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Articles

Treatment of Respiratory Distress Syndrome with Single Recombinant Polypeptides that Combine Features of SP-B and SP-C

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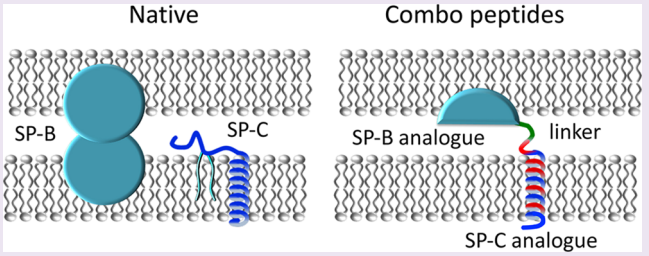
ABSTRACT: Treatment of respiratory distress syndrome (RDS) with surfactant replacement therapy in prematurely born infants was introduced more than 30 years ago; however, the surfactant preparations currently in clinical use are extracts from animal lungs. A synthetic surfactant that matches the currently used nature-derived surfactant preparations and can be produced in a cost-efficient manner would enable worldwide treatment of neonatal RDS and could also be tested against lung diseases in adults. The major challenge in developing fully functional synthetic surfactant preparations is to recapitulate the properties of the hydrophobic lung surfactant proteins B (SP-B) and SP-C. Here, we have designed single polypeptides that combine properties of SP-B and SP-C and produced them recombinantly using a novel solubility tag based on spider silk production. These Combo peptides mixed with phospholipids are as efficient as nature-derived surfactant preparations against neonatal RDS in premature rabbit fetuses.



INTRODUCTION

Pulmonary surfactant is essential for normal respiration by increasing lung compliance and preventing alveolar collapse and it also contributes to lung host defense. Natural surfactant is a complex mixture of about 80% phospholipids, 10% neutral lipids, and 10% proteins.[1] Unlike other phospholipid membranes, almost half of the phospholipids are fully saturated, and the main constituent is 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC). Surfactant contains four specific proteins: surfactant proteins A (SP-A), SP-B, SP-C, and SP-D. SP-A and SP-D are water-soluble collagenous lectins that play important roles in lung innate immunity,[2] while SP-B and SP-C are membrane-associated and hydrophobic proteins that require organic solvents for solubilization.[3] SP-B and SP-C promote phospholipid spreading to the alveolar air−liquid interface and thereby mediate a reduction in surface tension so that lung compliance is increased and alveolar collapse at the end-expiration is avoided.[4,5]

Premature infants with insufficient amounts of pulmonary surfactant develop respiratory distress syndrome (RDS).[6] This life-threatening disease is being treated since the 1980s by airway instillation of nature-derived surfactant preparations extracted from animal lungs,[7−9] which reduced the mortality and morbidity in neonatal RDS markedly.[10,11] However, there is still room for improvement since the development of synthetic surfactants that are similar to the nature-derived surfactants would be potentially beneficial, could reduce production costs, improve the batch-to-batch reproducibility, eliminate the risk of transmitting disease, and allow efficient large-scale production that is necessary if adults with RDS (ARDS) secondary to surfactant dysfunction or other diseases turn out to benefit from surfactant instillation.[11]

Nature-derived surfactant preparations are obtained by extractions and purification steps in organic solvents and thus essentially contain phospholipids and 1−2% (by weight) of SP-B and SP-C, while the hydrophilic SP-A and SP-D and neutral lipids are removed.[12] Simple phospholipid mixtures together with SP-B and SP-C, or analogues thereof, are capable of establishing lung gas volumes at end-expiration and tidal volumes that are similar to the ones obtained with the nature-derived surfactant poractant alfa in a premature rabbit model of RDS.[13] Importantly, synthetic surfactants that contain both SP-B and SP-C, or analogues thereof, are superior to surfactants with only one of these protein components. SP-B, or analogues thereof, is essential for establishing high lung gas volumes at the end-expiration in the absence of positive end-expiratory pressure (PEEP) during ventilation.[14,15]

SP-B and SP-C have several unusual features that make it difficult to produce them synthetically or by recombinant methods. SP-B contains seven Cys residues engaged in three intramolecular and one intermolecular disulfide bonds, the latter covalently linking the native 17 kDa SP-B homodimer.[16]

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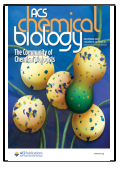

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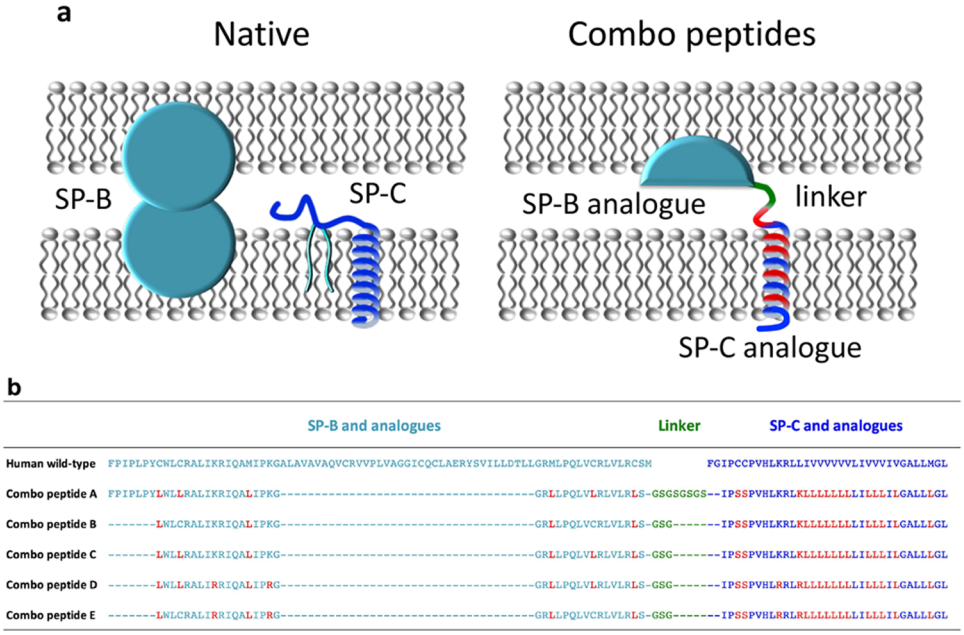

