Mixtures of Low Molecular Weight Surfactant Proteins and Dipalmitoyl Phosphatidylcholine Duplicate Effects of Pulmonary Surfactant *in Vitro* and *in Vivo*

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ABSTRACT. Pulmonary surfactant proteins SP-B and SP-C were isolated from lavage fluids of bovine lungs and recombined (lipid/proteins, 9/1, wt/wt) with dipalmitoyl phosphatidylcholine for testing *in vitro* and in surfactant-deficient adult rats. Using a pulsating bubble surfactometer, we found that inflation pressures of bubbles at minimum radii in these mixtures were 0.34 ± 0.05 cm H₂O ($\pm$ SD, $n = 24$) after 1 min. These values were not affected by increasing amounts of surfactant protein relative to dipalmitoyl phosphatidylcholine (DPPC). Minimum inflation pressures were similar to those of modified bovine surfactant, surfactant Tokyo Akita (TA) (0.33 ± 0.05 cm H₂O, $n = 7$). *In vivo* testing was carried out in adult rats made surfactant deficient by repeated lavage and ventilated with 100% oxygen. Rats received tracheal instillations of either air, DPPC, DPPC/SP-B,C (9:1), or surfactant TA at 50 mg/kg body weight. Surfactant TA and DPPC/SP-B, SP-C mixtures resulted in similar immediate and sustained improvements in arterial oxygenation (308 ± 66 torr, $n = 10$ and 312 ± 101 torr, $n = 6$ at 30 min posttreatment) that were significantly greater than those of sham (76 ± 24 torr, $n = 17$) and DPPC-treated rats (64 ± 32 torr, $n = 7$). Rats treated with either DPPC/SP-B,C mixtures or surfactant TA showed similar postmortem static lung compliances (2.3 ± 0.8 ml/cm H₂O/kg, $n = 8$ and 1.9 ± 0.4 ml/cm H₂O/kg, $n = 5$, respectively) that were significantly larger than sham (1.3 ± 0.3 ml/cm H₂O/kg, $n = 14$) and DPPC-treated rats (1.2 ± 0.2 ml/cm H₂O/kg, $n = 6$). We conclude that simple mixtures of DPPC and SP-B,C duplicate results found with more complex mixtures of pulmonary surfactants. (*Pediatr Res* 23: 484-490, 1988)

SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis

Many recent reports emphasized the functional importance, both *in vitro* and *in vivo*, of several pulmonary surfactant proteins with molecular weights that range between 5-26 kDa (1-11). These proteins are markedly hydrophobic (1, 5, 7-9, 13-23), coisolate with lipids in commonly used organic solvents (1, 4-8, 11, 13, 15-17, 20) and may represent monomeric and oligomeric forms of two distinct peptides—SP-B and SP-C (12, 16)—that are derived through proteolytic processing of larger precursor proteins (1, 21-24). Various reports indicate that SP-B and SP-C, together or separately, augment phospholipid recycling in cell cultures (13, 14, 19), enhance absorption (1, 5-8, 10), surface spreading and dynamic respreading (6, 7, 9, 10), and surface tension-lowering properties of synthetic phospholipid mixtures or protein-free lipid extracts of natural surfactant *in vitro* (3-11). SP-B and SP-C have also been reported to enhance the abilities of defined lipid mixtures to improve pulmonary compliance in the lungs of premature rabbits (2, 4, 6, 7, 9). Many of these studies have only been published recently in preliminary form, and it remains unclear whether good separation techniques are readily available for separation of these two surfactant proteins, one from the other.

We have extended these studies by isolating SP-B,C from bovine lung lavage, combining these proteolipids with DPPC and testing the efficacy of these mixtures *in vitro* and in surfactant-deficient adult rats. Comparisons were made with DPPC alone and with a modified bovine surfactant used clinically for treatment of respiratory distress syndrome (surfactant TA, Tokyo Tanabe, Co., Tokyo).

Abbreviations

SP-A, relatively hydrophilic 30-35 kDa surfactant protein.
SP-B,C, hydrophobic surfactant proteins with m.w. ranging between 5,000 to 26,000 Da.
DPPC, dipalmitoyl phosphatidylcholine.
TA surfactant, Tokyo Akita surfactant, Tokyo Tanabe Company, Tokyo, Japan.

