Effect of Cuban Porcine Pulmonary Surfactant (Surfacen) and rCmPI-II Protease Inhibitor on Neutrophil Elastase Activity

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ABSTRACT

INTRODUCTION In inflammatory respiratory diseases, the imbalance between proteases and endogenous protease inhibitors leads to an exacerbated activity of human neutrophil elastase (a protease that destroys the extracellular matrix and stimulates proinflammatory cytokine release). Elastase is considered a target in the search for therapeutic treatments for inflammatory respiratory diseases. Pulmonary surfactant is a promising product for this purpose, because in addition to its biophysical function, it has anti-inflammatory properties.

OBJECTIVE Evaluate effect of the Cuban porcine pulmonary surfactant (Surfacen), the rCmPI-II elastase inhibitor, and the Surfacen/rCmPI-II combination on activated neutrophil elastase activity in vitro, and determine if Surfacen’s interface property changes in the presence of the inhibitor.

METHODS The anti-elastase effect of Surfacen, rCmPI-II and the Surfacen/rCmPI-II combination was evaluated in an in vitro model of activated neutrophils, previously purified from the blood of healthy subjects. The cells were stimulated with LPS/MLP and were incubated with different concentrations of Surfacen, rCmPI-II and the Surfacen/rCmPI-II combination. Elastase activity was measured. The interface property was determined on a Langmuir surface balance.

RESULTS Surfacen at 10 mg/mL inhibited 71% of stimulated neutrophil elastase activity. rCmPI-II at 0.1 μM reduced 20% of elastase activity; at 200 μM—the maximum concentration evaluated—inhibition was 68%. Both products had a dose-dependent effect. The Surfacen/inhibitor combination (0.5 mg/mL/80 μM) did not affect the surfactant interface property or the inhibitory activity of rCmPI-II against human neutrophil elastase.

CONCLUSIONS Surfacen and the rCmPI-II inhibitor have an anti-elastase effect on an activated neutrophil model. rCmPI-II does not affect Surfacen’s interface property and, therefore, both can be evaluated for combined use in treating inflammatory lung diseases.

KEYWORDS Pulmonary surfactants, elastase inhibitor, drug carriers, neutrophils, Cuba

INTRODUCTION

Endogenous pulmonary surfactant is a complex mixture of lipids and proteins. It is synthesized and secreted into the alveolar space by type II pneumocytes. Its main function is to reduce surface tension at the air–liquid interface in the alveolus, preventing alveolar collapse and reducing respiratory effort. Pulmonary surfactant is essential and its absence, deficiency or inactivation is associated with several pulmonary diseases, such as neonatal respiratory distress syndrome (NRDS, or hyaline membrane disease), acute respiratory distress syndrome (ARDS), meconium aspiration syndrome, asthma and chronic obstructive pulmonary disease.[1] In addition to improving pulmonary function and oxygenation, surfactant is a key modulator of innate and acquired pulmonary immunity.[2,3]

Natural exogenous surfactant preparations are quite safe and effective standard therapy in newborns with NRDS.[4] The preparations used in neonatology services come from bovine or porcine surfactant extracts. Surfactants for clinical use are obtained from bronchoalveolar lavage or from lung tissue. Hydrophobic lipid and protein fractions, which contain the surfactant’s main tensoactive components, are extracted using organic solvents, usually chloroform/methanol. Five surfactant preparations have been marketed by large pharmaceutical companies, all registered for use in NRDS: Curosurf (Chiesi Farmaceutici, Italy); Survanta (AbbVie, USA); BLES (BLES, Canada); Infasurf (ONY Biotech, USA), and Alveofact (Boehringer-Ingelheim, Germany).[5] Cuba also markets a registered surfactant preparation called Surfacen.[6] WHO includes these kinds of products on its Essential Medicines List.

