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Bile salt enhancers for inhalation: correlation between *in vitro* and *in vivo* lung effects

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Abstract

The lungs have potential as a means of systemic drug delivery of macromolecules. Systemic delivery requires crossing of the air-blood barrier, however with molecular size-dependent limitations in lung absorption of large molecules. Systemic availability after inhalation can be improved by absorption enhancers, such as bile salts. Enhancers may potentially interfere with the different constituents of the lungs, e.g. the lung surfactant lining the alveoli or the lung epithelium. We used two *in vitro* models to investigate the potential effects of bile salts on lung surfactant function (with the constrained drop surfactometer) and on the epithelium in the proximal airways (with the MucilAir^{TM} cell system), respectively. In addition, we measured direct effects on respiration in mice inhaling bile salt aerosols. The bile salts inhibited lung surfactant function at different dose levels, however they did not affect the integrity of ciliated cells at the tested doses. Furthermore, the bile salt aerosols induced changes in the breathing pattern of mice indicative of pulmonary irritation. The bile salts were ranked according to potency *in vitro* for surfactant function disruption and *in vivo* for induction of pulmonary irritation. The ranking was the same, suggesting a correlation between the interference with lung surfactant and the respiratory response.

Keywords

Lung surfactant, constrained drop surfactometer, inhaled pharmaceutical enhancer, bile salt, 3D human airway *in vitro* model, MucilAir^{TM}, alternative method.

Chemical compounds studied in this paper

Sodium deoxycholate (PubChem CID: 23668196); Sodium taurodeoxycholate (PubChem CID: 23664773); Sodium glycocholate (PubChem CID: 23670522); Sodium taurocholate (PubChem CID: 23666345)

Abbreviations

ADSA: axisymmetric drop shape analysis, ATP: adenosine triphosphate, APIs: active pharmaceutical ingredients, BPM: breaths per minute, bw: body weight, CDS: constrained drop surfactometer, CMC: critical micelle concentration, DMSO: dimethylsulfoxid, GLP: good laboratory practice, IP: impregnation product, kDa: kilo Dalton, LDH: lactate dehydrogenase, LS: lung surfactant, NA: not applicable, NaDCA: sodium deoxycholate, NaGCA: sodium glycocholate, NaTCA: sodium taurocholate, NaTDCA: sodium taurodeoxycholate, OPS: optical particle sizer, PBS: Phosphate-buffered saline, RSB: rapid shallow breathing, sd: standard deviation, TB: time of break, TE: time of expiration, TEER: trans-epithelial electrical resistance, TI: time of inspiration, TP: time of pause, VT: tidal volume

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1. Introduction

Inhalation of therapeutic agents is essential for treatment of respiratory diseases, where local delivery targets the molecules to the diseased organ whilst limiting systemic exposure. Furthermore, inhalation is promising as a means of systemic delivery of active pharmaceutical ingredients (APIs), such as biomolecules. In this case fast and efficient absorption is essential. Drugs can be delivered to the airways via dry powder aerosols, these typically contain excipients, such as carriers. To optimize gas-exchange, the lungs have a large surface area and a thin barrier between the air and the systemic circulation. The lungs are however also designed as a barrier to foreign substances as the continuous branching of the airways constitutes an efficient filter for particles entering the lungs. Generally, large particles are retained in the upper/central airways, and removed by constant upward movement by mucus covered ciliated cells, while smaller particles (<2 μm in aerodynamic diameter) reach the respiratory region of the lungs, the alveoli and respiratory bronchioles (Pulliam et al., 2007).

To enter the blood stream, APIs need to cross not only the alveolar-capillary cell barrier, but also the layer of lung surfactant (LS) that covers the alveolar epithelium. The movement of large biomolecules across the air-blood barrier is restricted, where the quantity of the biomolecule that transfers from the lung lumen to the blood is inversely proportional to size. These larger molecules (defined as >40 kDa) will predominantly be retained in the lungs and will not reach the systemic circulation (Hastings et al., 2004; Pfister et al., 2014). To make inhaled macromolecules systemically bioavailable, absorption enhancers can be added to the formulation (Hussain et al., 2004). Identifying enhancers that do not evoke adverse effects on the respiratory tract will greatly improve the potential for pulmonary drug delivery of systemically acting APIs.

Development of new inhalation therapeutics is complex and attrition during the development process has historically been high. Analysis of the AstraZeneca drug portfolio between 2005 and 2010 showed that safety attrition of inhaled candidate drugs due to toxicity to the respiratory system accounted for about 30% of the project closures related to adverse respiratory effects (Cook et al., 2014). These closures occurred relatively late in the development process, i.e. at the stage of comprehensive regulatory GLP toxicology studies or during clinical evaluation. The high rate of attrition highlights the lack of available predictive *in*

vitro and *in vivo* tools for screening for toxicity in the respiratory tract. Absorption enhancers work by altering the local physiology of the air-blood barrier and their invasive mode of action may pose an especially high potential for induction of unwanted reactions in the lungs. These effects could include sustained alterations in the mucus layer in the upper airways or opening of tight junctions between epithelial cells, direct damage of epithelial cells by solubilization of membrane phospholipids, or increased transfer of other biomolecules than the intended API.

In the present study we have focused on bile salts as enhancers for systemic delivery of APIs via the lungs. Bile salts are amphipathic cholesterol derivatives that increase the dispersion of lipids and facilitate their absorption by the intestinal mucosa in the small intestine. Based on these properties, bile salts have been investigated for their applicability as enhancers in inhaled formulations of peptide- and protein-based drug formulations (Okumura et al., 1992; Komada et al., 1994; Yamamoto et al., 1994; Bäckström et al., 1996; Yamamoto et al., 1997; Griese et al., 1998; Herting et al., 2001; Johansson et al., 2002; Gross et al., 2006; Lopez-Rodriguez et al., 2011). Our use of bile salts was primarily related to a principle evaluation of the models, rather than an exact characterization of dose-relationship for any specific application.

