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SYNTHETIC PULMONARY SURFACTANT: EFFECTS OF SURFACTANT PROTEINS B AND C AND THEIR ANALOGUES

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To my wonderful family: Milena, Axel and Filippa

ABSTRACT

Pulmonary surfactant is a lipid/protein mixture lining the air-liquid interface in the alveoli. Its main function is to lower surface tension during respiration and thereby prevent alveolar collapse at end-expiration. Surfactant deficiency, especially common in prematurely born babies, is the main cause of respiratory distress syndrome (RDS). This disease is treated with exogenous surfactant replacement using animal-derived modified natural surfactants. Production of these is quite expensive and the supply is limited. In addition there is a possible risk of transmitted infectious agents, which is why synthetic alternatives are under development.

We investigated the effect of an SP-C analogue, SP-C33, in phospholipids as a synthetic alternative. By circular dichroism and infrared spectroscopy we found that SP-C33 shows secondary structure and orientation in a phospholipid bilayer similar to SP-C. 1-2% of this analogue in a mixture of dipalmitoylphosphatidylcholine (DPPC)/palmitoylloleoylphosphatidylglycerol (POPG) (68:31 by weight) showed tidal volumes similar to those obtained by the modified natural surfactant Curosurf when used in ventilated prematurely born rabbits. When ventilated without positive end-expiratory pressure, SP-C33 surfactant shows lower lung gas volumes (LGV) compared to Curosurf, indicating that some component in the latter is needed to stabilize the lung at end-expiration. Our study shows that inclusion of both SP-C33 and SP-B, or an analogue thereof, significantly increases LGV. This indicates that SP-B and SP-C exerts different tasks in surfactant and that both proteins are necessary to obtain alveolar stability. The SP-B analogue Mini-B shows good surfactant activity both *in vitro* and *in vivo* and may be a good replacement in synthetic surfactant.
C-terminal modifications of SP-C33 do not alter its surfactant properties, indicating that mobility inside the membrane probably is not necessary for surfactant activity.

A synthetic surfactant consisting of 2% SP-C33 (by weight) in 80mg/ml DPPC/POPG (68:31 w/w) and an SP-B analogue, possibly Mini-B, may be a good replacement for modified natural surfactant in future treatment of neonatal RDS.

LIST OF PUBLICATIONS

I. Johansson J, Some M, Linderholm B-M, Almlén A, Curstedt T, Robertson B. A synthetic surfactant based on a poly-Leu SP-C analog and phospholipids: effect on tidal volumes and lung gas volumes in ventilated immature newborn rabbits. J Appl Physiol 2003; 95: 2055-2063

II. Almlén A, Stichtenoth G, Robertson B, Johansson J, Curstedt T. Concentration dependence of a poly-leucine surfactant protein C analogue on in vitro and in vivo surfactant activity. Neonatology 2007; 92: 194-200

III. Almlén A, Vandenbussche G, Linderholm B, Haegerstrand-Björkman M, Johansson J, Curstedt T. Alterations of the C-terminal end do not affect in vitro and in vivo activity of SP-C analogues. Submitted

IV. Almlén A, Stichtenoth G, Linderholm B, Haegerstrand-Björkman M, Robertson B, Johansson J, Curstedt T. Surfactant proteins B and C are both necessary for alveolar stability at end expiration in premature rabbits with respiratory distress syndrome. J Appl Physiol 2008; 104: 1101-1108

V. Almlén A, Walther FJ, Waring AJ, Robertson B, Johansson J, Curstedt T. Synthetic surfactant based on analogues of SP-B and SP-C is superior to single-peptide surfactants in ventilated premature rabbits. Neonatology 2010; 98: 91-99

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LIST OF ABBREVIATIONS

Three- and one-letter codes for the twenty gene-encoded amino acids.

Amino acid	Three-letter code	One-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

CBS	Captive bubble surfactometer
CD	Circular dichroism
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
FRC	Functional residual capacity
FTIR	Fourier transform infrared
LGV	Lung gas volume
PA	Palmitic acid
PBS	Pulsating bubble surfactometer
PC	Phosphatidylcholine
PEEP	Positive end-expiratory pressure
PG	Phosphatidylglycerol
PIP	Peak inspiratory pressure
POPG	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol
RDS	Respiratory distress syndrome
SP	Surfactant protein
SFTPB	Surfactant protein B gene
SFTPC	Surfactant protein C gene
V_T	Tidal volume

1 INTRODUCTION

1.1 BACKGROUND

With a surface area of about 1 m^2/kg bodyweight (*1*), the lungs constitute the largest contact between man and the environment. This large surface is required to facilitate gas exchange and is obtained by branching of the airways from trachea through bronchi to the fine structure of the alveolus. Because of the high surface tension of the liquid lining the alveoli, the lungs would have a natural propensity to collapse at the end of expiration without the presence of pulmonary surfactant in the alveoli. Pulmonary surfactant (SURFace ACTive AgeNT) is a complex mixture that acts at the air/liquid interface and lowers the surface tension and thus preventing the lung from collapsing. The first article addressing this phenomenon was published in 1929 by von Neergaard who discovered that a larger pressure was needed to fill the lungs with air than with liquid. From this he hypothesized that a surface tension lowering substance was stabilizing the alveolar lining (*2*). Later, in the 1950's the properties and function of surfactant were further described by Pattle and Clements. In 1955 Pattle proposed that the alveolar lining had its origin within the lungs (*3*). Two years later, Clements found that the surface-active material was essential for stabilizing the lung (*4, 5*). By studying lung extracts from newborn infants who died from respiratory distress syndrome (RDS, at that time called hyaline membrane disease) Avery and Mead showed that the minimum surface tension of the lung material was increased and concluded that the disease was associated with the deficiency of surfactant (*6*). Starting in the 1970s, King and Clements noticed that surface active material from lung lavage behaved as a lipoprotein. Some year later they identified non-serum proteins with a molecular weight of about 34,000 and 10,000, with high affinity for phospholipids in canine surface active material (*7-9*). In addition to the ability to decrease surface tension, a recent study proposes that surfactant might be important for facilitating oxygen transport through the air/liquid interface (*10*).

Surfactant is synthesized by alveolar type II cells, which represent about 10% of the total area lining the alveolar surface. After being synthesized, surfactant is stored in closely packed multiple bilayers called lamellar bodies. The lamellar bodies are secreted into the alveolar space where their content is rearranged into a lattice-like

structure called tubular myelin. From the tubular myelin, the surfactant lipids and proteins are spread at the air-liquid interface where they form an interfacial monolayer that reduces surface tension (Fig.1). Surfactant is removed from the interfacial lining as unilamellar vesicles. These vesicles are phagocytized by macrophages or degraded and recycled into lamellar bodies in the type II cell (*11, 12*).

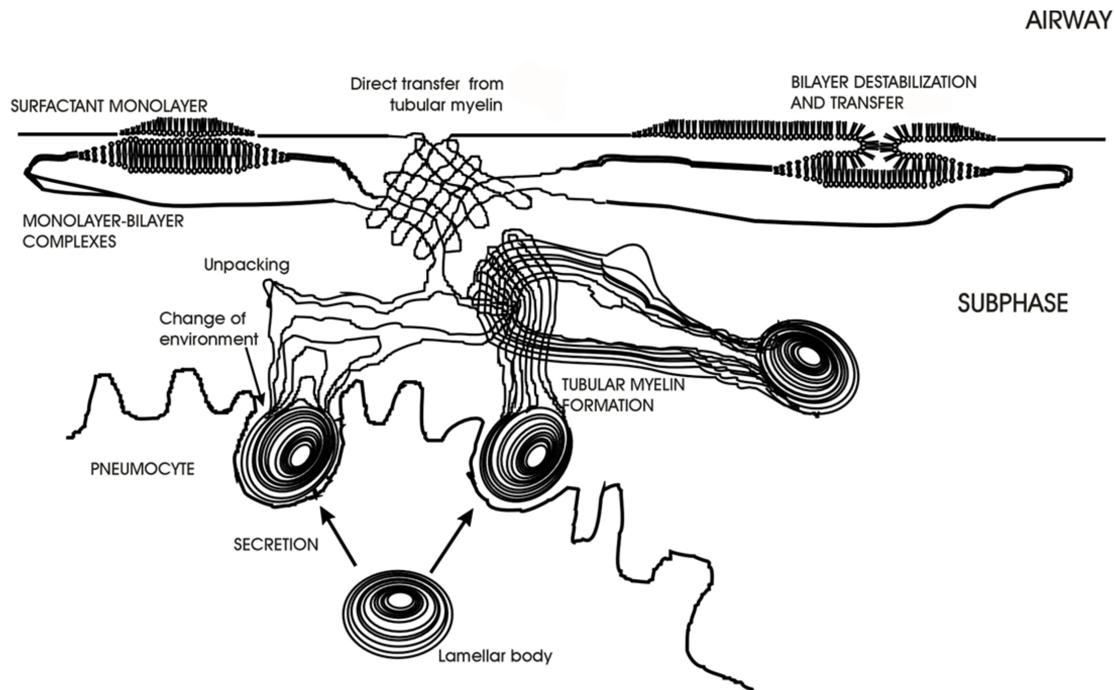


Figure 1. Surfactant transfer to the air/liquid interface

Lamellar bodies are secreted into the alveolar space and rearranged into tubular myelin from which the surfactant lipids and proteins are spread at the air-liquid interface. Figure from (*13*)

1.2 COMPOSITION OF SURFACTANT

The main constituents of surfactant can be divided into lipids and surfactant-specific proteins. Approximately 90% of surfactant mass consists of lipids whereof 80-90% is represented by phospholipids, whereas the remaining part includes cholesterol and other neutral lipids. Of the phospholipids, the dominant species, about 80% of total phospholipid content, is phosphatidylcholine (PC) and in particular the saturated 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) which is also mainly responsible for decreasing surface tension. The second most abundant of the anionic phospholipid, with ~10% of the phospholipids, is phosphatidylglycerol (PG) (Figure 2) (*14*). This is possibly related to the concentrating effect of SP-B on the distribution of anionic phospholipids (*15, 16*).

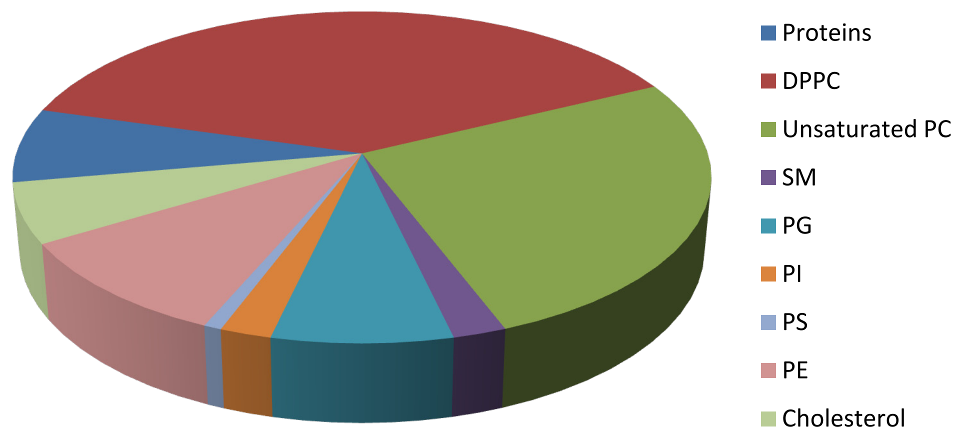
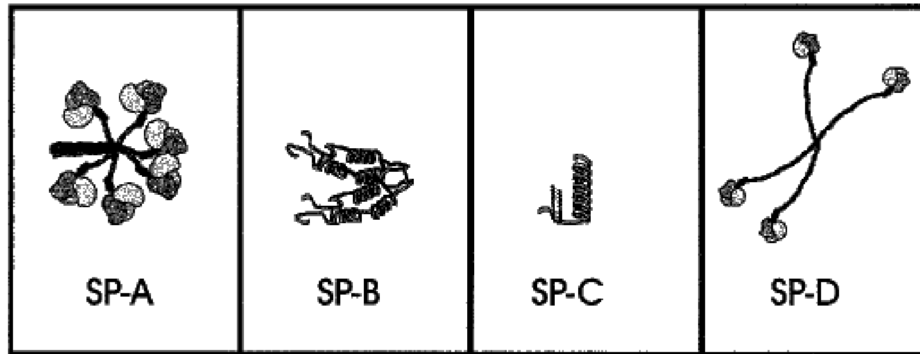


Figure 2. Composition of human pulmonary surfactant
DPPC: Dipalmitoylphosphatidylcholine; PC: phosphatidylcholine; SM: Sphingomyelin; PG: phosphatidylglycerol; PI: Phosphatidylinositol; PS: phosphatidylserine; PE: phosphatidylethanolamine. Data from (*14*).

The protein part of surfactant (~10%, w/w) contains four different surfactant-specific proteins, (SP)-A, SP-B, SP-C and SP-D (*17*)(Figure 3).



	Hydrophilic	Hydrophobic	Hydrophobic	Hydrophilic
M_W (monomer)	26-38 kDa	8.7 kDa	4.2 kDa	43kDa
Quaternary structure	Octadecamer	Dimer	Monomer	Dodecamer
Homologues	Collectins	Saposins	-	Collectins

Figure 3. Surfactant proteins

Main structural features of surfactant-associated proteins. Figure modified from (*13*).

1.2.1 SP-A and SP-D

The hydrophilic surfactant proteins, SP-A and SP-D are also expressed in several other tissues, for example gastric and intestinal mucosae (*18-20*). SP-A and SP-D, are members of the collectin (collagenous carbohydrate-binding proteins) family and important parts of the host defense of the lung (*21*) due to their ability to bind multiple pathogens, e. g. viruses, bacteria and fungi (*22, 23*). Collectins have a primary structure containing four specific structures:

- An N-terminal domain rich in cysteines
- A collagenous domain
- An α-helical neck domain
- A lectin or carbohydrate recognition domain

SP-A and SP-D counteract pathogens by aggregation of pathogens, stimulation of phagocytosis and modulation of the inflammatory response (*21*).

SP-A is the main protein component in surfactant, ~5-6% (dry weight) and is also important for phospholipid reuptake and secretion, and formation of tubular myelin. The presence of SP-A is, however, not essential for normal respiration *in vivo* (*24*). In a recent study, SP-D was shown to regulate the surfactant pool size in the newborn period. By interacting with PI-rich, newly secreted surfactant SP-D causes lysis of surfactant lipid membranes, and thereby converts the lipid forms into smaller surfactant lamellated structures that are critical for surfactant uptake by type II cells and normal surfactant homeostasis(*25*).