MATERIALS AND METHODS

Isolation of proteins. SP-B,C were isolated from bronchoalveolar lavage of excised lungs from freshly sacrificed calves. Each lung was lavaged four to six times with approximately 0.3 liters of 0.15 M NaCl (4° C) that was poured intratracheally and removed by gravity flow with gentle massaging. Cells and debris were removed by centrifugation for 10 min at 500 or 1000 $\times$ g (4° C). The resulting supernatant was centrifuged at 20,000 $\times$ g for 1 h (4° C). The pelleted surfactant complex was extracted overnight three to four times with stirring in chloroform/methanol (2:1, vol/vol; 3 mg phospholipid/ml). Insoluble material was removed by centrifugation at 10,000 $\times$ g for 30 min (4° C).

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The supernatant from the final extraction was concentrated by partial evaporation of the solvent under nitrogen, then applied to either an analytic column (1 × 50 cm) or to a preparative column (2.5 × 100 cm) of Sephadex LH-20 that had been equilibrated in chloroform/methanol (2:1, vol/vol). Loads of approximately 35 mg/0.5 ml or 400 mg/4 ml were applied to the columns, respectively. Elution with the same solvent was carried out at a flow rate of 0.34 ml/min (20° C). The effluent was monitored at 280 nm. Two-min fractions were collected and analyzed for protein by the methods of Lowry as modified by Peterson (26) or by amino acid analysis and by one-dimensional, SDS-PAGE (27). The fractions containing protein from each chromatograph were collected and pooled with similar fractions from successive runs. The other lipid-rich fractions containing protein were also collected and rerun on the column.

Characterization of proteins. PAGE with SDS was carried out as previously described (15, 27, 28) using 15% acrylamide and 1.5 mm-thick slab gels with 3.5% acrylamide stacking gels. The gels were stained with silver stain kits from N.E. Nuclear (Boston, MA). Molecular weight markers included myoglobins: III (2.5 kDa), II (6.2 kDa), I (8.2 kDa), I and II (14.4 kDa) from LKB Laboratories (Paramus, NJ) and lactalbumin (14.2 kDa), trypsin inhibitor (20.0 kDa), trypsinogen (24.0 kDa), carbonic anhydrase (29.0 kDa), and glyceraldehyde-3 phosphate dehydrogenase (36.0 kDa), albumin (egg) (45.0 kDa), and albumin (bovine) (66.0 kDa) from Sigma Chemical Co. (St. Louis, MO). Electrophoretic transfer and immunostaining of the proteins was done according to the methods of Burnette (29) as modified by Phelps *et al.* (28) using an immunoblotting apparatus (Bio-Rad, Richmond, CA) and nitrocellulose paper (Schleicher Schuell, Keene, NH) with a pore size of 0.05 μ m. We used polyclonal rabbit antisera raised against surfactant TA proteins as previously described (34) to identify SP-B,C. We also used polyclonal rabbit antisera to the hydrophilic surfactant protein, SP-A, partially purified from pulmonary lavage of human patients with alveolar proteinosis (28, 30).

Amino acid composition was determined from protein-rich LH-20 fractions that were hydrolyzed in 6 N HCl at 110° C for 24 h. Free amino acid composition was determined with a Beckman 6300 Amino Acid analyzer. The quantity of protein used in recombinant studies was determined either by amino acid analysis or by Lowry assay with values normalized to amino acid equivalents.

Lipid analysis. Measurements of phosphorus in crude surfactant, organic solvent soluble extracts, and Sephadex LH-20 eluates were carried out after Folch extraction (31) and quantified by the methods of Weinhold and Villet (32). Samples of LH-20 eluates for lipid analysis were dried under nitrogen and redissolved in small volumes of chloroform/methanol (2:1, vol/vol). The lipid components were separated by thin-layer chromatography on silica gel by using chloroform/methanol/2-propanol/water/triethylamine (30:9:25:7:25, by volume) (33). After chromatography, solvents were removed by brief air drying followed by evaporation in an evacuated chamber for approximately 2 h. The plates were then sprayed with 70% sulfuric acid and charred overnight at 180° C.