The LS layer is the very first barrier any inhaled substance meets in the alveoli. This thin film coats the respiratory regions of the lungs. The LS lowers the surface tension at the air-liquid interface, but also provides some protection against hazardous inhaled substances (Zuo et al., 2008). Under normal conditions, the surface tension changes during the breathing cycle and makes breathing effortless. However, if the LS is damaged, the surface tension does not decrease during the expiratory compression, and the alveoli may collapse and the respiratory bronchioles fill with liquid, making breathing labored. The bile salts can potentially disrupt the structure of the LS film and thereby potentially inhibit its function.

Here we used the constrained drop surfactometer (CDS) to explore the effect of bile salts on LS function by exposing a drop of LS to increasing amounts of the different bile salts. This approach has previously been used in investigations of nanomaterials, pharmaceuticals and impregnation products (Valle et al., 2014; Valle

et al., 2015; Sørli et al., 2015a; Sørli et al., 2017). Importantly disruption of LS function *in vitro* correlates strongly with respiratory effects *in vivo* for impregnation products (Sørli et al., 2015b; Sørli et al., 2017).

The effect of bile salts was also assessed in a 3D human airway *in vitro* model (MucilAir^{TM}), representative of the central airways. Respiratory toxicity was evaluated by measuring cell viability and barrier integrity by trans-epithelial electrical resistance (TEER) after bile salt exposure. A reduction in TEER indicates that the epithelial barrier integrity is disrupted by the test chemical. The model has earlier been used to test compounds with known inhalation toxicity profiles, where it was found that cell barrier integrity and viability were predictive of *in vivo* toxicity (Balogh Sivars et al., 2018).

Furthermore, we used measurements of breathing patterns in mice to evaluate acute respiratory reactions to inhaled bile salts. Mice in head-out plethysmographs were exposed to increasing aerosol concentrations of bile salts and their breathing pattern was compared to baseline values. The method is a standard method for measuring airway irritation, and has previously been used to test airway irritation potential of industrial chemicals (Alarie, 1973; Nielsen et al., 2005; ASTM International, 2012).

As described above, bile salts may potentially be hazardous to the airways. We therefore hypothesized that bile salts could cause inhibition of LS function in the CDS set-up, alter the TEER and viability of lung epithelial cells and affect the breathing pattern in mice at different doses. We furthermore hypothesized that the compounds could be ranked according to toxicity and that the ranking *in vitro* and *in vivo* would be correlated.

2. Materials and methods

2.1 Chemicals

The following chemicals were bought from Sigma-Aldrich: cadmium chloride, dimethylsulfoxid (DMSO), Triton™ X-100, sodium taurocholate, sodium glycocholate, sodium taurodeoxycholate and sodium deoxycholate. The abbreviations, hydroxyl positions and structures of the bile salts are summarized in Table

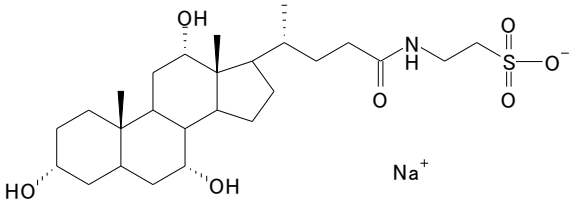
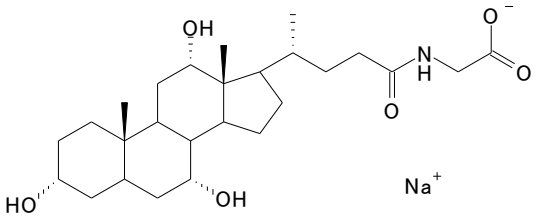
1. The concentration of bile salts was below the critical micelle concentration (CMC) reported in the literature for the *in vitro* experiments, and below or on the CMC for the *in vivo* experiments (Reis et al., 2004; Monte et al., 2009). Micronized lactose (Lactohale®300) was a kind gift from DFE pharma, (Goch, Germany). MucilAir™ culture medium was purchased from Epithelix Sàrl and PBS from Thermo Fisher.

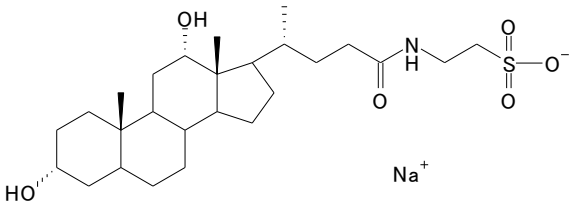
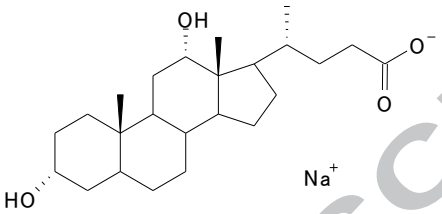
Curosurf® (Chiesi, Parma, Italy) was used as a model LS for all the experiments. It is made from solvent extracted minced porcine lung tissue and contains ~99% w/w phospholipids and 1% w/w hydrophobic surfactant-associated proteins (SP-B and SP-C) (Zhang et al., 2011). Curosurf® was diluted to 0.5 mg/mL in a buffer containing 0.9% NaCl, 1.5 mM $CaCl_2$, and 2.5 mM HEPES, adjusted to pH 7.0 (Valle et al., 2015).

2.2 pH measurements

LS is sensitive to extreme pH (the optimal range is between pH 4 and 7) (Amirkhanian and Merritt, 1995). Bile salts were diluted in water to a final concentration of 1 mg/mL. The pH of the solutions was measured using pH indicator strips (Alkalit, Merck). The pH of all bile salt solutions was between 5.5 and 6.