No direct role of SP-A or SP-D in the surface tension reducing action of surfactant has been shown. SP-A has, however, shown ability to increase surfactant resistance against inhibition both *in vitro* (*26, 27*) *and in* vivo (*28-31*). This thesis is concentrated on synthetic surfactant containing SP-B and SP-C and their analogues.

1.2.2 SP-B and SP-C

SP-B and SP-C are hydrophobic polypeptides involved in lowering the surface tension by promoting rapid adsorption of phospholipids into the air-liquid interface as well as maintaining and stabilizing the interfacial film during respiration (*32*).
SP-B and SP-C are both membrane proteins. Membrane proteins may have very diverse functions and properties and can be bound to either only the membrane surface (peripheral membrane proteins) or have a region buried within the membrane and domains on one or both sides of it (integral membrane proteins). Protein domains on the extracellular membrane surface are generally involved in cell-cell signaling or interactions, while domains within the membrane, particularly those that form channels and pores, may transport molecules across the membrane. Domains connected to the cytosolic side of the membrane may have a wide range of functions, from anchoring proteins to the membrane to triggering intracellular signaling pathways (*33*).

SP-B is a 17kDa homodimer with a tertiary structure that likely includes four to five α-helices with antiparallel arrangements per subunit. Three intramolecular disulphide bridges between the helices give the monomer the form of three loops and an intermolecular bridge give rise to the homodimeric structure (*34*). No three-dimensional protein structure for SP-B has been determined but being part of the saposin-like protein (SAPLIP) family (*35*), which has well conserved structural

elements, and having a helical content similar to another SAPLIP, the antimicrobial peptide NK-lysin, SP-B is expected to also have a similar structure (*36, 37*). The hydrophobicity of SP-B ensures a constant association with the lipid membrane but the exact orientation has not yet been determined. However, in a proposed model, the polar side of the amphipathic helices is interacting with the phospholipid headgroup and the positively charged amino acids interact specifically with PG (*16*).

The other hydrophobic surfactant protein, SP-C, is a 4.2 kDa peptide consisting of a dipalmitoylated part at the N-terminal end and an α-helical part in the C-terminal end (*38*). The amino acid sequence is highly preserved among different species. Although there are some minor differences, the characteristic poly-valine α-helix is intact. The helix, stretching from residue 9 to 34 is perfectly suited to span a DPPC bilayer and is confirmed to obtain a transmembrane orientation when inserted in a lipid membrane (*39, 40*). The charged residues at the N-terminal part may anchor the helix at the lipid headgroups while the C-terminal end is free to move (*41, 42*). In most species, dog and mink excepted, SP-C is palmitoylated at the two cysteines in the N-terminal region (Figure 4). The function of the palmitoyl groups is unclear. They have, however, been shown to be of importance for lipid respreading, surface stability and association of subphase lipids to the interfacial monolayer (*43*), possibly by retaining the peptide inside the film at high surface pressures (*44*). The palmitoyl groups have also been shown to stabilize the helix (*45*) and additionally seem to mediate interactions between adjacent layers and induce piling of bilayers (*46*). In a recent study, the palmitoylation of SP-C is proposed to promote and facilitate association of SP-B and SP-C-containing membranes with ordered lipid structures found in highly compressed surfactant films (*47*).

Both SP-B and SP-C are synthesized as larger precursor proteins that are cleaved in several steps into mature proteins (*32*). Mature SP-B is formed from a 42 kDa proprotein which is proteolytically cleaved at both the C- and N-terminal ends in at least three steps (*49-52*). Mature SP-C is produced from a 21 kDa proprotein which also is cleaved in both C- and N-terminal ends (*53-55*).

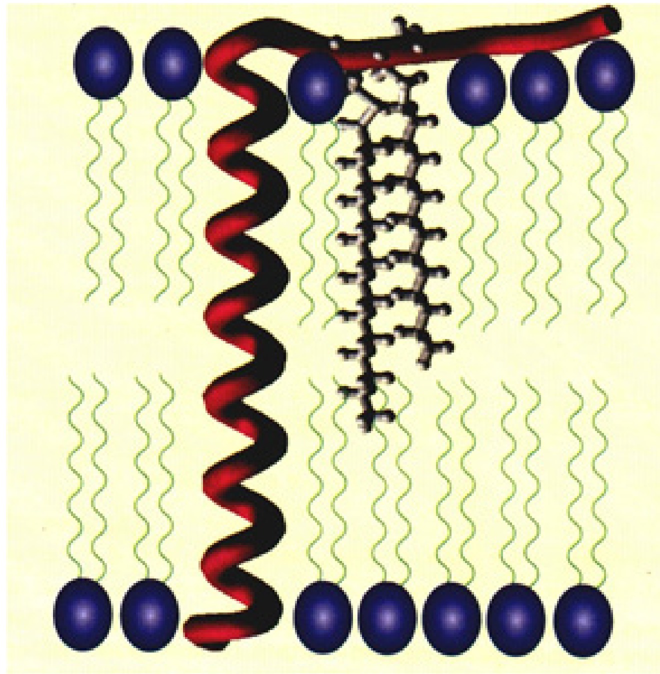


Figure 4. SP-C in a phospholipid bilayer with a proposed orientation of the palmitoyl groups. Figure from (*48*)

As mentioned earlier, the main surface tension reducing agent in pulmonary surfactant is DPPC. A DPPC layer can attain surface tensions close to 0 mN/m under compression (*56-58*). Unlike the other surfactant phospholipids, DPPC has a rather high melting point (~41°C) which means that it is present in a crystalline gel state at physiological temperature. In this state phospholipids are tightly packed and by expelling water molecules at the interface, i.e. decreasing the number of water molecules exposed to the air, low surface tension is obtained. Unfortunately, the rate of adsorption of DPPC in the crystalline gel state is very low due to its rigidity and it spreads very slowly at the air/liquid interface (*56, 59*). The adsorption process of surfactant can be divided into two steps, diffusion close to the interface followed by fusion with the interface (*60, 61*). The phospholipids are held together by multiple interactions, and an energy barrier must be overcome to expose the hydrophobic fatty acid chains to the air (*61*). To some extent the unsaturated phospholipids help to increase the fluidity but the main agents to improve the adsorption are SP-B and SP-C (*13, 62*). Studies indicate that the formation of a fusion neck or pore may play an important role for the adsorption process (*63*), possibly performed by SP-B and/or SP-C (Figure 5).

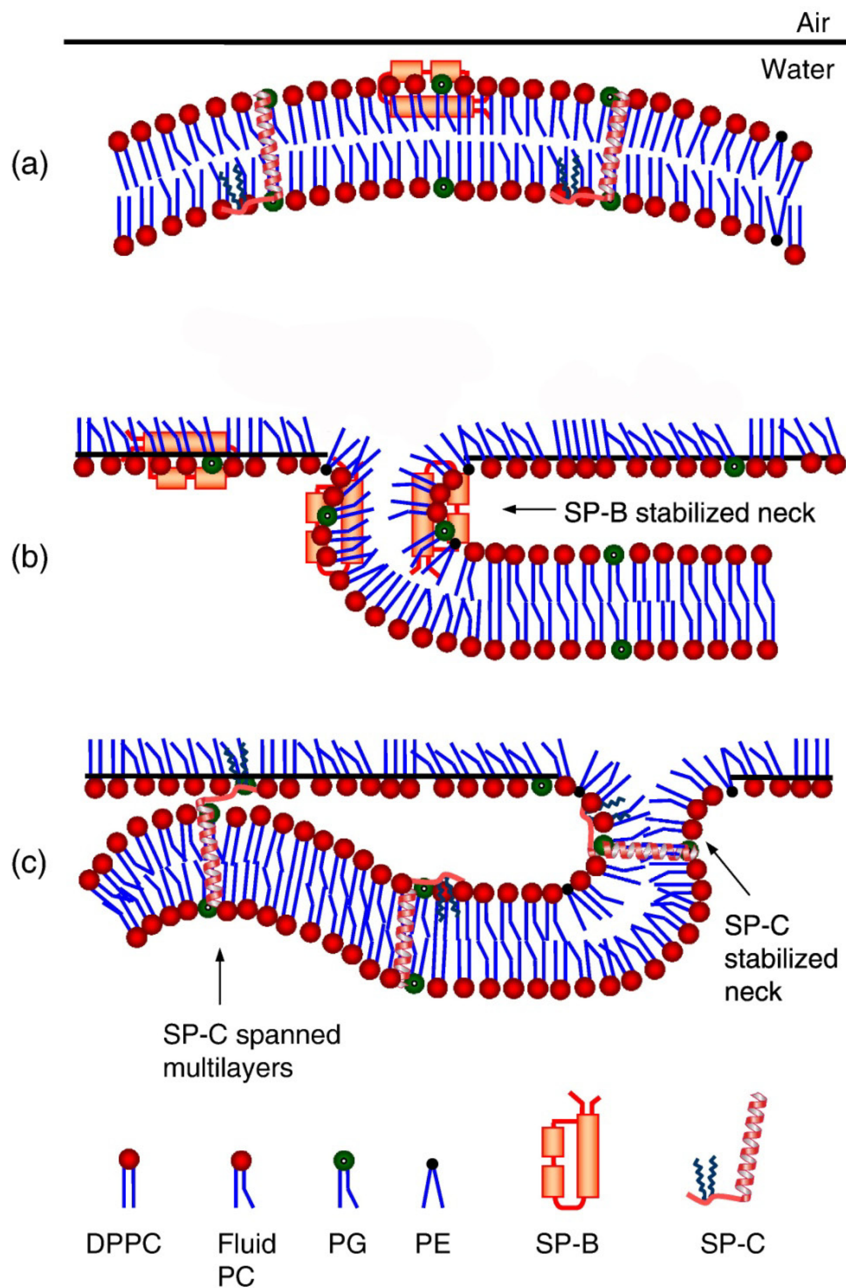


Figure 5. Schematic possible mechanism of surfactant adsorption.

(a) Diffusion of a surfactant vesicle/bilayer towards the air/liquid interface. The surfactant bilayers consist mainly of DPPC, fluid PC (such as POPC), PG (DPPG and POPG) and PE. (b) Fusion of the vesicle/bilayer with the interface stabilized by SP-B. Note that figure only shows monomeric SP-B. (c) Fusion of the vesicle/bilayer with the interface stabilized by SP-C. Note that the bilayer can also be connected to the interfacial monolayer by a SP-C molecule. Figure adopted from (*64*).

Although apparently important for the activity of pulmonary surfactant, the mechanisms of SP-B or SP-C action are not known, but several different hypotheses have been put forward.

When phospholipid monolayers are compressed into the liquid-expanded to liquid-condensed transition plateau, a coexistence of highly condensed domains floating in a background of less packed (liquid-expanded) lipids are present. SP-B has been observed to partition strictly into the liquid-expanded areas of these films (*65*). The presence of SP-B increases the number and reduces the size of condensed regions (*56, 66*), which leads to a structural stabilization of the films during compression (*56*).

When presented with phospholipid bilayers, SP-B is placed with the axes of the helices nearly parallel to the interface of the membranes (*67*). In a proposed hypothesis, the more hydrophobic face of each helix interacts deeper into the bilayers, while the nine positive charged residues of the protein would interact with the polar headgroup of anionic phospholipids. It has been reported that SP-B interacts selectively towards PG (*16, 68*) and that it has the ability to produce aggregation, fusion and lysis of phospholipid vesicles (*69-73*).

The N-terminal tail of SP-C was earlier assumed to be a “flexible disordered” segment (*38*) but later studies indicate that it seems to adopt an amphipathic conformation, with strong tendency to partition into the interface of phospholipid membranes and monolayers, even in the absence of palmitoylated cysteines (*74, 75*). This region perturbs lipid packing by interacting with membranes leading to changes in membrane permeability. It is therefore proposed that the N-terminal segment of SP-C would be the most dynamic part of the molecule and the main motif responsible for the activity of SP-C to promote interfacial adsorption of phospholipids from bilayers into the air/liquid interface. In this regard, SP-C seems to have an activity which is partly redundant, although with lower efficiency, with that of SP-B.

It has been shown that SP-C disorders the acyl chains of a phospholipid bilayer, while the head groups are stabilised, possibly due to its mobility gradient across the bilayer (*76, 77*). This mobility gradient has been suggested to derive from interactions of the Lys-Arg pair in the N-terminal end of the transmembrane α-helix with phospholipid head-groups, combined with the lack of charged residues in the C-terminal end thereby able to disturb interactions between 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 (*78-81*). SP-C is

also thought to facilitate the reinsertion of surface active molecules into the interface from surface-associated reservoirs during alveolar expansion (*82*).

SP-B and SP-C have also been shown to decrease the energy barrier limiting adsorption of phospholipids to the air/liquid interface, preferably in the presence of anionic phospholipids (such as PG), possibly by introducing electrostatic interactions (*60, 61, 83*)

1.3 SP-B AND SP-C MUTATIONS

The surfactant protein B gene (SFTPB) encodes a 2 kb mRNA transcript that is translated into a 381 amino acid proprotein. This proprotein is glycosylated and undergoes several proteolytic cleavages before it turns into mature SP-B (*84, 85*). Over 30 mutations in SFTPB that result in partial or complete SP-B absence have been identified and the most common, the 121ins2 mutation, is responsible for approximately 70% of the cases of SP-B deficiency (*86, 87*). This mutation causes a frame-shift that result in absence of pro-SP-B. The absence of SP-B gives rise to abnormal surfactant composition, decreased surfactant function and lack of lamellar bodies (*88*). Moreover, the absence of proSP-B also leads to incomplete processing of proSP-C to mature SP-C (*89*). Instead an intermediate, SP-C_i, which does not improve spreading of phospholipids (*90*) is accumulated.

In studies using SP-B knock-out mice, no mature lamellar bodies, deviantly processed SP-C and enlarged multivesicular bodies with small lipid vesicles were observed (*91, 92*) and respiratory failure was observed in conditional SP-B knock-out mice following a decrease of SP-B concentration below 25% of normal levels (*93*).

The surfactant protein C gene (SFTPC) encodes a 0.9 kb transcript that is translated into a 191- or 197 amino acid proprotein. The proprotein is palmitoylated and proteolytically cleaved in several steps, both in the N- and C-terminal end to yield mature SP-C (*54, 94, 95*). For SFTPC, over 35 mutations associated with acute and chronic lung diseases have been identified (*96-99*). About 55% of these arise spontaneously while the remainders are inherited. Mutations in SFTPC are believed to result in misfolded proSP-C that accumulates within cellular quality control pathways,

resulting in activation of cell stress responses, cellular injury and apoptosis (*94, 100-102*).

