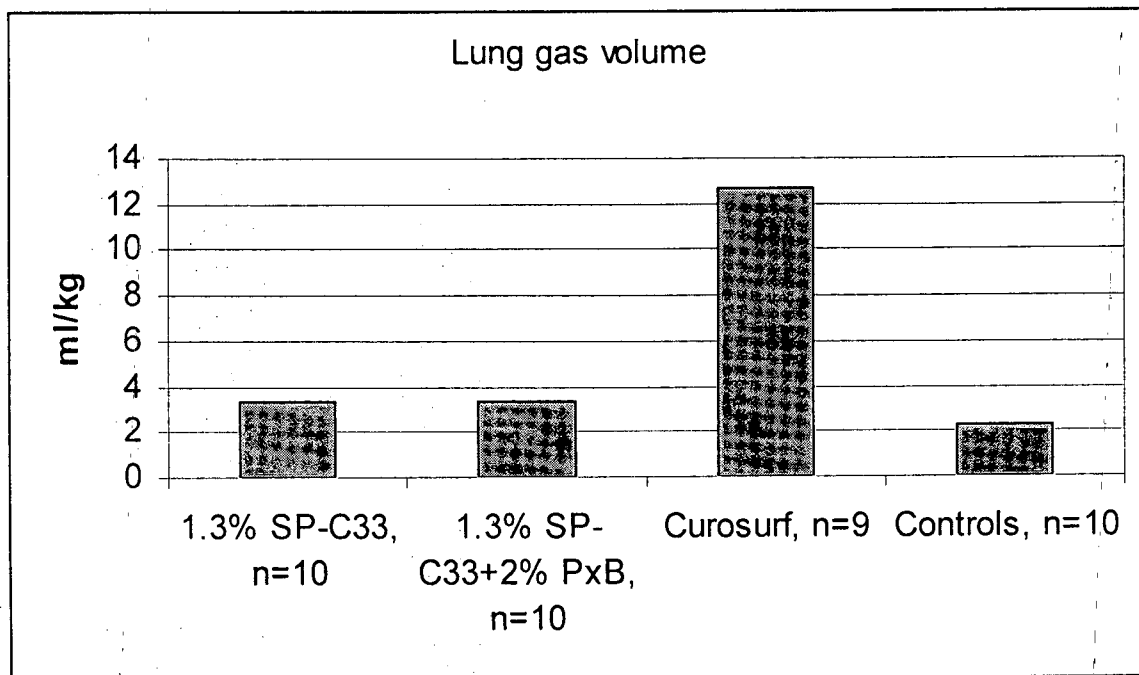
(Curstedt et al.).

5. Curstedt et al. provides a strong suggestion to use a polymyxin, preferably polymyxin B, as the component corresponding to SP-B or an active analog thereof. See page 8, lines 14-16.

6. The following experiment was carried out by me or under my direct supervision and control.

Immature new-born rabbits (gestational age 27 days) were treated at birth with 200 mg/kg (80 mg/ml) of 1.3% SP-C33 in DPPC: 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC):1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (SAPC):DPPG:POPG: (45: 30:10:5:10, by weight) or the same mixture with the addition of 2% polymyxin B (PxB). Animals receiving the same dose of Curosurf served as positive and non-treated littermates as negative controls. The new-born rabbits were ventilated in parallel with a standardized sequence of peak insufflation pressures. To open up the lungs, pressure was first set at 35 cmH₂O for 1 minute. After this recruitment maneuver, the pressure was lowered to 25 cmH₂O for 15 minutes and further on to 20 and 15 cm H₂O. Finally, the pressure was raised again to 25 cmH₂O for 5 minutes, after which the lungs were ventilated for additional 5 minutes with nitrogen and then excised for gas volume measurements. The experiments were performed without PEEP. Lung gas volumes, given as median values, are reported in Figure 1.

Figure 1

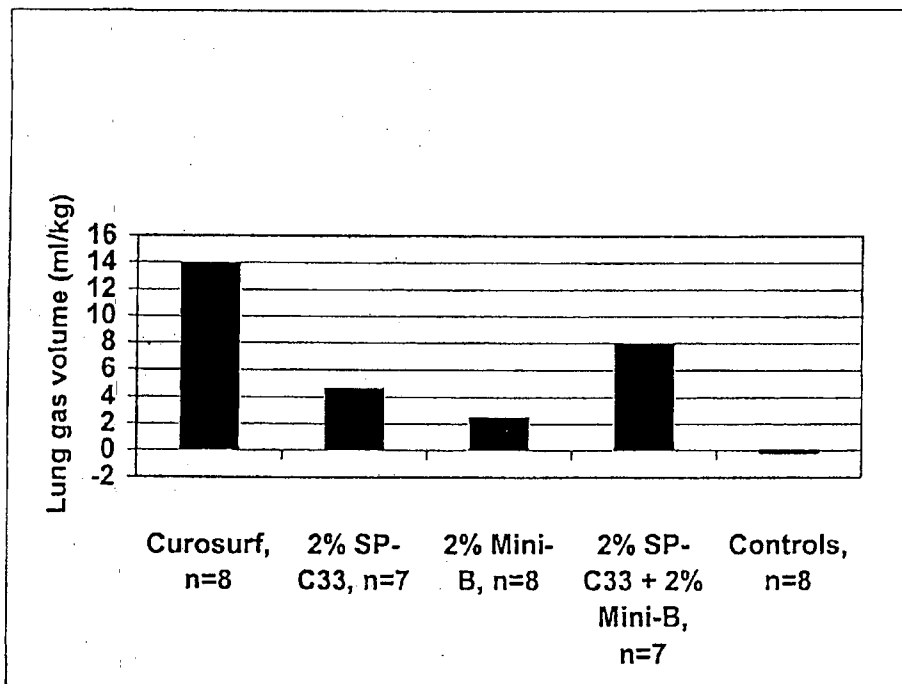


7. The results in Figure 1 show that the effect of a combination of the peptide SP-C33 with polymyxin B (PxB) on the lung gas volume is not significantly different from that of SP-C33 alone.