Table 1: Name, abbreviation and structure of bile salts used

Compound	Abbreviation	Position of hydroxyls	Structure
Sodium taurocholate	NaTCA	3α7α12α	
Sodium glycocholate	NaGCA	3α7α12α	

Sodium taurodeoxycholate	NaTDCA	3α12α	
Sodium deoxycholate	NaDCA	3α12α	

2.3 *In vitro* determination of lung surfactant inhibition in the CDS model

Inhibition of LS function *in vitro* was tested using the constrained drop surfactometer (Sørli et al., 2015a; Valle et al., 2015) by exposing a drop of LS to increasing amounts of the different bile salts. A drop of LS (Curosurf®, 10 µL of 0.5 mg/mL) was placed on a hollow based pedestal with a sharp edge, and was dynamically cycled at 20 cycles/min and <30% compression rate (baseline). The LS was cycled prior to exposure to obtain a baseline value. Any baseline experiment with a minimum surface tension >5 mN/m and compression >35% was discarded. Images were continuously taken of the drop and analyzed by axisymmetric drop shape analysis (ADSA) software (Yu et al., 2016). The output was, among others, surface tension of the drop. The CDS was kept at 37°C inside a heating box.

The effect of the bile salts (1 mg/mL) on LS function was tested in 2 ways: by injecting a bile salt solution into the cycling drop or by spreading of the bile salt solution on top of the cycling drop as follows. A drop of surfactant was cycled during the entire experiment. After a 15 sec baseline, 0.5 µl of bile salt solution was injected into the drop or spread on the drop, respectively (see Fig. 1). A new injection/spreading was repeated every 30 sec. After 12.5 minutes of injection/spreading, the drop was cycled for 7.5 min (i.e. each experiment took 20 min). The experiment was repeated 5 times for each bile salt and technique. The dose of bile salt leading to persistent inhibition of LS function (minimum surface tension >10 mN/m) was defined as the inhibitory dose, calculated as µg bile salt per mg LS. Lactose was used as negative control and the

experiments were done as for the bile salts. We have previously shown that lactose does not inhibit LS function (Sørli et al., 2015a).

2.4 3D human airway i*n vitro* model

Primary airway cells were acquired from human patients undergoing surgical lobectomy where the study was conducted according to the declaration of Helsinki on biomedical research (World Health Organization, 2001) and received approval from the local ethics commission. The experimental procedures were explained in full and all subjects provided informed consent. MucilAir™ (Epithelix Sàrl) airway epithelia was reconstituted of a mixture of human tracheal and bronchial cells and cultured at the air liquid interface (ALI) in MucilAir™ culture medium (Epithelix Sàrl), in 24-well plates with 6.5-mm Transwell® inserts (Corning). At each sampling point, or every 2-3 days for those wells not being sampled, the cell culture medium was changed.

NaTCA, NaGCA, NaTDCA and cadmium chloride were dissolved in DMSO and diluted further in a buffered saline solution (0.9% NaCl, 1.25 mM $CaCl_2$, 10 mM HEPES) to obtain a total concentration of 0.8% DMSO (v/v). To mimic inhalation *in vivo*, bile salts and cadmium chloride (10 µL) were applied daily on the apical surface of the MucilAir™ cultures at 1, 10 and 100 µM and left to incubate for 6 h prior to replacing with fresh medium. The procedure was repeated for a period of 48 hours and each treatment was run as four replicates for each bile salt concentration.

Barrier integrity was assessed by measuring TEER as previously described (Huang et al. 2017). Cell viability was determined by quantitation of ATP in cell lysates at termination by CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions. Cadmium chloride and 1% Triton™ X-100 diluted in PBS (without $MgCl_2$ and $CaCl_2$) were used as positive controls.

2.5 Mouse bioassay

2.5.1 Animals

A total of 52 inbred BALB/cJ male mice aged 5-8 weeks at arrival were purchased from Taconic M&B (Ry, Denmark) and housed in polypropylene cages (380x220x150 mm) furnished with aspen bedding (Tapvei, Estonia), enriched with a mouse house (80-ACRE011, Techniplast, Italy) and small aspen blocks (Tapvei, Estonia). The photo-period was from 06:00 to 18:00, and the temperature was 21°C and relative humidity in the animal room was 55%. The inhalation exposures were done between 09:00 and 15:00. Cages were sanitized twice weekly. Food (Altromin no. 1324, Altromin, Lage, Germany) and municipal tap water were available *ad libitum*. The mice were randomly assigned to cages, 3-4 mice per cage, and acclimatized for a minimum of one week. Mice from the same cage were used in the same experiment.

2.5.2 Ethical statement

Treatment of the animals followed procedures approved by The Animal Experiment Inspectorate, Denmark (Permissions No. 2014-15-2934-01042-C2). All experiments were performed by trained personnel and conformed to the Danish Regulations on Animal Experiments (LBK nr. 474 af 15/05/2014 and BEK nr. 1589 af 11/12/2015), which include guidelines for care and use of animals in research. Anesthesia was not used during the experiments, because the bioassay depends on the animals being fully awake with uncompromised breathing. The exposure was stopped if the tidal volume (VT) was reduced by >30% compared to baseline or after 1h of exposure. The exact number of animals used to test the toxicity of each bile salt can be found in Supplementary table 1.

2.5.3 Generation of bile salt aerosols for mouse bioassay

Dry powder aerosol exposure was initially tried using a Rotating brush generator (Seipenbusch particle engineering, Kuppenheim, Germany), but the generator could not deliver stable aerosols (Supplementary fig. 1). Therefore the salts were dissolved in deionized water at a concentration of 10 mM (except NaDCA that was tested at 5 mM), droplets were generated by a jet nebulizer (Wong and Alarie, 1982) and the water content was reduced by passage through a diffusion drier (Diffusion Dryer 3062, TSI Inc., Shoreview, MN,

USA), before entering the 20 L mouse exposure chamber (Clausen et al., 2003) (see Supplementary fig. 2 for drying of aerosols). The drying of the bile salt droplets produced stable aerosols, and the exposure concentration could be increased stepwise in a reproducible manner (the relationship between the infusion rate into the nebulizer and the particle concentration in the exposure chamber and reproducibility between 2 exposures can be seen in Supplementary fig. 3). As the bile salts were diluted in water no vehicle control was included in the experiments to limit the number of mice used for the assessment. Outlet air was passed through a series of particle- and active coal-filters before the exhaust to the atmosphere.