Several respiratory diseases characterized by a strong inflammatory response involve an imbalance between proteolytic enzymes and their inhibitors. Neutrophils (and especially neutrophil elastase) are the main inflammatory mediators involved in acute alveolar injury and interstitial edema related to an increase in vascular permeability in ARDS.[7] In other chronic inflammatory lung conditions (cystic fibrosis, chronic obstructive pulmonary disease and bronchiectasis), abundant neutrophils are present in lung tissue. Excessive release of neutrophil elastase accelerates lung tissue damage, which causes a decrease in pulmonary function.[8]

Uncontrolled neutrophil elastase proteolytic activity is regulated by various endogenous protein inhibitors; however, this enzyme can evade this regulation using several pathways.[9] Recognizing neutrophil elastase as a therapeutic target in inflammatory lung diseases has led to discovery of new elastase inhibitors.[10–12] now undergoing clinical studies.[13,14]
Recently, two surfactants in clinical use, Alveofact and Curosurf, were shown to inhibit formation of neutrophil extracellular traps (NETs) by decreasing elastase, myeloperoxidase and cell-free DNA (cfDNA) levels.[15]

Surfacen is a natural porcine surfactant, approved for use in Cuba in 1995 by the country’s regulatory agency, the Center for State Control of Medicines, Equipment and Medical Devices (CECMED) (health registration No. 0800).[6] It was introduced into medical practice in all neonatal intensive care units to treat hyaline membrane disease or NRDS in preterm infants.[16] Later, in 2011, Surfacen was approved for ARDS treatment in adults,[17] and in 2014, for ARDS in pediatric patients.[18] Surfacen is a safe product; no adverse reactions have been reported in any of the clinical trials conducted, and it has a safety profile similar to the other surfactants marketed internationally.[19] In the Cuban protocol for clinical management of patients with COVID-19 published in 2020, Surfacen is one of the medicines recommended for use.[20]

In addition to its biophysical function, Surfacen has an in vitro anti-inflammatory effect, decreasing tumor necrosis factor-alpha (TNF alpha) and interleukin 6 (IL-6).[21] and has shown an anti-inflammatory and anti-allergic immunomodulatory effect in an animal model of allergen-induced asthma.[22]

Pulmonary surfactant has been evaluated as a drug carrier or vehicle since the 1990s;[23] it has been used as a budesonide carrier in premature newborns at risk of developing bronchopulmonary dysplasia and is currently undergoing clinical trials. [24,25] Those studies provide the first clinical proof of concept for a surfactant-drug combination and justify research on its possible use in combination therapies with other drugs.

Given the importance of neutrophil elastase in respiratory diseases, the combination of Surfacen with inhibitors of this enzyme could lead to new therapeutic options for these diseases. CmPI-II, a Kazal-type serine protease inhibitor, is isolated from the Cenchritis muricatus marine snail. CmPI-II is a strong inhibitor of serine proteases, especially human neutrophil elastase.[26,27] The recombinant variant of this inhibitor (rCmPI-II) has functional and molecular characteristics similar to the natural inhibitor. Its ability to inhibit human neutrophil elastase has suggested important biomedical applications, including in inflammatory diseases.[28]

Very few in vitro and in vivo studies have been conducted on the combination of surfactants and protease inhibitors. Belai[29] demonstrated that use of Survanta pulmonary surfactant with alpha-1 antitrypsin had a positive effect on oxygenation and surfactant metabolism in surfactant-deficient rats. Cochrane[30] demonstrated that the combination of elafin and a surfactant in an animal model of lung damage decreased the lung damage.

The objective of this study was to evaluate the effect of Surfacen pulmonary surfactant, the rCmPI-II elastase inhibitor, and the Surfacen/rCmPI-II combination on elastase activity in an in vitro model of activated neutrophils, as well as the surfactant’s interface property when combined with the inhibitor.

METHODS

EVALUATED PRODUCTS

Surfacen Natural surfactant obtained from porcine pulmonary lavage. The final product is supplied as a sterile white lyophilized powder in a 6R vial containing 50 mg total phospholipids. Composition: phospholipids (95%), hydrophobic proteins (SP-B and SP-C, 1.5%) and other lipids (3.5%).[6,31] Surfacen is produced in the National Center for Animal and Plant Health (CENSA) in collaboration with the National Biopreparations Center (BIOCEN), both in Cuba. Each vial is reconstituted in 2 mL of water for injection, obtaining a phospholipid concentration of 25 mg/mL.

