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Evaluation of the toxicity of sodium dodecyl sulphate (SDS) in the MucilAir™ human airway model *in vitro*

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ABSTRACT

The aim of the study was to use multiple *in vitro* assays to assess the effects of a model irritant, sodium dodecyl sulphate (SDS) (≤10 mM (0.29 %, w/v)), on an *in vitro* model of the airway, MucilAir™. The use of MucilAir™ in recovery studies was also explored.

A 24 h exposure increased IL-8 release at an SDS concentration ≥0.63 mM (0.018 %, w/v). Mucin secretion increased and transepithelial electrical resistance (TEER) decreased at SDS concentrations ≥1.25 mM (0.04 %, w/v). Cytotoxicity (lactate dehydrogenase (LDH) release into basolateral chamber) was observed at SDS concentrations of ≥2.5 mM (0.07 %, w/v). The sensitivity of the assays was IL-8 release > TEER = mucin secretion > LDH release.

After 7 days, full or partial recovery was observed for intermediate concentrations of SDS using all assays but not at 5 and 10 mM SDS. Morphologically, erosion and cell loss were observed at these concentrations. Resazurin metabolism at 7 days tended to decrease in a dose-dependent manner at SDS concentrations above 2.5 mM (0.07 %, w/v).

Together, these data support a No Observable Effect Level of 0.31 mM (0.009 % w/v) SDS and the use of MucilAir™ as a relevant model for airway toxicity studies.

1. Introduction

The airway epithelial barrier has a role in airway protection and is a key target site for respiratory tract toxicity. The Organisation for Economic Co-operation and Development (OECD) define acute inhalation toxicity as the total adverse effects caused by exposure to a material for <24 h (Oecd, 2009). Historically, assessment of acute inhalation toxicity was generated by exposing animals to inhaled doses of a substance and then assessing adverse effects. However, the primary endpoint and categorisation tool for these acute tests was often lethality, with little or no investigation into mechanisms of toxicity. Development of non-animal testing methods that can provide mechanistic insight into inhalation toxicity will support the development of Adverse Outcome Pathway (AOP) models. The data from assays predicting the steps or processes modelled by the AOPs will provide more information to the risk assessor than an observation of lethal doses (Clippinger et al., 2018).

The respiratory tract is a complex organ comprised of numerous cell types located within specific regions. Due to this complexity, the creation of a single *in vitro* lung model is challenging, and currently not technically feasible. To date, much of the published literature describes studies using immortalized/transformed cell lines such as PTBE, BEAS-2B, A549, PSAE, Met-5A, and Calu-3, due to their accessibility and relatively low cost (Lujan et al., 2019). However, gene expression and biological function in these simplified, immortalized, models can differ from the true *in vivo* phenotype; therefore, organotypic multi-cell type models for specific regions of the airway have been developed. Currently available commercial *in vitro* upper airway models include MucilAir™, SmallAir™ (Epithelix Sàrl) and EpiAirway™ (MatTek Corporation); these consist of ciliated cells, mucus secreting goblet cells and basal cells (Balharry et al., 2008; Huang et al., 2013a) and have been demonstrated to reflect the physiological conditions in the relevant region of the lung. This allows the study of cell-cell interactions (Clippinger et al., 2018), and is likely to play a powerful role in future *in vitro* assessment of

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Abbreviations

ANOVA	analysis of variance
AOP	Adverse Outcome Pathway
AU	Absorbance Units
ELISA	enzyme-linked immunosorbent assay
ELLA	enzyme linked lectin assay
EPA	United States Environmental Protection Agency
IL-6	interleukin-6
IL-8	interleukin-8
LDH	lactate dehydrogenase
LoRM	lower limit of reliable measurement
OECD	Organisation for Economic Co-operation and Development
PBS	phosphate buffered saline
PBST	phosphate buffered saline containing Tween 20
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
TEER	transepithelial electrical resistance
WGA	wheat germ agglutinin
ZO-1	zonula occludens-1

respiratory toxicity.

MucilAir™ has been shown to have the potential to predict the *in vivo* airway toxicity of inhaled drugs for respiratory disease (Balogh Sivars et al., 2018). Likewise, both the MucilAir™ and EpiAirway™ models have been widely used in the assessment of the toxic effects of cigarette smoke and related products (Balharry et al., 2008; Iskandar et al., 2013; Neilson et al., 2015). Both models are well characterised in terms of structure, barrier properties, expression of tissue -relevant markers and *in vivo*-like behaviour (Balogh Sivars et al., 2018; Baxter et al., 2015; Huang et al., 2017; Iskandar et al., 2013; Zavala et al., 2016). In addition to tissue inserts derived from healthy donors, MucilAir™ and EpiAirway™ tissues are also available from donors with airway diseases such as cystic fibrosis (Beubler et al., 2016), asthma (Chortarea et al., 2017) and COPD. This may be beneficial in the development of new treatments for these conditions, or for risk assessments relevant to these particularly vulnerable groups.

Due to the emphasis on acute inhalation toxicity for risk assessment, there is increasing interest in the use of models such as MucilAir™ and EpiAirway™ for the determination of acute respiratory damage and contact irritancy. Such models can support *in vivo* testing by elucidating mechanistic processes and can be used in screening to deselect particularly toxic compounds and formulations. Alternatively, they can replace *in vivo* testing completely (Clippinger et al., 2018). The United States Environmental Protection Agency (EPA) has considered the use of these *in vitro* models for screening or replacement of animal tests for agrochemical safety assessment for the pesticide, chlorothalonil (Epa, 2018) leading, after further work, to a revised human health draft risk assessment for chlorothalonil (Epa, 2021).

This work utilises one such upper respiratory tract model, MucilAir™. MucilAir™ is a ciliated epithelial model, derived from healthy human donor airway cells cultured at the air interface. The culture process reconstructs a functional model of human tracheobronchial epithelium, exhibiting a pseudostratified, ciliated epithelium which secretes mucus (Huang et al., 2013a). This model is increasingly used in inhalation toxicity and pharmaceutical lead optimisation development and testing to identify potential airway toxicants, primarily irritants, and facilitate *in vivo* dose range finding. A major advantage of MucilAir™ is its long -life span (Baxter et al., 2015) in comparison to many cell -line models. This enables it to be used to evaluate damage upon long term exposure (Cervena et al., 2019) or repeated exposure (Anderson et al., 2013; Ito et al., 2018; Rossner et al., 2019) and also to monitor recovery (George et al., 2019). The model has the potential to further reduce animal use prior in *in vivo* inhalation studies since it can be used to predict non-toxic starting doses or identify toxic liabilities.