2.5.4 Aerosol exposure monitoring

The bile salt concentrations in the exposure chamber were monitored by gravimetric filter sampling and by measurement of the aerosol particle size distribution. Gravimetric filter sampling was done essentially as described in the standard DS/EN 481 (Dansk Standard, 1994). Shortly, aerosols were collected on pre-weighed Teflon filters (Flouropore™ Membrane filters, pore size 0.45 µm, Millipore A/S, Denmark) placed in a closed-face 25-mm cassette. Filter sampling was done at the same time as the mice were exposed and for the whole duration of the exposure period (45-60 min). The aerosols were drawn through the filter by an Apex2™ personal sampling pump (Casella, Buffalo, USA) at a flow rate of 2 L/min. The particle size distributions were measured by NanoScan (TSI Inc., Shoreview, MN, USA; particles ranging from 0.01 to 0.36 µm) and optical particle sizer (OPS, TSI Inc., Shoreview, MN, USA; particles from 0.32 to 10 µm). The aerodynamic diameter of the majority of the generated particles was <0.7 µm according to the particle number size distribution. The relationship between the concentrations measured by aerosol sampling by Nanoscan and OPS compared to the concentration calculated from filter sampling can be found in Supplementary fig. 4. In 3 cases, the concentration in the chamber exceeded the upper capacity of the OPS (marked in Fig. 2 as “not applicable”, NA). These points were at the 4th and highest exposure level for the study of NaGCA, NaTCA and NaTDCA.

The chamber concentrations calculated from bile salts collected on filters were in most cases higher than estimated by the aerosol measurements (Supplementary fig. 4). This is likely because the concentrations

became too high to be measured by the OPS at the highest level of exposure. The aerosol concentration measured by NanoScan and OPS were used to estimate the deposited dose at each exposure level.

2.5.5. Calculation of deposited dose from aerosol concentrations

The estimated deposited doses in Fig. 2 are calculated by using the following assumptions. Firstly, that all of the particles at each aerosol concentration level could be inhaled deeply into the lungs, this is likely as measurements made by NanoScan and OPS showed that the majority of the particles were <0.7 μm in aerodynamic diameter. Secondly, that 10% of the inhaled particles deposited in the lungs of the mice, and finally, that the deposited dose accumulated over the time of the exposure. The estimated deposited dose was divided by the body weight of the mouse resulting in a dose of x μg deposited bile salt per kg body weight of the mouse (Fig. 2 and Table 4). To estimate the deposited dose at each exposure level the aerosol concentration was multiplied with the tidal volume, breathing frequency and duration of exposure. This number was multiplied by 0.1, as a deposition of 10% was assumed, and divided by the weight of the mouse. As we assumed that the bile salts accumulated during the duration of exposure, the deposited doses calculated for any previous exposure levels were added to the calculated deposited dose.

2.5.6 Bile salt exposure in the mouse bioassay

To assess the acute effects of bile salts on respiration, groups of mice (n =3-7, Supplementary table 1) were placed in individual, head-out plethysmograph tubes and exposed. First, a 15 min baseline period was recorded for each mouse while breathing clean air. Then, the mice were exposed to the bile salt aerosol until the breathing pattern was affected (≥30% reduction of VT compared to baseline), or for a maximum of 60 min. If the breathing pattern of the mice was affected by the exposure, the mice were exposed to clean air for 15 min after end of exposure, to assess for reversibility of the respiratory effects. The lowest observed adverse effect dose (LOAED) was found by range-finding experiments.

The range-finding experiments were done as follows: a group of mice (n=3-7) was exposed to increasing aerosol concentrations of the bile salt in question. The start concentration was set based on data from the CDS, i.e. NaDCA was tested at a lower start concentration than the 3 other bile salts. This was done to

ensure that the first concentration would not cause acute lung effects. The start concentration was used during the first 15 minutes of exposure. If no effect was observed at this concentration, the infusion flowrate was doubled every 15 min. If no effect was observed after a total of 60 min of exposure, a new group of mice was used for a second range-finding experiment until the highest concentration that could be generated in the system was reached. If no effect occurred during any of the range-finding experiments, the concentration of the bile salt solution was increased, i.e. NaTCA was subsequently tested at a concentration of 30 mM in the same dose-increase setup until the LOAED was identified.

2.5.7 Collection of respiratory parameters

The Notocord Hem (Notocord Systems SA, Croissy-sur-Seine, France) data acquisition software was used to collect and calculate several mouse respiratory parameters. We used the tidal volume (VT, mL), respiratory frequency (BPM, breaths/min), time of break (TB, ms), time of pause (TP, ms), time of expiration (TE, ms), and time of inspiration (TI, ms). Comprehensive descriptions of the breathing parameters and their interpretation have been made elsewhere (Alarie, 1973; Vijayaraghavan et al., 1993; Larsen and Nielsen, 2000). Data acquisition and calculations were performed as described previously (Larsen et al., 2004).

2.5.8 Evaluation of acute respiratory effects

Prior to exposing groups of mice to the bile salt aerosol, the mice breathed clean air, only mice that had a stable baseline continued through to the exposure phase of the experiment. To assess effects related to exposure, the respiratory parameters during exposure were compared to baseline levels, i.e. each mouse served as its own control. For each mouse, the mean values of each minute during the experiment were calculated and the change compared to baseline was calculated. To estimate the effect of each bile salt concentration the final 10 min of each 15 min period was plotted against the minute of exposure. The accumulated deposited dose is indicated above each exposure period (Fig. 2). Adverse effect caused by the exposure to an aerosol of a bile salt was defined as a reduction in tidal volume (VT) to ≤90% of baseline (Fig. 2). A change of 10% compared to baseline was chosen because this could be distinguished from changes to the respiration caused by stress, e.g. induced by handling and restraint in the plethysmograph. This reduction in VT coincided with an increase of breathing frequency (BPM) (Fig. 2) and reduction of TE

and TI (not shown) compared to baseline, resulting in the characteristic breathing pattern: rapid shallow breathing (RSB). After the exposure stopped, respiratory data was collected for 15 min more as the mice were breathing clean air to determine if the changes to respiration were reversible.