SP-C knock-out mice show only small decrease in lung function, decreased stability of the surfactant film at low lung volumes and progressive inflammatory lung disease (*103, 104*). Hence, while mutations in SFTPB lead to respiratory failure that is lethal in the neonatal period, mutations in SFTPC cause interstitial lung disease of varying severity and age of onset.

1.4 RESPIRATORY DISTRESS SYNDROME

Surfactant deficiency, most commonly occurring in prematurely born infants (*105, 106*), causes respiratory distress syndrome (RDS). The increased alveolar surface tension found in babies suffering from RDS leads to alveolar collapse and reduced lung gas volumes. This may lead to insufficient oxygenation producing alveolar epithelial injury. RDS may also occur in full-term babies due to genetic abnormalities in surfactant metabolism (*107-111*) and studies indicate that mutations in the ABCA3 transporter gene are a significant cause of this (*112*). ABCA3 is a protein of the ATP binding cassette transporter family. It is expressed in type II epithelial cells of the lung and is found in the limiting membranes of the lamellar bodies. ABCA3 has recently been shown to be necessary for lamellar body biogenesis, SP-B processing and lung development late in gestation (*113*).

In addition to neonatal RDS, sometimes referred to as primary surfactant deficiency, there is secondary surfactant deficiency which is caused by inactivation of the surfactant system by, for example, plasma proteins, proteases/lipases, oxidants or gastric contents. Secondary surfactant deficiency may eventually lead to acute (adult) RDS (ARDS).

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1.5 SURFACTANT REPLACEMENT THERAPY

Before clinical routine to treat with animal-derived surfactant preparations, RDS used to be the major cause of mortality of neonates. During the past 25 years, surfactant replacement therapy has been available and from have been a common cause of mortality and morbidity of prenatal infants, it is now possible to rescue babies born from week 22 (*114*).The morbidity of these extremely prematurely born babies is, however, high (*115*). The first attempt with surfactant replacement therapy (using aerosolized DPPC) in premature infants with RDS was reported in the 1960s (*116*). The trial showed low effect, possible due to the gel state of DPPC at body temperature and the fact that no spreading agent was present. In 1980 the potential of surfactant therapy was shown by Fujiwara et al who gave premature infants with RDS modified natural surfactant by tracheal instillation (*117*).

Clinical studies have shown that a prophylactic treatment is more effective than waiting until RDS is developed (*118*). In addition, surfactant replacement therapy may enhance maturation of type II cells to varying extent, depending on which surfactant preparation is used (*119*). No direct adverse effects of treatment with exogenous surfactant have been shown, although the need of intubation and positive pressure ventilation may give lung injuries as shown in immature lambs (*120*). The surfactants clinically used are modified natural surfactants originating from animals, most commonly bovine or porcine preparations (Table 1).

These animal derived preparations, containing phospholipids together with SP-B and SP-C, are quite expensive and supply is relatively limited. There is also a presumptive risk of transmitted infectious agents. Consequently, synthetic alternatives are desirable and presently under development.

Treatment of patients with ARDS is more troublesome since the surfactant used must be resistant to inactivation due to the inhibitors present in the alveoli (*121*).

Table 1. Modified natural surfactants

Origin and manufacturer of commercial animal-derived surfactants

Bovine		Porcine	
Name	**Producer**	**Name**	**Producer**
Survanta	Abbott Nutrition, USA	Curosurf	Chiesi Pharmaceutici, Italy
Alveofact	Boehringer Ingelheim GmbH, Germany	Surfacen	CENSA, Cuba
Infasurf	ONY, Inc. USA	HL-10	Leo Pharma A/S, Denmark (Discontinued)
Newfacten	Yuhan Co Ltd Korea		

1.6 SYNTHETIC SURFACTANT

Development of synthetic surfactant has been in progress since the 1960:s (*116*). The apparently simple task to copy natural surfactant has turned out to be quite a challenge. In total, surfactant contains over 50 species of phospholipids which would not be industrially applicable to exactly mimic. Thus a simpler, yet satisfying, lipid mixture is desirable and different approaches regarding synthetic surfactant are being evaluated.

Early synthetic surfactants were protein-free. In Exosurf the main constituent was DPPC combined with hexadecanol that acts as a spreading agent for DPPC. Included was also Tyloxapol, a polymeric long-chain repeating alcohol and a nonionic surfactant, which acts to disperse both DPPC and hexadecanol (*122*). Another early surfactant, ALEC (artificial lung expanding compound), consisted of the phospholipids DPPC/PG exclusively. These preparations have limited acute effects in infants with established RDS but may slowly improve lung function when recycled in the type II cells (*123*).

1.7 SURFACTANT PROTEIN ANALOGUES

Of the four surfactant proteins, only SP-B and/or SP-C are believed to be necessary constituents of surfactant preparations.

1.7.1 SP-B analogues

Synthetic full-length SP-B (SP-B 1-78) has, by fluorescence measurements, been shown to promote ordering of the lipid bilayers at the air-water interface (*124*). It does not, however, reach the same extent of surface ordering as native SP-B, possibly due to its inability to adopt the complex tertiary structure of SP-B. The complicated homodimeric structure with several disulphide bridges has forced development of SP-B analogues to concentrate on single parts.

SP-B analogue, SP-B(1-25) (*125*) has been shown to have surfactant properties similar to synthetic full-length SP-B 1-78 protein (*126*). A dimeric form, dSP-B(1-25) (Table 2) was shown to further increase surfactant activity *in vitro* compared to monomeric SP-B(1-25) (*127*).

Another approach is based on oligo-N-substituted glycines, called peptoids, with simple, repetitive sequences designed to form amphiphilic helices (*128*). The characteristic difference between peptides and peptoids is that the latter have their sidechains attached to the amide nitrogens instead of the α-carbon. This placement of the side chain makes peptoids protease resistant (*129*), and although the N-substituted glycine backbone is achiral, stable helical secondary structure can be induced sterically through the inclusion of chiral side chains (*130, 131*). These peptoids have shown *in vitro* surfactant activity similar to that of SP-B(1-25) and KL_4 (see 1.7.3). In a recent study a helical and cationic peptoid-based SP-B mimic was modified by SP-C-like N-terminus alkylation with octadecylamine. These “hybridized” mono- and dialkylated peptoids significantly decreased the maximum surface tension of the lipid film during cycling on the pulsating bubble surfactometer relative to the unalkylated variant (*132*). A possible explanation may be that the alkylated chain(s) ensure that the molecule is attached to the interface, or remains predominantly lipid-associated.

The same group also presents another non-natural approach to SP-B mimicry. This mimic is based on copolymers with random sequences containing cationic and

lipophilic subunits. Pulsating bubble surfactometry data indicate that these polymers achieve adsorptive and dynamic-cycling properties that exceed those of natural peptides intended to mimic SP-B. Furthermore, attachment of an N-terminal octadecanoyl unit to the nylon-3 copolymers, as with the peptoids, further improves in vitro surfactant activity by reducing the surface area compression needed to reach low minimum surface tension (*133*).

Mini-B is another analogue based on segments found in native SP-B. It is a 34-residue peptide where the N- and C-terminal helices have been fused by an engineered loop. Mini-B also contains two intramolecular disulphide bridges present in native SP-B (Figure 6) (*134*). Mini-B possesses a strikingly amphipathic surface with a large hydrophobic patch localized to one face of the peptide and most of the positively charged residues on the opposite face. This marked partitioning is likely a key in the mechanism by which Mini-B, and presumably SP-B, functions (*135*).

The N-terminal helical domain was recently shown to be required for the fusogenic, lytic and surface activity properties of SP-B (*73*) so a further developed analogue, Super Mini-B, with native SP-B residues 1-7 attached to the N-terminus of Mini-B has been studied. Super Mini-B gave higher oxygenation and dynamic compliance, compared to Mini-B, in a model with lung-lavaged rats. The increased surface activity most likely depends on self-assembly of Super Mini-B into a dimer (*136*).

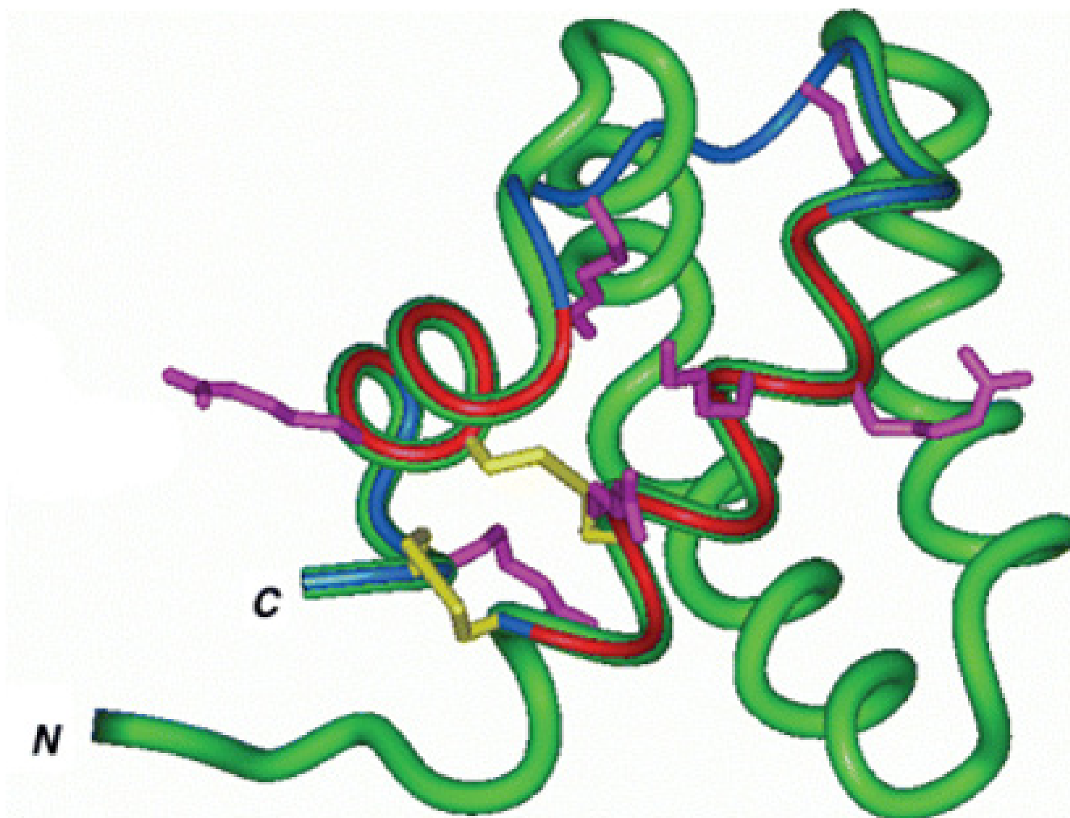

Figure 6. Mini-B structure in methanol

Helical segments in red, the engineered loop in blue and the disulfide linkages in yellow. Mini-B is overlaid on the corresponding N- and C-terminal regions of the full-length SP-B protein (green). Figure from (*134*).

Electron spin resonance and fluorescence spectroscopy of SP-B(1-25) indicate a surface location in lipid bilayers for the N-terminal helix (corresponding to Mini-B residues 8-25) (*137, 138*), while two-dimensional NMR spectroscopy of the C-terminal SP-B(63-78) in SDS micelles showed that its helix (i.e. Mini-B residues 24–34) was restricted to the water interface (*139*). The surface location for native SP-B in lipid bilayers (*140*) may be due to the resistance of these charged amphipathic domains to penetrate into the bilayer.

Table 2. Primary structures of SP-B and SP-B analogues

Underlined residues indicate changed amino acids compared to primary structure of native SP-B

SP-B

```
         10         20         30
FPIPLPYCWL CRALIKRIQA MIPKGALAVA
         40         50         60
VAQVCRVVPL VAGGICQCLA ERYSVILLDT
         70
LLGRMLPQLV CRLVLRCSM -COOH
```

SP-B(1-25)

```
FPIPLPYCWL CRALIKRIQA MIPKG -COOH
```

dSP-B(1-25)

```
FPIPLPYCWL ARALIKRIQA MIPKG -COOH
       |
FPIPLPYCWL ARALIKRIQA MIPKG -COOH
```

(The A at position 11 is underlined in both chains.)

Mini-B

```
CWLCRALIKR IQAMIPKGGR MLPQLVCRLV LRCS -COOH
```

Super Mini-B

```
FPIPLPYCWL CRALIKRIQA MIPKGGRMLP QLVCRLVLRC S -COOH
```

1.7.2 SP-C analogues

Synthetic SP-C has, due to its poly-valine part, a propensity to form β-sheets instead of the native α-helix (*42, 141*). Due to the discordant sequence, past attempts to synthesize SP-C using the native sequence have not yielded the expected secondary structure (*141*), why different approaches to obtain an SP-C mimic have been attempted.

The work on developing synthetic SP-C analogues preceding this thesis has undergone several stages. To avoid the formation of β-sheet aggregates associated with the native primary structure, the poly-valine stretch was substituted with leucines, which are more prone to form helices (*142*). This peptide, SP-C(Leu), had secondary structure similar to SP-C and also had comparable surface properties *in vitro.* However, attempts to suspend SP-C(Leu)/lipids at higher concentrations (> 20mg/ml) for use *in vivo* failed since the suspensions tended to become viscous, possibly due to peptide assembly into oligomers (*142*). To avoid SP-C(Leu) to turn into oligomers a modified variant, SP-C(LKS), containing three additional lysines (Table 3) that decreased peptide-peptide interactions was made. SP-C(LKS) combined the properties of SP-C(Leu) with the ability to make lipid suspensions at higher concentrations. Surfactant based on SP-C(LKS) showed fast adsorption *in* vitro but did not improve lung function in premature rabbits(*143*)[Unpublished observations]. A possible explanation is that the positively charged lysines in the N-terminal nonpolar region disrupt the SP-C dipole moment and alter the protein-membrane interactions.

In addition to the poly-val to poly-leu substituted SP-C analogues SP-C33 (paper I), and SP-CL16(6-28)(*144*), there are helical peptoid SP-C mimics using poly-N-substituted glycines (*145*). In a recent study, it was shown that an SP-C-like peptoid based on aromatic residues had superior surface activity, as measured using a pulsating bubble surfactometer, compared to aliphatic-based peptoids (*146*).