The anionic surfactant sodium dodecyl sulphate (SDS, CAS No. 151-21-3, also known as sodium lauryl sulphate) is known to cause acute respiratory toxicity and skin and respiratory irritation. SDS is routinely utilised as an *in vitro* positive control for acute skin irritation (Oecd, 2020). However, to date there has been no detailed *in vitro* analysis of the mechanisms through which it causes acute respiratory toxicity.

The aim of this study was to establish the number of assays that could be reliably used in a single MucilAir™ insert to determine toxicity/irritancy and to explore the use of the model to study recovery. SDS was used as a "model" acute respiratory irritant over a range of concentrations to demonstrate the utility and performance of MucilAir™, and to provide information on its mechanism of action that is lacking from *in vivo* models.

2. Materials and methods

2.1. MucilAir™ culture and exposure

MucilAir™ tissues prepared from the nasal tissue of three male non–smoking donors, with no pathology noted, aged between 34 and 54 years (batch numbers MD023401 (henceforth termed batch 1), MD024001 (henceforth termed batch 2) and MD025501 (henceforth termed batch 3) and MucilAir™ medium were obtained from Epithelix Sàrl, 14 Chemin des Aulx, CH 1228 Plan-Les-Ouates, Geneva, Switzerland. SDS was obtained from Sigma -Aldrich, Dorset, UK (CAS No. 151-21-3, catalogue no. L6026). CytoTox ONE™ Homogeneous Membrane Integrity Assay kit was obtained from Promega, Delta House, Southampton Science Park, Southampton, UK. Quantikine® Human IL-6 (Catalogue No. D6050) and Human CXCL8/IL-8 (Catalogue No. D8000C) immunoassay kits were obtained from R&D Systems Europe Ltd, Abingdon Science Park, Abingdon, UK. All other materials were obtained by Charles River and were analytical or tissue culture grade, as appropriate.

On delivery, MucilAir™ inserts were aseptically transferred into 24-well plates containing proprietary serum-free MucilAir™ medium (700 μL). The tissues were allowed to recover in a humidified incubator set to maintain a temperature of 37 °C with a 5 % CO_2 environment (standard conditions) for *ca* 1 week. The medium was replaced at 2–3 day intervals.

Cultures were dosed with SDS (30 μL; 0.16–10 mM *i.e.*, 0.005–0.29 %, w/v) in physiological saline (0.9 %, w/v; control cells were dosed with 30 μL saline) applied to the apical surface of the cells for 24 h in standard incubator conditions. The actual SDS concentrations tested were in doubling increments: 0.16, 0.31, 0.63, 1.25, 2.5, 5 and 10 mM. After 24 h incubation, SDS and control solutions were removed from the apical chambers by rinsing with saline. Each MucilAir™ insert was moved to fresh MucilAir™ medium and the spent medium retained for analysis. MucilAir™ inserts were then maintained in culture until 168 h post dose (seven days) to study recovery. Five inserts were exposed to each concentration of SDS (and control condition), one from batch 1 and two each from batches 2 and 3.

2.2. Experimental protocol summary

The study design is summarised as follows:

Batch 1 (MD023401) (n = 1)	Batch 2 (MD024001) (n = 2)	Batch 3 (MD025501) (n = 2)
TEER LDH Resazurin IL-8, IL-6		

(*continued on next page*)

(*continued*)

Batch 1 (MD023401) (n = 1)	Batch 2 (MD024001) (n = 2)	Batch 3 (MD025501) (n = 2)
Mucin ELLA v1 (WGA lectin; 6 μg/mL)		Optimised Mucin ELLA v2 (WGA lectin; 2 μg/mL)
Histopathology		SEM

Full details of each experimental procedure followed are provided in the relevant assay specific sections. 24 hours before the experiment, mucus was removed from the apical surface of the cells by lavage and inserts were placed in wells containing fresh culture medium. This was to ensure that samples collected immediately pre dosing corresponded to 24 h analyte release. Prior to the application of SDS, mucus was collected from the apical chamber by rinsing the cells with saline. After the final rinse, the inserts were placed in wells containing saline and the TEER of each insert was measured. The inserts were then transferred to wells containing fresh medium. SDS in saline or saline alone (controls) was applied to the apical surface of the cells (0 h). At 24 and 168 h, mucus and media samples were collected and TEER measured using the same methods. Following the 24 h measurements, tissues were returned to fresh media for the recovery period to 168 h. No further media changes were conducted during this time. At 168 h, a resazurin assay was also carried out and selected inserts were fixed for histological examination.

Following transfer of MucilAir™ tissues to fresh medium or saline, the spent basolateral medium remaining in the wells was retained for lactate dehydrogenase (LDH), Interleukin-8 (IL-8) and Interleukin-6 (IL-6) analysis. Samples were analysed for LDH release within 1 h of collection. Samples analysed for IL-6 and IL-8 were stored at −80 °C, until required for analysis.

2.3. Transepithelial electrical resistance (TEER)

To measure TEER, MucilAir™ inserts were transferred into 24-well plates containing saline (700 μL/well) and an aliquot of saline (200 μL) was added to each apical chamber. TEER was then measured using a Millicell®-ERS meter (MilliporeSigma™ MERSSTX01) with chopstick electrodes, and saline was maintained at ca 37 °C. TEER was measured immediately before SDS exposure (pre dose (0 h)) and at 24 and 168 h post dose. Measured TEER values were adjusted for the resistance of the MucilAir™ support membrane according to the manufacturer's instructions (corrected value = measured value −100 Ω), then corrected for the tissue surface area (0.33 cm^2) and reported as (Ω x cm^2).