2.5.9 Statistical evaluation of respiratory data

The respiration parameter at each exposure level was graphed and statistically analyzed using R (version 3.4.3). For each bile salt, changes in tidal volume (VT) and breathing frequency (BPM) were expressed as a percentage of the baseline value. Analysis of variance with pairwise comparison and Bonferroni adjustment were performed to compare the changes in breathing pattern for each dose compared to baseline. A significant difference between the breathing parameter at a specific dose and baseline has been marked with 1, 2 or 3*, indicating a significant difference from the mean of the baseline of $p<0.05$, 0.01 and 0.001 respectively (Fig. 2). Linear regressions of the changes in breathing pattern over time were fitted to the data set and plotted as a red line in Fig. 2.

3. Results

3.1 *In vitro* determination of LS inhibition in the CDS model

The bile salts were tested for LS inhibition *in vitro* using the CDS setup. The minimum surface tension was chosen as the endpoint. Inhibition of LS function was defined as an increase in the minimum surface tension above 10 mN/m that persisted over time. Each bile salt was tested 5 times with each method (injection or spreading). Lactose was included in the experiments as a negative control. The dose for inhibition was calculated as µg bile salt per mg LS.

Table 3: Lung surfactant was exposed to 4 bile salts and lactose either by injection or spreading to determine the dose that inhibited LS function (i.e. a persistent change in minimum surface tension above 10 mN/m).

	Injection	Spreading
Substance	Inhibitory dose (µg/mg LS)	
NaDCA	220±45	300±122

NaTDCA	360±55	440±207
NaGCA	360±55	1140±270
NaTCA	1100±200	No inhibition*
Lactose	No inhibition	No inhibition

Data is given as (mean ± sd). *in 1 of the 5 repeats, LS was inhibited by the highest dose of NaTCA (2500 µg/mg LS).

The bile salts were ranked according to the dose where a persistent inhibition occurred.

3.2 3D human airway *in vitro* model

The barrier integrity as measured by TEER of the human lung epithelial cell layer did not change after exposure (2 exposures over 48 hours, at 1, 10 or 100 µM) to NaTDCA, NaGCA or NaTCA. Moreover, the bile salts did not alter the cell viability as measured by ATP in cell lysates.

3.3 Breathing pattern analysis in the *in vivo* mouse bioassay

Overall, the breathing patterns of the mice used for these experiments were similar during baseline to those measured by other research groups using the same setup and the same or other strains of mice (Supplementary table 2).

An adverse effect of the exposure to an aerosol of a bile salt was defined as the onset of rapid shallow breathing (RSB). RSB was defined as reduction in tidal volume (VT) to ≤90% of baseline (Fig. 2). This change coincided with an increase compared to baseline in breathing frequency (BPM) (Fig. 2), time of expiration (TE), and time of inspiration (TI) (not shown). The accumulated deposited dose has been indicated above each exposure level (µg/kg bw) in Fig. 2. With increasing bile salt aerosol concentrations, the effect on the breathing parameters became larger, reaching a maximum of approximately 40% reduction in VT and 40% increase in breathing frequency (Fig. 2). After end of exposure the change to the breathing parameters reverted towards baseline rapidly, however the baseline values were not reached within the 15 min recovery period (Fig. 2).

Table 4: Assuming that all particles of the aerosol were inhaled, that 10% deposited in the lungs and that the bile salts accumulated during the duration of the exposure, the lowest dose causing onset of rapid shallow breathing (RSB) was calculated as µg/kg body weight. Using the same assumptions the dose was also estimated in relation to the lung surfactant pool (µg/ mg of LS). The estimation of dose/amount of LS was based on the assumption that the mouse lung contains approximately 0.15 mg of LS.

	LOAED, accumulated deposited lung dose	
Substance	Related to mouse weight (µg/kg bw)*	Related to LS pool (µg/mg LS) ¤
NaDCA	0.88	0.12
NaTDCA	3.65	0.49
NaGCA	6.07	1.30
NaTCA	9.34	2.23

* based on 10% deposition, ¤ based on 10% deposition and that a mouse has 0.15 mg LS. LOAED: lowest observed adverse effect dose, LS: lung surfactant, NaDCA: sodium deoxycholate, NaGCA: sodium glycocholate, NaTCA: sodium taurocholate, NaTDCA: sodium taurodeoxycholate,

The dose that induced RSB in exposed mice was used to rank the bile salts *in vivo*.

4. Discussion

The lungs have potential as route of delivery for systemically acting biomolecules, but supplementation with absorption enhancers may be needed. To our knowledge, there are currently no enhancers applied in inhaled formulations and the intended interaction with the air-blood barrier is associated with risks for adverse effects. This could include harmful impact on surfactant function and/or direct epithelial toxicity. When identifying novel excipients and enhancers, it is highly desirable to introduce model systems that can address some of these risks and thereby enable exclusion of harmful substances at an early stage. *In vitro* tests followed by acute *in vivo* tests addressing direct respiratory responses may identify the most harmful

substances before they proceed into more comprehensive *in vivo* assessment. In this investigation we used two *in vitro* techniques that mimic two different compartments and toxicological targets in the lung that potentially could be affected by absorption enhancers, followed by assessment of acute effects on respiratory function *in vivo*. Firstly, we simulated the LS layer in the alveolar region by assessing how the LS function was affected by our test substances, four different bile salt enhancers. Secondly, we recreated the conductive airways by applying air-liquid interphase cultures and exposed ciliated and mucus producing epithelial cells. Thirdly, we studied *in vivo* whether acute airway responses in mice following inhalation of enhancers correlated with the *in vitro* response and ranking.