rCmPI-II Elastase inhibitor obtained at the University of Havana Biology Faculty’s Protein Studies Center (CEP) via recombinant pathway in Pichia pastoris yeast. Its characteristics are similar to those of the CmPI-II natural protein from the Cenchritis muricatus marine snail. The inhibitor concentration was determined by measuring absorbance at 280 nm using the coefficient of extinction ε1%, 280 nm = 16.1, previously determined for the natural inhibitor. The concentration was 2 mg/mL. Molecular mass of rCmPI-II is 5485 Da.[32]

HUMAN NEUTROPHIL PURIFICATION FROM HUMAN BLOOD

Neutrophils were obtained from 10 mL of venous blood from healthy volunteers using Ficoll density gradient cell separation (Ficoll-Histopaque-10771, density: 1.077 g/mL, Sigma-Aldrich, USA).[33] First, blood was collected in EDTA tubes (Vacuette 4 mL K3EDTA, Greiner Bio-One, Germany) and centrifuged at 1500 rpm (DL6M centrifuge, Kaida, China) for 15 minutes at 20 ºC. Plasma was collected and centrifuged at 2500 rpm for 5 minutes at 20 ºC, to obtain a platelet-poor supernatant, which was remixed with the rest of the blood. After gravity sedimentation gradient was performed using Ficoll-Histopaque, plasma was collected and put through a second centrifugation gradient at 2500 rpm for 30 minutes at 20 ºC with Ficoll-Histopaque. To eliminate the remaining Ficoll, the precipitate obtained was resuspended in 5 mL of phosphate-buffered saline and centrifuged at 2500 rpm for 5 minutes at 20 ºC. Finally, the precipitate was collected and resuspended in 1 mL of pH 7.2 phosphate-buffered saline (PBS) solution supplemented with 0.1% bovine serum albumin (BSA, Sigma-Aldrich, USA). Purity of purified neutrophils (>95%) was determined in the MaccsQuant 10 flow cytometer (Milteny, Germany) by fluorescence-activated cell sorting (FACS) using anti-CD45-FITC and anti-CD3-PE antibodies (Milteny, Germany).

NEUTROPHIL ACTIVATION ASSAY

Purified neutrophils were adjusted to a concentration of 1×10⁷ cells per mL in PBS solution supplemented with 0.1% BSA (Sigma-Aldrich, USA). Then, 100 mL of the neutrophil suspension was activated for 10 minutes through incubation with 10 mL of cytochalasin B from Drechslera (Sigma-Aldrich, USA) at a final assay concentration of 5 μg/mL, and then stimulated for 5 minutes through the addition of 10 mL lipopolysaccharides (LPSs) from Escherichia coli O55:B5 (Sigma-Aldrich, USA) at a final assay concentration of 1 μg/mL. Simultaneously, 10 mL of the N-formylmethionyl leucyl-phenylalanine (fMLP, Sigma Aldrich, USA) stimulant was added at a final assay concentration of 50 nM, along with the compounds to be evaluated (surfactant and inhibitor) and incubated for 30 minutes at 37 ºC while mixing in a thermo shaker (TS 100, Fisher, USA). Surfacen was used at the final assay concentrations of 0.25 mg/mL to 10 mg/mL, and rCmPI-II was used at final concentrations of 0.1 μM to 200 μM. In the Surfacen/rCmPI-II com-
bination assays, 0.5 mg/mL of Surfacen and 80 μM of inhibitor were used. The final assay volume was adjusted to 200 mL with PBS. Samples corresponding to the different designs were centrifuged at 2500 rpm for 10 minutes at 22 °C, and the supernatant was used to determine elastase activity.

In parallel, a positive control was performed with activated/stimulated neutrophils, and a negative control was performed with untreated neutrophils. To evaluate the effect of Surfacen and rCmPI-II, these were treated with neutrophils that were not activated/stimulated. The maximum elastase percentage present in neutrophils was determined by lysing activated/stimulated cells with 0.1% hexadecyltrimethylammonium bromide (HTAB) detergent (Sigma-Aldrich, USA) for 15 minutes at 4 °C.