Despite the intrinsic propensity of SP-C to form β-sheets, recombinant SP-C expressed in bacteria as a fusion protein has the helical structure preserved. (*147, 148*). In a recent trial, rSP-C surfactant (Venticute) was shown to improve oxygenation in patients with ARDS (*149*).

There are several reports on synthetic analogues of SP-C improving lung function in premature animals (*145, 150, 151*). One common observation, though, is that ventilation with positive end-expiratory pressure (PEEP) is necessary to obtain these improvements (*148, 152*).

Table 3. Primary structure of SP-C and SP-C analogues

Underlined residues indicate changed amino acids compared to primary structure of native SP-C. PA: Palmitic Acid

	PA PA (positions 5, 6); 10; 20; 30
Native SP-C	FGIPCCPVHL KRLLIVVVVV VLIVVVIVGA LLMGL-COOH
SP-CL16(6-28)	CPVHL KRLLLLLLLL LLLLLLLL -COOH
rSP-C	FGIPFFPVHL KRLLIVVVVV VLIVVVIVGA LLMGL-COOH
SP-C(Leu)	FGIPSSPVΔL KRLLILLLLL LLILLLILGA LLMGL-COOH
SP-C(LKS)	FGIPSSPVHL KRLLILKLLL LKILLLKLGA LLMGL-COOH
SP-C33	IPSSPVHL KRLKLLLLLL LLILLLILGA LLMGL-COOH

1.7.3 Other analogues

The synthetic surfactant peptide KL_4 is based on a C-terminal helical region present in human SP-B. With its repetitive lysine-leucine sequence it has been proposed to stabilize the interfacial lipid layers by interactions between lysines and phospholipid headgroups, and between leucines and fatty acyl chains (*153*). KL_4 has shown *in vitro* surfactant activity and has also shown effect *in vivo* in treatment of RDS in animals (*154-157*).

There is also an analogue based on a SP-B N-terminal segment, Hel 13-5. Hel 13-5 is an amphiphilic α-helical peptide synthesized to mimic the structural balance and function of SP-B (*158*). Fluorescence- and atomic force microscopy studies shows that Hel 13-5 interacts specifically with PG during the "squeeze-out" action (*159*), in resemblance with native SP-B (*82*).

Table 4. Primary structure of KL4 and Hel 13-5

KL4
KLLLLKLLLL KLLLLKLLLL K-COOH
Hel 13-5
KLLKLLLKLW LKLLKLL-COOH

2 SCOPE OF THE PRESENT STUDY

2.1 GENERAL AIM

The aim of the research was to further investigate SP-B, SP-C and their analogues in order to develop a synthetic alternative to the animal-derived surfactants that are clinically used today to treat RDS.

2.2 SPECIFIC AIMS

- to evaluate an analogue of SP-C that has the desired secondary structure and together with a simple phospholipid mixture is able to decrease surface tension *in vitro* and *in vivo* to the same extent as natural-derived surfactant.

- investigate if modifications of the C-terminal end of SP-C affect its *in vitro* and *in vivo* function.

- to investigate if the inclusion of both SP-B and SP-C analogues is superior to a single-peptide preparation.

- to study if Mini-B could mimic native SP-B in a synthetic surfactant.

3 RESULTS

3.1 PAPERS I-III

In paper I, properties of SP-C(Leu) and SP-C(LKS) were combined into SP-C33 which was evaluated both *in vitro* and *in vivo* in its nonpalmitoylated as well as dipalmitoylated form.

The *in vitro* surface activity measurements were performed using a pulsating bubble surfactometer (PBS) where palmitoylated and non-palmitoylated SP-C33 were introduced in different lipid mixtures. By keeping the DPPC to unsaturated phospholipids ratio constant, the impact of varying the unsaturated lipid species (POPC and POPG) was investigated. The results showed that a minimum of 20% POPG in DPPC with or without POPC is necessary to obtain minimum surface tension below 5mN/m.

When comparing SP-C33 surfactant (2% (w/w) in DPPC/PG/PA 68:22:9) with the modified natural surfactants Curosurf and Survanta in premature rabbits, all preparations gave similar tidal volumes. However, to make the lipid mixture more similar to natural surfactant (with respect to ratio of saturated/unsaturated phospholipids and other phospholipid classes) and also further evaluate the importance of lipid composition, different compositions were tested. The conclusion was that the mixture of DPPC/POPG (68:31 by weight) is the most efficient, considering preparation and surfactant activity, for making a synthetic surfactant (Table 5).

It was also noticed that by applying PEEP during ventilation the lung gas volumes increase significantly in animals treated with SP-C33 surfactant. The lung gas volumes are comparable to those obtained in animals treated with Curosurf. This indicates that some components in Curosurf, possibly SP-B, help stabilize the lungs at end of expiration.

Table 5. Surface activity of SP-C33 in different lipid mixtures

2% SP-C (by weight) suspended in lipids (10mg/ml *in* vitro and 80mg/ml *in vivo*)

γ_{min} and γ_{max} : minimum and maximum surface tension after 5 min pulsation in a pulsating bubble surfactometer. VT_{30min}: Tidal volume after 30 min ventilation in prematurely born rabbits. Mean values shown

Surfactant mixture	γ_{min}	γ_{max}	VT_{30min} (ml/kg)
DPPC/POPG (68:31)	1	37	25
DPPC/POPC/POPG (6:2:2)	1	37	15
DPPC/PG/PA (68:22:9)	2	48	17

In paper II, we investigated the amount of SP-C33 necessary to achieve an active surfactant. The SP-C analogue SP-C33 was mixed at 0, 0.5, 1, 1.5 and 2% (by weight) with DPPC/POPG (66:31, w/w) and evaluated *in vitro* in the CBS. Although no statistically significant differences were shown, a linear trend showed that both minimum and maximum surface tension decreased and the adsorption speed increased with higher peptide amounts. Due to the small differences between surfactant preparations containing 0.5, 1 and 1.5% SP-C33 *in vitro* only preparations containing 0, 1 or 2% were studied further *in vivo* in a model using premature rabbits. In this study the animals were treated with a PEEP of 3cm H_2O. For the first 25 minutes of ventilation tidal volumes for Curosurf were significantly higher than all other groups, but after 30 minutes no significant differences were seen between the groups treated with SP-C33 surfactant and Curosurf. During this time, a drop in ventilation pressure was performed to investigate the ability of the preparations to reopen the lungs. The result indicates that SP-C33 surfactant may need a longer time of initial opening pressure to give maximum effect.

All surfactant-treated groups showed significantly higher tidal volumes than the group receiving only phospholipids. Curosurf gave significantly higher lung gas volumes than all other treatments and surfactants containing 1 or 2% SP-C33 gave significantly

higher lung gas volumes than the control group and the group treated with only phospholipids. Treatment with PEEP had a positive effect on lung gas volume, especially for the synthetic surfactants with 1 or 2% SP-C33. Taken together, the study shows that 1% (w/w) of SP-C33 is necessary to obtain an active surfactant.

In paper III 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 surface activity of a synthetic surfactant preparation. SP-C33 modified in three different ways was studied *in vitro*, by CBS and *in vivo* in a rabbit model using a PEEP of 3-4 cmH_2O. SP-C30 is a truncated variant that is assumed to be too short to fully span a DPPC bilayer. The other two variants are full-length with exchanged amino acid residues in the C-terminal part of the α-helix, SP-C33M31K and SP-C33M31I (Table 6).

Table 6. Primary structure of modified SP-C33 analogues

SP-C33 M31K	NH_2-IPSSPVHLKRLKLLLLLLLLILLLILGALLKGL-COOH
SP-C33 M31I	NH_2-IPSSPVHLKRLKLLLLLLLLILLLILGALLIGL-COOH
SP-C30	NH_2-IPSSPVHLKRLKLLLLLLLLILLLILGALL-COOH

To determine the secondary structure and orientation of SP-C33 we used circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy. Analysis of secondary structure, by CD and FTIR, showed that SP-C33 forms mainly α-helical structure. Further FTIR analysis revealed that SP-C33 adopts a transmembrane orientation when inserted in a DPPC/PG environment.

The *in vitro* surfactant activity was equal for all tested preparations, γ_{min} around 1-2 mN/m and γ_{max} between 22-27 mN/m. Additionally, the degree of compression required to reach 5 mN/m was comparable (22-32%) with no significant differences between tested preparations.

In line with the *in vitro* results, all protein-containing preparations behaved similarly *in vivo*. Rabbits ventilated with a standardised sequence of insufflation pressure showed similar tidal volumes throughout the entire experiment. After 35 minutes of

ventilation tidal volumes around 12-15 ml/kg were observed. Likewise, the lung gas volumes measured after the experiments were similar for all surfactant-treated groups.

3.2 PAPERS IV-V

In paper IV we investigated if addition of native SP-B to SP-C or SP-C33 surfactant would increase alveolar stability. The *in vivo* experiments in these papers were performed without PEEP.

Treatment groups receiving either 2% SP-C, 2% SP-B or 2% SP-C33 showed lung gas volumes significantly lower than those treated with SP-B+SP-C surfactant, while tidal volumes were comparable. In addition, alveolar expansion was higher in animals treated with surfactant containing both SP-B and SP-C33 (Figure 7).

To ensure that these findings were not related to total peptide amount, preparations including 2% and 4% SP-C33 were compared. No differences were found and the conclusion was that addition of SP-B to surfactant containing SP-C, or SP-C33, will increase lung gas volumes at end-expiration and that SP-B and SP-C perform different functions. Furthermore, surfactant containing both SP-B and SP-C has better surface activity *in vitro* regarding both adsorption and minimum and maximum surface tension measured in the CBS. The effect of SP-B on surfactant stability was studied by long-time (up to 7 days) surface area change by rotation in sealed glass tubes. Surface activity was then measured in a CBS giving the result that SP-B makes the surfactant more resistant.

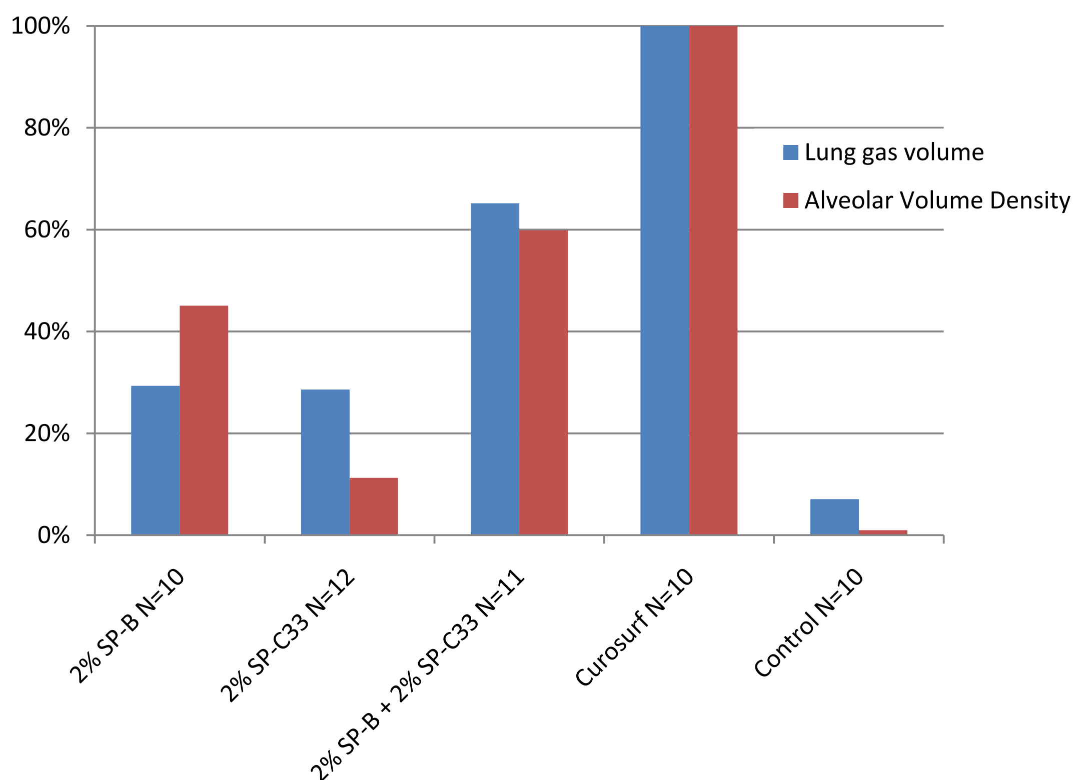

Figure 7. Lung gas volumes and alveolar expansion
of synthetic surfactants in DPPC/POPG (68:31 by weight) in relation to Curosurf (Curosurf set to 100%). Data presented as medians (lung gas volumes) or mean (alveolar volume density). Data from paper IV.

In paper V we investigated the effect on lung gas volumes of Mini-B in synthetic surfactant in prematurely born rabbits. The experiment showed that surfactant containing both SP-C33 and Mini-B gives lung gas volumes significantly higher than single-peptide surfactants but they do not reach as high volumes as Curosurf. This indicates that Mini-B holds properties similar to those of SP-B. To evaluate the importance of the amphiphilic character, two model peptides were used in the preparations. $KL_{2,3}$ has a basic/hydrophobic amphiphilic α-helix (*142*) and KLK3 that contains three clusters of basic residues separated by two hydrophobic 7-residue segments. When tested in the rabbit model, neither of these peptides showed any improvement on lung gas volume when added to SP-C33 surfactant.

4 DISCUSSION

Development of synthetic surfactant has been ongoing during the past 45 years. The components of this complex lipid/protein mixture have complicated the progress due to structural properties of the proteins and the multitude of phospholipids. The research has evolved from simple phospholipid preparations to more advanced surfactants including analogues of either SP-B or SP-C.

