Cellular Physiology and Biochemistry

Cell Physiol Biochem 2019;53:1015-1028
DOI: 10.33594/000000194
Published online: 20 December 2019
Accepted: 12 December 2019

Original Paper

Clinical Development of Sphingosine as Anti-Bacterial Drug: Inhalation of Sphingosine in Mini Pigs has no Adverse Side Effects

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Key Words
Sphingosine • Inhalation • Lung • Side effects • Inflammation • Cell death

Abstract
Background/Aims: Pulmonary infections with *Pseudomonas aeruginosa* (*P. aeruginosa*) or *Staphylococcus aureus* (*S. aureus*) are of utmost clinical relevance in patients with cystic fibrosis, chronic obstructive pulmonary disease, after trauma and burn, upon ventilation or in immuno-compromised patients. Many *P. aeruginosa* and *S. aureus* strains are resistant to many known antibiotics and it is very difficult or often impossible to eradicate the pathogens in patient´s lungs. We have recently shown that the sphingoid base sphingosine very efficiently kills many pathogens, including for instance *P. aeruginosa*, *S. aureus* or *Acinetobacter baumannii*, *in vitro*. *In vivo* experiments of our group on cystic fibrosis mice indicated that inhalation of sphingosine prevents or eliminates existing acute or chronic pneumonia with *P. aeruginosa* or *S. aureus* in these mice. We also demonstrated that sphingosine is safe to use for inhalation up to high doses, at least in mice. To facilitate development of sphingosine to an anti-bactericidal drug that can be used in humans for inhalation, safety data on non-rodents,

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larger animals are absolutely required. ***Methods:*** Here, we inhaled mini pigs with increasing doses of sphingosine for 10 days and analyzed the uptake of sphingosine into epithelial cells of bronchi as well as into the trachea and lung and the systemic circulation. Moreover, we measured the generation of ceramide and sphingosine 1-phosphate that potentially mediate inflammation, the influx of leukocytes, epithelial cell death and disruption of the epithelial cell barrier. ***Results:*** We demonstrate that inhalation of sphingosine results in increased levels of sphingosine in the luminal membrane of bronchi and the trachea, but not in systemic accumulation. Inhaled sphingosine had no side effects up to very high doses. ***Conclusion:*** In summary, we demonstrate that inhalation of sphingosine results in an increase of sphingosine concentrations in the luminal plasma membrane of tracheal and bronchial epithelial cells. The inhalation has no systemic or local side effects.

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) and *Staphylococcus aureus* (*S. aureus*) are ubiquitous and opportunistic pathogens that cause severe respiratory tract and systemic infections, especially among patients with cystic fibrosis (CF), previous viral infections, burn wounds, trauma, or sepsis [1-6]. Worldwide, *P. aeruginosa* has become the most common gram-negative pathogen associated with community-acquired pneumonia (17% of all cases), nosocomial pneumonia (25% of all cases) [7], and ventilator-associated pneumonia in intensive care units (28% of all cases) [8]. Reported mortality rates associated with ventilator-associated pneumonia range from 33% to 72% [8]. Chronic airway infections with *P. aeruginosa* are also common among patients with bronchiectasis and advanced-stage chronic obstructive pulmonary disease (COPD) [9], which affects approximately 15% of the populations of North America and Europe. Intermittent colonization with *P. aeruginosa* is observed in approximately 30% of patients with COPD. Chronic *P. aeruginosa* airway infections that lead to substantial morbidity and mortality rates occur among 5% of COPD patients [10].

However, most important are *P. aeruginosa* and *S. aureus* infections among patients with CF. CF is the most common recessively inherited disorder in North America and Europe [11, 12]: more than 80, 000 persons with CF live in the United States and Europe alone [https://www.cff.org]. The most frequent cause of morbidity and mortality among CF patients is chronic pulmonary infection with bacterial pathogens, in particular *P. aeruginosa* [13, 14]. Approximately 80% of all CF patients are chronically infected with *P. aeruginosa* by the age of 25. Many strains of *P. aeruginosa* and *S. aureus* are highly resistant to existing antibiotics, and attempts to eradicate pulmonary *P. aeruginosa* or *S. aureus* among CF or COPD patients usually fail [15, 16]. Thus, it is important to develop novel strategies for treating pulmonary infections caused by *P. aeruginosa* and *S. aureus*.

We and others have recently reported that the lipid sphingosine, a sphingoid long-chain base that is generated from ceramide via the enzyme (acid) ceramidase, efficiently kills many bacterial species *in vitro* and *in vivo*, including *P. aeruginosa*, *S. aureus* (even MRSA), *Acinetobacter baumannii*, *Escherichia coli*, and *Neisseria meningitides* [17-23]. Our studies demonstrated that sphingosine is abundantly expressed on the luminal surface of human nasal epithelial cells obtained from healthy subjects and on epithelial cells of trachea and conducting bronchi in healthy humans and wild-type mice, whereas it is almost undetectable on the surface of nasal epithelial cells from CF patients and on tracheal and bronchial cells from CF mice [17, 18, 22]. Thus, CF mice and patients exhibit a severe reduction of sphingosine concentrations in airway epithelial cells. Mechanistically, we have shown that the initial accumulation of ceramide in bronchial and tracheal epithelial cells seen in CF results in trapping and ectopic surface expression of β1 integrins in these epithelial cells, and that these integrin molecules, via still-unknown mechanisms, mediate a downregulation of acid ceramidase expression [18]. This downregulation of the expression of acid ceramidase, which converts ceramide into sphingosine, results in a feed-forward cycle of additional

ceramide accumulation and β1-integrin clustering and in a further marked reduction in sphingosine levels [18].

Most importantly, treating CF mice with inhaled sphingosine eliminated existing chronic pulmonary *P. aeruginosa* infections and prevented new *P. aeruginosa* or *S. aureus* infections in these mice [17, 18], a finding demonstrating that sphingosine plays a key role in the innate and immediate defense of the upper respiratory tract. Likewise, the inhalation of recombinant human acid ceramidase by CF mice restored epithelial airway sphingosine levels and reversed acute and chronic infection with *P. aeruginosa* [18].

These studies indicate that sphingosine efficiently kills pathogens, but they did not investigate toxic effects of sphingosine. To develop sphingosine to a drug against bacterial pneumonia, it is essential to test for any side effects of inhaled sphingosine in different animal models. Here, we inhaled mini pigs with increasing doses of sphingosine and determined sphingosine uptake into bronchial epithelial cells, a possible generation of ceramide and sphingosine 1-phosphate, death of bronchial epithelial cells, influx of inflammatory cells into the lung and blood parameters. Sphingosine was incorporated into bronchial epithelial cells after inhalation. We did not observe any toxic or pro-inflammatory side effects of inhaled sphingosine.

