Peptide-based synthetic pulmonary surfactant for the treatment of respiratory distress disorders

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Abstract

KL₄ (Sinapultide) represents the first peptide-based replacement for surfactant protein B in pulmonary surfactant (PS) therapies approved for clinical use. Surfaxin, its formulation with PS lipids, shows the promise of synthetic PS for replacing animal-derived PS in the treatment of respiratory distress syndromes and for treating acute lung injury. Efforts to characterize the molecular basis for KL₄ function have revealed the peptide exhibits a helical structure which differentially partitions in response to both lipid saturation levels and pH. The penta-residue repeat of KL₄ leads to adaptive peptide helicity, varying with partitioning depth, and suggests structural plasticity may represent an important mechanism for differential trafficking of lipids, particularly in intra-alveolar surfactant for the formation of stable DPPC monolayers at air-water interfaces.

Graphical abstract

Pulmonary surfactant (PS) is a lipid-rich fluid which forms the inner lining of the alveoli; its primary functions are to minimize surface tension at the alveolar air-fluid interface and to provide a barrier against disease [1-6]. Inadequate levels of PS or breakdown of PS causes respiratory distress syndromes (RDS). PS is produced in alveolar epithelial type II (AE2) cells (Figure 1A) and secreted into the alveolar space as multilamellar vesicles. Extracellular surfactant serves as a reservoir for lipids which are cycled to a monolayer at the air-water interface; PS lipid reserves are turned over every 5-10 hours [7]. The phospholipid composition of PS is unusual with much higher levels of DPPC and PG than typically observed in cellular lipid bilayers. DPPC, a fully saturated lipid, can withstand high lateral surface pressures and lowers surface tension at the air-water interface [8]. Equally critical to the function of PS is surfactant protein B (SP-B), a lipophilic protein essential for the packaging of intracellular PS as lamellar bodies which convert to tubular myelin on secretion and form the multilayer surface film observed at the air-water interface (Figure 1B). It is thought that DPPC may be specifically enriched at the air-water interface by trafficking and sorting of lipids in bulk PS by SP-B. Insufficient PS levels are a leading cause of RDS and death in premature infants, with infants born at 26-28 weeks gestation having a 50% incidence of RDS and at 30-31 weeks gestation a 25% incidence. SP-B mutations or deletions lead to disorganization of lamellar bodies, an abnormal morphology for alveolar type II cells, and loss of tubular myelin organization preventing proper lung function and viability [9-13].

Identification and development of PS peptides for treatment of RDS

Characterization of the functional properties of SP-B resulted in identification of fragments of the native sequence which retain these functions, particularly peptides corresponding to the N- and C-termini. The N-terminal 25-amino acid segment of SP-B, SP-B₁₋₂₅, is surface active [18], and has been shown to interact with specific anionic lipids to facilitate squeeze-out of lipids [19-21]. The C-terminal segment of SP-B, SP-B₅₉₋₈₀, has shown efficacy in *in vitro* assays measuring surface tension [22]. Synthetic, peptide-based SP-B replacements can remove the immunologic risks associated with animal-derived surfactant and potentially allow for greater therapeutic consistency. The possibility also exists for tailoring specific PS formulations to different respiratory diseases. Of particular clinical interest, the charge distribution of the last 22 amino acids of SP-B served as the template for designing the KL₄ peptide [23], known clinically as sinapultide. To date, KL₄ has enjoyed the most clinical success as a synthetic peptide replacement for SP-B, discussed below, including approval by the US Food and Drug Administration in 2012 for prevention of RDS in at-risk premature infants.

FPIPLPYCWL**CRALIKRIQAMIPKG****ALAVAVAQVCRVVLVAGGICQCLAERYSVILLDTLLGRMLPQLVCRLVLRCSMD**
 SP-B₁₋₂₅ **FPIPLPYCWL****CRALIKRIQAMIPKG**
 SP-B₅₉₋₈₀ **DTLLGRMLPQLVCRLVLRCSMD**
 Sinapultide (KL₄) **KLIIIIKIIIIKIIIIKIIIIK**
 Mini-B **CWL****CRALIKRIQAMIPKG** **GRMLPQLVCRLVLRCS**

Figure 2. SP-B and fragments of SP-B important to function with charged amino acids (blue cationic/red anionic), polar amino acids (purple), cysteines (yellow orange), and prolines (green) highlighted. Brackets indicate disulfide bonds. The functional peptide mimic of SP-B₅₉₋₈₀, known as Sinapultide or KL4, utilized in Surfaxin is also shown.