2.4. LDH release assay

Cytotoxicity was assessed at 0, 24 and 168 h by measurement of LDH release into the medium (Decker and Lohmann-Matthes, 1988; Korzeniewski and Callewaert, 1983) using the Promega CytoTox ONE™ Homogeneous Membrane Integrity Assay kit. The maximal LDH release value for healthy cells was assayed in untreated MucilAir™ inserts disrupted with 10 % (w/v) Triton X-100 Lysis Solution applied apically, before samples were incubated at *ca* 37 °C for 3 h. Duplicate aliquots (100 μL) of medium were transferred into 96-well plates. An additional set of control wells containing fresh MucilAir™ culture medium was also prepared to allow correction for background fluorescence. Cytotoxicity was then assessed according to the manufacturer's instructions. Fluorescence of wells was read at 590 nm with excitation at 544 nm ($544_{Ex}/590_{Em}$), using a Thermo Scientific Fluoroskan Ascent® FL microplate fluorimeter, within 2 h of stopping the reaction.

2.5. Measurement of interleukins by enzyme-linked immunosorbent assay (ELISA)

R&D Quantikine® immunoassays were used to measure the IL-6 and IL-8 content of collected media samples. The manufacturers recommended protocols were followed throughout. Optical absorbance at 450 nm with correction at 550 nm was measured for collected samples (MRX microplate reader, Dynex Technologies, Praha, Czech Republic). IL-6 and IL-8 content was calculated by reference to a calibration curve run in each assay plate.

2.6. Measurement of mucin by enzyme-linked lectin assay (ELLA) assay

Mucus was collected immediately prior to dosing (0 h) and at 24 and 168 h after dosing. To ensure that each collection (0, 24 and 168 h) corresponded to 24 h of secretion, mucus was removed by lavage 24 h prior to each collection. Mucus was collected by dispensing saline (200 μL) into the apical compartments, gently flushing the apical surface 3 to 5 times, repeating this a total of 3 times at *ca* 5–10 min intervals. MucilAir™ tissues were then transferred to wells containing fresh medium.

Mucus samples were assayed for mucin content using a sandwich ELLA assay (Kishioka et al., 1997) which relies on the affinity of mucin glycoprotein carbohydrate residues for *Triticum vulgaris* lectin (wheat germ agglutinin, WGA) (Piqué and De Servi, 2018).

For analysis of samples from batches 1 and 2, Nunc Maxisorp™ plates were first coated with WGA lectin (Sigma L0636, 6 μg/mL in phosphate buffered saline (PBS), pH 6.8; 60 μL/well) for 2 h at 37 °C. Plates were washed three times with PBS (200 μL) containing Tween®-20 (Sigma P1379, 0.05 % (v/v); PBST) before applying samples (50 μL) and incubating for 30 min at 37 °C. Plates were again washed with PBST, then incubated with bovine serum albumin (Calbiochem 12659, 0.1 % (w/v) in PBS; 100 μL/well) for 30 min at 37 °C. Following another wash, plates were incubated for 30 min at 37 °C with soybean lectin labelled with horseradish peroxidase (Sigma L2650, 1 μg/mL in PBS; 50 μL/well). After a final wash, horseradish peroxidase detection was performed using BD OptEIA™ 3,3′,5,5′-tetramethylbenzidine substrate (50 μL/well) incubated at ambient temperature for 15 min, stopped with sulphuric acid (2 N; 50 μL/well). Mucin content was determined by optical absorbance (450 nm) (MRX microplate reader, Dynex Technologies).

An in–house optimised sandwich ELLA method was used to analyse samples from batch 3. Wells were coated with lower concentration of WGA lectin (Sigma L0636, 2 μg/mL in PBS, pH 6.8; 60 μL/well) for 2 h at 37 °C. After washing, plates were incubated with PBST (300 μL) for 30 min at 37 °C and then treated as described previously.

2.7. Resazurin metabolism cytotoxicity assay

The metabolic competence of cells was assessed by measuring the reduction of resazurin to resorufin by the MucilAir™ cells (O'brien et al., 2000). MucilAir™ inserts were transferred into 24-well plates containing resazurin solution (Sigma R7017, 6 μM in saline; 500 μL/well). A further aliquot of resazurin solution (200 μL) was applied to the apical surface of each insert. Plates were then incubated for 1 h in standard conditions. After incubation, duplicate samples (90 μL) were collected for analysis from the apical chamber, transferred into 96-well plates and resorufin was measured by fluorescence at ($544_{Ex}/590_{Em}$) (Thermo-Scientific Fluoroskan Ascent® FL microplate fluorimeter). Additional control wells for the background absorbance of untreated resazurin solution were included with each analysis. Resazurin metabolism was calculated from background corrected fluorescence readings as a percentage of the vehicle control as nominal 100 % metabolism.

2.8. Histology

Following the 168 h endpoint assessment of cytotoxicity and metabolic competence, MucilAir™ inserts from batch 1 were fixed by submerging in two changes of neutral buffered formalin (10 %, v/v). Following fixation for at least 24 h, fixed tissues were paraffin embedded and sections were stained with haematoxylin-eosin according to standard industry established methods. Images from histological analysis were acquired using a Leica DM2500 microscope with DFC310 FX digital camera. Images were saved electronically as high-resolution TIFF files (1392 × 1040 pixels).

2.9. Scanning Electron Microscopy

Following the 168 h measurements, MucilAir™ inserts from batch 3 were fixed by incubation in buffered glutaraldehyde (5 %, v/v, in sodium cacodylate (0.1 M), sucrose (Sigma S7903, 0.2 M), pH 7.4) for *ca* 22 h in a refrigerator set to maintain a temperature of 5 °C. Fixed samples were rinsed in buffered sodium cacodylate (sodium cacodylate (0.1 M), sucrose (0.2 M), pH 7.4) for *ca* 30 min then in sterile ultrapure water for *ca* 5 min. Samples were then dehydrated by passing through a graded series of ethanol dilutions (ethanol from Hayman, F20023B: 33.3, 66.6, 85, 95 and 100 % (v/v) in sterile ultrapure water). Dehydrated samples were submerged in hexamethyldisilazane (Sigma 379212) for *ca* 5 min, air dried in a ventilated hood for *ca* 30 min and then stored in a desiccator until analysis. Scanning electron microscopy was performed at the Grant Institute of Earth Science, University of Edinburgh. Samples were mounted on aluminium stubs with conductive adhesive; sputter coated with gold and viewed using a Philips XL30CP scanning electron microscope. The microscope was operated using 10 kV accelerating voltage and a 4.0 nA electron beam. Images from SEM analysis were saved electronically as high-resolution TIFF files (1424 × 1064 pixels) and a proprietary IMG format. Image files in TIFF format were converted to JPEG format using Microsoft Office 2007 Picture Manager (12.0.6413.1000).