Materials and Methods

Mini pigs

Goettingen mini pigs were obtained at an age between 15 and 25 weeks from Ellegaard Minipigs A/S, Soroe Landevej 302, DK-4261 Dalmose, Denmark. The pigs were allowed to adapt onto the new environment for at least 7 days. Animals were investigated according to FELASA criteria and were free of pig-specific pathogens (SPF-status). The health status was again tested upon arrival. Pigs were further tested for nasal methicillin-resistant *S. aureus* as well as fecal Campylobacter, Salmonella, Shigella, Aeromonas and Yersinia species. All tests were negative except a colonization of two pigs with *Aeromonas caviae*. The two pigs did not show any symptoms of a gastrointestinal infection and no further treatment was initiated. We also investigated for gastrointestinal parasites. These tests were all negative. Animals were prophylactically treated with Baycox (Toltrazuril, 20 mg/kg) against Coccidiosis. In one pig we detected Coccidia oocytes in the feces, which rapidly disappeared after treatment.

Pigs were housed within the pig facility of the University Hospital Essen, University of Duisburg-Essen, Germany. All procedures performed on the animals were approved by the LANUV, Recklinghausen, Germany, under the permission number # 84-02.04.2017.A266.

Inhalation experiments

Sphingosine (SPH) (Avanti Polar Lipids, Alabaster, USA) was resuspended as a 20 mM stock solution in distilled water containing 10% octylglucopyranoside (OGP) (Sigma). Prior to use, the sphingosine stock was sonicated in a bath sonicator for 10 min to promote the formation of micelles. The sphingosine stock was diluted in 0.9% saline to a 10 μM, 125 μM, 250 μM or 1 mM solution. Pigs inhaled 3 mL of these 10 μM, 125 μM, 250 μM or 1 mM sphingosine solution in 0.9% NaCl/0.005%, 0.0625%, 0.125% or 0.5% OGP, respectively. Control pigs were inhaled with 0.9% NaCl only or with 0.125% OGP in 0.9% NaCl or left untreated. Pigs were inhaled using a NEBUTEC device (# DM-2000/SaDoMa®-II, Germany), which produced particles of 3.54 μm size, which corresponds to the size often used in inhalation devices for treatment of cystic fibrosis patients. Pigs were inhaled twice daily for 14 days, except on weekends. This regimen is similar to planned clinical phase I and II studies on volunteers and patients, in which we would inhale 1-2-times per day over a period of 14 days with a break on the weekends for safety reasons. Pigs were placed in a hammock for inhalation and manually held in the hammock. The entire inhalation procedure was trained with the pigs using 0.9% NaCl for inhalation for at least 21 days prior to inhalation of sphingosine. This period of training allowed us to perform the subsequent experimental inhalation without stressing the animals and without any sedation, which may impact the inhalation.

Biopsies

Biopsies from the bronchi were obtained 17 hrs after the last inhalation of sphingosine. To this end, the pigs were sedated using intramuscular injection of Ketamine (30 mg/kg body weight) and Xylazine (2 mg/kg body weight) and anesthetized by intravenous injection of Ketamine and Midazolam until disappearance of relevant reflexes has been achieved. Pigs were then subjected to a bronchoscopy employing a fiberoptic videoscope from Ambo A/S, Baltorpbakken 13, DK-2750 Ballerup, Denmark. An illuminated spattle was used to push tongue and larynx caudally to allow intubation of the trachea. The bronchial system was examined for pathological changes especially signs of inflammation, epithelial disintegration and tumors. The videoscope was then positioned in target areas. Biopsies from the larger bronchi for histological and biochemical studies were taken using toothed (alligator) forceps and biopsies. The biopsy specimens were immediately fixed in 4% PFA for 40 hrs or shock-frozen in liquid nitrogen.

Uptake studies

To determine the uptake of sphingosine into the lung, circulation and other organs, we inhaled one group of pigs (n=4) once with 3.8 mL of 125 µM deuterated sphingosine (D-erythro-sphingosine-d7, Avanti Polar Lipids, # 860657P). The pigs were sacrificed 60 mins after this inhalation and tissue samples were obtained from the trachea, lungs (bronchi, alveoli in the periphery), liver, kidney, spleen and blood. The samples were immediately shock-frozen and analyzed by mass spectrometry as described below.

Blood samples

In addition to obtaining tissue biopsies, we collected venous blood samples from the ear veins of the pigs and analyzed blood cell counts and a panel of blood chemistry values.

Mass spectrometry

Biopsies were shock-frozen for mass spectrometry.

Sphingolipids. Porcine tissue was homogenized in aqueous buffered solution with a Bead Ruptor 12 (Omni International, Kennesaw, USA). Aliquots of the homogenates were subjected to lipid extraction with 1.5 mL methanol/chloroform (2:1, v:v) as described [24]. The extraction solvent contained C_{17}-ceramide (Cer17), C_{16}-d_{31}-sphingomyelin (d_{31}-SM16), d_7-sphingosine (d_7-SPH) and d_7-sphingosine 1-phosphate (d_7-S1P) (all Avanti Polar Lipids) as internal standards. To study the uptake of inhaled deuterated sphingosine, the latter two internal reference compounds were replaced by C_{17}-sphinganine (C17 dhSPH) (Avanti Polar Lipids). Lipid extracts were subjected to LC-MS/MS analysis using a 1260 Infinity HPLC coupled to a 6490 triple-quadrupole mass spectrometer (both Agilent Technologies, Waldbronn, Germany) operating in the positive electrospray ionization mode (ESI+). Sphingolipids and their deuterated analogues (*m/z* ratios given in parentheses) were analyzed by selected reaction monitoring (SRM) using the following mass transitions: *m/z* 288.5 → 270.5 for C17 dhSPH, *m/z* 300.3 (307.3) → 282.3 (289.3) for SPH, *m/z* 380.3 (387.3) → 264.3 (271.3) for S1P, *m/z* 520.5 (527.6) → 264.3 (271.3) for Cer16, *m/z* 534.5 → 264.3 for Cer17, *m/z* 548.5 (555.6) → 264.3 (271.3) for Cer18, *m/z* 576.6 (583.6) → 264.3 (271.3) for Cer20, *m/z* 604.6 (611.6) → 264.3 (271.3) for Cer22, *m/z* 630.6 (637.7) → 264.3 (271.3) for Cer24:1, *m/z* 632.6 (639.7) → 264.3 (271.3) for Cer24, *m/z* 703.6 (710.6) → 184.1 for SM16, *m/z* 731.6 (738.7) → 184.1 for SM18, *m/z* 734.8 → 184.1 for d_{31}-SM16, *m/z* 759.6 (766.7) → 184.1 for SM20, *m/z* 787.7 (794.7) → 184.1 for SM22, *m/z* 813.7 (820.7) → 184.1 for SM24:1 and *m/z* 815.7 (822.7) → 184.1 for SM24. Quantification was performed with MassHunter Software (Agilent Technologies). Determined sphingolipid amounts were normalized to the actual protein content of the tissue homogenate used for lipid extraction.