Analysis of surface activity. The protein rich fractions in chloroform/methanol (2:1, vol/vol) from column chromatography were mixed with synthetic DPPC (99 + % purity, Sigma) in chloroform/methanol (2:1, vol/vol) to achieve ratios ranging between 100:1 and 5:1 (wt/wt) of DPPC to protein. All suspensions except surfactant TA were briefly vortexed, dried under nitrogen, dispersed in distilled water with brief vortexing, frozen (−70° C), lyophilized, and stored at −20° C until use. Surfactant TA was the generous gift of Tokyo Tanabe Co. Its composition has been described by us and others (34–36). It was stored as a lyophilized powder at −70° C until use. All mixtures were dispersed in 0.15 M saline with sonication at 20% output (Branson Sonifier, model 185 with microtip) for three, 3-s bursts to a final

concentration of 2.0 mg/ml. The dispersions were tested in a pulsating bubble surfactometer (37). This instrument measures inflation pressures for a bubble in communication with ambient air in a surfactant suspension at 37° C. The bubble is made to pulsate at 20 cycles/min between minimum and maximum radii of 0.40 and 0.55 mm. We monitored inflation pressures for a 5-minute period after bubble creation.

In vivo testing. We adopted, with modification, the methods of Lachman *et al.* (38) and Tausch *et al.* (34) for exogenous surfactant treatment of rats made surfactant deficient by multiple lavage. Adult, male Sprague-Dawley white rats weighing 0.355 ± 0.062 kg were anesthetized with an intraperitoneal injection of pentobarbital (35 mg/kg) followed 20 min later by a subcutaneous injection of ketamine (80 mg/kg). A 14-gauge catheter was inserted into the trachea and tied (Fig. 1). The left common carotid artery was then ligated, and a 22-gauge catheter was inserted proximal to the ligation into the artery for use in blood gas sampling, pressure monitoring, and fluid and drug administration. The animal was paralyzed with pancuronium bromide (0.1 mg/kg) given intraarterially. Anesthesia was maintained with hourly injection of ketamine (12 mg/kg) and injections of pancuronium (0.08 mg/kg) every 30 min and when paralysis declined. Body temperature was maintained using a heating pad (Delta Phase, Braintree Scientific, Braintree, MA) placed under the animal and with an overhead heat lamp. Physiologic saline with heparin (1000:1) was infused into the arterial catheter at 1.0 ml/h. Blood removed for analysis was returned and saline was injected periodically to replace blood volume that was lost.

Ventilation was maintained with oxygen (100%) at flow rates of 0.75 l/min (6.2 ml stroke volume) using a time-cycled ventilator (34) or by means of a volume-controlled ventilator (Harvard Apparatus Co., South Natick, MA, model 683) using stroke volumes of 7.2 ml/kg. Ventilator rates were adjusted to maintain the pCO_2 between 25 and 55 torr, our goal being 40–45 torr.

Arterial blood gases were obtained immediately before paral-

Fig. 1. Schematic of the experimental set-up used for *in vivo* testing of surfactant.

ysis and artificial ventilation, 5 min after ventilation, and at 10-min intervals until blood gas concentrations were stable. Thirty min later the animals were given several sigh breaths ($2 \times V_t$) and lavaged four times by 20 ml/kg instillation and aspirations of saline (37°C). Additional lavages were performed if arterial paO_2 remained more than 120 torr after the first four lavages. Sigh breaths were given before each lavage and after the last lavage. After the last lavage, positive end expiratory pressure of 4 cm H_2O was applied and maintained for the duration of the experiment. The animals received instillations of either air (sham treatment), DPPC, surfactant TA, or DPPC/protein (9:1) after 30 min of stable blood gases.

The surfactants were prepared at a concentration of either 30 or 15 mg/ml 0.15 M saline and dispersed by brief sonication. Dilution to 15 mg/ml saline was done to reduce the viscosity of the DPPC and DPPC/protein mixtures (9, 39). Surfactant TA, DPPC/protein, and DPPC-treated animals received dosages of 50 mg/kg. These instillations were carried out over a 5-min period in quarter doses with rats positioned supine, prone, and on either side to distribute the instillate. Sigh breaths were given after each quarter dose. Blood gases were obtained 5 min after surfactant administration and at 30-min intervals for up to 2 1/2 h.