Figure 1. Hypothetical model and sequences of Combo peptides. (a) Schematic structures and hypothetical interactions of native SP-B and SP-C and Combo peptides with phospholipids. (b) Amino acid sequences of the Combo peptides using one-letter abbreviations. The positions that differ between the Combo peptides and the native human SP-B and SP-C are marked in red.

SP-B belongs to the SAPLIP (saposin-like) family of lipid-interacting proteins and is a peripheral membrane protein; however, in contrast to other SAPLIPs, it is not water-soluble.[17] No experimentally determined structure of SP-B is available, and several models of its structure have been put forward.[18−20] A common feature of such models is that the SP-B homodimer is able to join two phospholipid layers and thereby promote the transfer of phospholipids between vesicles in the subphase and a surface-active layer at the alveolar air−liquid interface.[20−22] SP-C is a 4 kDa lipopeptide with one or two (depending on species) palmitoylated Cys and features a unique transmembrane α-helix composed essentially of poly-Val.[23−25] Val strongly promotes β-strand conformation and biosynthesis of helical SP-C requires the presence of a chaperone domain in proSP-C, and mutations in this domain result in lethal amyloid lung disease.[26−29] In line with these observations, the chemical synthesis of SP-C results in the formation of insoluble β-sheet aggregates and amyloid-like fibrils.[29−31]

To overcome the complications to produce native SP-B and SP-C, attempts to design analogues that act as natural counterparts but are easier to produce have been made. Mini-B and Mini-BLeu are 34 residue peptides that correspond to the first and last predicted α-helices of the native 79-residue SP-B linked by a loop, while the predicted central helices of SP-B have been omitted. The helices of Mini-B and Mini-BLeu are linked by two intramolecular disulfides.[32,33] Adding the N-terminal seven residues of native SP-B to Mini-B, resulting in Super Mini-B, was hypothesized to facilitate its homodimerization and thereby promote surfactant adsorption and spreading.[19,34] The N-terminal segment of SP-B has also been reported to work as a membrane insertion sequence.[35] Super Mini-B mixed with phospholipids increases oxygenation and compliance in rat and rabbit models of ARDS.[19,35,36]

The SP-C analogues SP-C33 and SP-C33Leu have been developed by us to overcome the amyloidogenic nature of the native poly-Val sequence, with the main difference that the poly-Val region of native SP-C has been substituted by a nonamyloidogenic poly-Leu stretch (see Figure 1).[37] We recently found that SP-C33Leu can be efficiently produced recombinantly in *Escherichia coli* by an innovative approach based on the high solubility of the N-terminal domain of spider silk proteins (NT).[38,39] It struck us that the same approach might be used to produce novel surfactant protein analogues that combine properties of SP-B and SP-C into a single polypeptide chain, called Combo peptides. Here, we design and produce several Combo peptides and evaluate their effects on lung function combined with simple phospholipid mixtures (DPPC/egg yolk-phosphatidylcholine (PC)/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), 50:40:10 (by weight) and DPPC/POPG, 50:50 (by weight)) in a rabbit model of neonatal RDS in the absence of PEEP.

RESULTS AND DISCUSSION

Design, Production, and Analysis of Combo Peptides.

The Combo peptides were designed by fusing an SP-B analogue, a linker, and an SP-C analogue into one polypeptide (Figure 1a). The linkers used were either short, GSG, or long, $(GS)_4$ (Figure 1b). The SP-C33Leu analogue was included in all Combo peptides, since synthetic or recombinant SP-C33Leu can be used to formulate synthetic surfactants that are efficient in animal models of neonatal RDS as well as ARDS and can be produced in high amounts.[38,40−43] All Combo peptides have an SP-B analogue with two predicted α-helices with similarities to Mini-BLeu, either without (Combo peptides A, C, and D) or with two (Combo peptides B and E) Cys residues. Combo peptide A is the only peptide containing the first seven residues of native SP-B. In Combo peptides D and E, all Lys are replaced by Arg to avoid inadvertent palmitoylation of lysine side-chain amino groups during purification.[44]

Combo peptides A−E were produced recombinantly and purified by two chromatography steps over consecutive Lipidex-5000 columns (Figure 2a). The first chromatographic