Development of sinapultide, early studies of efficacy, and preclinical studies

Cochrane and co-workers pursued functional studies of peptide variants based on the SP-B C-terminus, SP-B₅₉₋₈₀, after establishing that the hydrophilic residues were necessary for its function. They made simplified versions in which charged and polar amino acids were uniformly converted to either arginine, lysine, or aspartic acid while hydrophobic amino acids were converted to leucines. Cationic peptides, termed RL₄ and KL₄, had similar activity to the original peptide while the anionic peptide, DL₄, was much less active [23]. Measurements with varying combinations of zwitterionic and anionic phospholipids led to the conclusion that the cationic amino acids could better interact with both PC and PG lipids giving RL₄ and KL₄ superior surface properties relative to DL₄ [23-25]. The lysine variant, later named sinapultide [26] and patented in 1995 [27], was ultimately selected for preclinical and clinical studies; in order to form active PS the peptide is combined with DPPC, POPG, and palmitic acid to form a suspension of peptide-containing liposomes in buffered saline. An initial study utilizing preterm infant rhesus monkeys showed efficacy for KL₄-surfactant which was superior to Exosurf, a non-peptide-containing synthetic surfactant [28]. An initial test of safety and efficacy for KL₄-surfactant in 47 premature human infants showed strong efficacy with only two enrolled participants requiring crossover to a previously FDA-approved surfactant (either Surfacta or Exosurf) during the study [29].

Further pre-clinical studies showed success in treatment of meconium aspiration syndrome (MAS) in adult rabbits and newborn rhesus monkeys following intratracheal instillation of human meconium to induce pulmonary injury [30], and that KL₄-surfactant exhibited an efficacy similar to porcine-derived surfactant in treating preterm lambs with severe RDS [31]. Several more recent studies indicate further improvements in methods of delivery may enhance the use and efficacy of KL₄-based therapies, enabling them to supplant animal-based surfactants as the clinical standard of care for RDS [32-36].

Sinapultide formulation and clinical development

KL₄, or sinapultide, is made synthetically and the purified peptide is combined with DPPC and POPG phospholipids and palmitic acid in a buffered saline suspension. This formulation, known as lucinactant and marketed by Discovery Laboratories, Inc., as Surfaxin, is administered intratracheally whereupon it spontaneously enters the alveoli and lowers surface tension, reducing the need for mechanical ventilation; it has shown efficacy in treating several respiratory conditions. While under development, lucinactant was designated as an Orphan Drug Product by the US FDA for the treatment or prevention of RDS in premature infants [37, 38], the prevention of bronchopulmonary dysplasia in premature infants [39], the treatment of meconium aspiration syndrome in newborn infants [40], and the treatment of acute respiratory distress syndrome in adults [41]. The necessity of developing a formulation containing phospholipids, in addition to the unusually high melting temperature of DPPC, led to unique challenges in developing a manufacturing process and clinical protocols sufficiently robust for FDA approval. It received authorization for clinical use in treating infant RDS in 2012 [42]. A lyophilized form of Surfaxin as well as an aerosolized version, known as Aerosurf, are currently in clinical trials for the treatment of RDS. An aerosol form is also in early development for the treatment of viral- or chemically-induced acute lung injury (ALI) [43]. The properties of lucinactant suggest it might have use in drug delivery applications in addition to the treatment of RDS and ALI [44]. Given the recent approval of lucinactant, large-scale clinical studies comparing its efficacy to animal-derived PS are still lacking and of significant interest to the pulmonology community [45, 46], particularly since *in vitro* studies suggest it might be more resistant to inactivation by oxidation or serum proteins [47, 48].