We found that all the tested bile salts inhibited the LS function *in vitro*. This allowed the bile salts to be ranked according to toxicity, and this ranking was the same as for the *in vivo* effects as assessed by the dose that induced rapid shallow breathing (RSB). None of the bile salts affected the barrier integrity or viability of the air-liquid interphase grown cells at the tested doses.

4.1 *In vitro* determination of lung surfactant inhibition in the CDS model

A drop of LS was exposed to bile salt solutions either by injecting bile salt into the cycling drop or by spreading the solution onto the cycling drop. The bile salts were ranked from the least to the most inhibitory in almost the same order by both means of administration; NaTCA<NaTDCA<NaGCA<NaDCA (by injection NaTDCA=NaGCA), leaving NaDCA as the most potent inhibitor of LS function. However, when the bile salt solution was spread onto the cycling drop, inhibition generally occurred at higher doses than for injection. For the least inhibitory bile salt, NaTCA, only 1 of 5 experiments yielded an inhibition when it was spread onto the LS drop (Table 3). These results suggest that the method of mixing affects the interaction of bile salt with the LS components and structures that exert the surface tension lowering effect. Also, structurally very similar compounds (Table 1) had different effects on the surfactant function, hence small changes in structure may have large effects on the LS. Further elucidation of these mechanisms would however require methods that specifically study the interaction between the LS components and bile salt, such as a CDS coupled to an atomic force microscopy (Valle et al., 2015). The pH of the dissolved salts was measured to exclude the possibility that the reaction was a result of different pH of the bile salts; it was

shown that all the bile salt had similar pH in solution. Lactose was used as a control for both the injection and spreading experiments as it is known to be extensively used in marketed inhaled formulations (Sørli et al., 2015a). Lactose did not inhibit the LS function by either mixing method, showing that the inhibition was not an effect of injecting or spreading a foreign substance into the cycling drop.

The interaction between bile salts and LS has been the focus on several publications, because of the severe disease meconium aspiration syndrome (MAS), which can affect newborns aspirating stained amniotic fluid during birth. The initial phase of MAS is characterized by obstructed airways and LS inhibition, followed by a phase dominated by airway inflammation (Lindenskov et al., 2015; Herting et al., 2001). Meconium is the first pass through the newborns digestive tract, and among many other components it contains bile salts, approximately 0.005 mg/mg meconium (Lopez-Rodriguez et al., 2011). Several groups have investigated the effect of meconium on LS function *in vitro* and found the inhibitory bile salt dose to be between 0.07 to 20 µg/mg LS (using the estimate of 0.005 mg/mg meconium)(Bae et al., 1998; Moses et al., 1991; Sun et al., 1993), i.e. much lower than the inhibitory doses of pure bile salts found in this study (220-1100 µg/mg LS). Possibly, components in the complex mixture of meconium work in synergy to inhibit LS function, but the high variance in inhibitory dose may also reflect the diversity in methods of detection, differences in LS preparation, and meconium sampling. The mechanisms underlying inhibition of LS by meconium are not clear. Bae *et al* (Bae et al., 1998) observed that meconium changed the ultrastructure of the LS from loosely stacked layers to spherical lamellar structures. Lopez-Rodriguez *et al* (Lopez-Rodriguez et al., 2011) suggest that bile salts can mobilize cholesterol into the air-liquid interphase and thereby alters LS function. The surfactant preparation used in the present study (Curosurf®) does however not contain cholesterol and thus this is likely not the explanation for loss of function in the present study.

4.2 3D human airway i*n vitro* model

The bile salts (NaTCA, NaGCA or NaTDCA) did not affect the TEER of human airway epithelial cells after 2 consecutive dosings over 48 hours with concentrations of up to 100 µM. These doses were selected in relation to previous knowledge regarding measured lung concentrations of inhaled agents in inhalation toxicology studies at doses causing lung pathology (Balogh Sivars et al., 2018).

NaTCA has previously been used to enhance the transport of insulin (approximately 6 kDa) across cells *in vitro* using Caco-2 cells grown on trans-well inserts (Johansson et al., 2002). The authors found that when the cells were exposed to concentrations between 20 and 30 mM NaTCA the TEER was low compared to cells exposed to lower concentrations, but the cells survived and the insulin was transported across the cell layer. Morimoto *et al* (Morimoto et al., 2000) used 10 mM NaGCA to enhance the permeation of insulin and thyrotropin-releasing hormone across excised rabbit trachea. Both research groups used much higher concentrations than those applied in the present setup.

Compared to the approximately 0.1µm thick barrier between the air and blood in the alveoli, the mucus covered ciliated cells of the upper airways would be expected to present a much thicker and relatively robust barrier to bile salts. Alveolar epithelial cells therefore would be a more physiological relevant model for testing potential hazardous interactions, however these cells are difficult to obtain and to maintain the alveolar epithelium phenotypes *in vitro*.

4.3 Breathing pattern analysis in the *in vivo* mouse bioassay

The mouse model explored in the present study has previously been used to assess the acute sensory airway irritation potential of industrial chemicals (Alarie, 1973; Nielsen et al., 2005) and is a standardized method (ASTM International, 2012). The test assesses whether test substances induce specific changes in the breathing pattern of mice upon inhalation (Alarie, 1973). As the bile salts are dissolved in water that is subsequently removed in the diffusion drier, there is no vehicle control group of mice for these experiments, and the mice served as their own controls. An adverse effect of the exposure to an aerosol of a bile salt was defined as the onset of rapid shallow breathing (RSB), an indicator of pulmonary sensory irritation. Pulmonary sensory irritation is divided into 2 phases. The first phase, P1, is characterized by rapid shallow breathing and can be identified by an increase in breathing frequency (BPM), in combination with a reduction of time of expiration and inspiration (TE and TI, respectively) and tidal volume (VT) (Nielsen et al., 1999). It can be followed by a second phase, P, which is characterized by an increase in time of pause (TP). All the bile salts caused signs of pulmonary sensory irritation, evident as induction of RSB, but the

effect occurred at different exposure levels. Elongation of TP was seen rarely and only at the highest doses (not shown).