Fatty aldehydes and fatty acids (d_7-SPH uptake study only). Aliquots of tissue homogenates prepared as described above were subjected to fatty acid and aldehyde extraction according to our optimized protocol [25]. Pentadecanal and heptadecanoic acid (both Sigma-Aldrich, Taufkirchen, Germany) served as internal standards. Dried organic extracts were derivatized using 2-diphenylacetyl- 1, 3-indandione-1-hydrazone (DAIH) and *N*-(3-dimethylaminopropyl)-*N*′-ethyl- carbodiimide hydrochloride (EDC hydrochloride) (both from Sigma-Aldrich) to form fatty aldehyde and acid derivatives ionizable by negative mode electrospray ionization (ESI-) [26]. To investigate whether inhaled d_7-SPH exited the sphingolipid metabolism pathway via S1P lyase-catalyzed S1P break-down, we analyzed the derivatized samples for (2*E*)-hexadecenal-d_7 (2*E*HD-d_7), (2*E*)-hexadecenoic acid-d_7 (2*E*HD-COOH-d_7) and hexadecanoic acid-d_7 (palmitate-d_7) using the above mentioned LC-MS/MS instrumentation. The following multiple reaction monitoring (MRM)

transitions were recorded: *m/z* 580.4 → 256.2 / 323.1 for 2*E*HD-d_7, *m/z* 596.4 → 159.1 / 402.4 for 2*E*HD-COOH-d_7 and *m/z* 598.4 → 159.1 / 404.4 for palmitate-d_7. Methodological details and MS/MS parameter of unlabeled fatty aldehydes and acids (including internal standards) that were measured simultaneously can be taken from ref. [26].

Antibodies and reagents

Ceramide or sphingosine stainings were performed with the monoclonal mouse anti-ceramide antibody clone S58-9 (#MAB_0011, Glycobiotech), or the monoclonal mouse anti-sphingosine antibody clone NHSPH (#ALF-274042010, Alfresa Pharma Corporation), which were visualized with Cy3 donkey anti-mouse IgM $F(ab)_2$ fragments (#715-166-020; Jackson ImmunoResearch).

Immunohistochemistry

Stainings were performed as previously reported [17, 18, 22, 27, 28]. The PFA-fixed tissue biopsies were serially dehydrated with an ethanol to xylol gradient and then embedded in paraffin. Samples were sectioned at 7 μm, dewaxed and rehydrated. To achieve retrieval of the antigens, sections were treated with pepsin (Digest All; #003009, Invitrogen) for 30 min at 37°C and then washed with distilled water. Unspecific binding sites were blocked by 10 min incubation with PBS, 0.05% Tween 20 (Sigma) and 5% fetal calf serum (FCS) at room temperature. The samples were then incubated with anti-ceramide- (1:100 dilution) or anti-sphingosine- (1:1000 dilution) antibodies in H/S (132 mM NaCl, 20 mM HEPES [pH 7.4], 5 mM KCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$, 0.8 mM $MgSO_4$) plus 1% FCS at room temperature for 45 min. Samples were then washed three times with PBS plus 0.05% Tween 20, 5 min per wash step, and once with PBS. The sections were secondarily labeled with Cy3-coupled anti-mouse IgM $F(ab)_2$ fragments (Jackson Immunoresearch) in H/S plus 1% FCS for 30 min. The sections were again washed three times with PBS plus 0.05% Tween 20, each 5 min, and once with PBS. The samples were embedded in Mowiol and analyzed on a Leica TCS-SP5 confocal microscope employing a 40 × lens. Images were analyzed with Leica LCS software version 2.61 (Leica Microsystems, Mannheim, Germany). All comparative samples were measured at identical settings.

All immunostainings were controlled with control antibodies that showed no or very weak staining. These were mouse IgM for the antibodies against sphingosine and ceramide. We also included controls with secondary Cy3-coupled antibodies only. These controls showed again no or very weak staining.

TUNEL assays

PFA-fixed sections were dewaxed and rehydrated as above. The sections were then treated in 0.1 M sodium citrate (pH 6.0) in a microwave at 450 W for 5 min, washed twice in PBS and the TUNEL reaction was performed with 5 μL TUNEL enzyme, 20 μl TMR label and 25 μL TUNEL dilution buffer following the instructions of the vendor (Roche). Samples were incubated for 60 min at 37°C, washed 3-times in PBS. The samples were then incubated for 10 min at 70°C in PBS to reduce background staining, washed once in PBS, and were finally embedded in Mowiol.

Hemalaun stainings

Lung paraffin sections were dewaxed, rehydrated and washed as above. The specimens were stained for 5 min with hemalaun. Samples were embedded in Mowiol and analyzed on a Leica DMIRE2. In the hemalaun staining studies we used a score for analysis: Grade 0: no change of the epithelial cell layer, basal membrane intact, no evidence of leukocyte influx, less than 2% pyknotic, i.e. dead epithelial cells. Grade 1: small disruptions of the epithelial cell layer, basal membrane intact, very minor leukocyte influx with few singular cells in the epithelial cell layer, less than 5% pyknotic, i.e. dead epithelial cells. Grade 2: Larger disruptions of the epithelial cell layer, basal membrane still intact, scattered leukocyte influx, less than 10% pyknotic, i.e. epithelial dead cells. Grade 3: Larger disruptions of the epithelial cell layer, disrupted basal membrane, massive leukocyte influx, more than 10% pyknotic, i.e. dead epithelial cells.

Quantification and statistical analysis

Data are expressed as arithmetic means ± SD. For the comparison of continuous variables from independent groups we used Student's t-test for two groups and one-way ANOVA for more than two groups followed by post-hoc Student´s t-tests for all pairwise comparisons applying Bonferroni correction for multiple testing. The p-values for the pairwise comparisons were calculated after Bonferroni correction. All

values were normally distributed. The statistical details (n-numbers, mean ± SD and tests) are given in the figure legends. The sample size planning was based on two-sided Wilcoxon-Mann-Whitney tests (software: G*Power Version 3.1.7 of the University of Duesseldorf, Germany). Investigators were blinded for histology analyses and animal identity. Ceramide and sphingosine fluorescence data were quantified using Image J and are expressed as arbitrary units (a.u.). In each specimen 5 randomly chosen areas with 20 different cells were quantified, i.e. 100 cells per specimen were analyzed. We analyzed 3 sections per animal, i.e. 300 cells per animal, in total 1200 cells from the 4 pigs per group. The fluorescence values were averaged with the values obtained in the other photos of the fluorescence microscopy studies. In the TUNEL and hemalaun stainings we analyzed again 300 epithelial cells per animal in 3 sections, a total of 1200 cells in all 4 pigs per group. The results are expressed as percentage of cells. For studying epithelial cell integrity, we used the above-described score.

Results

Inhalation of mini pigs with sphingosine for 14 days, i.e. 10 days of inhalation and breaks on the weekends, did not result in an obvious change of the health status of the mini pigs. We did not observe any inflammation in the nose, mouth or eyes, nor any systemic changes such as lack of activity, reduced food intake, weight loss or any other change of the general health status. We also did not observe any adverse behavior of the pigs during the inhalation.

Analysis of the histological sections by immunofluorescence microscopy demonstrated an accumulation of sphingosine specifically in bronchial epithelial cells after inhalation. Interestingly, we observed a maximum of the local sphingosine concentration with inhalation of a 125 μM sphingosine suspension (Fig. 1A and B). The inhalation of 250 μM and 1 mM sphingosine suspension did not result in a further increase of sphingosine in the epithelial cell layer (Fig. 1A and B).

In contrast to the accumulation of sphingosine in bronchial epithelial cells we did not observe an increase of ceramide in bronchial epithelial cells by immunofluorescence microscopy (Fig. 2A and B).