In vitro mechanistic studies of PS formulations

SP-B in native PS functions at a concentration of ~400-800 lipids per SP-B monomer; Surfaxin contains a molar ratio of lipids/sinapultide of >100:1. A primary focus in early PS research was the functional properties of SP-B and synthetic peptides in lipid monolayers at air-water interfaces. Their ability to facilitate, at low peptide/protein concentrations, the formation of stable lipid monolayers which can withstand much higher lateral pressures than monolayers of pure DPPC or lipid mixtures remains a hallmark of peptides targeted for PS-based therapies. Their biophysical properties preclude characterizing them under functional conditions by standard structural biology techniques, such as x-ray crystallography or solution NMR. Based on circular dichroism measurements, FTIR studies, and molecular modelling, SP-B is thought to form amphiphilic helices linking lipid mono- and bilayer interfaces to regulate their physical properties and lipid trafficking [49-51]. Partitioning and folding of amphiphilic peptide helices at membrane interfaces is a well-recognized motif in structural biology; amphiphilic helices play key roles in protein and membrane regulation, including gating of ion channels and activation of G-protein coupled receptors. Simple examples have primary sequences of periodically spaced hydrophobic and hydrophilic residues that are easily segregated on the faces of an α -helix

by assuming a canonical 3.6 residues/turn and $i \rightarrow i+4$ hydrogen bonding. KL₄ has also been shown by CD and FTIR measurements to be primarily helical in a lipid environment [52-55]. However, the periodicity of cationic lysines in KL₄ rules out a structural model in which the peptide binds to lipid interfaces as a classic, amphiphilic α -helix (Figure 3a), and its sequence is intermediate in hydrophobicity (24% charged/polar residues) relative to typical amphiphilic α -helices (~50% charged/polar residues), such as antimicrobial peptides, and fully hydrophobic transmembrane α -helices, which are important in cellular regulation. Low resolution studies attempting to qualitatively determine the structure and orientation of KL₄ in lipid environments have yielded conflicting interpretations regarding the secondary structure of KL₄ as well as its orientation in a lipid environment [52, 53, 56, 57]. Some of this divergence between studies is due to differences in sample preparation, lipids utilized, and experimental configuration, as CD spectra as a function of negatively charged lipids clearly show [58].

Figure 3. A) Helical wheel plots for KL₄ with varying pitch. Wheels generated assuming a canonical α -helix (left); using the average torsion angles determined in POPC/POPG vesicles, pH 7.4 (middle; 4.1 residues/turn) [54]; and using the average torsion angles suggested by preliminary NMR experiments with DPPC/POPG vesicles, pH 7.4 (right; 4.6 residues/turn). Arrows indicate the net hydrophobic moments resulting from the distribution of charged lysine sidechains on the helix surface. The first lysine is omitted in NMR-based models as data indicate the N-terminus is less structured relative to the rest of the peptide. **B)** CD spectra of KL₄ in POPC:POPG lipid vesicles as a function of buffer pH. KL₄ structure in DPPC:POPC lipid vesicles also exhibits a pH dependence. The pH shifts from 7.3 to 6.8 on secretion from type II pneumocytes into the extracellular PS reservoir [59, 60].