At the termination of the experiment (approximately 2 h after instillation) the trachea was clamped and the lungs degassed (40). Immediately after death, lung-thorax closed chest compliance was measured at one-half full inflation (8 ml) (41) and normalized to body weight.

Statistical analyses. Evaluation of statistical significance between groups was carried out by one-way analysis of variance with $p < 0.01$ accepted as significant.

RESULTS

Protein isolation. The chloroform/methanol soluble, bovine lung surfactant lipid extract contained approximately 1% protein (by Lowry assay) relative to phospholipids by weight. Figure 2 illustrates the elution patterns of the proteins and phospholipids present in the lipid extracts after Sephadex LH-20 liquid chromatography. The proteins appeared slightly earlier than the main lipid peak that contained primarily phosphatidylcholine (fractions 11–15), sphingomyelin (fractions 11–15), and phosphatidylethanolamine (fractions 15–19) (Fig. 3). The more acidic phospholipids: phosphatidylserine (fraction number 30), phosphatidylinositol (fraction 57), and phosphatidylglycerol (fraction numbers 57 and 70) eluted later. In two pooled samples of protein-rich fractions, some lipid phosphorus was present giving an estimated phospholipid to protein weight (wt/wt) ratio of 2.4 (assuming phosphorus as 4% of phospholipid weight). Thin-layer chromatography of one of these samples showed that the principal lipid was phosphatidylcholine and there was some streaking of lipid through the remainder of the plate. Some charring occurred near the solvent front indicating the possibility that neutral lipids may have been present in the protein-rich fractions.

In the protein-rich fractions from Sephadex LH-20 chromatography, three major bands at 6, 14, and 18–26 kDa could be identified under nonreducing conditions by SDS-PAGE (Fig. 2). When 2-mercaptoethanol was added to samples before electrophoresis, we observed almost no 18- to 26-kDa band and increased density in the 5- to 6-kDa and 14-kDa bands. When smaller amounts of protein were used for SDS-PAGE, bands only at 18–26 kDa could be seen under nonreducing conditions and at 5–6 kDa under reducing conditions. In other work, we

Fig. 2. Elution profile of the proteins and phospholipid in Sephadex LH-20 eluates of lipid extract of whole bovine lung surfactant. Chloroform/methanol (2:1: vol/vol) was used as solvent and mobile phase. Inset are electrophoretic patterns of the eluting proteins with (lane 1) and without the presence of a reducing agent (lane 2). Surfactant proteolipids appear in fractions 8–15 and the major surfactant lipids appear in fractions 11–23. The ordinate indicates μg of either phosphorous or protein.

Fig. 3. Absorbance profile for lipid extracts of whole lung surfactant after chromatography using Sephadex LH-20. Fraction numbers are the same as in Figure 2. Superimposed according to fraction number are results from thin-layer chromatography of selected fractions. The migration patterns for the various lipids are given at right. The ordinate indicates absorbance at 280 nm. SPH, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; FA, free fatty acids; TAG, triacylglycerols. The lipid most closely associated with the hydrophobic surfactant proteins consistently appears to be phosphatidylcholine.

have determined that the unreduced band at 18–26 kDa contains SP-B and SP-C, and the unreduced bands at 5 and 14 kDa are SP-C (16). These proteins were immunoreactive with antibodies to surfactant TA. SP-A was not detected in liquid chromatography fractions by SDS-PAGE with silver staining or immunoblot analysis.

Pulsating bubble surfactometer. Suspensions of DPPC alone showed poor adsorption to the air-liquid interface (Fig. 4) and were ineffective at reducing the inflating pressures of the bubbles in the pulsating bubble surfactometer up to 5 min after bubble creation (Fig. 5, Table 1). The addition of 10% by weight of SP-B,C to DPPC resulted in increased adsorption rates (Fig. 4) and lower surface tension (Fig. 5) that were similar to those exhibited by surfactant TA (Fig. 4; Table 1). No additional reduction in surface tensions were seen when SP-B,C were increased to 20% by weight of DPPC. Based on these results, we used DPPC/SP-B,C (9:1) for testing in surfactant-deficient adult rats.