2.10. Data analysis

For each SDS concentration, measurements were performed on five MucilAir™ tissues: one from batch 1 and two inserts each from batches 2 and 3.

Assay data were transferred to Microsoft Office Excel 2016, Graphpad Prism 6.00 and GraphPad Instat 3.0 for further analysis.

For each numerical end-point, variation between batches pre dosing was compared using a Kruskal-Wallis non-parametric ANOVA followed by a Dunn's multiple comparison post-test. This test was also used to compare the effect of SDS on resazurin metabolism.

To compare the effect of each treatment on TEER, LDH, IL-8, IL-6 and mucin release each insert was used as its own control and a repeated-measures ANOVA was used to test for changes in response due to SDS application at each time point. Where normality of data could be shown, comparisons between batches were made using a parametric repeated-measures ANOVA followed by a Bonferroni multiple comparisons post-test. Where normality could not be shown, comparisons between batches were made using a non-parametric repeated measures ANOVA (Friedman Test) followed by a Dunn's multiple comparison post-test. $P < 0.05$ was considered statistically significant.

To permit a meaningful graphical comparison of the data, the assay endpoint of each insert was normalised to the average assay endpoint of its batch prior to dosing.

3. Results

3.1. Effect of SDS on MucilAir™ transepithelial electrical resistance

Prior to dosing, TEER values were similar for MucilAir™ tissues within each batch; the TEER values of batches 1, 2 and 3 were 499 ± 34, 346 ± 14 and 638 ± 81 Ω cm^2 respectively (mean ± SD; n = 8 (batch 1) and n = 16 (batches 2 and 3)) (Table 1). However, the mean TEER of the batches were significantly different to each other ($P < 0.05$). Qualitatively, the TEER of each batch of cells responded similarly to the application of SDS. By normalising the TEER of each insert to the average TEER of the batch prior to dosing, it was possible to make a graphical comparison of the effect of the different concentrations of SDS on TEER (Fig. 1). To enable statistical comparison of batches, the TEER values measured at 24 and 168 h were compared to the TEER value measured pre dosing for that insert at any one concentration.

At 24 h post dosing, exposure to SDS concentrations equal to or lower than 0.63 mM demonstrated no significant change to the barrier function of the cultures in any of the batches ($P > 0.05$). However, at an SDS concentration of 1.25 mM, there was a significant reduction in barrier integrity for all batches of cells ($P < 0.05$) at 24 h. Concentrations greater than 2.5 mM caused extensive to complete loss of barrier function (TEER ≤21 Ω cm^2) when compared to controls ($P < 0.05$). By 168 h, some functional integrity was regained in MucilAir™ tissues damaged by intermediate concentrations of SDS, with tissues exposed to SDS between 1.25 mM and 2.5 mM achieving pre-treatment TEER values or greater. Tissues exposed to SDS concentrations of 5.0 and 10 mM did not recover their barrier function after six days (168 h) ($P < 0.05$). For inserts undamaged by SDS at 24 h, no difference was observed between the endpoints at 168 h and before dosing ($P > 0.05$).

3.2. Effect of SDS on LDH release from MucilAir™

Some concentrations of SDS caused more LDH release from the MucilAir™ inserts than the positive control (Triton X-100; 10 %, w/v) provided with the kit. The lysed Triton X-100 positive control samples were performed according to the manufacturer's instructions to demonstrate the assay was successfully identifying LDH release. However, it appeared that complete lysis of the cells was not achieved, and it was, therefore, not possible to compare the release of LDH caused by SDS with a value of 100 % release. As such, the values presented for LDH release are those of the fluorescence of the samples (Table 2). By normalising the fluorescence value of each insert to the average fluorescence value of its batch prior to dosing, it was possible to make a graphical comparison of the effect of the different concentrations of SDS on LDH release (Fig. 2).

Prior to dosing, the release of LDH from the cells in the previous 24 h period was measured. The mean LDH release (fluorescence of samples) was 5.1 ± 1.2, 17.9 ± 5.1 and 4.9 ± 1.7 relative fluorescence units (RFU) from batches 1, 2 and 3 respectively (mean ± SD; n = 8 (batch 1) and n = 16 (batches 2 and 3)) (Table 2). The mean LDH release from batch 2 was significantly higher than that from batches 1 and 3 ($P < 0.05$). Qualitatively, the responses of the different batches to SDS were similar. Therefore, the normalised LDH release from each tissue at 24 and 168 h was compared with the pre dosing release from the same batch (Fig. 2).

At 24 h post dose, treatment with SDS up to 1.25 mM showed no significant change in LDH release compared to pre dose values ($P > 0.05$) indicating no cytotoxic effects at these concentrations. However, at SDS concentrations of 2.5 mM and 5.0 mM, there was a significant increase in LDH release relative to untreated controls ($P < 0.05$). The highest SDS concentration tested (10 mM) showed only a small increase in LDH release compared to control tissues ($P > 0.05$).

Following a six-day recovery period, by 168 h, LDH release remained at pre dose values for those inserts unaffected by exposure to SDS (concentrations of SDS up to 1.25 mM) ($P > 0.05$). The exception was 1.25 mM SDS where LDH release at 168 h exceeded its release pre dosing. For 2.5 mM SDS, the tissues appeared to recover as the LDH release returned to pre dose levels ($P > 0.05$). However, at higher concentrations of SDS (5 and 10 mM), the LDH release tended to be lower than pre dose levels. At concentrations of SDS of 2.5 mM and greater, the release of LDH at 168 h were significantly less than the release at 24 h (P

Table 1
Effect of SDS on TEER (Ω x cm^2) across individual MucilAir™ inserts from three batches (1, 2 and 3) at 0 h, 24 h post dose exposure and 168 h post dose.