In paper I- III, we investigated SP-C33 *in vitro* and *in vivo* in different lipid mixtures and at different amounts to find the most effective, yet simple preparation, followed by studies on how modifications of the C-terminal end of SP-C33 affect surfactant activity. As starting point we chose to suspend the protein in the DPPC/PG/PA (68:22:9 by weight). Although only present in trace amounts in natural surfactants, the inclusion of PA was early shown to improve *in vitro* properties of surfactant preparations (*160*). As well as being difficult to suspend at higher concentrations, especially together with dipalmitoylated SP-C33, our tests showed that inclusion of PA resulted in lower tidal volumes. This is in contradiction with the results obtained in a study using SP-C peptoids (*161*). Here DPPC/POPG/PA exhibited higher activity than DPPC/POPG and DPPC/POPC/palmitoyloleoylphosphatidylethanolamine (POPE)/ palmitoyloleoyl phosphatidylserine (POPS)/Cholesterol. This study, however, was performed *in vitro* using Langmuir-Wilhelmy balance and PBS. It is important to remember that surfactant may act differently *in vivo* and *in vitro.*

Natural surfactant contains 2-3% SP-C (*82*) while the modified natural surfactant, Curosurf, only contains ~1%(*162*). In paper II, we found that a minimum of 1% SP-C33 was necessary to obtain adequate tidal volumes and that an increase of SP-C33 content to 2% showed no improvement. Still, in our continuous work, we chose to use 2% SP-C33 to have an apparent excess of peptide as a safety margin. Used surfactant is degraded and partly phagocytized and since no de novo synthesis is present in babies suffering from neonatal RDS a surplus may reduce the number of surfactant instillations.

The investigation of secondary structure of SP-C33, made by CD and FTIR, revealed a mainly α-helical structure. This was expected due to the exchange of the discordant amino acid sequence. The C-terminally modified variants of SP-C33 were not studied

by spectroscopy, but taking into consideration that all so far analyzed SP-C variants with a poly-leucine sequence have very similar helical contents, their secondary structures were assumed to be identical to that of SP-C33. This assumption is also supported by the observation of an α-helical structure of the synthetic analogues SP-C(CC) and SP-C(1-21) (*41*). In resemblance with native SP-C they adopt a transmembrane orientation when inserted in a phospholipid bilayer. Modifications resulting in hypothetically changed mobility properties of the C-terminal end of SP-C33 do not alter its surface tension decreasing capabilities, not *in vitro* nor *in vivo.* This indicates that the mechanism of action of SP-C is not dependent of the proposed mobility of the C-terminal end.

The two surfactant proteins SP-B and SP-C have been thought to have overlapping properties since absence of either one has still shown surfactant activity (*163*). In paper IV however, surfactant containing both SP-B and SP-C or SP-C33 was shown to better stabilize the lung at end-expiration than single-peptide preparations why synthetic surfactants ideally should contain representatives of both hydrophobic surfactant proteins. This is in agreement with the behavior of SP-B deficient mice but is not expected from studies of SP-C deficient mice. Experiments show that SP-C knock-out mice have normal respiration at birth but develop progressive pneumonitis (*104*). These results indicate that SP-B is able to induce low alveolar surface tension by itself. This discrepancy is not easily explained. A possible reason may be that the multistep isolation and purification of porcine SP-B in some way alter its original, rather complex, structural and functional properties.

Due to the complex tertiary structure of SP-B it is hard to synthesize. Instead, research has been concentrated in finding functional analogues based on different structural parts of native SP-B. These analogues show promising results (*125, 126, 134, 136, 153*) in vitro and inclusion of Mini-B to SP-C33 surfactant was shown to stabilize the alveoli. These simplified SP-B analogues are, however, still inferior to native SP-B. The lower surfactant activity of these analogues indicates that some crucial properties of SP-B have to be revealed and further reinforce the difficulties of developing a synthetic surfactant.

In Mini-B the N- and C-terminal helices of native SP-B are fused. In this way, Mini-B shows a higher structural resemblance to native SP-B compared to other, shorter,

analogues. This is probably an advantage since the complicated structure seems to be of great importance for the activity and Mini-B has also shown to reproduce some of the properties performed by SP-B. It is, nevertheless, important to remember that even though the structures resemble, Mini-B lacks parts of the primary structure and only are present as monomers. The evolved analogue, Super Mini-B, was found to form dimers and also have surfactant activity in lung-lavage rats with ARDS(*136*) and could therefore improve surfactant activity of SP-C33 surfactant.

A new approach using non-natural SP-B mimics show biophysical *in vitro* surfactant activity (*128, 132, 133*). Depending on results from *in vivo* experiments, this may be an interesting alternative to peptide analogues. The advantage of these mimics may be their ability to circumvent peptide-associated problems like misfolding and irreversible aggregation together with low cost of production. These non-natural mimics may also have the advantage of resistance against inhibition due to their insensitivity to proteases which may help in treatment of ARDS. On the other hand, this may cause a problem of accumulated protein mimics in the lung.

Clinical studies of KL_4-surfactant, Surfaxin, have shown results similar to those obtained by modified natural surfactants (*164*). Surfaxin was, however, recently rejected approval for treatment of RDS by the FDA (*165*) why commercial availability has been postponed.

While RDS is successfully treated with exogenous surfactant, there is no treatment of ARDS. Together with the higher incidence compared to neonatal RDS, this is a big opening for synthetic surfactant. Venticute, based on recombinant SP-C, was studied in a phase III clinical study on patients with ARDS. Adding treatment with Venticute to standard therapy showed no improvement compared to standard therapy alone (*166*). Post hoc analysis showed that reduced mortality associated with surfactant treatment was obtained in patients with severe respiratory insufficiency due to pneumonia or aspiration. These patients are the focus of an ongoing randomized, blinded, clinical trial with Venticute (*149*).

In addition to the trials of synthetic surfactants in ARDS treatment, a study using the modified natural surfactant HL-10 has been performed. Like the synthetic preparations no positive effect was seen after HL-10 treatment. On the contrary, a trend towards increased mortality and adverse effects was seen (*167*).

Due to its heterogenous character, treatment of ARDS is difficult to master. It was found that ARDS originating from pneumonia or aspiration was most likely to benefit surfactant replacement therapy so maybe inclusion of either SP-A or SP-D would make an improvement.

5 CONCLUSIONS

Development of a synthetic surfactant is in progress. By replacing the poly-valine α-helical part of native SP-C with leucines, thereby circumventing the propensity to form oligomers, an analogue (SP-C33) with similar properties and activity was found. Surfactant preparations containing 1-2% SP-C33 (by weight) in 80 mg/ml DPPC/POPG (68:31, by weight) function as well as modified natural surfactant preparations regarding tidal volumes in premature newborn rabbits. However, the animals treated with these synthetic preparations must be ventilated with PEEP in order to stabilize the alveoli at end-expiration. With an applied PEEP of 3-4 cmH_2O, lung gas volumes in animals treated with SP-C33 surfactant were similar to those obtained in Curosurf-ventilated animals. To obtain these volumes without PEEP, inclusion of SP-B or an analogue thereof is probably required. This indicates that SP-B and SP-C exert different physiological functions in surfactant.

Our results show that the surfactant properties of SP-C are not dependent on the assumed mobility of the C-terminal end of the peptide as modifications herein do not alter its secondary structure or surfactant activity *in vitro* nor *in vivo*. Addition of native SP-B to SP-C/SP-C33 surfactant increases the stabilization of the alveoli at end-expiration in experiments performed without PEEP but the results are still inferior to those obtained by treatment with Curosurf. This discrepancy may depend on the differences in phospholipid composition between synthetic and natural surfactants. The SP-B analogue Mini-B, which is a 34-residue peptide containing the N- and C-terminal helices of SP-B, has similar effect as native SP-B in animal experiments and may act as a replacement in synthetic surfactants.

The results in this thesis shows that a synthetic surfactant consisting of 2% SP-C33 together with 2% Mini-B in 80 mg/ml DPPC/POPG (68:31, by weight) has similar activity to modified natural surfactants and indicates that surfactants containing analogues of both SP-B and SP-C are superior to single-peptide surfactants and may replace natural surfactants for treatment of RDS in the near future.

6 MATERIALS AND METHODS

6.1 MODIFIED NATURAL SURFACTANT

Curosurf (Chiesi Pharmaceutici, Parma, Italy) is a surfactant suspension derived from minced porcine lung tissue. Its constituents are approximately 99% phospholipids and 0.7% and 0.6% SP-B and SP-C by weight, respectively (*162*).

6.2 PURIFICATION OF NATIVE SP-B AND SP-C

SP-B and SP-C were isolated from surfactant extracted from minced pig lungs. The proteins were separated from phospholipids by size-exclusion chromatography using a Sephadex LH-20 column in chloroform/methanol (2:1, by volume)(*168*) and thereafter separated from each other on a Sephadex LH-60 column in chloroform/methanol/0.1M hydrochloric acid (19:19:2, by volume)(*169*). Determination of protein amount was performed by amino acid analysis (*162*).

6.3 PULSATING BUBBLE SURFACTOMETER

In vitro surfactant activity was performed using a pulsating bubble surfactometer (*170*). In the PBS an air-bubble, connected to the ambient air by a chimney, placed in a test chamber filled with surfactant was studied. The bubble radius was changed from 0.55 to 0.40 mm (corresponding to an area compression of 50%) in a cyclic manner at a rate of 40/minute. By recording the pressure, surface tension was calculated using the law of Laplace. The equilibrium surface tension was measured after 2 minutes of adsorption and the surface tension at minimum and maximum bubble size (γ_{min} and γ_{max}) was recorded.

6.4 CAPTIVE BUBBLE SURFACTOMETER

The captive bubble surfactometer was introduced in 1989 as an air-tight, leakage-proof alternative to the PBS. An air bubble was inserted into a sucrose-filled chamber where it was placed, by buoyancy, next to an agarose gel plug. Subsequently, the sample solution was introduced and adsorbed at the air-liquid interface. By compressing the chamber a pressure was applied that alters the surface area of the bubble. The bubble was monitored and by measuring the width-to-height ratio of the bubble, surface tension could be calculated (*171*).

6.5 CIRCULAR DICHROISM SPECTROSCOPY

Left and right circularly polarized light interacts differently with chiral molecules, like all α-amino acids except glycine. Wavelength-dependent difference of absorption yields a CD spectrum. Secondary structure of proteins can be determined by CD spectroscopy in the "far-UV" spectral region (190-250 nm). α-Helix, β-sheet, and random coil structures each give rise to a characteristic shape and magnitude of a CD spectrum. α-Helix will give a maximum at 192 nm and double minima at 208 and 222nm, β-sheet show maximum at 195nm and minimum at 217 while random coil have a minimum at 197nm and maximum at 220nm (Figure 8) (*172, 173*).

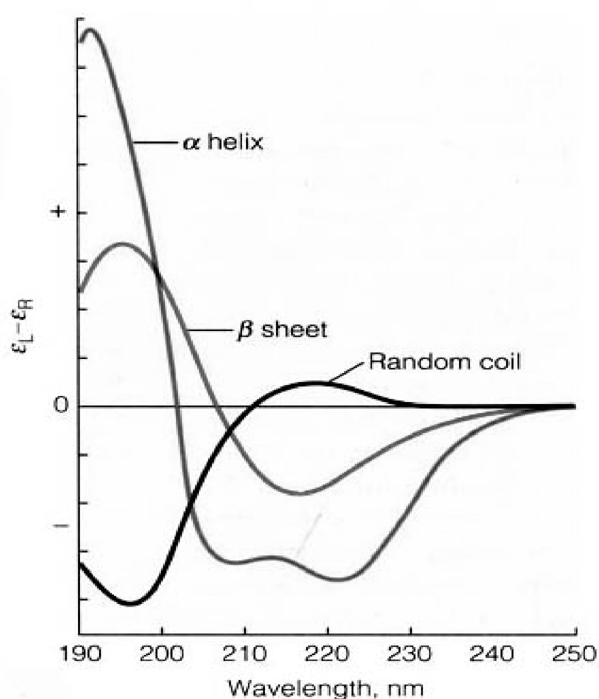

Figure 8. Circular dichroism spectra of α-helix, β-sheet and random coil

The approximate fraction of each secondary structure type that is present in any protein can be determined by analyzing its far-UV CD spectrum as a sum of fractional multiples of reference spectra for each structural type. The CD signal reflects an average of the entire molecular population. Thus, while CD-spectroscopy determines the percentage of different structures, it cannot determine which specific residues are involved in each type.

6.6 ATTENUATED TOTAL REFLECTION FOURIER TRANSFORM INFRARED SPECTROSCOPY

Fourier transform infrared (FTIR) spectroscopy is a measurement technique for collecting infrared spectra. Instead of recording the amount of energy absorbed when the frequency of the infra-red light is varied, the pattern of interference between two or more light waves is studied. Performing a Fourier transform on this signal data results in a spectrum identical to that from conventional (dispersive) infrared spectroscopy. By studying the frequence dependent IR absorption, secondary structure content of proteins can be determined (*174*). Additionally, it is possible to determine orientation of peptides in a phospholipid bilayer by analyzing the orientation of peptide bond C=O groups relative to phospholipid C=O group. This is done by recording spectra with parallel and perpendicular polarized light with respect to the incidence plane.
Using hydrogen/deuterium exchange of accessible amide protons, dynamics of the polypeptide backbone can be studied (*39*).

6.7 *IN VIVO* ANIMAL EXPERIMENT

While *in vitro* studies give detailed biophysical data, one cannot simply convert these into physiological properties. Consequently, the *in vivo* experiments were performed to evaluate the physiological effects of the surfactant preparations. Preterm newborn rabbits obtained at a gestational age of 27 days (term 31 days), were kept in whole body plethysmograph boxes at 37°C and ventilated in parallel. Depending on the experimental design the ventilation was performed with or without PEEP (3-4 cmH_2O). During ventilation, individual tidal volumes (V_T) were recorded. At the end of the experiment lungs were excised and weighed. Lung gas volumes, an equivalent to functional residual capacity (FRC), were determined by water displacement technique (*175*)(paper IV). While V_T is the normal volume of air displaced between normal inspiration and expiration, FRC is defined as the amount of air remaining at the end of normal respiration which in turn is highly dependent on the ability of the alveoli to withstand collapse at end-expiration.