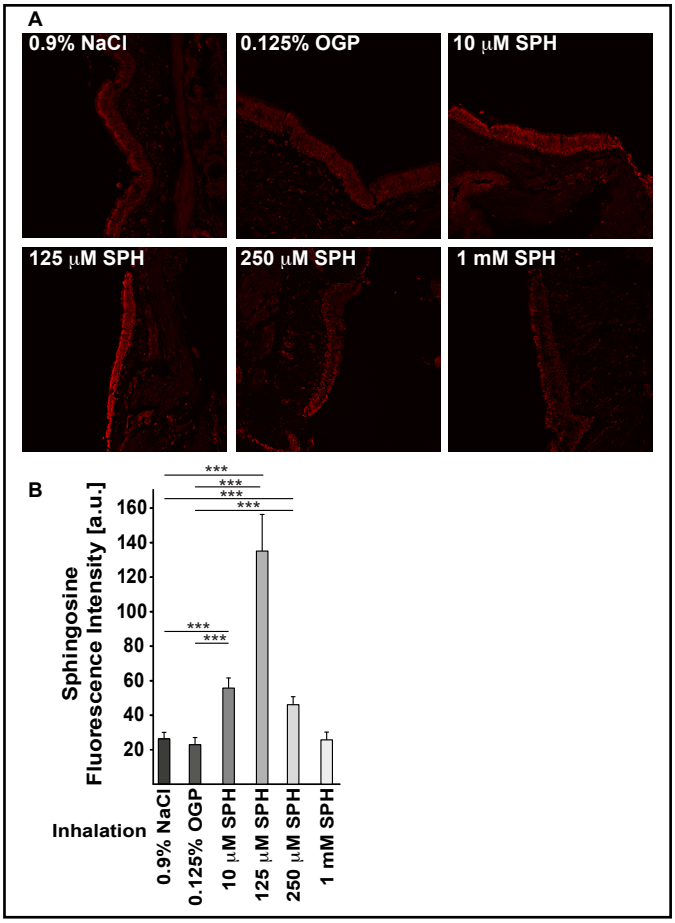

Fig. 1. Histological studies demonstrate an accumulation of sphingosine in bronchial epithelial cells after inhalation. Mini pigs were inhaled with sphingosine at the indicated concentration, with 0.9% NaCl alone or with 0.125% octylglucopyranoside (OGP) as controls. The pigs were subjected to bronchoscopy 17 hrs after the last sphingosine inhalation, biopsies were fixed in paraformaldehyde, embedded in paraffin and sectioned. Sections were stained with Cy3-coupled anti-sphingosine antibodies. Shown are representative immune stainings (A) and the quantitative analysis of the fluorescence intensity of a total of 1200 cells per group (B). Given is the mean ± SD, *p<0.05, **p<0.01, ***p<0.001, ANOVA.

Next, we investigated the distribution and metabolism of inhaled sphingosine applying deuterated (d7) sphingosine and performing measurements after 60 minutes. Mass spectrometry analysis of the biopsies from pigs that were inhaled with 3.8 mL of a 125 μM suspension of deuterated (d7) sphingosine revealed a rapid metabolism of sphingosine. We detected deuterated sphingomyelin and very low levels of deuterated ceramide in the trachea and the lung (Table 1), but did not detect deuterated sphingosine, sphingosine 1-phosphate, (2*E*)-hexadecenal or its fatty acid derivatives. We also detected some very low amounts of deuterated sphingomyelin in inner organs such as liver, heart, kidney and spleen (Table 2), although the levels compared to endogenous levels of sphingomyelin were very low (Table 2). Very low levels of d7-ceramide were also detected in the lung, trachea and liver, but not in other organs (Table 3). We only detected d7-C16-ceramide and no d7-C18-, C20-, C22-, C24- and C24:1-ceramide in these tissues. We did not detect deuterated sphingosine or sphingosine 1-phosphate in inner organs.

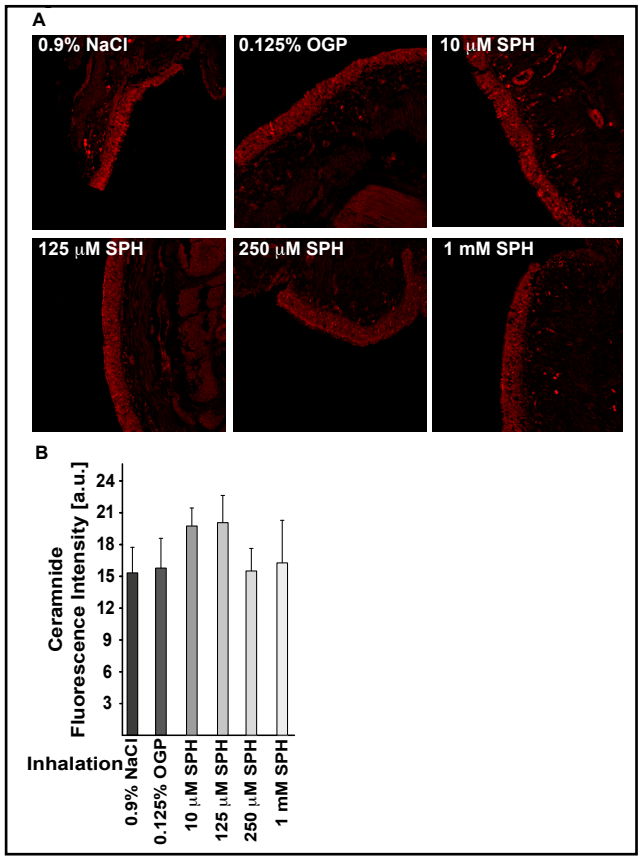

Fig. 2. Ceramide does not increase in bronchial epithelial cells upon inhalation of sphingosine. Paraffin sections from biopsies of the pigs were obtained as above and stained with Cy3-labelled anti-ceramide antibodies. Displayed are representative immune stainings from 4 pigs (A) and the quantitative analysis of the fluorescence intensity of a total of 1200 cells per group (B). Given is the mean ± SD, *p<0.05, **p<0.01, ***p<0.001, ANOVA.

Table 1. Mass spectrometry analysis of sphingomyelin in the biopsies from pigs after inhalation of deuterated d7-sphingosine. Mass spectrometry analysis of the biopsies from pigs that inhaled 3.8 mL of a 125 μM suspension of deuterated d7-sphingosine indicated that inhaled sphingosine reached the trachea and the lung after inhalation of deuterated d7-sphingosine, but is rapidly degraded. Samples were collected post mortem from the pigs 1 h after inhalation of d7 sphingosine. No deuterated sphingosine, sphingosine 1-phosphate or its break-down products (d7-labeled (2E)-hexadecenal, (2E)-hexadecenoic acid or palmitate) were detected in the trachea or lung. We only detected d7-sphingomyelin. Deuterated d7-sphingomyelin was determined in the upper and lower half of the trachea and the lung (Table 1) as well as in spleen, kidney, liver and heart (Table 2). Compared to endogenous levels of sphingomyelin the concentrations of d7-sphingomyelin were very low. Analyzed were 9 samples from the lung, i.e. each 2 or 3 samples per pig and each 4 samples (one from each pig) for the upper and lower half of the trachea, spleen, kidney, liver and heart. Given is the mean ± SD of each parameter, *p < 0.05, **p < 0.01, ***p < 0.001, ANOVA

Parameter	Lung	Trachea upper half	Trachea lower half
d7- sphingomyelin	3.58 ± 1.18 pmol/mg protein	9.73 ± 7.15 pmol/mg protein	6.95 ± 3.96 pmol/mg protein
Total sphingomyelin	35 097 ± 6364 pmol/mg protein	20 558 ± 4378 pmol/mg protein	15 825 ± 5 608 pmol/mg protein

Consistent with these studies, we did not detect a significant change of sphingosine, ceramide, sphingomyelin or sphingosine 1-phosphate in lung biopsies after inhalation of 10 μM, 125 μM, 250 μM or 1 mM sphingosine compared to pigs that inhaled only 0.9% NaCl solution (Table 4).