Research in my group has focused on characterizing the molecular mechanisms of lipid dynamics and interactions with KL₄ to provide guidance for the development of next generation synthetic PS therapies. To examine both the peptide and its effects on PS lipids at clinically relevant concentrations in lipid environments mimicking native PS and lucinactant, we have developed and applied solid state NMR and EPR spectroscopic techniques. This enables a systematic approach to understanding the relationship between peptide partitioning and structure as a function of lipid environment and to measuring peptide-mediated changes in lipid dynamics to discern its effects on specific lipid species, particularly DPPC. Initially, we characterized the structure of KL₄ in a model lipid system containing monounsaturated POPC and POPG and found that it forms a helical structure with torsion angles at the sites of measurement that deviate significantly from a classic α -helix [54]. A comparison of its effects on DPPC/POPG vs. POPC/POPG lipid vesicles indicate the binding of KL₄ is dependent on the level of mono-unsaturation in the fatty acid chains, with KL₄ partitioning more peripherally in a fully mono-unsaturated lipid environment and more deeply inserting into a DPPC-rich environment [55]. Addition of KL₄ increases collective motions in POPC/POPG lipid bilayers and decreases collective motions in DPPC/POPG lipid bilayers while attenuating local lipid dynamics in both environments, consistent with different peptide partitioning depths between the two lipid bilayer systems [61]. These observations suggest that the addition of KL₄ leads to positive curvature strain in the POPC/POPG lipid system but stabilizes the DPPC/POPG lipid bilayers. Near the phase transition temperature of the DPPC/POPG lipid mixtures, which is just below the physiologic temperature of lung surfactant, KL₄ causes phase separation with the DPPC remaining in a gel phase and the POPG partitioned between gel and fluid phases. The partitioning and orientation of KL₄ in DPPC/POPG and POPC/POPG phospholipid bilayers, as inferred from the leucine side chain dynamics and EPR power saturation measurements for spin-labeled variants of KL₄, is consistent with monomeric KL₄ lying in the plane of the bilayers and adopting an adaptive helical structure which confers amphiphilicity and allows partitioning into the lipid hydrophobic interior [61, 62]. The structural plasticity of KL₄ and its adaptive behavior in differentially partitioning into lipid lamellae containing varying levels of saturation, leading to subsequent changes in curvature strain, suggest a mechanism for its role in restoring lung compliance in which it differentially traffics DPPC to form a stable monolayer at the air-water interface (Figure 4). More recently we have observed, via CD measurements, its helical structure is pH dependent over the pH range experienced by PS on secretion from type II pneumocytes into the extracellular space (Figure 3b) [59, 60]. This observation suggests peptide partitioning into the hydrophobic lipid bilayer interior shifts the pK_a values of charged moieties within KL₄ to a physiologically relevant regime, with changes in pH also affecting

peptide partitioning depth and lipid curvature strain. If a similar phenomenon exists with SP-B, it would provide an explanation for the lipid morphology changes observed during PS secretion and re-uptake, warranting a more thorough investigation of peptide/protein partitioning and structure with respect to pH to direct further development of synthetic replacements.

Figure 4. KL₄ Models built using backbone torsion angle restraints from ssNMR measurements. (A) A KL₄ conformer from measurements with POPC-rich samples; (B) a second conformer from measurements with DPPC-rich samples. Models created using PyMol [63] and Membrane Builder [64]

Other PS active peptides and future directions

While KL₄ was developed based on the hydrophilic/ hydrophobic pattern in SP-B₅₉₋₈₀, their primary sequences have only modest similarity raising the question of whether KL₄ and SP-B₅₉₋₈₀ are comparable in their function in a lipid environment. NMR and CD studies indicate SP-B₅₉₋₈₀ exhibits structural plasticity which is lipid dependent and has similar effects on PS lipid dynamics [65]. However, SP-B₅₉₋₈₀ contains a proline residue in the middle of the sequence leading to a helix-turn-helix structure in which the turn structure changes with environment, suggesting that while its function may be similar to KL₄ its mechanism of action may be quite different [66]. SP-B₁₋₂₅, which also exhibits functional activity, does not exhibit structural adaptation to different lipid environments. However, its effects on lipid dynamics, including the induction of non-lamellar lipid polymorphisms, differ markedly from SP-B₅₉₋₈₀ and KL₄, and it also specifically affects DPPC dynamics in PS lipid mixtures [67, 68]. SP-B₁₋₂₅ also differs from SP-B₈₋₂₅ in its ability to alter lipid morphology, suggesting that the N-terminal, proline-rich segment is critical to how the amphiphilic helix encompassing residues 9 through 25 partitions into the membrane and influences lipid organization. A hybrid peptide, termed Mini-B which contains SP-B residues 8-25 and 63-78 and disulfide bridges similar to the native protein, has been demonstrated to have *in vitro* activity similar to full SP-B [69, 70]. It is considerably more active in its oxidized form, and it out-performs a physical mixture of N-terminal and C-terminal peptides. These observations suggest the N- and C-termini of SP-B play complimentary roles in lipid organization and trafficking, and that future research developing chimeric constructs of SP-B₁₋₂₅ combined with either SP-B₅₉₋₈₀ or KL₄ variants for clinical use is merited.

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