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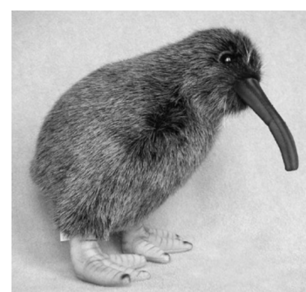

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8 REFERENCES

1. Parmigiani, S., Solari, E., and Bevilacqua, G. (2005) Current concepts on the pulmonary surfactant in infants, *J Matern Fetal Neonatal Med 18*, 369-380.
2. von Neergaard K, W. K. (1929) Neue Auffassungen über einen Grundbegriff der Atemmechanik, *T Gesamte Exp Med*, 374-394.
3. Pattle, R. E. (1955) Properties, function and origin of the alveolar lining layer, *Nature 175*, 1125-1126.
4. Clements, J. A. (1957) Surface tension of lung extracts, *Proc Soc Exp Biol Med 95*, 170-172.
5. Clements, J. A., Brown, E. S., and Johnson, R. P. (1958) Pulmonary surface tension and the mucus lining of the lungs: some theoretical considerations, *J Appl Physiol 12*, 262-268.
6. Avery, M. E., and Mead, J. (1959) Surface properties in relation to atelectasis and hyaline membrane disease, *Am J Dis Child 97*, 517-523.
7. King, R. J., and Clements, J. A. (1972) Surface active materials from dog lung. II. Composition and physiological correlations, *Am J Physiol 223*, 715-726.
8. King, R. J., and Clements, J. A. (1972) Surface active materials from dog lung. I. Method of isolation, *Am J Physiol 223*, 707-714.
9. King, R. J., Klass, D. J., Gikas, E. G., and Clements, J. A. (1973) Isolation of apoproteins from canine surface active material, *Am J Physiol 224*, 788-795.
10. Olmeda, B., Villen, L., Cruz, A., Orellana, G., and Perez-Gil, J. (2010) Pulmonary surfactant layers accelerate O(2) diffusion through the air-water interface, *Biochim Biophys Acta.* In press.
11. Jobe, A. H., and Ikegami, M. (1993) Surfactant metabolism, *Clin. Perinatol. 20*, 683-696.
12. Wright, J. R., and Clements, J. A. (1987) Metabolism and turnover of lung surfactant, *Am Rev Respir Dis 136*, 426-444.
13. Pérez-Gil, J., and Keough, K. M. (1998) Interfacial properties of surfactant proteins, *Biochim Biophys Acta 1408*, 203-217.
14. Veldhuizen, R., Nag, K., Orgeig, S., and Possmayer, F. (1998) The role of lipids in pulmonary surfactant, *Biochim Biophys Acta 1408*, 90-108.
15. Creuwels, L. A., van Golde, L. M., and Haagsman, H. P. (1996) Surfactant protein B: effects on lipid domain formation and intermembrane lipid flow, *Biochim Biophys Acta 1285*, 1-8.
16. Perez-Gil, J., Casals, C., and Marsh, D. (1995) Interactions of hydrophobic lung surfactant proteins SP-B and SP-C with dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol bilayers studied by electron spin resonance spectroscopy, *Biochemistry 34*, 3964-3971.
17. Haagsman, H. P., and Diemel, R. V. (2001) Surfactant-associated proteins: functions and structural variation, *Comp Biochem Physiol A Mol Integr Physiol 129*, 91-108.
18. Herias, M. V., Hogenkamp, A., van Asten, A. J., Tersteeg, M. H., van Eijk, M., and Haagsman, H. P. (2007) Expression sites of the collectin SP-D suggest its importance in first line host defence: power of combining in situ hybridisation, RT-PCR and immunohistochemistry, *Mol. Immunol. 44*, 3324-3332.
19. Stahlman, M. T., Gray, M. E., Hull, W. M., and Whitsett, J. A. (2002) Immunolocalization of surfactant protein-D (SP-D) in human fetal, newborn, and adult tissues, *J Histochem Cytochem 50*, 651-660.

20. Madsen, J., Tornoe, I., Nielsen, O., Koch, C., Steinhilber, W., and Holmskov, U. (2003) Expression and localization of lung surfactant protein A in human tissues, *Am J Respir Cell Mol Biol 29*, 591-597.
21. Crouch, E., and Wright, J. R. (2001) Surfactant proteins a and d and pulmonary host defense, *Annu Rev Physiol 63*, 521-554.
22. Brummer, E., and Stevens, D. A. (2010) Collectins and fungal pathogens: roles of surfactant proteins and mannose binding lectin in host resistance, *Med Mycol 48*, 16-28.
23. Haagsman, H. P., Hogenkamp, A., van Eijk, M., and Veldhuizen, E. J. (2008) Surfactant collectins and innate immunity, *Neonatology 93*, 288-294.
24. Korfhagen, T. R., Bruno, M. D., Ross, G. F., Huelsman, K. M., Ikegami, M., Jobe, A. H., Wert, S. E., Stripp, B. R., Morris, R. E., Glasser, S. W., Bachurski, C. J., Iwamoto, H. S., and Whitsett, J. A. (1996) Altered surfactant function and structure in SP-A gene targeted mice, *Proc Natl Acad Sci USA 93*, 9594-9599.
25. Ikegami, M., Grant, S., Korfhagen, T., Scheule, R. K., and Whitsett, J. A. (2009) Surfactant protein-D regulates the postnatal maturation of pulmonary surfactant lipid pool sizes, *J Appl Physiol 106*, 1545-1552.
26. Cockshutt, A. M., Weitz, J., and Possmayer, F. (1990) Pulmonary surfactant-associated protein A enhances the surface activity of lipid extract surfactant and reverses inhibition by blood proteins in vitro, *Biochemistry 29*, 8424-8429.
27. Venkitaraman, A. R., Hall, S. B., Whitsett, J. A., and Notter, R. H. (1990) Enhancement of biophysical activity of lung surfactant extracts and phospholipid-apoprotein mixtures by surfactant protein A, *Chem Phys Lipids 56*, 185-194.
28. Sun, B., Curstedt, T., Lindgren, G., Franzen, B., Alaiya, A. A., Calkovska, A., and Robertson, B. (1997) Biophysical and physiological properties of a modified porcine surfactant enriched with surfactant protein A, *Eur Respir J 10*, 1967-1974.
29. Yamada, T., Ikegami, M., Tabor, B. L., and Jobe, A. H. (1990) Effects of surfactant protein-A on surfactant function in preterm ventilated rabbits, *Am Rev Respir Dis 142*, 754-757.
30. Yukitake, K., Brown, C. L., Schlueter, M. A., Clements, J. A., and Hawgood, S. (1995) Surfactant apoprotein A modifies the inhibitory effect of plasma proteins on surfactant activity in vivo, *Pediatr Res 37*, 21-25.
31. Sato, A., Whitsett, J. A., Scheule, R. K., and Ikegami, M. (2010) Surfactant protein-d inhibits lung inflammation caused by ventilation in premature newborn lambs, *Am J Respir Crit Care Med 181*, 1098-1105.
32. Weaver, T. E., and Conkright, J. J. (2001) Function of surfactant proteins B and C., *Annu Rev Physiol 63*, 555-578.
33. Lodish, H., Berk, A., Lawrence Zipursky, S., Matsudaira, P., Baltimore, D., and Darnell, J., (Eds.) (2000) *Molecular Cell Biology*, 4th ed., W. H. Freeman and Company.
34. Johansson, J., Curstedt, T., and Jörnvall, H. (1991) Surfactant protein B: disulfide bridges, structural properties, and kringle similarities, *Biochemistry 30*, 6917-6921.
35. Munford, R. S., Sheppard, P. O., and O'Hara, P. J. (1995) Saposin-like proteins (SAPLIP) carry out diverse functions on a common backbone structure, *J Lipid Res 36*, 1653-1663.
36. Liepinsh, E., Andersson, M., Ruysschaert, J. M., and Otting, G. (1997) Saposin fold revealed by the NMR structure of NK-lysin, *Nat Struct Biol 4*, 793-795.

37. Patthy, L. (1991) Homology of the precursor of pulmonary surfactant-associated protein SP-B with prosaposin and sulfated glycoprotein 1, *J Biol Chem 266*, 6035-6037.
38. Johansson, J., Szyperski, T., Curstedt, T., and Wuthrich, K. (1994) The NMR structure of the pulmonary surfactant-associated polypeptide SP-C in an apolar solvent contains a valyl-rich alpha-helix, *Biochemistry 33*, 6015-6023.
39. Vandenbussche, G., Clercx, A., Curstedt, T., Johansson, J., Jörnvall, H., and Ruysschaert, J. M. (1992) Structure and orientation of the surfactant-associated protein C in a lipid bilayer, *Eur J Biochem203*, 201-209.
40. Pastrana, B., Mautone, A. J., and Mendelsohn, R. (1991) Fourier transform infrared studies of secondary structure and orientation of pulmonary surfactant SP-C and its effect on the dynamic surface properties of phospholipids, *Biochemistry 30*, 10058-10064.
41. Clercx, A., Vandenbussche, G., Curstedt, T., Johansson, J., Jornvall, H., and Ruysschaert, J. M. (1995) Structural and functional importance of the C-terminal part of the pulmonary surfactant polypeptide SP-C, *Eur J Biochem229*, 465-472.
42. Johansson, J. (1998) Structure and properties of surfactant protein C, *Biochim Biophys Acta 1408*, 161-172.
43. Gustafsson, M., Palmblad, M., Curstedt, T., Johansson, J., and Schurch, S. (2000) Palmitoylation of a pulmonary surfactant protein C analogue affects the surface associated lipid reservoir and film stability, *Biochimica et Biophysica Acta Biomembranes 1466*, 169-178.
44. Bi, X., Flach, C. R., Perez-Gil, J., Plasencia, I., Andreu, D., Oliveira, E., and Mendelsohn, R. (2002) Secondary structure and lipid interactions of the N-terminal segment of pulmonary surfactant SP-C in Langmuir films: IR reflection-absorption spectroscopy and surface pressure studies, *Biochemistry 41*, 8385-8395.
45. Gustafsson, M., Griffiths, W. J., Furusjö, E., and Johansson, J. (2001) The palmitoyl groups of surfactant protein C reduce unfolding into a fibrillogenic intermediate, *J Mol Biol 310*, 937-950.
46. Na Nakorn, P., Meyer, M. C., Flach, C. R., Mendelsohn, R., and Galla, H. J. (2007) Surfactant protein C and lung function: new insights into the role of alpha-helical length and palmitoylation, *Eur Biophys J 36*, 477-489.
47. Plasencia, I., Baumgart, F., Andreu, D., Marsh, D., and Perez-Gil, J. (2008) Effect of acylation on the interaction of the N-Terminal segment of pulmonary surfactant protein SP-C with phospholipid membranes, *Biochim Biophys Acta 1778*, 1274-1282.
48. Robertson, B., Johansson, J., and Curstedt, T. (2000) Synthetic surfactants to treat neonatal lung disease, *Mol Med Today 6*, 119-124.
49. Brasch, F., Ochs, M., Kahne, T., Guttentag, S., Schauer-Vukasinovic, V., Derrick, M., Johnen, G., Kapp, N., Muller, K. M., Richter, J., Giller, T., Hawgood, S., and Buhling, F. (2003) Involvement of napsin A in the C- and N-terminal processing of surfactant protein B in type-II pneumocytes of the human lung, *J Biol Chem 278*, 49006-49014.
50. Ueno, T., Linder, S., Na, C.-L., Rice, W. R., Johansson, J., and Weaver, T. E. (2004) Processing of pulmonary surfactant protein B by napsin and cathepsin H, *J Biol Chem 279*, 16178-16184.
51. Weaver, T. E., Lin, S., Bogucki, B., and Dey, C. (1992) Processing of surfactant protein B proprotein by a cathepsin D-like protease, *Am J Physiol 263*, L95-103.

52. Weaver, T. E., and Whitsett, J. A. (1989) Processing of hydrophobic pulmonary surfactant protein B in rat type II cells, *Am J Physiol 257*, L100-108.
53. Wang, W. J., Russo, S. J., Mulugeta, S., and Beers, M. F. (2002) Biosynthesis of surfactant protein C (SP-C). Sorting of SP-C proprotein involves homomeric association via a signal anchor domain., *J Biol Chem 277*, 19929-19937.
54. Johnson, A. L., Braidotti, P., Pietra, G. G., Russo, S. J., Kabore, A., Wang, W. J., and Beers, M. F. (2001) Post-translational processing of surfactant protein-C proprotein: targeting motifs in the NH(2)-terminal flanking domain are cleaved in late compartments., *Am J Respir Cell Mol Biol 24*, 253-263.
55. Brasch, F., ten Brinke, A., Johnen, G., Ochs, M., Kapp, N., Muller, K. M., Beers, M. F., Fehrenbach, H., Richter, J., Batenburg, J. J., and F., B. (2002) Involvement of cathepsin H in the processing of the hydrophobic surfactant-associated protein C in type II pneumocytes., *Am J Respir Cell Mol Biol 26*, 659-670.
56. Cruz, A., Worthman, L. A., Serrano, A. G., Casals, C., Keough, K. M., and Perez-Gil, J. (2000) Microstructure and dynamic surface properties of surfactant protein SP-B/dipalmitoylphosphatidylcholine interfacial films spread from lipid-protein bilayers, *Eur Biophys J 29*, 204-213.
57. Nag, K., Munro, J. G., Inchley, K., Schurch, S., Petersen, N. O., and Possmayer, F. (1999) SP-B refining of pulmonary surfactant phospholipid films, *Am J Physiol 277*, L1179-1189.
58. Wustneck, R., Perez-Gil, J., Wustneck, N., Cruz, A., Fainerman, V. B., and Pison, U. (2005) Interfacial properties of pulmonary surfactant layers, *Adv Colloid Interface Sci 117*, 33-58.
59. Cruz, A., Casals, C., Keough, K. M., and Perez-Gil, J. (1997) Different modes of interaction of pulmonary surfactant protein SP-B in phosphatidylcholine bilayers, *Biochem J 327*, 133-138.
60. Ross, M., Krol, S., Janshoff, A., and Galla, H. J. (2002) Kinetics of phospholipid insertion into monolayers containing the lung surfactant proteins SP-B or SP-C, *Eur Biophys J 31*, 52-61.
61. Walters, R. W., Jenq, R. R., and Hall, S. B. (2000) Distinct steps in the adsorption of pulmonary surfactant to an air-liquid interface, *Biophys J 78*, 257-266.
62. Veldhuizen, E. J., Batenburg, J. J., van Golde, L. M., and Haagsman, H. P. (2000) The role of surfactant proteins in DPPC enrichment of surface films, *Biophys J 79*, 3164-3171.
63. Perkins, W. R., Dause, R. B., Parente, R. A., Minchey, S. R., Neuman, K. C., Gruner, S. M., Taraschi, T. F., and Janoff, A. S. (1996) Role of lipid polymorphism in pulmonary surfactant, *Science 273*, 330-332.
64. Zuo, Y. Y., Veldhuizen, R. A., Neumann, A. W., Petersen, N. O., and Possmayer, F. (2008) Current perspectives in pulmonary surfactant--inhibition, enhancement and evaluation, *Biochim Biophys Acta 1778*, 1947-1977.
65. Nag, K., Taneva, S. G., Perez-Gil, J., Cruz, A., and Keough, K. M. (1997) Combinations of fluorescently labeled pulmonary surfactant proteins SP-B and SP-C in phospholipid films, *Biophys J 72*, 2638-2650.
66. Cruz, A., Vazquez, L., Velez, M., and Perez-Gil, J. (2004) Effect of pulmonary surfactant protein SP-B on the micro- and nanostructure of phospholipid films, *Biophys J 86*, 308-320.
67. Vandenbussche, G., Clercx, A., Clercx, M., Curstedt, T., Johansson, J., Jörnvall, H., and Ruysschaert, J. M. (1992) Secondary structure and orientation of the surfactant protein SP-B in a lipid environment. A Fourier transform infrared spectroscopy study, *Biochemistry 31*, 9169-9176.