In vivo comparisons. Sham- and DPPC-treated animals generally showed no improvement in arterial oxygenation after treatment (Fig. 6). Animals receiving either surfactant TA or DPPC/SP-B,C (9:1) mixtures showed improvements in arterial oxygenation at 5 min after treatment that were generally sustained for the experimental period. DPPC/SP-B,C and surfactant TA-treated rats generally maintained stable, normal blood pCO_2 , and pH levels after treatment and had lungs that appeared well aerated on postmortem inspection. Static lung compliance measures for surfactant TA- (1.9 ± 0.2 ml/cm $\text{H}_2\text{O/kg}$, $n = 8$) and DPPC/SP-B,C-treated rats (2.3 ± 0.4 ml/cm $\text{H}_2\text{O/kg}$, $n = 5$) were not significantly different from each other but were significantly greater than those of sham- (air) (1.3 ± 0.1 ml/cm $\text{H}_2\text{O/kg}$) and DPPC-treated rats (1.2 ± 0.1) ($p < 0.01$).

DISCUSSION

We have tested the *in vitro* and *in vivo* efficacy of mixtures of DPPC and the small, hydrophobic proteins (SP-B,C) unique to

pulmonary surfactant. Simple mixtures similar to those studied herein have been variously studied by a number of other investigators (Table 2) and have been shown to have many biophysical properties similar to natural surfactants. Because of different techniques for isolation and measurement of these proteins, the reported number and molecular weights of these proteins have varied. However, recently two small hydrophobic surfactant proteins with molecular weights of approximately 5 kDa under reducing conditions have been cloned (18, 21, 23) and their amino acid sequences reported (18, 20–23, 25). These two proteins have N-terminal amino acids of phenylalanine and leucine/isoleucine when isolated from bovine lung, and evidence suggests that amino acid sequences are similar when these are proteins isolated from different species. Our test mixtures contained both SP-B (Phe) and SP-C (Leu/Ile) (16). We and others suspect that the native low molecular weight surfactant proteins are heterooligomers of these two gene products (12, 16). For this reason (and because we were unable to isolate SP-B and SP-C separately in sufficient amounts for the *in vivo* studies), we used both SP-B and SP-C together in these studies of function. Our observation that the presence of SP-B,C enhances *in vitro* surface activity of DPPC is in qualitative agreement with others who have studied similar mixtures (5, 6, 9). Our study is unique in that serial measures of gas exchange and measurement of static lung compliance were carried out in surfactant-deficient animals after treatment with DPPC/SP-B,C. Our study differs from some other studies in that we chose to use SP-B,C mixed with DPPC alone, without addition of other surfactant lipids. By so doing, it is possible that we needed a higher protein:DPPC ratio than others have found necessary when other protein and/or lipid surfactant constituents are included (2, 10).

Whereas others (2, 4, 6, 7, 9) have tested mixtures similar to ours in premature rabbit fetuses, the rabbit model does not readily allow measurements of both gas exchange and lung mechanics over time. Nonetheless, the surfactant-deficient adult

Fig. 4. Representative recordings of inflation pressures of bubbles in solutions of DPPC, surfactant TA, and DPPC/SP-B,C (9:1) assayed by a pulsating bubble surfactometer. The bubble is created at time zero with pulsation at 20 cycles/min between radii of 0.55 mm (max) and 0.40 mm (min) begun after 10 s. All mixtures were at concentrations of 2.0 mg/ml (37° C).

Fig. 5. Inflation pressures of DPPC or mixtures of DPPC and SP-B,C were measured on a pulsating bubble surfactometer. Means $\pm$ SEM are given for bubbles at maximum (0.55 mm) and minimum radius (0.40 mm) after 20 inflation cycles (1.0 min). $n = 13$ for DPPC alone and $n = 3$ for all others.

Table 1. Surface tension (mN/m)* of DPPC, surfactant TA, and DPPC/SPL5-26 solutions

	<i>n</i>	15 s	1 min	3 min	5 min
DPPC	13	29 $\pm$ 2†	26 $\pm$ 2†	22 $\pm$ 1†	21 $\pm$ 2†
Surfactant TA	7	10 $\pm$ 1	6 $\pm$ 1	5 $\pm$ 0.5	5 $\pm$ 0.5
DPPC/SPL5-26 (9:1)	24	13 $\pm$ 1 NS	7 $\pm$ 1 NS	4 $\pm$ 1 NS	3 $\pm$ 1 NS

* Means $\pm$ SEM for three to six measurements/sample at a minimum bubble radius of 0.4 mm using a pulsating bubble surfactometer. Times indicate the period of bubble pulsation.