Concentration of SDS (mM)	TEER (Ω x cm^2)														
	0 h					24 h					168 h				
Batch number	1	2	2	3	3	1	2	2	3	3	1	2	2	3	3
0.00	469	331	367	538	577	524	434	409	275	250	499	345	321	630	630
0.16	463	350	335	739	630	519	473	345	426	402	508	269	279	861	865
0.31	521	340	324	591	573	614	410	413	575	375	391	268	310	759	851
0.63	463	351	353	696	640	556	317	269	439	623	198	319	273	809	944
1.25	550	367	363	673	729	138	35	40	100	239	600	377	338	1244	957
2.50	487	343	349	686	766	12	12	14	19	23	408	18	29	1990	2284
5.00	537	335	359	545	667	8	14	15	12	11	12	7	8	21	17
10.00	502	331	336	481	673	11	8	16	15	12	−5	8	9	15	10

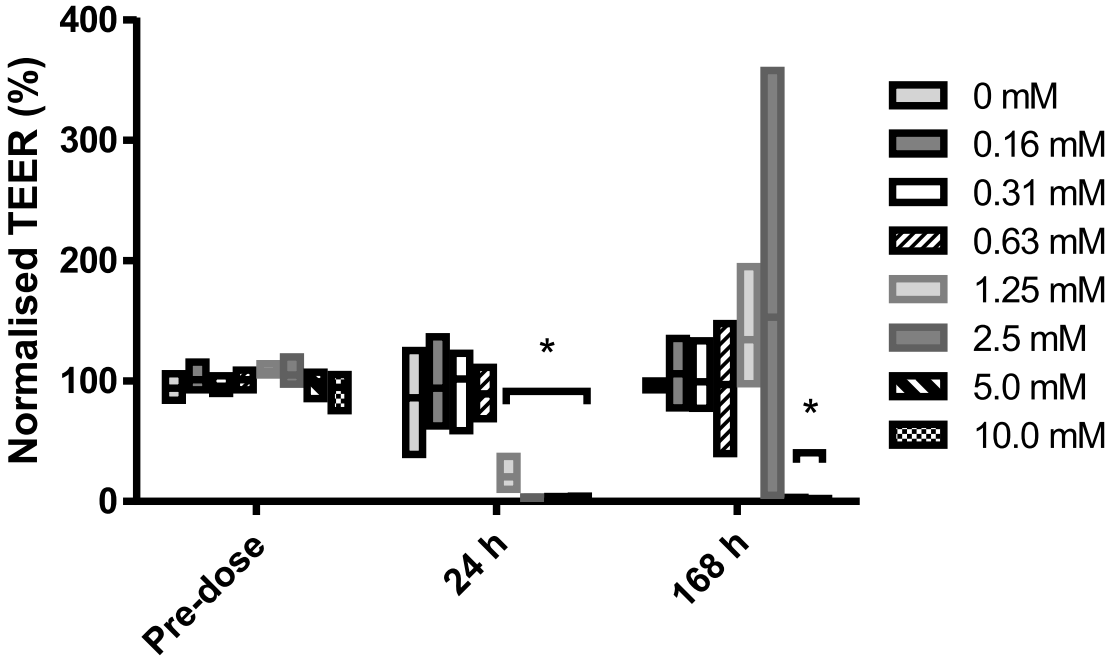

Fig. 1. Effect of SDS on normalised TEER across MucilAir™ inserts at 0 h (pre dose baseline values at each concentration), 24 h post dose exposure and 168 h post dose (i.e., after a recovery of 144 h). Mean ± SD; n = 5. * TEER statistically different to pre dose values ($P < 0.05$).

Table 2
Effect of SDS on LDH release (fluorescence of samples) from individual MucilAir™ inserts from three batches (1, 2 and 3) at 0 h, 24 h post dose exposure and 168 h post dose.

Concentration of SDS (mM)	LDH release (fluorescence of samples)														
	0 h					24 h					168 h				
Batch number	1	2	2	3	3	1	2	2	3	3	1	2	2	3	3
0.00	7.88	17.1	18.1	5.68	10.7	4.00	6.46	9.68	4.00	4.09	5.20	34.4	34.0	9.86	9.34
0.16	4.67	18.8	29.1	5.60	5.21	4.26	6.52	14.9	4.36	4.98	0.66	26.4	31.2	11.2	9.80
0.31	4.34	25.1	24.0	3.86	3.89	3.70	15.2	17.1	2.06	2.55	1.97	28.2	34.4	9.14	10.4
0.63	4.85	14.9	21.8	4.75	5.07	3.20	8.37	13.0	2.71	2.78	0.00	32.8	39.9	8.65	5.88
1.25	4.22	11.6	21.3	3.53	4.35	3.60	24.1	19.1	2.81	2.74	6.13	57.0	40.8	7.77	6.94
2.50	4.45	13.0	16.1	3.70	3.90	28.5	92.4	121	9.47	12.0	4.44	39.1	43.6	3.96	3.23
5.00	5.68	14.6	13.1	3.75	3.96	27.9	87.7	60.3	39.6	19.0	0	9.98	5.90	2.33	2.70
10.00	4.38	12.7	15.3	5.45	5.22	3.21	13.4	32.6	12.3	15.1	0	0	13.0	0	0

< 0.05).

3.3. Effect of SDS on Interleukin-8 release from MucilAir™

Prior to dosing, baseline IL-8 secretion was 4.14 ± 0.61, 4.96 ± 1.67 and 8.19 ± 3.29 ng/mL from batches 1, 2 and 3 respectively (mean ± SD; n = 8 (batch 1) and n = 16 (batches 2 and 3)) (Table 3). The mean IL-8 release from batch 3 was significantly higher than that from batches 1 and 2 ($P < 0.05$). Qualitatively, the responses of the different batches were similar (Fig. 3). IL-8 release from each insert at 24 and 168 h was compared with the pre dosing release from the same insert. At 24 h post dose, there was a significant increase in IL-8 secretion from MucilAir™ inserts exposed to SDS concentrations of 0.63, 1.25 and 2.5 mM ($P < 0.05$). Although the increase in IL-8 secretion was smaller at the lower dose level of 0.63 mM, 5 mM SDS caused an increase in IL-8 secretion but this was not significant ($P > 0.05$). Conversely, 10 mM SDS caused a significant decrease in the release of IL-8 ($P < 0.05$). By 168 h, IL-8 levels were observed to have either returned to their pre-exposure values (or