Next, we determined death of bronchial epithelial cells, integrity of the bronchial epithelial cell layer and influx of leukocytes into the epithelial cell layer by TUNEL and hemalaun stainings. The results revealed that inhaled sphingosine did not induce any cell death (Fig. 3) and did not disturb the epithelial cell integrity (Fig. 4A and B). Likewise, we did not observe an influx of leukocytes into the epithelial cell layer (Fig. 4A and C).

Table 2. Mass spectrometry analysis of sphingomyelin in the biopsies from pigs. For detailed information see Table 1. Given is the mean ± SD of each parameter, *p < 0.05, **p < 0.01, ***p < 0.001, ANOVA

Parameter	Spleen	Kidney	Liver	Heart
d7-sphingomyelin	0.14 ± 0.03 pmol/mg protein	4.7 ± 1.08 pmol/mg protein	10.25 ± 5.25 pmol/mg protein	1.07 ± 0.34 pmol/mg protein
Total sphingomyelin	3 490 ± 720 pmol/mg protein	30 294 ± 4071 pmol/mg protein	5 256 ± 1 186 pmol/mg protein	3 365 ± 1 719 pmol/mg protein

Table 3. Mass spectrometry analysis of ceramide in the biopsies from pigs after inhalation of deuterated d7-sphingosine. Levels of total deuterated d7-ceramide in the upper and lower half of the trachea, the lung and inner organs were very low compared to endogenous ceramide levels after inhalation of d7-sphingosine. We only detected deuterated d7-ceramide in the trachea, lung and liver, but not in heart, kidney and spleen. d7-C16-ceramide was the only detectable labeled ceramide species. Analyzed were 8 samples from the lung, i.e. each 2 samples per pig and each 3 samples for the trachea and liver. Samples were collected post mortem from the pigs 1 h after inhalation of d7 sphingosine. The levels of d7-ceramide were under the detection level in further 4 samples from lung, 5 samples from trachea, 1 sample from liver and in each 4 samples from kidney, heart and spleen. Those negative samples were not included in the calculation of the mean ± SD. Given is the mean ± SD of each parameter, *p < 0.05, **p < 0.01, ***p < 0.001, ANOVA

Parameter	Lung	Trachea	Liver
d7- ceramide	0.44 ± 0.15 pmol/mg protein	1.65 ± 0.67 pmol/mg protein	0.33 ± 0.03 pmol/mg protein
Total ceramide	2 233 ± 710 pmol/mg protein	31 152 ± 782 pmol/mg protein	3 365 ± 1 719 pmol/mg protein

Table 4. Mass spectrometry analysis of endogenous sphingosine, ceramide and sphingosine 1-phosphate in biopsies from pigs after inhalation of increasing doses of sphingosine. Levels of sphingosine, ceramide and sphingosine 1-phosphate (S1P) were determined in bronchoscopy biopsies from pigs 17 hrs after inhalation of 3.8 mL each of 0.9% NaCl, 0.125 % octylglucopyranoside (OGP), 10 μM, 125 μM, 250 μM and 1 mM sphingosine (SPH). Analyzed were each 1 sample/pig with 4 pigs per group. Protein concentrations on the small biopsies were too low to get reliable protein values and we therefore normalized for sphingomyelin, since the studies with d7-sphingosine show that inhalation of sphingosine does not result in a significant change of sphingomyelin. Given is the mean ± SD of each parameter from 4 pigs per group, *p < 0.05, **p < 0.01, ***p < 0.001, ANOVA

Inhalation	Sphingosine	Ceramide	S1P
0.9% NaCl	17.6 ± 9.0 fmol/pmol SM	139.2 ± 92.2 fmol/pmol SM	3.08 ± 4.8 fmol/pmol SM
0.125% OGP	20.5 ± 6.3 fmol/pmol SM	195.1 ± 37.3 fmol/pmol SM	1.46 ± 1.49 fmol/pmol SM
10 μM Sphingosine	23.3. ± 7.5 fmol/pmol SM	114.6 ± 58.1 fmol/pmol SM	1.88 ± 1.65 fmol/pmol SM
125 μM Sphingosine	10.4 ± 2.3 fmol/pmol SM	195.1 37.3 fmol/pmol SM	2.85 ± 2.81 fmol/pmol SM
250 μM Sphingosine	13.8 ± 6.6 fmol/pmol SM	101.5 ± 28.1 fmol/pmol SM	0.58 ± 0.41 fmol/pmol SM
1 mM Sphingosine	12.3 ± 2.9 fmol/pmol SM	101.1 ± 21.1 fmol/pmol SM	0.43 ± 0.15 fmol/pmol SM

In addition, blood studies showed that inhalation of sphingosine had no effects on blood parameters (Table 5). We also did not observe any indications of local effects such as an increase of amylase in the blood (Table 5), for instance by affecting the salivary glands.

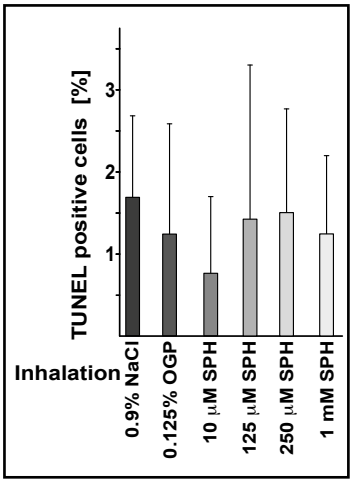

Fig. 3. Inhalation of sphingosine does not induce cell death in bronchi. Paraffin sections from bronchial biopsies from pigs that were inhaled with sphingosine, 0.9% NaCl with 0.125% octylglucopyranoside (OGP) as control or left untreated were stained with TUNEL to determine cell death. The results revealed that inhaled sphingosine did not induce any cell death. Shown is the quantitative analysis of TUNEL stainings of a total of 1200 cells per group. Given is the mean ± SD, *p<0.05, **p<0.01, ***p<0.001, ANOVA.