† In comparison to surfactant TA, $p < 0.01$.

Fig. 6. Serial measurements of arterial oxygen levels in surfactant-deficient adult rats before ventilation (*pre-vent.*), before lavage (*pre-lav.*), and pre- and posttreatment are illustrated. The dosage for all treated animals was 50 mg/kg at concentrations of either 30 or 15 mg/ml saline. Sample sizes given are for each group up to 90 min posttreatment with $n = 33$ at prelavage and $n = 39$ at 5 min posttreatment due to sampling errors. Deaths occurred as follows: sham (1), DPPC (1), and surfactant TA (2) at 120 min posttreatment; sham (1), and DPPC (1) at 150 min posttreatment. Mean $\pm$ SEM given at prelavage as three to four measurements per rat (each separated by at least 10 min) were averaged; means $\pm$ SD are given for all other points. Values for DPPC/protein SPL5-26-treated rats are significantly different from sham- and DPPC-treated animals for all time points posttreatment and are not significantly different from surfactant TA-treated animals at any time point. Values for surfactant TA are significantly different at all time points from sham-treated animals and at all time points except 150 min posttreatment from DPPC-treated animals.

rat model has limitations as well, 1) an absence of lung immaturity, 2) possible damage to lung epithelium caused by the lavage procedure, and 3) potential effects of residual lavage fluid on the response by the lung to test mixtures. Using immature sheep (42, 43) or primates (44) may be ideal models in which to test these mixtures, but limitations include the need for larger amounts of test material and expense.

Table 2. Efficacy of low m.w. surfactant proteins when mixed with surfactant phospholipid(s)*

Reference	Source	Lipids	Proteins (m.w., red)	Measurements	
				<i>In vitro</i>	<i>In vivo</i>
Hawgood <i>et al.</i> (1, 2)	Dog	DPPC/PG	5, 8 kDa	K/C	PIP, C/L
Yu and Poss- mayer (11)	Calf	DPPC/PG	6	P/S	
Revak <i>et al.</i> (4)	Human	DPPC/PG	6	P/S	
Suzuki <i>et al.</i> (9)	Pig	DPPC/PG	<15	P/S W/B	P/V hist.
Takahashi and Fujiwara (5)	Cow	DPPC	5	P/S	
Tanaka <i>et al.</i> (6, 7)	Cow	DPPC	10	K/C W/B	P/V
Notter <i>et al.</i> (10)	Cow	DPPC/PG+	6	P/S	
Current	Cow	DPPC	5-26 (SP-B,C)	P/S	G/E C/L

* PIP, peak inspiratory pressure; PG, phosphatidyl glycerol; C/L, compliance of lung; P/S, pulsating surfactometer; K/C, King/Clements' measure of adsorption rate; W/B, Wilhelmy balance measure of surface tension; P/V, pressure/volume measures of lung; G/E, gas exchange; hist, histology; MW red, m.w. after reduction of sulfhydryl bonds. All *in vivo* studies with the exception of the current one were carried out in premature rabbits.

We have considered the possibility that our results with DPPC/SP-B,C are due in part to the presence of lipid that coisolate with these proteins. It is not yet known whether part of the hydrophobicity of these proteins is due to covalent binding of lipid, *i.e.* whether these proteins are proteolipids. The only lipid that we could identify that coisolated with SP-B,C was phosphatidylcholine, and that was found in varying amounts in different batches. Based on these findings and the work of others, we think it unlikely that lipid bound to SP-B,C, rather than SP-B,C per se, is responsible for alteration of the biophysical behavior of the DPPC/SP-B,C dispersions.

In summary, our results clearly indicate that a simple aqueous dispersion of hydrophobic low molecular weight surfactant proteins and DPPC have *in vitro* and *in vivo* characteristics that are similar to a more extensively tested "natural" surfactant. Further testing of simpler surfactant mixtures may allow insights into mechanisms of action and metabolism of alveolar surfactant, and may offer new approaches for improved treatment of surfactant deficiency states.

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