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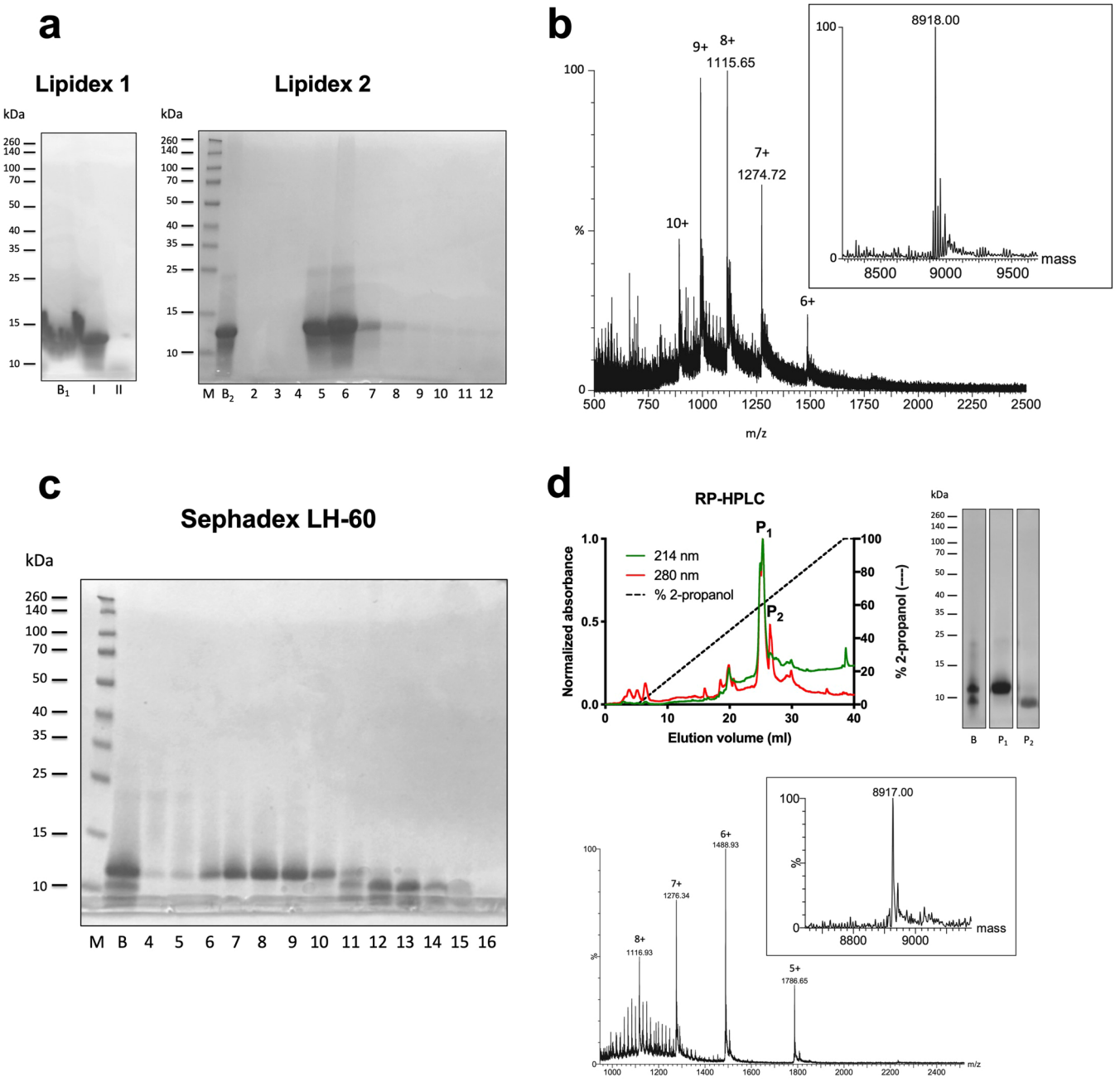

Figure 2. Purification and analysis of Combo peptides. (a) Two consecutive Lipidex chromatographic steps were used for purification. (b) Mass spectrum of the pooled Combo peptide A fractions obtained from the second Lipidex chromatography with the number of charges is indicated as well as the spectrum after deconvolution (inset). (c) Sephadex LH-60 chromatography of the Combo peptide E containing fraction obtained after Lipidex chromatography and identification with SDS-PAGE. (d) RP-HPLC of Combo peptide A eluted from the second Lipidex column. The peaks P_1 and P_2 were analyzed by SDS-PAGE and the peak P_1 by mass spectrometry as in panel (b).

separation was performed to reduce the amount of NaCl and to get rid of unpolar lipids in the sample. The first fraction collected from this column contained most of the eluted peptide as shown by SDS-PAGE, as well as polar lipids and corresponded to 18 ± 6 (mean ± SD, n = 12) % of the weight of the sample loaded. This peptide-containing fraction was applied to a second, longer Lipidex-5000 column for the separation of peptides from the lipids. The fractions containing peptides, which eluted before the lipids, were used to produce the synthetic surfactants containing Combo peptides A−E and correlated to 37 ± 13 (mean ± SD, n = 7) % of the mass applied to the column. The final purified Combo peptides comprised 4.7 ± 2.4 (mean ± SD, n = 7) % of the initial weight before the two Lipidex chromatography steps. Overall, the yields of purified Combos peptides A−E were between 0.3 and 0.7 mg per liter of bacterial shake-flask culture.