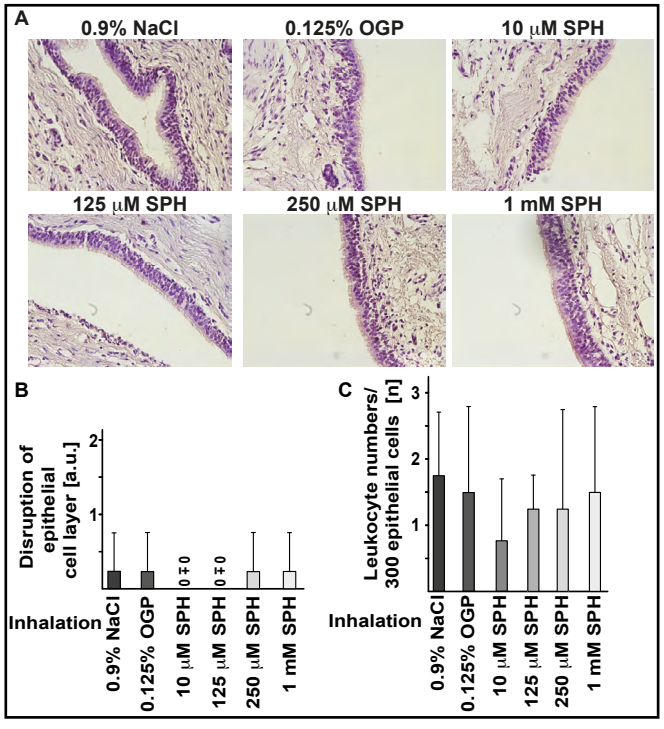

Fig. 4. Inhalation of sphingosine does not affect epithelial cell integrity in bronchi. Paraffin sections from bronchial biopsies from pigs that were inhaled with sphingosine, 0.9% NaCl with 0.125% octylglucopyranoside (OGP) as control or left untreated were stained with hemalaun to analyze the integrity of the bronchial epithelial cell layer and influx of leukocytes into the epithelial cell layer. The studies demonstrated that sphingosine inhalation did not affect epithelial cell integrity (A, B). Sphingosine inhalation also did not induce an influx of leukocytes into the epithelial cell layer (A, C). To determine epithelial cell integrity, we employed the following score: Grade 0: no change of the epithelial cell layer, basal membrane intact, no evidence of leukocyte influx, less than 2% pyknotic, i.e. dead epithelial cells. Grade 1: small disruptions of the epithelial cell layer, basal membrane intact, no evidence of leukocyte influx, less than 5% pyknotic, i.e. dead epithelial cells. Grade 2: Larger disruptions of the epithelial cell layer, basal membrane still intact, no evidence of leukocyte influx, less than 10% pyknotic, i.e. dead epithelial cells. Grade 3: Larger disruptions of the epithelial cell layer, disrupted basal membrane, leukocyte influx, more than 10% pyknotic, i.e. dead epithelial cells. Shown are representative hemalaun stainings from 4 pigs (A) and the quantitative analysis of the epithelial cell integrity (B). In panel C, the number of leukocytes in the epithelial cell layer (total of 300 epithelial cells/pig) was counted. Given are the means ± SD, *p<0.05, **p<0.01, ***p<0.001, ANOVA.

Table 5. Blood parameters after sphingosine inhalation. Blood studies showed that inhalation of sphingosine had not effects on blood parameters. WBC - White blood counts in 10^3 cells/μL, RBC - Red blood counts in 10^6 cells/μL, Hb - Hemoglobin in g/dL, HCT - hematocrit in %, PLT - platelets in 10^3 cells/μL, MCV - mean cell volume in μm^3, MCH - mean cell hemoglobin in pg, MCHC - mean cell hemoglobin concentration in g/dL, T-Pro - total protein in g/dL, Alb - Albumin in g/dL, T-Bil - total bilirubin in mg/dL, GOT - glutamic oxaloacetic transaminase in IU/L, GPT - glutamic pyruvic transaminase in IU/L, LDH - lactate dehydrogenase in IU/L, BUN - blood urea nitrogen in mg/dL, γGT - γ-glutamyltransferase in IU/L, CPK - creatinine-phosphokinase in IU/L, Glucose in mg/dL, Creatinine in in mg/dL, Amylase in IU/L. Given are the means ± SD of each parameter from 4 pigs per group *p < 0.05, **p < 0.01, ***p < 0.001, compared to 0.9% NaCl, ANOVA

Parameter	0.9% NaCl	0.125% OGP	10 μM SPH	125 μM SPH	250 μM SPH	1 mM SPH
WBC	6.2 ± 2.6	6.7 ± 1.4	8.1 ± 3.8	6.1 ± 1.4	6.7 ± 3.1	6.3 ± 1.3
RBC	5.5. ± 1.4	6.2 ± 0.5	6.7 ± 1.2	7.1 ± 0.9	7.5 ± 1.0	7.0 ± 0.9
Hb	11.8 ± 1.0	10.6 ± 0.4	11.0 ± 0.5	10.2 ± 1.3	12.3 ± 2.0	14.2 ± 0.9
HCT	36.5 ± 2.8	32.8 ± 1.3	35.4 ± 2.1	35.5 ± 4.2	40.1 ± 5.8	42.6 ± 2.2
PLT	466 ± 48	522 ± 38	378 ± 29	385 ± 151	455 ± 63	447 ± 33.8
MCV	58.8 ± 5.3	53.3 ± 2.5	54 ± 7	49.8 ± 0.5	54.8 ± 3.1	62.5 ± 5.9
MCH	19.1 ± 2.0	17.3 ± 1.0	16.9 ± 2.6	14.3 ± 0.3	16.3 ± 0.9	20.7 ± 2.2
MCHC	32.4 ± 1.3	32.4 ± 0.6	31.2 ± 0.4	28.6 ± 0.5	30.0 ± 0.17	33.2 ± 0.9
T-Pro	5.6 ± 0.3	5.9 ± 0.3	5.7 ± 0.4	5.3 ± 0.3	5.3 ± 0.2	5.6 ± 0.3
Alb	3.6 ± 0.2	3.7 ± 0.13	3.9 ± 0.06	3.4 ± 0.3	3.3 ± 0.15	3.9 ± 0.2
T-Bil	0.275 ± 0.09	< 0.2*	0.3 ± 0	< 0.2	< 0.2	< 0.2
GOT	< 10	< 10	< 10	< 10	< 10	< 10
GPT	38.8 ± 12.3	31.0. ± 12.8	42.0 ± 13.0	30.8 ± 7.8	30.5 ± 6.3	30 ± 9.8
LDH	750 ± 59	834 ± 152	768 ± 25	947 ± 66*	951 ± 117*	763 ± 51
BUN	6.5 ± 1.7	7.5 ± 1.0	6.0 ± 1.0	5.8 ± 1.0	6.5 ± 1.0	8.3 ± 5.2
γGT	90.8 ± 10.0	67.5 ± 3.1*	82.0 ± 2.5	75.8 ± 8.8	89 ± 14	86.5 ± 5.2
CPK	416 ± 182	601 ± 260	393 ± 48	436.5 ± 295.8	584 ± 193	389 ± 106
Glucose	95 ± 10.8	102.3 ± 14.3	102 ± 42	114 ± 27	97.5 ± 21.6	97.0 ± 18.0
Creatinine	0.63 ± 0.1	0.6 ± 0.0	0.6 ± 0.2	0.6 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
Amylase	2376 ± 303	3265 ± 393*	2844 ± 1051	3404 ± 1550	3365 ± 1062	2788 ± 831

Discussion

In the present study, we demonstrate that inhalation of sphingosine in healthy mini pigs has no side effects in the trachea and the lung. We also did not detect any systemic side effects of sphingosine. These studies suggest that sphingosine is a safe drug to treat bacterial pneumonia. Future studies on infected mini pigs are required to confirm this notion in infected animals and to prove the efficacy of sphingosine to eliminate bacteria in mini pig lungs *in vivo*.