68. Baatz, J. E., Elledge, B., and Whitsett, J. A. (1990) Surfactant protein SP-B induces ordering at the surface of model membrane bilayers, *Biochemistry 29*, 6714-6720.
69. Shiffer, K., Hawgood, S., Duzgunes, N., and Goerke, J. (1988) Interactions of the low molecular weight group of surfactant-associated proteins (SP 5-18) with pulmonary surfactant lipids, *Biochemistry 27*, 2689-2695.
70. Oosterlaken-Dijksterhuis, M. A., van Eijk, M., van Golde, L. M., and Haagsman, H. P. (1992) Lipid mixing is mediated by the hydrophobic surfactant protein SP-B but not by SP-C, *Biochim Biophys Acta 1110*, 45-50.
71. Poulain, F. R., Allen, L., Williams, M. C., Hamilton, R. L., and Hawgood, S. (1992) Effects of surfactant apolipoproteins on liposome structure: implications for tubular myelin formation, *Am J Physiol 262*, L730-739.
72. Chang, R., Nir, S., and Poulain, F. R. (1998) Analysis of binding and membrane destabilization of phospholipid membranes by surfactant apoprotein B, *Biochim Biophys Acta 1371*, 254-264.
73. Ryan, M. A., Qi, X., Serrano, A. G., Ikegami, M., Perez-Gil, J., Johansson, J., and Weaver, T. E. (2005) Mapping and analysis of the lytic and fusogenic domains of surfactant protein B, *Biochemistry 44*, 861-872.
74. Plasencia, I., Keough, K. M., and Perez-Gil, J. (2005) Interaction of the N-terminal segment of pulmonary surfactant protein SP-C with interfacial phospholipid films, *Biochim Biophys Acta 1713*, 118-128.
75. Plasencia, I., Rivas, L., Keough, K. M., Marsh, D., and Perez-Gil, J. (2004) The N-terminal segment of pulmonary surfactant lipopeptide SP-C has intrinsic propensity to interact with and perturb phospholipid bilayers, *Biochem J 377*, 183-193.
76. Horowitz, A. D., Elledge, B., Whitsett, J. A., and Baatz, J. E. (1992) Effects of lung surfactant proteolipid SP-C on the organization of model membrane lipids: a fluorescence study, *Biochim Biophys Acta 1107*, 44-54.
77. Johansson, J., Szyperski, T., and Wüthrich, K. (1995) Pulmonary surfactant-associated polypeptide SP-C in lipid micelles: CD studies of intact SP-C and NMR secondary structure determination of depalmitoyl-SP-C(1-17), FEBS Lett *362*, 261-265.
78. Veldhuizen, E. J., and Haagsman, H. P. (2000) Role of pulmonary surfactant components in surface film formation and dynamics, *Biochim Biophys Acta 1467*, 255-270.
79. Bangham, A. D., Morley, C. J., and Phillips, M. C. (1979) The physical properties of an effective lung surfactant, *Biochim Biophys Acta 573*, 552-556.
80. Goerke, J. (1998) Pulmonary surfactant: functions and molecular composition, *Biochim Biophys Acta 1408*, 79-89.
81. Yu, S. H., and Possmayer, F. (1992) Effect of pulmonary surfactant protein B (SP-B) and calcium on phospholipid adsorption and squeeze-out of phosphatidylglycerol from binary phospholipid monolayers containing dipalmitoylphosphatidylcholine, *Biochim Biophys Acta 1126*, 26-34.
82. Serrano, A. G., and Perez-Gil, J. (2006) Protein-lipid interactions and surface activity in the pulmonary surfactant system, *Chem Phys Lipids 141*, 105-118.
83. Rodriguez-Capote, K., Nag, K., Schurch, S., and Possmayer, F. (2001) Surfactant protein interactions with neutral and acidic phospholipid films, *Am J Physiol Lung Cell Mol Physiol 281*, L231-242.
84. Guttentag, S. H., Beers, M. F., Bieler, B. M., and Ballard, P. L. (1998) Surfactant protein B processing in human fetal lung, *Am J Physiol 275*, L559-566.

85. Weaver, T. E., and Whitsett, J. A. (1991) Function and regulation of expression of pulmonary surfactant-associated proteins, *Biochem J 273(Pt 2)*, 249-264.
86. Nogee, L. M., Wert, S. E., Proffit, S. A., Hull, W. M., and Whitsett, J. A. (2000) Allelic heterogeneity in hereditary surfactant protein B (SP-B) deficiency, *Am J Respir Crit Care Med 161*, 973-981.
87. Nogee, L. M., Garnier, G., Dietz, H. C., Singer, L., Murphy, A. M., deMello, D. E., and Colten, H. R. (1994) A mutation in the surfactant protein B gene responsible for fatal neonatal respiratory disease in multiple kindreds, *J. Clin. Invest. 93*, 1860-1863.
88. Beers, M. F., Hamvas, A., Moxley, M. A., Gonzales, L. W., Guttentag, S. H., Solarin, K. O., Longmore, W. J., Nogee, L. M., and Ballard, P. L. (2000) Pulmonary surfactant metabolism in infants lacking surfactant protein B, *Am J Respir Cell Mol Biol 22*, 380-391.
89. Clark, J. C., Wert, S. E., Bachurski, C. J., Stahlman, M. T., Stripp, B. R., Weaver, T. E., and Whitsett, J. A. (1995) Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice, *Proc Natl Acad Sci USA 92*, 7794-7798.
90. Li, J., Ikegami, M., Na, C. L., Hamvas, A., Espinassous, Q., Chaby, R., Nogee, L. M., Weaver, T. E., and Johansson, J. (2004) N-terminally extended surfactant protein (SP) C isolated from SP-B-deficient children has reduced surface activity and inhibited lipopolysaccharide binding, *Biochemistry 43*, 3891-3898.
91. Tokieda, K., Whitsett, J. A., Clark, J. C., Weaver, T. E., Ikeda, K., McConnell, K. B., Jobe, A. H., Ikegami, M., and Iwamoto, H. S. (1997) Pulmonary dysfunction in neonatal SP-B-deficient mice, *Am J Physiol 273*, L875-882.
92. Vorbroker, D. K., Profitt, S. A., Nogee, L. M., and Whitsett, J. A. (1995) Aberrant processing of surfactant protein C in hereditary SP-B deficiency, *Am J Physiol 268*, L647-656.
93. Melton, K. R., Nesslein, L. L., Ikegami, M., Tichelaar, J. W., Clark, J. C., Whitsett, J. A., and Weaver, T. E. (2003) SP-B deficiency causes respiratory failure in adult mice, *Am J Physiol Lung Cell Mol Physiol 285*, L543-549.
94. Beers, M. F., and Mulugeta, S. (2005) Surfactant protein C biosynthesis and its emerging role in conformational lung disease., *Annu Rev Physiol 67*, 663-696.
95. Whitsett, J. A., and Weaver, T. E. (2002) Hydrophobic surfactant proteins in lung function and disease, *N Engl J Med 347*, 2141-2148.
96. Nogee, L. M., Dunbar, A. E., 3rd, Wert, S., Askin, F., Hamvas, A., and Whitsett, J. A. (2002) Mutations in the surfactant protein C gene associated with interstitial lung disease., *Chest 121*, 20S-21S.
97. Nogee, L. M., Dunbar, A. E., 3rd, Wert, S. E., Askin, F., Hamvas, A., and Whitsett, J. A. (2001) A mutation in the surfactant protein C gene associated with familial interstitial lung disease., *N Engl J Med 344*, 573-579.
98. Thomas, A. Q., Lane, K., Phillips, J., 3rd, Prince, M., Markin, C., Speer, M., Schwartz, D. A., Gaddipati, R., Marney, A., Johnson, J., Roberts, R., Haines, J., Stahlman, M., and Loyd, J. E. (2002) Heterozygosity for a surfactant protein C gene mutation associated with usual interstitial pneumonitis and cellular nonspecific interstitial pneumonitis in one kindred., *Am J Respir Crit Care Med 165*, 1322-1328.
99. Hamvas, A., Nogee, L. M., White, F. V., Schuler, P., Hackett, B. P., Huddleston, C. B., Mendeloff, E. N., Hsu, F. F., Wert, S. E., Gonzales, L. W., Beers, M. F., and Ballard, P. L. (2004) Progressive lung disease and surfactant dysfunction with a deletion in surfactant protein C gene, *Am J Respir Cell Mol Biol 30*, 771-776.

100. Kabore, A. F., Wang, W. J., Russo, S. J., and Beers, M. F. (2001) Biosynthesis of surfactant protein C: characterization of aggresome formation by EGFP chimeras containing propeptide mutants lacking conserved cysteine residues., *J Cell Sci 114*, 293-302.
101. Wang, W. J., Mulugeta, S., Russo, S. J., and Beers, M. F. (2003) Deletion of exon 4 from human surfactant protein C results in aggresome formation and generation of a dominant negative., *J Cell Sci 116*, 683-692.
102. Mulugeta, S., Nguyen, V., Russo, S. J., Muniswamy, M., and Beers, M. F. (2005) A surfactant protein C precursor protein BRICHOS domain mutation causes endoplasmic reticulum stress, proteasome dysfunction, and caspase 3 activation, *Am J Respir Cell Mol Biol 32*, 521-530.
103. Glasser, S. W., Burhans, M. S., Korfhagen, T. R., Na, C. L., Sly, P. D., Ross, G. F., Ikegami, M., and Whitsett, J. A. (2001) Altered stability of pulmonary surfactant in SP-C-deficient mice, *Proc Natl Acad Sci USA 98*, 6366-6371.
104. Glasser, S. W., Detmer, E. A., Ikegami, M., Na, C. L., Stahlman, M. T., and Whitsett, J. A. (2003) Pneumonitis and emphysema in SP-C gene targeted mice., *J Biol Chem 278*, 14291-14298.
105. Zimmermann, L. J., Janssen, D. J., Tibboel, D., Hamvas, A., and Carnielli, V. P. (2005) Surfactant metabolism in the neonate, *Biol Neonate 87*, 296-307.
106. Lynch, R. G. (2004) Surfactant and RDS in premature infants, *FASEB J 18*, 1624.
107. Nogee, L. M. (1998) Genetics of the hydrophobic surfactant proteins, *Biochim Biophys Acta 1408*, 323-333.
108. Haataja, R., Marttila, R., Uimari, P., Lofgren, J., Ramet, M., and Hallman, M. (2001) Respiratory distress syndrome: evaluation of genetic susceptibility and protection by transmission disequilibrium test, *Hum Genet 109*, 351-355.
109. Hallman, M., Haataja, R., and Marttila, R. (2002) Surfactant proteins and genetic predisposition to respiratory distress syndrome, *Semin Perinatol 26*, 450-460.
110. Hallman, M., Marttila, R., Pertile, R., Ojaniemi, M., and Haataja, R. (2007) Genes and environment in common neonatal lung disease, *Neonatology 91*, 298-302.
111. Marttila, R., Haataja, R., Ramet, M., Lofgren, J., and Hallman, M. (2003) Surfactant protein B polymorphism and respiratory distress syndrome in premature twins, *Hum Genet 112*, 18-23.
112. Whitsett, J. A., Wert, S. E., and Trapnell, B. C. (2004) Genetic disorders influencing lung formation and function at birth, *Hum Mol Genet 13 Spec No 2*, R207-215.
113. Cheong, N., Zhang, H., Madesh, M., Zhao, M., Yu, K., Dodia, C., Fisher, A. B., Savani, R. C., and Shuman, H. (2007) ABCA3 is critical for lamellar body biogenesis in vivo, *J Biol Chem 282*, 23811-23817.
114. Fellman, V., Hellstrom-Westas, L., Norman, M., Westgren, M., Kallen, K., Lagercrantz, H., Marsal, K., Serenius, F., and Wennergren, M. (2009) One-year survival of extremely preterm infants after active perinatal care in Sweden, *JAMA 301*, 2225-2233.
115. The_Express_group. (2010) Incidence of and Risk Factors for Neonatal Morbidity after Active Perinatal Care: Extremely Preterm Infants Study in Sweden (EXPRESS), *Acta Paediatr*. In Press.
116. Robillard, E., Alarie, Y., Dagenais-Perusse, P., Baril, E., and Guilbeault, A. (1964) Microaerosol administration of synthetic beta-gamma-dipalmitoyl-L-alpha-lecithin in the respiratory distress syndrome: A preliminary report, *Can Med Assoc J 90*, 55-57.