Pulmonary irritation has been studied with the same setup and strain of mice for several chemicals, e.g. ozone (Nielsen et al., 1999; Currie et al., 1998) and propranolol (Vijayaraghavan et al., 1993). Ozone caused both P1 (RSB) and P (elongated TP) sensory irritation in the same strain of mice used in this study (Nielsen et al., 1999). Currie et al (Currie et al., 1998) found that RSB persisted 24h after ozone inhalation, and that ozone inhalation impaired LS function, caused by water soluble proteins in the LS. Of note, ozone induces vascular leakage and pulmonary edema (Nielsen et al., 1999). We did not perform histopathological evaluation of lungs from mice exposed to the bile salts, however there was no persistent effects on respiratory parameters (Fig. 2). The response to propranolol, on the other hand, is more similar to that of the bile salts. Propranolol causes RSB in mice and Guinea pigs (P phase) at lower doses and elongated TP (P1) at higher doses, and the breathing pattern rapidly reverses to normal during the recovery phase (Vijayaraghavan et al., 1993). The bile salts caused RSB but only occasionally elongated TP at the highest dose level and after 45 min exposure. As for propranolol, the breathing pattern rapidly returned towards baseline during the recovery phase (Fig. 2).

Bile salts (an unspecified mix) have been studied as an enhancer for inhaled insulin in humans. The human subjects inhaled a total of 1028 µg bile salt. There were no adverse events after the inhalation and the mixture was well tolerated (Heinemann et al., 2000). Assuming that 30% of the inhaled powder reached the alveoli (Hirst et al., 2001; Thorsson et al., 2001) and that the average person has 1000 mg LS in the lungs (Sørli et al., 2015a) this would result in a calculated dose of 0.31 µg bile salt/mg LS. At this dose, 1 bile salt (NaDCA) induced RSB in our experiments when the dose was related to the amount of LS in mice (Table 4). As we do not know which bile salts were in the inhaled mixture it is difficult to make further comparison. NaGCA has been studied as an enhancer for macromolecules (insulin or calcitonin), in rats (Yamamoto et al., 1994; Yamamoto et al., 1997; Okumura et al., 1992; Bäckström et al., 1996; Morita et al., 1994). In all cases the bile salt enhanced bioavailability. No adverse advents were observed, however, as the rats were anesthetized during the exposure, effects on the breathing pattern would not have been possible to observe.

We have used LS function assessment in the CDS in combination with mouse breathing pattern analysis while studying lung toxicity of 21 impregnation products (IP). The *in vitro* method could predict IP toxicity in mice with 100% sensitivity and 63% specificity. However, the toxicological profile of the IPs in mice was qualitatively different from that of the bile salts. Toxic IPs induced a very rapid and steep reduction in VT (Sørli et al., 2017) that has been shown to cause spontaneous death if the mice are not immediately killed (Nørgaard et al., 2010; Nørgaard et al., 2014; Duch et al., 2014; Sørli et al., 2015b). In comparison the mice exposed to bile salts rapidly recovered from the RSB when the exposure was stopped, and they were allowed to breathe clean air for 15 min (Fig. 2), after which they were killed. We can therefore not exclude that there were long term effects of the inhaled bile salts, however, the immediate reversibility of the RSB supports that the sensory irritation was transient and caused by interaction with LS.

4.4 Ranking and dose considerations

When the bile salts were ranked by their ability to inhibit LS function *in vitro* and their ability to induce RSB in exposed mice, the *in vitro* and *in vivo* ranking was the same. Notably the assessment of LS function in the CDS is cell-free, indicating that there may be a component of LS interaction in the induction of RSB.

The dose per mg LS in the CDS *in vitro* system and per body weight and in relation to the LS pool in the *in vivo* bioassay are presented in Table 3 and 4 respectively. It is apparent that the estimated dose causing effects on RSB is considerably lower than the dose estimate from the *in vitro* CDS setup. However, caution should be taken in over interpreting this difference; the CDS experiment is limited to *in vitro* application and a selective measurement of surface tension, while animals demonstrate a physiological response upon aerosol exposure. There are aspects of exposure conditions, dose estimates, distribution and concentration at site of action, which could contribute to this difference. In addition, in the lung, bile salts will interact with components not represented *in vitro*, such as vagal nerve endings in the alveoli region. Atelectasis can activate the pulmonary stretch receptors (Alarie, 1973) and this presents a likely link between LS inhibition and RSB onset in the animals. Nevertheless, the *in vitro* LS inhibition experiment has the potential to rank the bile salts in the same order as the *in vivo* experiment.

5. Conclusion

The ranking of the bile salts was the same when tested *in vitro* for LS function in the CDS and the *in vivo* mouse bioassay. Even if the bile salts did not affect the barrier integrity or viability of human airway epithelial cells at the tested doses, it has earlier been proved to be a strong tool for identifying hazardous substances, hence suggesting no overt toxicity of bile salts on this cell type. The correlation between the ranking by LS inhibition and the ranking of induction of RSB *in vivo* strongly suggests that the physiological response is dependent upon LS function *in vivo*.

These types of models can be applied to address plausible respiratory liabilities with molecules like absorption enhancers, APIs or other chemicals, with a potential to grade responses, but also to separate direct and transient effects on surfactant function from toxicity to epithelial cells. Staging of the *in vitro* approaches can reduce the need for experiments on animals and enhance selection of agents without respiratory liabilities. Future research could include inclusion of molecules for which absorption enhancement is confirmed to enable assessment of potential separation between enhancing effect and any adverse consequence. Morphological evaluation of respiratory tissue could also broaden the understanding of any consequence related to the interaction with surfactant function.