Our data are consistent with previous data obtained in mice [29]: In these studies healthy wild-type mice were inhaled with sphingosine in 0.9% NaCl at concentrations ranging from 10 μM to 1 mM twice daily for 14 days. Similar to the present results, we did not observe any side effects, i.e. induction of inflammation, influx of leukocytes, cell death or disruption of the epithelial cell layers [29].

The local immune defense requires sphingosine in the luminal layer of the tracheal and bronchial epithelial cells [17, 18]. Thus, it is very important to determine the local

concentration of sphingosine in the epithelial cell layer, which we performed by immune stainings of bronchial biopsies. The confocal microscopy studies indicate that inhalation of 10 μM sphingosine results in an approximately 3-fold increase of the surface sphingosine concentration in the bronchial epithelial cell layer. The inhalation of 125 μM sphingosine resulted in an approximately 7-fold increase of the local sphingosine concentration. These concentrations were previously shown to be sufficient to eliminate *P. aeruginosa* and *S. aureus* in pulmonary infected cystic fibrosis mice [17, 18, 22].

Surprisingly, we did not observe a significant increase of sphingosine in bronchial epithelial cells when we inhaled higher doses of sphingosine, i.e. 250 μM or 1 mM sphingosine. It might be possible that sphingosine forms larger micelles at higher concentration that remain in the nose and larynx and/or the mucus and do not reach the epithelial cell layer. In addition, higher concentrations of sphingosine might also trigger the expression of sphingosine kinases and sphingosine 1-phosphate lyase resulting in rapid degradation of sphingosine. This means that the optimal dose for a treatment with inhaled sphingosine would be a 100 - 125 μM suspension.

The confocal microscopy and mass spectrometry data consistently showed that the concentrations of ceramide did not significantly change after inhalation. The mass spectrometry data also excluded an accumulation of sphingosine 1-phosphate in the trachea and lung after inhalation of sphingosine.

Previous studies from our laboratory indicated that a relatively large portion of sphingosine remains in the mucus on top of the epithelial cell layer [30]. In these experiments, sphingosine was locally applied onto the surface. It is technically very difficult to measure the exact concentration of sphingosine in the mucus after inhalation, because the mucus is washed off during fixation and dehydration of histology sections. Biopsies contain a small surface portion and most of the tissue in biopsies consists of submucosal tissue; thus, sphingosine in mucus on the surface is difficult to be determined in biopsies. Lavage fluid would also not reflect the local concentration of sphingosine in the luminal membrane of bronchial epithelial cells, which are not present in the lavage fluid. Further, the lavage fluid contains surface mucus from different areas of the bronchial tree and even alveoli. The local surface concentration of sphingosine could be determined in frozen sections that are subjected to immune fluorescence staining or, as best option, the local surface concentration of sphingosine could be determined by a surface kinase assay specifically measuring the concentration of sphingosine in the mucus and the luminal membrane of the epithelial cell layer in the trachea and the bronchi as previously described [18, 30].

The mass spectrometry experiments using deuterated d7-sphingosine suggested that the half-life time of sphingosine in the respiratory tract is very short and that most of the inhaled sphingosine is rapidly consumed. We did not detect a significant difference of d7-sphingomyelin amounts between the center of the lung and the periphery of the lung, indicating that we achieve a relatively homogenous distribution of the inhaled d7-sphingosine. The trachea and the lung together contain approximately 20 pmol d7-sphingomyelin/mg protein. The pieces of tissue, which we investigated for mass spectrometry contained approximately 1 cm^2 epithelial cell layer and certainly much more other cells from the submucosa, cartilage, etc. We inhaled 475 nmol deuterated sphingosine. If we assume that the lung of a pig has a surface of only 10 m^2 (the surface of the human lung is 100 m^2 and the pigs have a weight of 20 kg and, thus, the surface of the lung is more likely close to 20 m^2) the local concentration of the d7-sphingosine would be approximately 5 pmol/cm^2. This suggests that most of the d7-sphingosine was inhaled, but rapidly degraded. It should be indicated that the organ concentrations of endogenous sphingomyelin are 500 - 20 000-fold higher than the amount of d7-sphingomyelin and, thus, they are not significantly changed by the inhalation.

It should be noted that the studies on biopsies shown in Table 4 were done with very small biopsies obtained by bronchoscopy, while the studies measuring d7 sphingosine in Table 3 were performed with larger specimen obtained after sacrificing the pigs. This very likely also explains the high standard deviations in the samples reported in Table 4, i.e. the

bronchoscopy biopsies. Further, the bronchoscopy biopsies might be more variable and contain variable amounts of epithelial cells.

The fluorescence microscopy studies that were performed on tissues from pigs that were inhaled 17 hrs prior to the biopsies show a higher concentration of sphingosine in luminal membranes of the bronchial epithelial cells. The discrepancy between the mass spectrometry and immunofluorescence studies suggests that surface sphingosine within the plasma membrane is relatively stable, while sphingosine that is taken up by cells and the tissue is rapidly consumed and degraded. However, the luminal surface concentration of sphingosine has been shown to be approximately 1 pmol per total trachea surface in mice [17, 18]. This is beyond the detection limit of the employed mass spectrometry. We have previously shown that the anti-sphingosine antibody specifically detects sphingosine and does not detect sphingomyelin [17, 18]. The exact metabolism of sphingosine after inhalation and in particular the local surface concentration need to be determined in future studies employing surface kinase assays measuring the local sphingosine concentration using the activity of a sphingosine kinase in the presence of [^{32}P]γATP as previously described [17, 18]. These studies should also include detailed kinetics of the sphingosine concentration in the epithelial cell layer and the mucus on top of the epithelial cell layer.

Conclusion

In summary, we demonstrate that inhalation of sphingosine results in an increase of sphingosine concentrations in the luminal plasma membrane of tracheal and bronchial epithelial cells. The inhalation has no systemic or local side effects.

Acknowledgements

Author Contributions

HC and AC performed the bronchoscopies, FS and BK the mass spectrometry. SK, MK, CK, CS, RV and AS inhaled the pigs, MK and CK performed blood studies. AW and MD supervised housing of the pigs and performed the anesthesia. GH, MJE, MK, KAB and EG designed the studies. EG initiated the studies, supervised research and analyzed the histology studies.

Ethical Statement

All animal experiments conform to internationally accepted standards and have been approved by the appropriate institutional review body, i.e. LANUV, Recklinghausen, Germany, under the permission number # 84-02.04.2017.A266.

Funding Sources

The study was supported by DFG grant GU 335/35-1.

Disclosure Statement

The authors have no conflicts of interest to declare.

References

1 Crouch Brewer S, Wunderink RG, Jones CB, Leeper KV Jr: Ventilator-assisted pneumonia due to *Pseudomonas aeruginosa*. Chest 1996;109:1019-1029.