**Elastase enzyme activity** Elastase enzyme activity was evaluated in 90 μL of the supernatant by measuring at 405 nm hydrolysis of methoxyxuccinyl-Ala-Ala-Pro-Val-p-nitroaniline substrate; 10 μL, 2.8 mM dissolved in dimethyl sulfoxide (DMSO, Calbiochem, Merck, Germany), in Tris HCl 0.02 mol/L, NaCl 0.5 mol/L pH 8.0 (100 μL), for 30 minutes, in a plate reader (ChemWell Manager, EIA mode, USA). Each reaction (200 μL) was done in triplicate in a 96-well plate. The initial velocity of the reaction was calculated using the slope of the curve (DDO/Dt) in the linear area. Enzyme activity was expressed in U/mL.

**Determining kinetics of spreading** Equilibrium surface pressure was determined on a Langmuir surface balance. Surfacen (25 mg/mL), rCmPI-II (10 and 80 μM), and the Surfacen/rCmPI-II combination were applied with a 10 μL syringe (Hamilton, USA) to the air-liquid interface in the shape of a T, from a Langmuir balance made with teflon (Nima Technology, UK). The subphase volume is 15 mL (Tris buffer 5 mM, NaCl 150 mM, pH 7.0). Samples were applied at one end of the container, while a pressure sensor with surface plate (typically a small piece of filter paper) monitored changes in surface pressure of the active surface material, as a function of time. The kinetics of spreading (p vs. time) was obtained at 37 ± 1 °C for 300 seconds.

**Statistical analysis** This was performed using GraphPad software version 5.0 (GraphPad Software, USA). The mean and standard deviation (SD) were used as descriptive statistics, and the groups were compared using analysis of variance and a posteriori comparisons using Tukey’s test. The p value of 0.05 was used as the statistical significance threshold.

**RESULTS**

Elastase enzyme activity present in the positive control after neutrophil lysis with the HTAB detergent was 0.35 ± 0.05 U/mL (Figure 1, bar A). This value corresponds to total elastase enzyme activity in the neutrophil and was considered the maximum enzymatic activity.

The amount of elastase released in response to activation with cytochalasin B and to LPS and fMLP stimuli (positive control, Figure 1, bar B) corresponds to about 45% of the maximum enzyme activity in neutrophils, determined by cell lysis. In the unstimulated, untreated neutrophils (negative control, Figure 1, bar C), spontaneous release of the enzyme was detected, representing about 10% of the positive control value (Figure 1, bar B). In the inactivated, unstimulated neutrophils treated with surfactant and elastase inhibitor (Figure 1, bars D and E), the enzyme activity value was similar to the negative control, which suggests that these compounds do not cause neutrophil activation.

**Effect of Surfacen, rCmPI-II, and the Surfacen/rCmPI-II combination on activated neutrophil elastase activity** At concentrations of 0.25, 0.5 and 4 mg/mL, Surfacen inhibited 45% to 57% of elastase activity. At 10 mg/mL, inhibition was 78% (statistically significant value compared with other studied concentrations) (Figure 2-A).

In activated and stimulated neutrophils, incubated with rCmPI-II, elastase activity was inhibited at a magnitude dependent on the inhibitor concentration (Figure 2-B). rCmPI-II at 0.1 μM reduced 20% of elastase activity; at the maximum concentration evaluated (200 μM), inhibition was 68%. rCmPI-II is a competitive inhibitor of this enzyme and, therefore, increasing its concentration increases its inhibition. This characteristic has been described for the inhibitor in an enzyme assay with a chromogenic substrate. To

![Figure 1: Neutrophil elastase enzyme activity](image-url)

The bars represent the means of four independent experiments in U/mL. In the analysis of variance with Tukey’s test, the significant differences are identified with asterisks:

***: p <0.001 (A vs. B, C, D, and E); ***: p <0.001 (B vs. C, D, and E); ns: nonsignificant (C vs. D, and E).

A: neutrophils activated and stimulated with cytochalasin B and LPS+fMLP and lysed with HTAB (maximum percentage of elastase present in neutrophils)
B: neutrophils activated and stimulated with cytochalasin B and LPS+fMLP (positive control)
C: untreated neutrophils (negative control)
D: unactivated, unstimulated neutrophils, treated with Surfacen
E: unactivated, unstimulated neutrophils, treated with rCmPI-II

Cyt-B: cytochalasin B; fMLP: formylmethionyl leucyl-phenylalanine; HTAB: hexadecyltrimethylammonium bromide; LPS: lipopolysaccharide; NØ: neutrophils; rCmPI-II: *Cenchritis muricatus* (Gastropoda, Mollusca) iso-