The Lipidex-purified peptides were analyzed by electrospray ionization mass spectrometry, analytical Sephadex LH-60 and reverse-phase (RP) chromatography, and N-terminal amino acid sequence analysis. The mass spectrum of Combo peptide A revealed a deconvoluted mass of 8,918 Da, which is similar to the calculated average mass of 8,914 Da (Figure 2b). Sephadex LH-60 chromatography of Combo peptide E (Figure 2c) and RP-HPLC of Combo peptide A (Figure 2d) show that

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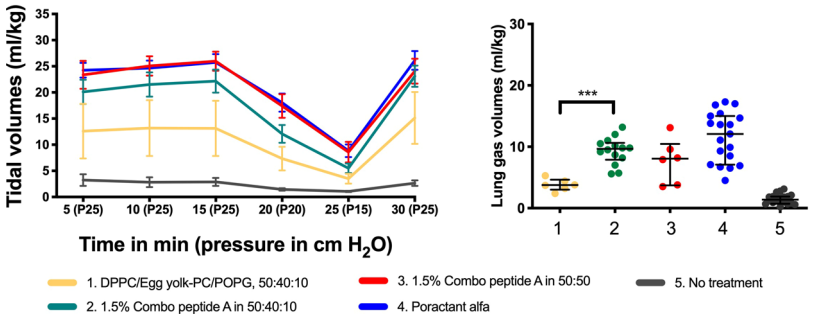

Figure 3. Activity of synthetic surfactants containing 1.5% of Combo peptide A in a rabbit model of neonatal RDS. Line graphs (left) show tidal volume mean ± SE during the 30 min ventilation period. Dot plots (right) present the lung gas volume median and interquartile ranges after 30 min of ventilation. Animals were treated with phospholipids only, Combo peptide A in DPPC/egg yolk-PC/POPG 50:40:10 (by wt) or in DPPC/POPG 50:50 (by wt), poractant alfa, phospholipids only, or untreated, as indicated under the graphs. ***$p \leq 0.001$.

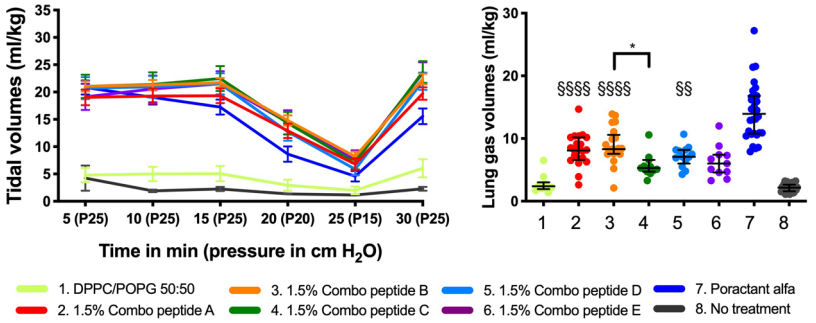

Figure 4. Activities of synthetic surfactants containing 1.5% of Combos A−E in a rabbit model of neonatal RDS. Line graphs (left) show tidal volume in mean ± SE during the 30 min ventilation period. Dot plots (right) present the lung gas volumes median and interquartile ranges after 30 min of ventilation. Animals were treated with DPPC/POPG 50:50 (wt/wt) only, 1.5% of Combo peptide A−E in DPPC/POPG 50:50 (by wt), poractant alfa, or untreated, as indicated under the graphs. *$p \leq 0.1$. §§$p \leq 0.01$, and §§§§$p \leq 0.0001$ versus phospholipids DPPC/POPG 50:50 (by wt) only.

the components that give rise to two bands observed by SDS-PAGE after Lipidex-5000 can be separated. Furthermore, mass spectrometry of the component migrating as the upper band of Combo peptide A gave a mass of 8,917 Da similar to its theoretical mass (Figure 2d).

An N-terminal sequence analysis of the upper SDS-PAGE band, migrating around 12 kDa, obtained after Sephadex LH-60 chromatography of Combo peptide E (Figure 2d), using Edman degradation for 10 cycles, gave the amino acid sequence LXLXRALIRR, where X denotes no identified residue. This is in good agreement with the N-terminal sequence of Combo peptide E (Figure 1b) considering that Trp2 and Cys4 are not detectable by Edman degradation due to oxidative destruction. The Edman degradation of the lower SDS-PAGE band migrating around 10 kDa, in contrast, did not yield any sequence, suggesting that it corresponds to nonprotein contaminants or an N-terminally blocked protein.

Activity in a Rabbit Model of Neonatal RDS. The purified Combo peptides were mixed with phospholipids to produce synthetic surfactant preparations that were tested in a rabbit model of neonatal RDS in the absence of PEEP. We compared the effects on tidal volumes and lung gas volumes of Combo peptide surfactant preparations, phospholipids only, poractant alfa, and untreated animals.

To first find a simple phospholipid composition, we used Combo peptide A (arbitrarily chosen as all Combo peptides have similar efficiencies, see below) to test its efficacy mixed with either DPPC/egg yolk-PC/POPG 50:40:10 (by wt) or DPPC/POPG 50:50 (by wt), the latter is a mixture used in other synthetic preparations.[11,33] There was no statistically significant ($p > 0.5$) difference between the results obtained with 1.5% Combo peptide A in DPPC/egg yolk-PC/POPG 50:40:10 and in DPPC/POPG 50:50 (Figure 3). Moreover, we observed that the instillation of only DPPC/egg yolk-PC/POPG 50:40:10 (Figure 3) or DPPC/POPG 50:50 (Figure 4) was not more effective than giving no treatment at all regarding lung gas volume but a positive effect on tidal volume could be obtained depending on phospholipid composition (Figures 3 and 4). Thus, in the further experiments with the Combo peptides A−E, we used the simplest phospholipid mixture with only two components, which is superior compared to more complex mixtures from a regulatory point of view.