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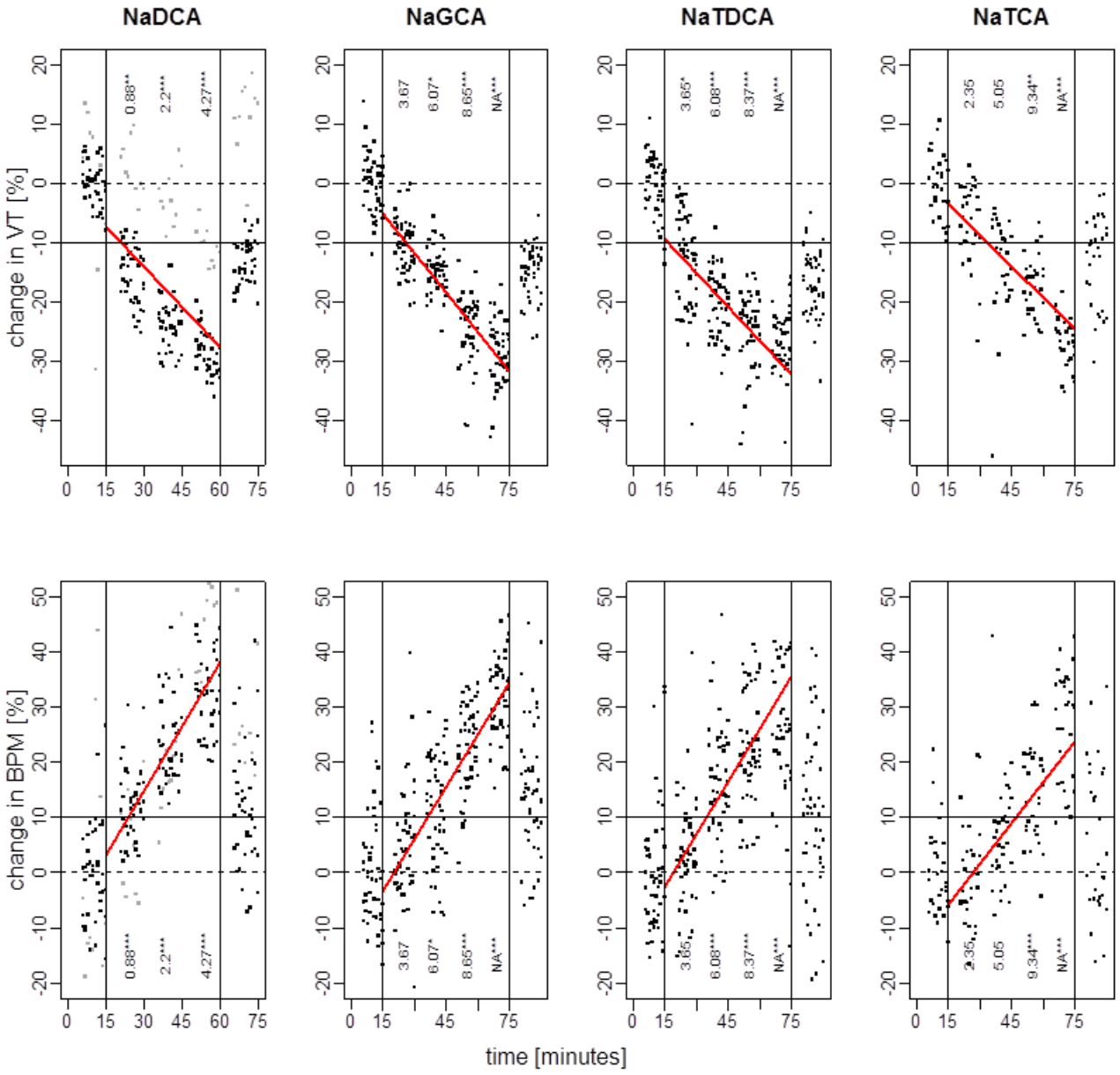
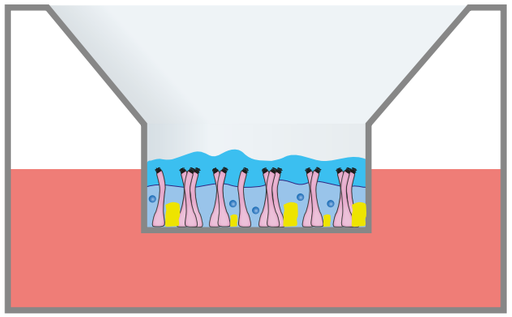
Figure 1: Examples of injection of bile salt solution into (left) and spreading of bile salt solution on to (right) a drop of LS.

Figure 2: The average minute breathing pattern for tidal volume (VT, top row) and frequency (BPM, bottom row) plotted against the minute of the experiment for each mouse in the experiment. During the first 15 minutes of an experiment the mice were breathing clean air. At 15 minutes the bile salt exposure started and the flow into the aerosol generator was doubled every 15 minutes. The calculated accumulated deposited dose in µg/kg bw (assuming 10% deposition and no clearance during the exposure period) has been indicated above (top panel) or below (bottom panel) each exposure level, except when the aerosol concentration exceeded the measuring range of the aerosol instrument (OPS) and the deposited dose could not be calculated (marked by NA). After exposure the mice breathed clean air for 15 minutes before the experiment was stopped. In the panels for NaDCA, one mouse reacted differently from the rest, it has been included in the analysis, but has been colored gray for identification. *, ** and *** indicate that the average mean is significantly different from the mean of the baseline with $p<0.05$, 0.01 and 0.001 respectively. Linear regression of the changes in breathing pattern over time were fitted to the data sets and plotted as a red line.





3D human airway *in vitro* model