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Figure 2: Effect of Surfacen (A), rCmPI-II (B) and the Surfacen/rCmPI-II combination (C) on activated and stimulated neutrophil elastase activity

The bars represent the mean ± standard deviation of four independent experiments. In the analysis of variance with Tukey's test, the significant differences are identified with asterisks: **: p <0.01, ***: p <0.001, ns: nonsignificant.

rCmPI-II: Cenchritis muricatus (Gastropoda, Mollusca) isolated elastase inhibitor obtained via recombinant pathway (Protein Studies Center, Faculty of Biology, University of Havana); Surfacen: natural porcine pulmonary surfactant (Censa, Cuba).

Figure 3: Spreading assays of Surfacen and Surfacen/rCmPI-II combination in a Langmuir surface balance

Vertical axis: interface pressure in millinewton per meter. Horizontal axis: time in seconds.

rCmPI-II: Cenchritis muricatus (Gastropoda, Mollusca) isolated elastase inhibitor obtained via recombinant (Protein Studies Center, Faculty of Biology, University of Havana); Surfacen: natural porcine pulmonary surfactant (Censa, Cuba).

study the effect of the product combination, the concentration that resulted in about 50% inhibition in the assays of each product was selected: for rCmPI-II, 80 μM, and for Surfacen, 0.5 mg/mL. The Surfacen/rCmPI-II combination did not affect the elastase inhibitor activity observed for both independently (Figure 2-C).

Effect of rCmPI-II on Surfacen interface properties Figure 3 compares the kinetics of spreading of Surfacen and the Surfacen/rCmPI-II combination, measured using changes in surface pressure (p) over time. Surfacen adsorbs very efficiently at the air-liquid interface, with an equilibrium surface pressure around 42 mN/m in less than 1 minute. The addition of rCmPI-II to Surfacen did not modify the kinetics of spreading. At the two concentrations evaluated, rCmPI-II did not modify the surface pressure.

DISCUSSION

Human pulmonary surfactant improves pulmonary function and oxygenation, and modulates innate and acquired immunity, regulating the lung’s inflammatory processes. Various surfactant preparations decrease respiratory burst cytokine release and nitric oxide production in inflammatory cells (neutrophils, monocytes and macrophages).[34,35] Among them, Surfacen, the Cuban porcine surfactant, decreases TNFa and IL-6 production in human monocytes responding to Staphylococcus aureus.[21]

There are few in vitro model studies on the modulatory effect of surfactants in clinical use on neutrophil activation. In 1996, Tegtmeier evaluated the modulatory effect of surfactants in clinical use on the function of neutrophils responding to different damage-inducing agents.[36] The surfactants tested did not produce neutrophil activation. However, when the neutrophils stimulated with interleukin-8 (IL-8), neutrophil-activating peptide-2 (NAP-2), or fMLP were incubated with different surfactant combinations, elastase release depended on the type of surfactant. Exosurf and Alveofact had little effect on the elastase release induced by the three mediators, while Curosurf and Survanta inhibited cell response in a dose-dependent manner. Schulz demonstrated that Curosurf and Alveofact had an inhibitory effect on in vitro NET formation in neutrophils stimulated with phorbol-12-myristate-13-acetate (PMA). In both preparations, the effect was dose- and time-dependent; the effect was stronger with Alveofact. When the neutrophils were incubated with these preparations before PMA activation, the inhibitory effect was higher, which suggests a preventive effect. The authors suggest that these preparations reduce neutrophil activation and highlight pulmonary surfactant as a possible candidate for attenuating inflammation.[15]