The surfactants containing 1.5% of Combo peptide A, B, or D all gave higher lung gas volumes than phospholipids alone ($p < 0.0001$, $p < 0.0001$, and $p = 0.0048$, respectively) (Figure 4). All of the synthetic surfactants containing Combo peptides gave higher tidal volumes than phospholipids alone during the experiment, except 1.5% Combo peptide D and E at 25 min. All of the surfactants containing 1.5% Combo peptides gave lower lung gas volumes, but not lower tidal volumes, than poractant alfa. We observed no differences among the tidal volumes and lung gas volumes obtained with Combo peptides except that the lung gas volumes for Combo peptide B were higher than that for Combo peptide C. From these experiments, it was not possible to conclude that any of the types of peptides (presence of N-terminal seven residues, or presence of Cys and/or Lys) represented by Combo peptides A−E were superior to the other types.

It has been shown that 2−3% of synthetic surfactant protein analogues appear necessary for optimal activity.[15,45] We

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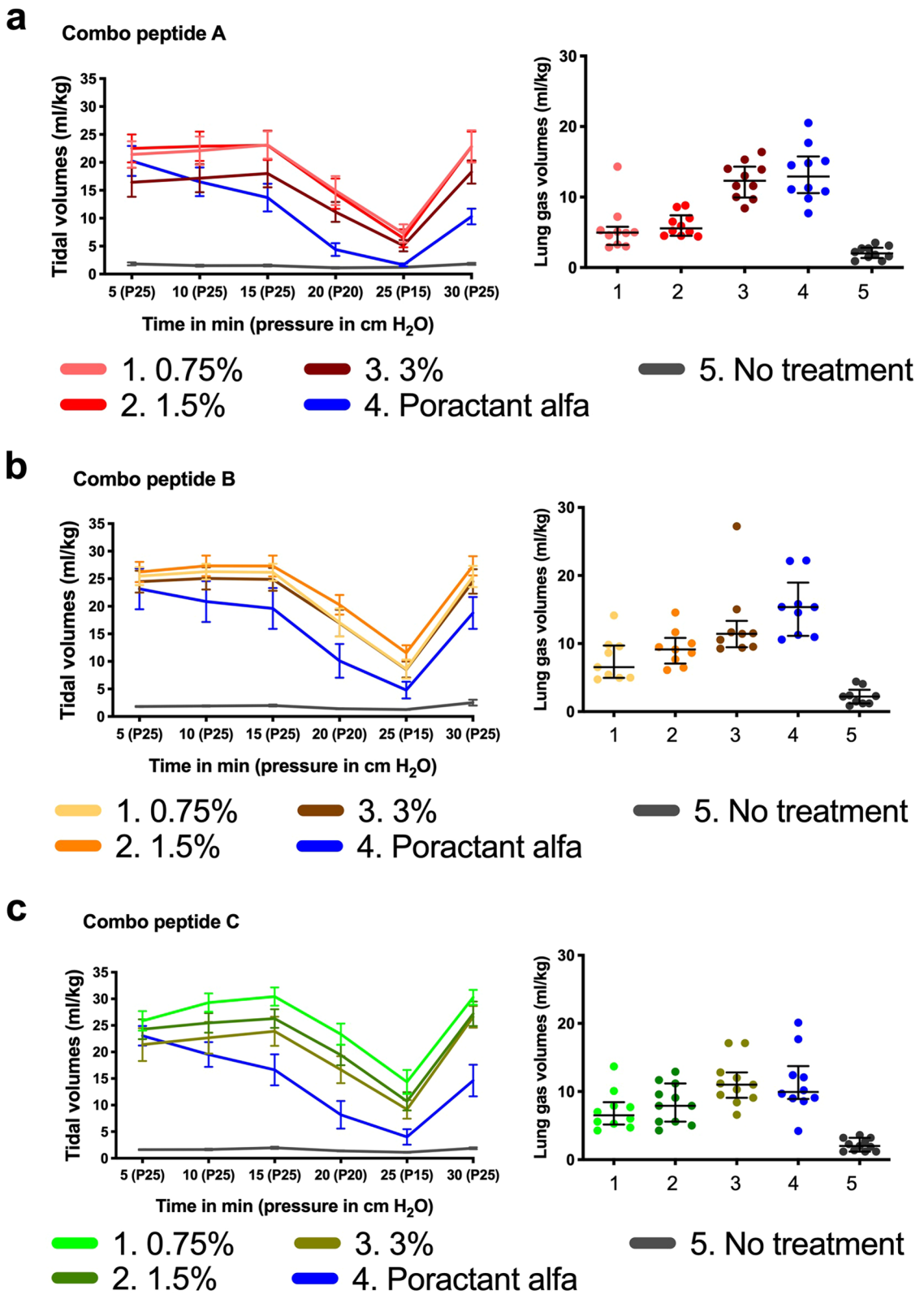

Figure 5. Combo peptide concentration effects on the efficacy in a rabbit model of neonatal RDS. Line graphs (left) show tidal volume mean ± SE during the 30 min ventilation period. Dot plots (right) present the lung gas volumes median and interquartile ranges after 30 min of ventilation. Animals were treated with 0.75, 1.5, or 3% (by wt) of Combo peptide A (a), B (b), or C (c), respectively, in DPPC/POPG 50:50 (by wt), poractant alfa, or untreated, as indicated under the graphs.

studied the dose dependency of each Combo peptide in DPPC/POPG 50:50 from 0.75 to 3% (by wt) (Figures 5 and 6). We observed a concentration dependency of the effect on lung gas volumes, and all of the synthetic surfactants gave higher lung gas volumes and tidal volumes than the untreated controls except for the tidal volumes obtained by 1.5% Combo