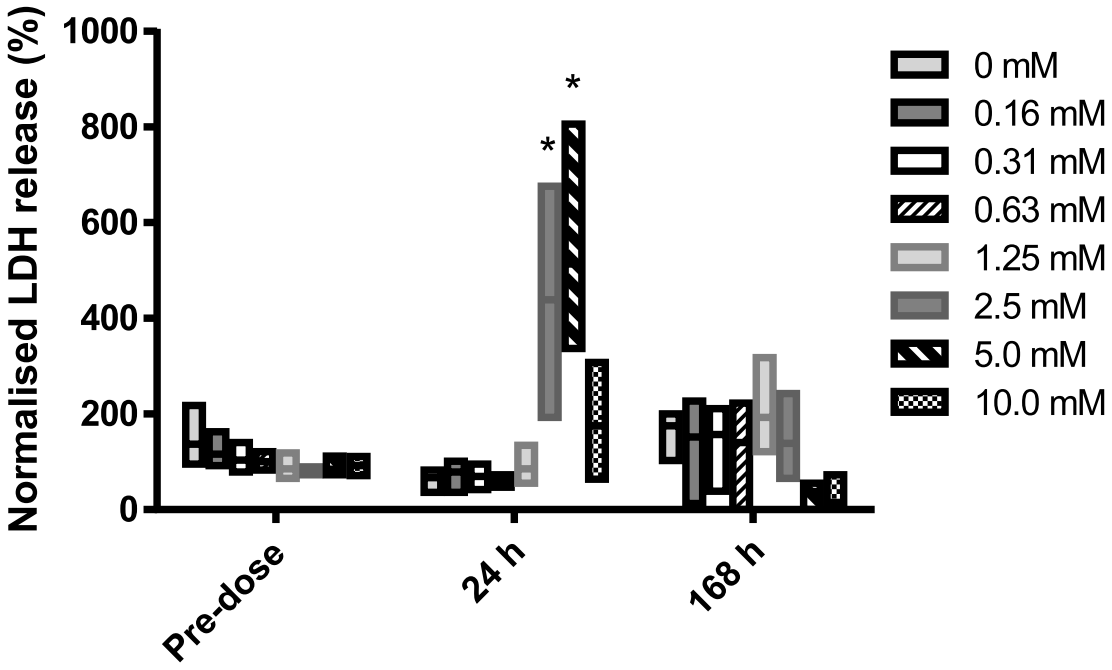

Fig. 2. Effect of SDS on normalised LDH release from MucilAir™ inserts at 0 h (pre dose baseline values at each concentration), 24 h post dose exposure and 168 h post dose (i.e., after a recovery of 144 h). Mean ± SD; n = 5. * LDH release statistically different to pre dose values ($P < 0.05$).

Table 3
Effect of SDS on IL-8 release (ng/mL) from individual MucilAir™ inserts from three batches (1, 2 and 3) at 0 h, 24 h post dose exposure and 168 h post dose.

Concentration of SDS (mM)	IL-8 release (ng/mL)														
	0 h					24 h					168 h				
Batch number	1	2	2	3	3	1	2	2	3	3	1	2	2	3	3
0.00	3.97	4.64	4.87	12.0	11.2	3.06	3.68	2.81	10.8	10.2	2.23	2.45	2.24	9.67	8.88
0.16	5.07	3.32	5.54	13.8	7.68	4.68	3.25	6.53	11.4	6.97	2.50	2.10	3.98	4.62	5.39
0.31	3.81	9.30	7.44	8.89	8.73	7.30	11.3	8.91	8.43	5.83	2.06	6.98	5.54	5.79	7.59
0.63	4.19	4.05	6.30	2.22	6.98	19.9	10.9	13.7	8.76	12.3	2.50	1.96	3.46	0.43	9.07
1.25	4.87	4.94	3.98	8.64	8.52	54.1	30.3	35.2	55.7	43.4	3.82	3.25	2.53	3.15	2.14
2.50	3.11	2.67	3.10	9.21	11.3	62.1	9.92	10.0	70.4	76.7	2.45	3.17	4.35	2.27	2.03
5.00	4.20	5.77	4.42	9.11	6.61	9.81	2.53	1.68	49.7	31.8	0.33	0.16	0.22	9.05	7.27
10.00	3.87	4.27	4.79	1.61	4.68	0.66	0.73	0.60	1.02	3.58	0.01	0.16	0.04	0.00	0.00

less) ($P > 0.05$) for all but the 10 mM SDS exposed MucilAir™, where IL-8 secretion remained negligible.

3.4. Effect of SDS on Interleukin-6 release from MucilAir™

This endpoint demonstrated the widest variation between the three MucilAir™ batches used. Prior to dosing, baseline IL-6 secretion was 119 ± 35.8, 205 ± 130 and 58.4 ± 26.0 pg/mL from batches 1, 2 and 3 respectively (mean ± SD; n = 8 (batch 1) and n = 16 (batches 2 and 3)) (Table 4). The mean IL-6 release from batch 3 was significantly lower than that from batches 1 and 2 ($P < 0.05$).

When comparing IL-6 release from each tissue at 24 and 168 h with the pre dosing release from the same tissue, no significant effects were seen at 24 h (Fig. 4) ($P > 0.05$). However, there was a slight increase in IL-6 release at SDS concentrations of 1.25 and 2.5 mM with a decrease in IL-6 release at 5 and 10 mM ($P > 0.05$). These differences were still evident at 168 h post dose.

3.5. Effect of SDS on mucin release by MucilAir™

Fig. 5 and Table 5 show the effect of SDS treatment on secretion of mucin from MucilAir™. The pre dose values for mucin release were significantly different ($P < 0.05$) for each batch of MucilAir™ inserts. The mean absorbance values of baseline mucin secretion were 0.10 ± 0.01, 0.31 ± 0.04 and 0.19 ± 0.05 AU for batches 1, 2 and 3 respectively (mean ± SD; n = 8 (batch 1) and n = 16 (batches 2 and 3)) (Table 5).

At 24 h post dose, mucin secretion was elevated at all concentrations although this increase was only significant at 1.25, 2.5 and 10 mM ($P < 0.05$). At 168 h, apart from 2.5 mM SDS, mucin secretion had returned to pre dosing levels ($P > 0.05$).

3.6. Effect of SDS on resazurin metabolism by MucilAir™

Resazurin metabolism in control cells was different in the different batches of cells; therefore, values for each tissue have been expressed as a percentage of the pre dose response for the relevant batch (Table 6). Although there was some inter-batch variability in response, the overall pattern displayed was of high levels of resazurin reduction to resorufin up to a concentration of SDS of 1.25 mM (Fig. 6). At SDS concentrations of 2.5 and 5 mM the metabolism of resazurin was reduced compared to the lower concentrations. However, the most pronounced reduction in resazurin metabolism was observed at 10 mM although there were no significant differences due to high data variability.