This study showed that Surfacen inhibits the activity of released elastase, present in activated/stimulated human neutrophils in vitro. Some surfactants show different effectiveness against this enzyme in vitro models of activated neutrophils, likely due to differences in its composition.[15,36] The inhibitory activity of Surfacen could be related to the release process of this enzyme by neutrophils once activated/stimulated. In this model, Surfacen’s elastase inhibition mechanism is unknown; however, for other surfactants (Survanta and Infasurf), the enzyme release mechanism is known to be modulated by their action on neutrophils, and it is mediated by depolarization and release of intracellular Ca++ through activation of the G protein pathway.[37] Recently, pulmonary surfactant preparations in clinical use have been shown to decrease expression of P2Y6, a G-coupled extracellular receptor that participates in NET formation by interacting with calcium pathways.[15]

rCmPI-II is an inhibitor of serine proteases, including human neutrophil elastase, that reduces elastase activity in an in vitro activated/stimulated neutrophil assay in a dose-dependent manner. This inhibitory activity is not affected by the presence of Surfacen. Several preclinical trials have investigated the therapeutic potential of neutrophil elastase inhibitors Sivelestat, Sirtinol, DX-890 and...
The mechanism of action of the synthetic inhibitor Sirtinol in an in vitro activated neutrophil model was to inhibit elastase enzyme activity, since its effect was not mediated by protein kinase A, or by calcium and kinase pathways regulated outside the cell; therefore, it did not directly affect neutrophil function. In an animal model of LPS-induced acute lung injury, Sirtinol reduced neutrophil recruitment and lung edema.[40]

DX-890, a peptide enzyme inhibitor, inhibits activity of elastase released by fMLP-stimulated neutrophils obtained from healthy individuals and patients with cystic fibrosis, inhibits neutrophil transmigration through epithelial cells, and decreases IL-8 secretion in nasal epithelial cells and sputum of patients with cystic fibrosis.[41] BAY 85-8501, another synthetic enzyme inhibitor, had a favorable safety and tolerance profile when administered over 28 days to patients with bronchiectasis not associated with cystic fibrosis; however, additional studies of prolonged treatment are needed to evaluate its clinical efficacy.[14]

rCmPI-II’s ability to reduce human neutrophil elastase activity, not only in experiments with a commercial enzyme,[26] but also in an in vitro model of activated/stimulated neutrophils obtained in this study, suggests that rCmPI-II might be used in inflammatory processes in which neutrophils are involved.

The combination of the surfactant Survanta and the alpha-1 antitrypsin elastase inhibitor in an in vivo model showed that the addition of alpha-1 antitrypsin improved oxygenation and surfactant metabolism in surfactant-deficient rats.[29] Here, Survanta is shown to be absorbed very efficiently into the air–liquid interface with an equilibrium surface pressure of about 42 mN/m in less than 1 minute. The results of this study show that rCmPI-II does not affect the Survanta surface properties.

Pulmonary surfactant may be combined with medications; to do so, both the surfactant and the drug transported by the surfactant must preserve their therapeutic functions. This study shows that the Survanta/rCmPI-II combination did not modify the properties of either one in vitro. Inhibition of neutrophil elastase did not increase with respect to the values obtained with each compound separately, which could be related to different mechanisms of action of these compounds. Understanding these mechanisms on activated/stimulated neutrophils would open the door to using the Survanta/rCmPI-II combination to treat inflammatory lung diseases.

Despite these advances, transferring the results with elastase inhibitors from the preclinical phase to the clinical phase is a challenge. Preclinical and clinical research continues, alongside development of new, more powerful and selective inhibitors.[42] Only two of these medications are used in clinical practice to date: alpha-1 antitrypsin (Prolastin), approved by the US Food and Drug Administration in 1987,[43,44] and Sivelestat (ONO-5046), approved for clinical use to treat ARDS and acute lung injury associated with systemic inflammatory response syndrome in Japan and South Korea.[13]

Respiratory failure is one of the causes of death in patients with COVID-19.[45,46] Clinical trials of pulmonary surfactants in COVID-19 are in process.[47,48] Surfactan is indicated in the Cuban COVID-19 Protocols for treatment of these patients.[6,49] The potential benefit of neutrophil elastase inhibitors in patients with severe COVID-19 is being investigated because these inhibitors could mitigate elastase damage on the lung connective tissue and limit the virus spreading capabilities by preventing S protein proteolytic activation.[50]

A limitation of this study is that it does not provide information about the mechanism of action of each of the compounds.

**CONCLUSION**

The anti-elastase effect of the two Cuban products, Survanta and the rCmPI-II inhibitor, is demonstrated in an activated neutrophil model. rCmPI-II does not affect Survanta’s interface property and, therefore, both can be evaluated for combined use in treating inflammatory lung diseases.

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45. Original Research
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