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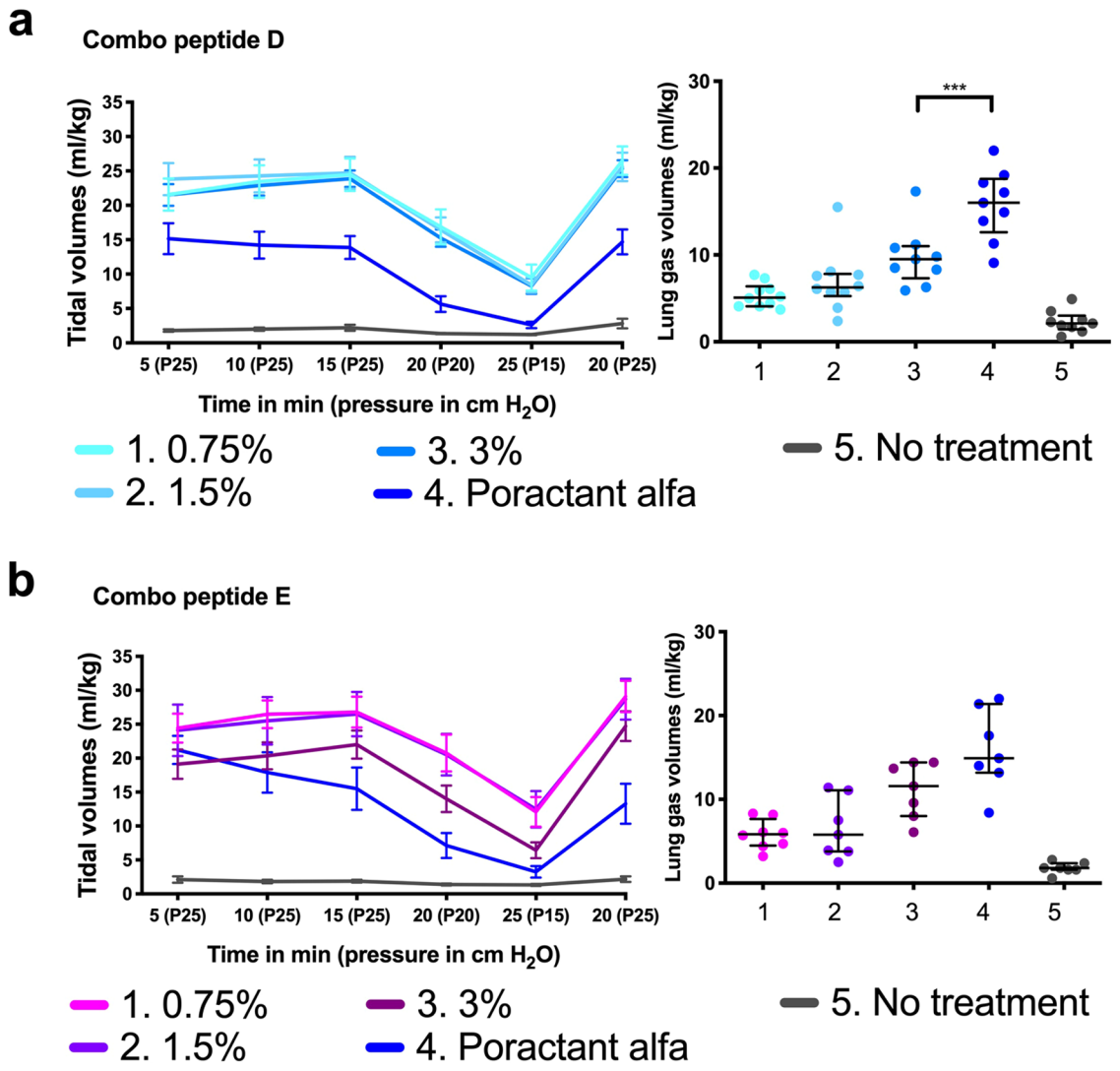

Figure 6. Combo peptide concentration effects on the efficacy in a rabbit model of neonatal RDS. Line graphs (left) show tidal volume mean ± SE during the 30 min ventilation period. Dot plots (right) present the lung gas volumes median and interquartile ranges after 30 min of ventilation. Animals were treated with 0.75, 1.5, or 3% (by wt) of Combo peptide D (a) or E (b) in DPPC/POPG 50:50 (by wt), poractant alfa, or untreated, as indicated under the graphs. ***$p \leq 0.001$.

peptide A at 25 min. Importantly, there were no statistical differences between the lung gas volumes after treatment with pulmonary surfactants containing 3% of any Combo peptide and poractant alfa, except for 3% Combo peptide D. All of the other surfactants containing 3% Combo peptide gave lung gas volumes equal or higher than 11 mL/kg. We recognized a trend where surfactants containing higher Combo peptide concentrations gave lower tidal volumes, but they were still higher than the tidal volumes for the poractantalfa-treated animals.

The animal model used herein employs constant pressure ventilation and no PEEP and is therefore suitable for screening different surfactant preparations. Treatment with fully active surfactant preparations gives tidal volumes well above the physiological range of 6−8 mL/kg, while untreated controls only reach tidal volumes of a few mL/kg.[46] Mature litters are thus easily identified since they have large tidal volumes even in the absence of treatment. Herein, the occurrence of untreated control animals with tidal volumes >5.5 mg/kg resulted in the whole litter being excluded from the study. Since no PEEP is used, the ability of the surfactant preparations to stabilize the alveoli at the end of expiration can be assessed in a robust manner.