117. Fujiwara, T., Maeta, H., Chida, S., Morita, T., Watabe, Y., and Abe, T. (1980) Artificial surfactant therapy in hyaline-membrane disease, *Lancet 1*, 55-59.
118. Soll, R. F., and Morley, C. J. (2001) Prophylactic versus selective use of surfactant in preventing morbidity and mortality in preterm infants, *Cochrane Database Syst Rev*, CD000510.
119. Amato, M., Petit, K., Fiore, H. H., Doyle, C. A., Frantz, I. D., 3rd, and Nielsen, H. C. (2003) Effect of exogenous surfactant on the development of surfactant synthesis in premature rabbit lung, *Pediatr Res 53*, 671-678.
120. Bjorklund, L. J., Ingimarsson, J., Curstedt, T., John, J., Robertson, B., Werner, O., and Vilstrup, C. T. (1997) Manual ventilation with a few large breaths at birth compromises the therapeutic effect of subsequent surfactant replacement in immature lambs, *Pediatr Res 42*, 348-355.
121. Robertson, B. (1998) Surfactant inactivation and surfactant therapy in acute respiratory distress syndrome (ARDS), *Monaldi Arch Chest Dis 53*, 64-69.
122. Phibbs, R. H., Ballard, R. A., Clements, J. A., Heilbron, D. C., Phibbs, C. S., Schlueter, M. A., Sniderman, S. H., Tooley, W. H., and Wakeley, A. (1991) Initial clinical trial of EXOSURF, a protein-free synthetic surfactant, for the prophylaxis and early treatment of hyaline membrane disease, *Pediatrics 88*, 1-9.
123. Morley, C. J., Greenough, A., Miller, N. G., Bangham, A. D., Pool, J., Wood, S., South, M., Davis, J. A., and Vyas, H. (1988) Randomized trial of artificial surfactant (ALEC) given at birth to babies from 23 to 34 weeks gestation, *Early Hum Dev 17*, 41-54.
124. Baatz, J. E., Sarin, V., Absolom, D. R., Baxter, C., and Whitsett, J. A. (1991) Effects of surfactant-associated protein SP-B synthetic analogs on the structure and surface activity of model membrane bilayers, *Chem Phys Lipids 60*, 163-178.
125. Bruni, R., Hernandez-Juviel, J. M., Tanoviceanu, R., and Walther, F. J. (1998) Synthetic mimics of surfactant proteins B and C: in vitro surface activity and effects on lung compliance in two animal models of surfactant deficiency, *Mol Genet Metab 63*, 116-125.
126. Gupta, M., Hernández-Juviel, J. M., Waring, A. J., Bruni, R., and Walther, F. J. (2000) Comparison of functional efficacy of surfactant protein B analogues in lavaged rats., *Eur Respir J. 16*, 1129-1133.
127. Veldhuizen, E. J., Waring, A. J., Walther, F. J., Batenburg, J. J., van Golde, L. M., and Haagsman, H. P. (2000) Dimeric N-terminal segment of human surfactant protein B (dSP-B(1-25)) has enhanced surface properties compared to monomeric SP-B(1-25), *Biophys J 79*, 377-384.
128. Seurynck, S. L., Patch, J. A., and Barron, A. E. (2005) Simple, helical peptoid analogs of lung surfactant protein B, *Chem Biol 12*, 77-88.
129. Miller, S. M., Simon, R. J., Ng, S., Zuckermann, R. N., Kerr, J. M., and Moos, W. H. (1995) Comparison of the proteolytic susceptibilities of homologous L-amino acid, D-amino acid, and N-substituted glycine peptide and peptoid oligomers, *Drug Dev Res 35*, 20-32.
130. Wu, C. W., Sanborn, T. J., Huang, K., Zuckermann, R. N., and Barron, A. E. (2001) Peptoid oligomers with alpha-chiral, aromatic side chains: sequence requirements for the formation of stable peptoid helices, *J Am Chem Soc 123*, 6778-6784.
131. Wu, C. W., Sanborn, T. J., Zuckermann, R. N., and Barron, A. E. (2001) Peptoid oligomers with alpha-chiral, aromatic side chains: effects of chain length on secondary structure, *J Am Chem Soc 123*, 2958-2963.

132. Dohm, M. T., Brown, N. J., Seurynck-Servoss, S. L., de la Serna, J. B., and Barron, A. E. (2010) Mimicking SP-C palmitoylation on a peptoid-based SP-B analogue markedly improves surface activity, *Biochim Biophys Acta.* In press.
133. Dohm, M. T., Mowery, B. P., Czyzewski, A. M., Stahl, S. S., Gellman, S. H., and Barron, A. E. (2010) Biophysical Mimicry of Lung Surfactant Protein B by Random Nylon-3 Copolymers, *J Am Chem Soc*. In press.
134. Waring, A. J., Walther, F. J., Gordon, L. M., Hernandez-Juviel, J. M., Hong, T., Sherman, M. A., Alonso, C., Alig, T., Braun, A., Bacon, D., and Zasadzinski, J. A. (2005) The role of charged amphipathic helices in the structure and function of surfactant protein B., *J Pept Res. 66*, 364-374.
135. Sarker, M., Waring, A. J., Walther, F. J., Keough, K. M., and Booth, V. (2007) Structure of mini-B, a functional fragment of surfactant protein B, in detergent micelles., *Biochemistry 46*, 11047-11056.
136. Walther, F. J., Waring, A. J., Hernandez-Juviel, J. M., Gordon, L. M., Wang, Z., Jung, C. L., Ruchala, P., Clark, A. P., Smith, W. M., Sharma, S., and Notter, R. H. (2010) Critical structural and functional roles for the N-terminal insertion sequence in surfactant protein B analogs, *PLoS One 5*, e8672.
137. Wang, Y., Rao, K. M., and Demchuk, E. (2003) Topographical organization of the N-terminal segment of lung pulmonary surfactant protein B (SP-B(1-25)) in phospholipid bilayers, *Biochemistry 42*, 4015-4027.
138. Gordon, L. M., Horvath, S., Longo, M. L., Zasadzinski, J. A., Taeusch, H. W., Faull, K., Leung, C., and Waring, A. J. (1996) Conformation and molecular topography of the N-terminal segment of surfactant protein B in structure-promoting environments, Protein Sci *5*, 1662-1675.
139. Booth, V., Waring, A. J., Walther, F. J., and Keough, K. M. (2004) NMR structures of the C-terminal segment of surfactant protein B in detergent micelles and hexafluoro-2-propanol, *Biochemistry 43*, 15187-15194.
140. Oosterlaken-Dijksterhuis, M. A., Haagsman, H. P., van Golde, L. M., and Demel, R. A. (1991) Characterization of lipid insertion into monomolecular layers mediated by lung surfactant proteins SP-B and SP-C, *Biochemistry 30*, 10965-10971.
141. Johansson, J., Nilsson, G., Strömberg, R., Robertson, B., Jörnvall, H., and Curstedt, T. (1995) Secondary structure and biophysical activity of synthetic analogues of the pulmonary surfactant polypeptide SP-C, *Biochem J 307*, 535-541.
142. Nilsson, G., Gustafsson, M., Vandenbussche, G., Veldhuizen, E., Griffiths, W. J., Sjövall, J., Haagsman, H. P., Ruysschaert, J. M., Robertson, B., Curstedt, T., and Johansson, J. (1998) Synthetic peptide-containing surfactants - Evaluation of transmembrane versus amphipathic helices and surfactant protein C poly-valyl to poly-leucyl substitution, *Eur J Biochem255*, 116-124.
143. Palmblad, M., Johansson, J., Robertson, B., and Curstedt, T. (1999) Biophysical activity of an artificial surfactant containing an analogue of surfactant protein (SP)-C and native SP-B, *Biochem J 339*, 381-386.
144. Otsubo, E., Takei, T., and Nomura, M. (2001) Synthesis, purification and surface activities of the human pulmonary surfactant protein-C (SP-C) analogue, SP-CL16 (6-28), *Biol Pharm Bull 24*, 1362-1365.
145. Wu, C. W., Seurynck, S. L., Lee, K. Y., and Barron, A. E. (2003) Helical peptoid mimics of lung surfactant protein C, *Chem Biol 10*, 1057-1063.
146. Brown, N. J., Wu, C. W., Seurynck-Servoss, S. L., and Barron, A. E. (2008) Effects of hydrophobic helix length and side chain chemistry on biomimicry in peptoid analogues of SP-C, *Biochemistry 47*, 1808-1818.

147. Lukovic, D., Plasencia, I., Taberner, F. J., Salgado, J., Calvete, J. J., Perez-Gil, J., and Mingarro, I. (2006) Production and characterisation of recombinant forms of human pulmonary surfactant protein C (SP-C): Structure and surface activity, *Biochim Biophys Acta 1758*, 509-518.
148. Hawgood, S., Ogawa, A., Yukitake, K., Schlueter, M., Brown, C., White, T., Buckley, D., Lesikar, D., and Benson, B. (1996) Lung function in premature rabbits treated with recombinant human surfactant protein-C, *Am J Respir Crit Care Med 154*, 484-490.
149. Taut, F. J., Rippin, G., Schenk, P., Findlay, G., Wurst, W., Hafner, D., Lewis, J. F., Seeger, W., Gunther, A., and Spragg, R. G. (2008) A Search for subgroups of patients with ARDS who may benefit from surfactant replacement therapy: a pooled analysis of five studies with recombinant surfactant protein-C surfactant (Venticute), *Chest 134*, 724-732.
150. Davis, A. J., Jobe, A. H., Hafner, D., and Ikegami, M. (1998) Lung function in premature lambs and rabbits treated with a recombinant SP-C surfactant, *Am J Respir Crit Care Med 157*, 553-559.
151. Otsubo, E., and Takei, T. (2002) Effects of the human pulmonary surfactant protein-C (SP-C), SP-CL16(6-28) on surface activities of surfactants with various phospholipids, *Biol Pharm Bull 25*, 1303-1306.
152. Davis, A. J., Jobe, A. H., Hafner, D., Ikegami, M., and Michna, J. (1998) Lung function in premature lambs and rabbits treated with a recombinant SP-C surfactant: Positive end-expiratory pressure preserves surfactant function in preterm lambs, *Am J Respir Crit Care Med 157*, 553-559.
153. Cochrane, C. G., and Revak, S. D. (1991) Pulmonary surfactant protein B (SP-B): structure-function relationships, *Science 254*, 566-568.
154. Walther, F. J., Hernandez-Juviel, J., Bruni, R., and Waring, A. J. (1998) Protein composition of synthetic surfactant affects gas exchange in surfactant-deficient rats, *Pediatr Res 43*, 666-673.
155. Walther, F. J., Hernandez-Juviel, J., Bruni, R., and Waring, A. J. (1997) Spiking Survanta with synthetic surfactant peptides improves oxygenation in surfactant-deficient rats, *Am J Respir Crit Care Med 156*, 855-861.
156. Cochrane, C. G., Revak, S. D., Merritt, T. A., Heldt, G. P., Hallman, M., Cunningham, M. D., Easa, D., Pramanik, A., Edwards, D. K., and Alberts, M. S. (1996) The efficacy and safety of KL4-surfactant in preterm infants with respiratory distress syndrome, *Am J Respir Crit Care Med 153*, 404-410.
157. Revak, S. D., Merritt, T. A., Cochrane, C. G., Heldt, G. P., Alberts, M. S., Anderson, D. W., and Kheiter, A. (1996) Efficacy of synthetic peptide-containing surfactant in the treatment of respiratory distress syndrome in preterm infant rhesus monkeys, *Pediatr Res 39*, 715-724.
158. Nakahara, H., Lee, S., Sugihara, G., and Shibata, O. (2006) Mode of interaction of hydrophobic amphiphilic alpha-helical peptide/dipalmitoylphosphatidylcholine with phosphatidylglycerol or palmitic acid at the air-water interface, *Langmuir 22*, 5792-5803.
159. Nakahara, H., Lee, S., and Shibata, O. (2009) Pulmonary surfactant model systems catch the specific interaction of an amphiphilic peptide with anionic phospholipid, *Biophys J 96*, 1415-1429.
160. Tanaka, Y., Takei, T., Aiba, T., Masuda, K., Kiuchi, A., and Fujiwara, T. (1986) Development of synthetic lung surfactants, *J Lipid Res 27*, 475-485.
161. Seurynck-Servoss, S. L., Brown, N. J., Dohm, M. T., Wu, C. W., and Barron, A. E. (2007) Lipid composition greatly affects the in vitro surface activity of lung surfactant protein mimics, *Colloids Surf B Biointerfaces 57*, 37-55.

162. Stark, M., Wang, Y., Danielsson, O., Jornvall, H., and Johansson, J. (1998) Determination of proteins, phosphatidylethanolamine, and phosphatidylserine in organic solvent extracts of tissue material by analysis of phenylthiocarbamyl derivatives, *J Lipid Res 265*, 97-102.
163. Possmayer, F., Nag, K., Rodriguez, K., Qanbar, R., and Schurch, S. (2001) Surface activity in vitro: role of surfactant proteins, *Comp Biochem Physiol A Mol Integr Physiol 129*, 209-220.
164. Lal, M. K., and Sinha, S. K. (2008) Surfactant respiratory therapy using Surfaxin/sinapultide, *Ther Adv Respir Dis 2*, 339-344.
165. Martino, M. (2009). "FDA rejects Discovery Labs Surfaxin." from http://www.fiercebiotech.com/story/fda-rejects-discovery-labs-surfaxin/2009-04-20.
166. Spragg, R. G., Lewis, J. F., Walmrath, H. D., Johannigman, J., Bellingan, G., Laterre, P. F., Witte, M. C., Richards, G. A., Rippin, G., Rathgeb, F., Hafner, D., Taut, F. J., and Seeger, W. (2004) Effect of recombinant surfactant protein C-based surfactant on the acute respiratory distress syndrome, *N Engl J Med 351*, 884-892.
167. Kesecioglu, J., Beale, R., Stewart, T. E., Findlay, G. P., Rouby, J. J., Holzapfel, L., Bruins, P., Steenken, E. J., Jeppesen, O. K., and Lachmann, B. (2009) Exogenous natural surfactant for treatment of acute lung injury and the acute respiratory distress syndrome, *Am J Respir Crit Care Med 180*, 989-994.
168. Pérez-Gil, J., Cruz, A., and Casals, C. (1993) Solubility of hydrophobic surfactant proteins in organic solvent/water mixtures. Structural studies on SP-B and SP-C in aqueous organic solvents and lipids, *Biochim Biophys Acta 1168*, 261-270.
169. Curstedt, T., Jörnvall, H., Robertson, B., Bergman, T., and Berggren, P. (1987) Two hydrophobic low-molecular-mass protein fractions of pulmonary surfactant. Characterization and biophysical activity., *Eur J Biochem 168*, 255-262.
170. Enhörning, G. (1977) Pulsating bubble technique for evaluating pulmonary surfactant, *J Appl Physiol 43*, 198-203.
171. Schürch, S., Bachofen, H., Goerke, J., and Possmayer, F. (1989) A captive bubble method reproduces the in situ behavior of lung surfactant monolayers, *J Appl Physiol 67*, 2389-2396.
172. Greenfield, N., and Fasman, G. D. (1969) Computed circular dichroism spectra for the evaluation of protein conformation, *Biochemistry 8*, 4108-4116.
173. Johnson, W. C., Jr. (1990) Protein secondary structure and circular dichroism: a practical guide, *Proteins 7*, 205-214.
174. Goormaghtigh, E., Cabiaux, V., and Ruysschaert, J. M. (1994) Determination of soluble and membrane protein structure by Fourier transform infrared spectroscopy. II. Experimental aspects, side chain structure, and H/D exchange, *Subcell Biochem 23*, 363-403.
175. Scherle, W. (1970) A simple method for volumetry of organs in quantitative stereology, *Mikroskopie 26*, 57-60.