2 McManus AT, Mason AD Jr, McManus WF, Pruitt BA Jr: Twenty-five year review of *Pseudomonas aeruginosa* bacteremia in a burn center. Eur J Clin Microbiol 1985;4:219-223.

3 Vidal F, Mensa J, Almela M, Martínez JA, Marco F, Casals C, Gatell JM, Soriano E, Jimenez de Anta MT: Epidemiology and outcome of *Pseudomonas aeruginosa* bacteremia, with special emphasis on the influence of antibiotic treatment. Analysis of 189 episodes. Arch Intern Med 1996;156:2121-2126.
4 Morrison AJ Jr, Wenzel RP: Epidemiology of infections due to *Pseudomonas aeruginosa*. Rev Infect Dis 1984;6:S627-642.
5 Currie AJ, Speert DP, Davidson DJ: *Pseudomonas aeruginosa*: role in the pathogenesis of the CF lung lesion. Semin Respir Crit Care Med 2003;24:671-680.
6 Rao S, Grigg J: New insights into pulmonary inflammation in cystic fibrosis. Arch Dis Child 2006;91:786-788.
7 Poch DS, Ost DE: What are the important risk factors for healthcare-associated pneumonia? Semin Respir Crit Care Med 2009;30:26-35.
8 American Thoracic Society, Infectious Diseases Society of America: Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. Am J Respir Crit Care Med 2005;171:388-416.
9 Murphy TF: *Pseudomonas aeruginosa* in adults with chronic obstructive pulmonary disease. Curr Opin Pulm Med 2009;15:138-142.
10 Martínez-Solano L, Macia MD, Fajardo A, Oliver A, Martinez JL: Chronic *Pseudomonas aeruginosa* infection in chronic obstructive pulmonary disease. Clin Infect Dis 2008;47:526-533.
11 Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL, et al.: Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 1989;245:1066-1073.
12 Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, Buchwald M, Tsui LC: Identification of the cystic fibrosis gene: genetic analysis. Science 1989;245:1073-1080.
13 Elborn JS: Cystic fibrosis. Lancet 2016;388:2519-2531.
14 Döring G, Gulbins E: Cystic fibrosis and innate immunity: how chloride channel mutations provoke lung disease. Cell Microbiol 2009;11:208-216.
15 Bhagirath AY, Li Y, Somayajula D, Dadashi M, Badr S, Duan K: Cystic fibrosis lung environment and *Pseudomonas aeruginosa* infection. BMC Pulm Med 2016;16:174.
16 Schaffer K: Epidemiology of infection and current guidelines for infection prevention in cystic fibrosis patients. J Hosp Infect 2015;89:309-313.
17 Pewzner-Jung Y, Tavakoli Tabazavareh S, Grassmé H, Becker KA, Japtok L, Steinmann J, Joseph T, Lang S, Tuemmler B, Schuchman EH, Lentsch AB, Kleuser B, Edwards MJ, Futerman AH, Gulbins E: Sphingoid long chain bases prevent lung infection by *Pseudomonas aeruginosa*. EMBO Mol Med 2014;6:1205-1214.
18 Grassmé H, Henry B, Ziobro R, Becker KA, Riethmüller J, Gardner A, Seitz AP, Steinmann J, Lang S, Ward C, Schuchman EH, Caldwell CC, Kamler M, Edwards MJ, Brodlie M, Gulbins E: β1-Integrin accumulates in cystic fibrosis luminal airway epithelial membranes and decreases sphingosine, promoting bacterial infections. Cell Host Microbe 2017;21:707-718.
19 Bibel DJ, Aly R, Shinefield HR: Antimicrobial activity of sphingosines. J Invest Dermatol 1992;98:269-273.
20 Fischer CL, Walters KS, Drake DR, Blanchette DR, Dawson DV, Brogden KA, Wertz PW: Sphingoid bases are taken up by *Escherichia coli* and *Staphylococcus aureus* and induce ultrastructural damage. Skin Pharmacol Physiol 2013;26:36-44.
21 Azuma MM, Balani P, Boisvert H, Gil M, Egashira K, Yamaguchi T, Hasturk H, Duncan M, Kawai T, Movila A: Endogenous acid ceramidase protects epithelial cells from *Porphyromonas gingivalis*-induced inflammation *in vitro*. Biochem Biophys Res Commun 2018;495:2383-2389.
22 Tavakoli Tabazavareh S, Seitz A, Jernigan P, Sehl C, Keitsch S, Lang S, Kahl BC, Edwards M, Grassmé H, Gulbins E, Becker KA: Lack of sphingosine causes susceptibility to pulmonary *Staphylococcus aureus* infections in cystic fibrosis. Cell Physiol Biochem 2016;38:2094-2102.
23 Becam J, Walter T, Burgert A, Schlegel J, Sauer M, Seibel J, Schubert-Unkmeir A: Antibacterial activity of ceramide and ceramide analogs against pathogenic Neisseria. Sci Rep 2017;7:17627.
24 Gulbins A, Schumacher F, Becker KA, Wilker B, Soddemann M, Boldrin F, Müller CP, Edwards MJ, Goodman M, Caldwell CC, Kleuser B, Kornhuber J, Szabo I, Gulbins E: Antidepressants act by inducing autophagy controlled by sphingomyelin-ceramide. Mol Psychiatry 2018;23:2324-2346.

25 Neuber C, Schumacher F, Gulbins E, Kleuser B: Mass spectrometric determination of fatty aldehydes exemplified by monitoring the oxidative degradation of (2E)-Hexadecenal in HepG2 cell lysates, in Wood P (ed.): Lipidomics, vol 125. Humana Press, New York, NY.

26 Neuber C, Schumacher F, Gulbins E, Kleuser B: Method to simultaneously determine the sphingosine 1-phosphate breakdown product (2E)-hexadecenal and its fatty acid derivatives using isotope-dilution HPLC-electrospray ionization-quadrupole/time-of-flight mass spectrometry. Anal Chem 2014;86:9065-9073.

27 Teichgräber V, Ulrich M, Endlich N, Riethmüller J, Wilker B, De Oliveira-Munding CC, van Heeckeren AM, Barr ML, von Kürthy G, Schmid KW, Weller M, Tümmler B, Lang F, Grassme H, Döring G, Gulbins E: Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis. Nat Med 2008;14:382-391.

28 Grassmé H, Jekle A, Riehle A, Schwarz H, Berger J, Sandhoff K, Kolesnick R, Gulbins E: CD95 signaling via ceramide-rich membrane rafts. J Biol Chem 2001;276:20589-20596.

29 Martin GE, Boudreau RM, Couch C, Becker KA, Edwards MJ, Caldwell CC, Gulbins E, Seitz A: Sphingosine's role in epithelial host defense: A natural antimicrobial and novel therapeutic. Biochimie 2017;141:91-96.

30 Seitz AP, Schumacher F, Baker J, Soddemann M, Wilker B, Caldwell CC, Gobble RM, Kamler M, Becker KA, Beck S, Kleuser B, Edwards MJ, Gulbins E: Sphingosine-coating of plastic surfaces prevents ventilator-associated pneumonia. J Mol Med (Berl) 2019;97:1195-1211.