In the absence of lung-stabilizing ventilation using PEEP, synthetic surfactants require 2% each of SP-B and SP-C analogues to achieve equal improvements in lung gas volumes as poractant alfa, which contains 0.7−0.8% each of SP-B and SP-C.[13,15] Three percent of Combo peptides A, B, C, or E mixed with only two synthetic phospholipids give as high lung gas volumes as poractant alfa (Figures 5 and 6), while synthetic surfactants that contain only an SP-B, or SP-C, analogue are inferior to poractant alfa in the absence of PEEP during ventilation.[14,15] Previously developed synthetic surfactants, which have not reached clinical use or are no longer available for clinical use, contained one peptide analogue only,[47] while CHF5633 that is currently in clinical development contains 1.5% SP-C analogue but only 0.2% SP-B analogue.[33,48,49] The analogue SP-C33Leu, which contributes the SP-C part in the Combo peptides, can be made by organic synthesis[33,37] but is also efficiently produced in *E. coli*.[38] In contrast, no recombinant production method for any SP-B analogue has so far been developed, leaving chemical synthesis as the only

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alternative, which is challenging and expensive. In particular, longer SP-B-derived peptides with several disulfide bridges are difficult to synthesize,[50] making Cys-free SP-B analogues attractive alternatives.[51] This has hampered the development of fully functional synthetic surfactants. The results presented herein show that Combo peptides afford a solution to the obstacles associated with the generation of SP-B analogues that can be produced recombinantly.

The Combo peptide approach simply combines SP-B and SP-C features into one single polypeptide chain that, like SP-C33Leu,[38] can be produced recombinantly in *E. coli*. Our approach to producing the Combo peptides employ the N-terminal domain (NT) from spider silk proteins (spidroins) as a solubility tag. In spiders, NT allows storage of spidroins at very high concentrations without aggregation;[39] and with this insight, we designed a mutant (NT*) that enables recombinant production of several aggregation-prone proteins, even at gram per liter yields.[38,52−56] Combo peptides A−E were produced recombinantly as fusion proteins to NT*, showing that Combo peptides, despite their length and marked hydrophobicity, are straightforward to produce.

Treatment of neonatal RDS with a nature-derived surfactant instillation is a very successful medical intervention but is currently only available to a fraction of all patients who need it.[11] Moreover, it has not yet been possible to fully evaluate whether treatment of ARDS, or acute lung injury, with an exogenous surfactant, or the use of surfactant as a drug carrier, is clinically viable. The main reasons for these shortcomings are that the nature-derived surfactant preparations are available in limited amounts and expensive to produce, while synthetic surfactants so far have been functionally inferior to the nature-derived counterparts, as evidenced by their inabilities to establish high lung gas volumes in the absence of an applied PEEP during ventilation.[13,15] Synthetic surfactants made from our designed recombinant Combo peptides are equally active as most used nature-derived surfactant for RDS, contain only three defined chemical components and can be produced in a cost-efficient manner. We believe that these features may allow Combo peptide-based surfactants to be further developed for the treatment of neonatal RDS and potentially additional lung diseases.

METHODS

Expression and Purification of Combo Peptides. Five Combo peptides were designed (Figure 1). The Combo peptides A−E (Figure 1) were expressed as fusion proteins with an N-terminal hexaHis-tag and a solubility tag (FlSpNT* from spider silk proteins,[53]). The genes coding for the fusion proteins (His_6-FlSpNT*-Met-Combo peptides A-E) were ligated into pT7 vectors and transformed into competent *E. coli* BL21(DE3)pLysS cells.[38] The fusion proteins were expressed at 20 °C for 17 h after induction at OD ≈ 0.9 with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were then collected by centrifugation and resuspended in 30 mL/L of bacterial culture 20 mM tris(hydroxymethyl)-ammoniummethane-HCl (Tris), pH 8.

The cells were disrupted by sonication using 85% amplitude, 1s on, 1s off for 4 min. After centrifugation at 30000 x *g* for 30 min at 4 °C, the soluble fraction was collected and stored at −20 °C. The thawed mixture was centrifuged at 25000 x *g* for 30 min at 4 °C and the pellet was collected and resuspended in 20 mM Tris pH 8 by mild sonication (60% amplitude, 1s on, 1s off during 3 min). The suspension was incubated overnight in 0.1 M HCl and 50 mM of cyanogen bromide (CNBr) at room temperature to cleave off the His_6 and solubility tag and the target Combo peptides were precipitated by centrifugation at 15000 x *g* for 30 min. The pellet was air-dried and weighed. Thereafter, 10 mg of the pellet was dissolved in 0.8 mL of methanol and 0.2 mL of 1 M KOH and after incubation at 40 °C for 60 min, the alkaline-treated samples were mixed with 1.6 mL of chloroform and 0.4 mL of H_2O, thereby creating a two-phase system. The mixture was centrifuged at 2000 x *g* for 5 min, the upper polar phase was discarded, and 1 mL of methanol/0.2 M KOH (1:1, by volume) was added to perform another two-phase separation. After centrifugation and discarding of the upper phase, this procedure was repeated once more. After centrifugation at 2000 x *g* for 5 min and discarding the upper phase, the lower organic phase was collected and mixed with methanol. A small amount of 12 M HCl was added to the sample to reduce the pH below 2. Then, the solvent mixture was gently evaporated under reduced pressure, and the product was resuspended in chloroform/methanol, 1:1 (by v), and centrifuged for 15 min at 3500 x *g*. The supernatant was collected and evaporated under reduced pressure and the dried sample was resuspended in methanol/ethylene chloride/0.1 M HCl, 85:10:5 (by v). The peptides were isolated by two consecutive Lipidex-5000 chromatography steps. The solvent system of both columns was methanol/ethylene chloride/0.1 HCl, 85:10:5 (by v). The sizes of the first and second columns were 8 × 2.5 cm and 45 × 1.1 cm, respectively. The dry weight of eluted fractions of both columns was calculated and after the second column chromatography, fractions containing Combo peptides were identified by SDS-PAGE, pooled, and used to prepare the synthetic surfactant mixtures. These fractions were also further analyzed by Sephadex LH-60 chromatography, reversed-phase (RP)-HPLC, N-terminal amino acid sequence analysis, and mass spectrometry.