3.7. Effect of SDS on MucilAir™ morphology

Visual effects on MucilAir™ were apparent following treatment with SDS by histology and electron microscopy (Fig. 7). Following

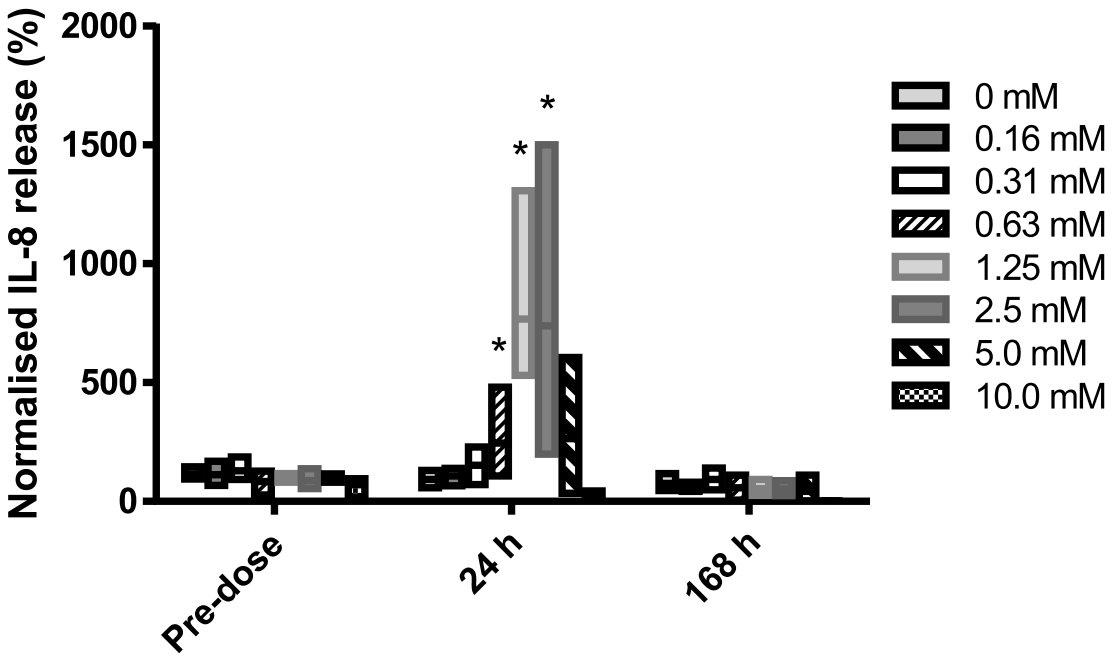

Fig. 3. Effect of SDS on normalised IL-8 release (ng/mL) from MucilAir™ inserts at 0 h (pre dose baseline values at each concentration), 24 h post dose exposure and 168 h post dose (i.e. after a recovery of 144 h). Mean ± SD; n = 5. * IL-8 release statistically different to pre dose values ($P < 0.05$).

Table 4
Effect of SDS on IL-6 release (pg/mL) from individual MucilAir™ inserts from three batches (1, 2 and 3) at 0 h, 24 h post dose exposure and 168 h post dose. * above limit of reliable measurement (LoRM) of 300 pg/mL. ** Two values omitted from Fig. 4 to aid clarity.

Concentration of SDS (mM)	IL-6 release (pg/mL)														
	0 h					24 h					168 h				
Batch number	1	2	2	3	3	1	2	2	3	3	1	2	2	3	3
0.00	138	129	111	82.2	49.6	45.0	106	89.5	23.4	13.3	29.4	111	59.6	13.5	3.4
0.16	146	145	436*	98.1	11.2	55.0	106	428*	30.5	9.4	32.9	88.3	401*	5.8	12.1
0.31	98.3	436*	435*	58.5	40.2	42.4	441*	445*	27.8	13.3	23.4	439*	431*	7.9	4.6
0.63	70.6	102	364*	25.7	102.7	106	199	406*	11.8	52.0	24.8	140	217	10.0	32.2
1.25	167	118	110	81.4	57.6	415*	428*	427*	86.6	51.1	39.1	271	176	2.8	6.7
2.50	115	112	147	51.4	60.8	475*	287	390*	154	167	31.4	453*	451*	798**	826**
5.00	146	162	170	51.4	24.2	238	203	79.4	115	72.9	301	18.1	11.0	7.9	4.9
10.00	73.8	117	176	81.7	58.2	95.7	33.3	100	59.6	48.8	1.58	1.3	17.6	4.3	2.1

histological investigation of treated tissues, when compared to the vehicle control dosed inserts, there was no observed damage in the samples exposed to SDS concentrations up to 0.31 mM. This pattern was also repeated in the observations from the electron micrographs with cilia clearly observed in all samples treated with these concentrations. The first clear effects of SDS exposure were observed in the 1.25 mM exposed histology samples with a thinning of cells. Cilia were stripped from the surface of these samples when observed in the electron micrographs. This thinning was more pronounced in the tissues exposed at an SDS concentration of 2.5 mM and there were no cilia observed in the electron micrograph.

Macroscopic observation revealed obvious erosion in tissues exposed to SDS concentrations of 5 and 10 mM exposure. Due to this extensive erosion and loss of cells from the supporting membrane, it was not possible to prepare sections for histological evaluation from these samples. When examining the electron micrographs at an SDS concentration of 5 mM, cells were observed to be retracting from the support membrane revealing patches of bare membrane. At 10 mM, there was very extensive loss of cells; the few remaining cells were rounded and no longer formed a monolayer. Electron micrographs for SDS at 5 and 10 mM are not shown for this reason.

4. Discussion

MucilAir™ has been widely used in studies of toxicity/irritancy (Sauer et al., 2013; Huang et al., 2013b; Anderson et al., 2013; Frieke Kuper et al., 2015; Kooter et al., 2017; Dankers et al., 2018; Balogh Sivars et al., 2018; Cervena et al., 2019, 2020; Rossner et al., 2019). Many of these studies used more than one cell model of the airway. Other models used alongside MucilAir™ include the primary cell culture model (Feng et al., 2015), EpiAirway™ (Sauer et al., 2013), and models derived from cell lines e.g., 16HBE14o- (with or without other cells) (Bisig et al., 2018), A549 (Anderson et al., 2013; Sauer et al., 2013), BEAS-2B (Frieke Kuper et al., 2015; Cervena et al., 2020; Rossner et al., 2019). Such cell lines tend to be more widely used in toxicity studies due to their accessibility, ease of use, reduced cost, and reduced variability (Lujan et al., 2019). However, depending on the degree of differentiation, not all cell lines are suitable for use with all toxicity assays. For instance, TEER is not a reliable measurement in BEAS-2B and A549 cells, which do not develop functional tight junctions (Winton et al., 1998; Stewart et al., 2012) and many airway cell lines e.g. BEAS-2B do not express MUC5AC (Stewart et al., 2012). MucilAir™ provides a fully differentiated airway epithelium (Huang et al., 2013a).