8. In sharp contrast, as demonstrated in Example 3 and Figure 3 of the above-identified application, a combination of SP-C33 and Mini-B provides a result which is significantly higher than that of SP-C33 alone.

9. Figure 3 of the above-identified application is reproduced below:

FIG. 3



10. The improved effect provided by the combination of SP-C33 and Mini-B as compared to the combination of peptide SP-C33 and polymyxin B is clinically significant.

11. There is no teaching in Curstedt et al. which would suggest that the combination of SP-C33 and Mini-B would provide an improved effect as compared to the combination of peptide SP-C33 and polymyxin B.

12. I declare further that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

13. Further, Declarant saith not.

Jan Johansson, Ph.D.

Date

EUROPEAN PATENT OFFICE
Erhardtstraße, 27
80331 MÜNCHEN
Germany

4 August 2008

SCB1113PCT/FM/cgr

By fax + DHL confirmation

Dear Sirs,

RE: International Application under the PCT
No. PCT/IB2007/002841 filed on 28 September 2007
In the name of CHIESI FARMACEUTICI S.p.A.

We hereby request the International Preliminary Examination of the above-identified PCT application, as per the enclosed demand. The International Preliminary Examination Fee and Handling Fee have been paid as per the enclosed voucher.

The applicant wishes to file a new set of claims, wherein new claim 1 differs from the original claim in the definition that the lipid carrier consists of phospholipids. This amendment is supported on page 16 lines 2-3 of the WO publication as well as in original claim 12 which has been accordingly deleted. The other claims have been re-numbered.

We believe that the new set of claims is inventive in view of the following discussion of the prior art.

The teaching of **D1** is directed to particular SP-C analogues '*showing particularly favourable properties for surface tension reduction*' (page 5, lines 17-18) and to the relevant reconstituted surfactant further comprising a lipid carrier and an SP-B analog.

As SP-B analog, **D1** gives an unambiguous hint to use a polymyxin, preferably polymyxin B (page 8, lines 14-16).

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The in vivo compliance in terms of **tidal volume** of an exemplary reconstituted surfactant comprising polymyxin B was assessed in an animal model and the results reported in Example 7.

D1 is silent about the effect on the **lung-gas volume** and on the alveolar patency at the end of expiration (for the definitions of tidal volume and lung gas volume see page 6, lines 23-28 of the PCT application).

However the applicant found that *'Reconstituted surfactants [of the prior art] gives rise to poor lung gas volumes and grade of alveolar patency at the end of expiration, and ventilation is required with a positive end expiratory pressure (PEEP) in order to achieve an in vivo activity comparable to that achieved with modified natural surfactants'* (page 3, lines 10-14).

Ventilation with PEEP in pre-term infants with respiratory distress syndrome (RDS), in turn, has long been recognized to contribute to lung injury which may lead to the development of pneumothorax and/or bronchopulmonary dysplasia and to other side-effects.

Therefore, starting from **D1**, the objective technical problem underlying the present invention is to provide *'a reconstituted surfactant preparation which is able of guaranteeing alveolar stability, and hence maintaining alveolar patency at the end of expiration without requiring ventilation with PEEP'* (page 3, lines 23-26).

The solution is offered by the reconstituted surfactant of new claim 1.

It has now been indeed found that *'particular analogues of the native SP-C protein, and preferably the polypeptides of WO 00/47623 can advantageously be combined with particular analogues of the native protein SP-B in order to provide a reconstituted surfactant preparation with improved properties in terms of lung compliance, and in particular capacity to efficaciously maintain alveolar patency at the end of expiration without requiring ventilation with PEEP'* (from page 3, line 28 to page 4, line 5)

In this respect we also observe that protein/peptide:lipid carrier system is a rather complex mixture whose properties greatly depend on the interaction of the components and cannot be predicted *"a priori"*.

Therefore, starting from the teaching of **D1**, which is silent about the problem of the alveolar patency, the skilled person would have had no hint for selecting the particular analog of **D2** among the several analogues disclosed in the art for making a reconstituted surfactant *able of guaranteeing alveolar stability, and hence maintaining alveolar patency at the end of expiration without requiring ventilation with PEEP*.

In particular, considering that **D1** gives an unambiguous hint to use polymyxin B as a functional substituent of SP-B and polymyxin B is smaller and much cheaper than the analog of **D2** as well, the skilled person would have no hint at all to replace polymyxin B with the analog of **D2**.

The same applies starting from the teaching of **D3**.

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In view of the above observations, it is requested that inventive step be recognised for the claimed subject matter.

I thank you for your kind attention and remain,

Yours faithfully,

BIANCHETTI BRACCO MINOJA
Fabrizio Minoja

Encls

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