Sephadex LH-60. A Sephadex LH-60 column of 45 × 1.06 cm was equilibrated with chloroform/methanol/0.1 HCl, 19:19:2 (by v), and the sample was loaded using the same solvent.[3] The fractions eluting were analyzed by SDS-PAGE and their dry weight was calculated.

RP-HPLC. RP-HPLC was performed using a Kromasil 100−5C18 250 × 4.6 mm column (AkzoNobel, Amsterdam, NL) and an ÄKTA pure chromatography system (GE Healthcare, Chicago, Illinois). The mobile phase was based on the solvent systems A (40% aqueous ethanol containing 0.1% trifluoroacetic acid (TFA)) and B (isopropanol/0.1% TFA). The column was equilibrated with solvent A, and after injection of the sample diluted in solvent A, the peptides were eluted with a linear gradient of solvent B. The eluted peptide was analyzed by SDS-PAGE and mass spectrometry.

Mass Spectrometry. Samples were directly infused into a Waters LCT ToF mass spectrometer equipped with an off-line nanospray source (MS Vision, Almere, NL) using coated borosilicate capillaries (Thermo Fisher Scientific). We found that the addition of formic acid to a final concentration of 5% greatly increased spectral quality. The capillary voltage was 1.5 kV, the source temperature was 80 °C, and the cone voltage was 200 V. The source pressure was maintained at 0.4 mbar. Spectra were acquired between 500 and 5000 m/z and analyzed using MassLynx 4.1 software (Waters Corp, Massachusetts).

N-Terminal Amino Acid Sequence Analysis. The samples obtained by Sephadex LH-60 chromatography and SDS-PAGE were transferred to polyvinyline difluoride (PVDF) membranes using a wet transfer system for 2 h at 200 mA. Coomassie-stained bands were cut out and analyzed by Edman degradation (Alphalyse A/S, Odense, DK).

Synthetic Surfactant Preparations. Synthetic surfactant preparations containing 0% (phospholipid only controls), 0.75, 1.5, or 3% (by wt) of Combo peptides in DPPC/POPG (Sigma-Aldrich, St. Louis, Missouri), 50:50 (by wt), or DPPC/egg yolk-PC/POPG, 50:40:10 (by wt) were prepared by mixing the phospholipids and the respective peptide in chloroform/methanol, 2:1 (by v), gently evaporating the solvents under reduced pressure, and finally resuspending the Combo peptide/phospholipid mixtures in physiological saline at 80 mg/mL of phospholipids.

Animal Experiments. New Zealand white rabbits at 27 gestational days (term 31 days) were delivered by caesarian section and anesthetized at birth with 2 μL/g body weight of Ketaminol 50 mg/mL/Domitor 1 mg/mL/physiological saline 4:1:15 (by v). After tracheostomy, the animals received 2.5 mL/kg body weight of

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poractant alfa, one of the synthetic Combo peptide surfactants, phospholipids only, or no treatment. The animals were placed in small plethysmograph chambers, connected to a ventilator, and ventilated without PEEP, with 21% oxygen, at a frequency of 40 breaths/min and an inspiration/expiration ratio of 1:1. The animals are ventilated with air since high oxygen levels correlate with higher morbidity and mortality.[57] After all the animals were placed in the ventilator, their lungs were opened with a peak inspiratory pressure (PIP) of 35 cm H_2O for 1 min, followed by 15 min at 25 cm H_2O, 5 min at 20 cm H_2O, 5 min at 15 cm H_2O, and finally 5 min at 25 cm H_2O. The animals were then ventilated for another 5 min at 25 cm H_2O with 100% N_2. The tidal volumes and the compliances were recorded every 5 min during the ventilation period. At the end of the ventilation, the animals were sacrificed and the lungs were removed and weighed. The lung gas volumes were calculated.[58] The entire experiment was excluded if the tidal volumes of the nontreated controls were more than 5.5 mL/kg body weight after 5 min of ventilation at 25 cm H_2O. The statistical analysis was performed using two-way ANOVA for the tidal volumes and one-way ANOVA for the lung gas volumes.

Ethical Permit. The animal studies were performed in accordance with ethical permits authorized by Stockholms Norra Djurförsöksetiska Nämnd (N275/09, N174/14, and 7308-2019).

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

SP(surfactant protein); RDS(respiratory distress syndrome); PEEP(positive end-expiratory pressure); DPPC(1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine); SAPLIP(Saposin-like protein); PC(phosphatidylcholine); POPG(1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol); SDS-PAGE(sodium dodecyl sulfate-polyacrylamide gel electrophoresis); CNBr(cyanogen bromide); NT(N-terminal domain); RP-HPLC(reversed-phase high-performance liquid chromatography); PIP(peak inspiratory pressure)

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