Donor differences is a recognised source of variation in *in vitro* experiments using primary cultures e.g., MucilAir™ and means that

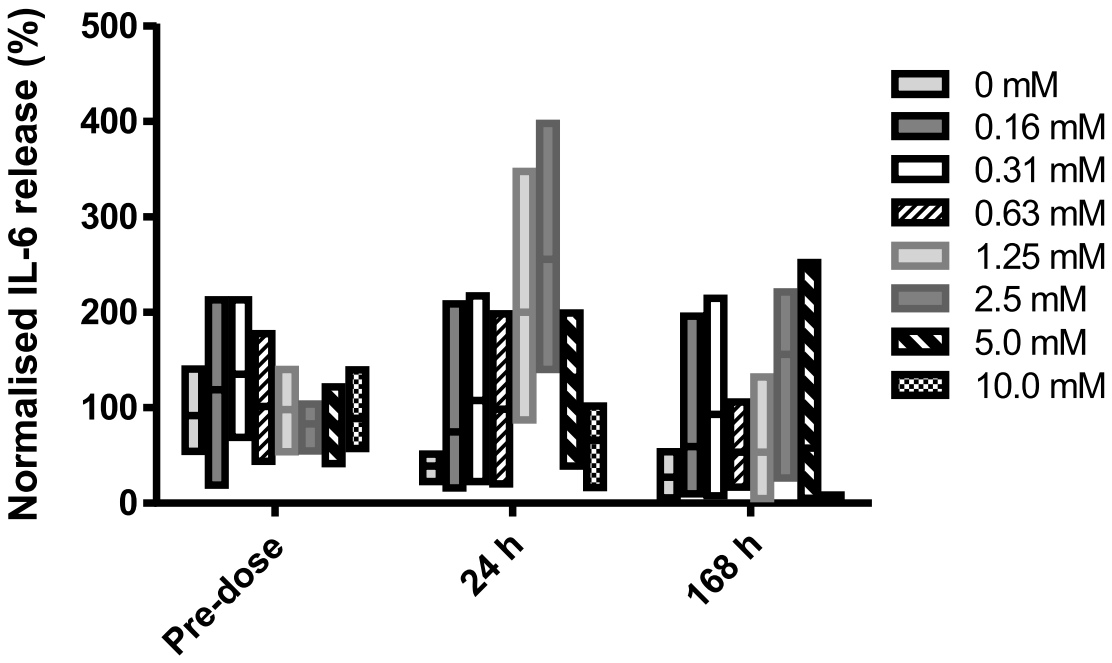

Fig. 4. Effect of SDS on normalised IL-6 release (ng/mL) from MucilAir™ inserts at 0 h (pre dose baseline values at each concentration), 24 h post dose exposure and 168 h post dose (i.e. after a recovery of 144 h). Mean ± SD; n = 5.

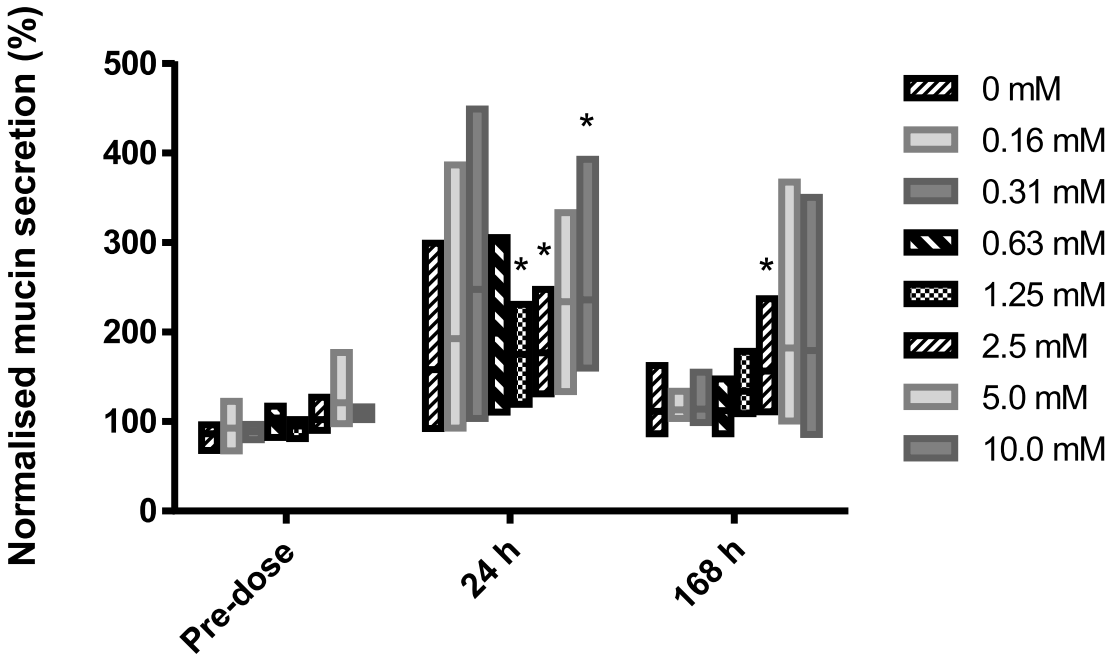

Fig. 5. Effect of SDS on normalised mucin release from MucilAir™ inserts at 0 h (pre dose baseline values at each concentration), 24 h post dose exposure and 168 h post dose (i.e. after a recovery of 144 h). Mean ± SD; n = 5. * Mucin release statistically different to pre dose values ($P < 0.05$).

careful statistical design of experiments is required particularly when testing substances with moderate to low toxicity when the variation in response could be close to the variation among donors. Kooter et al. (2017) considered this in detail in their study of aerosol application of nanoparticles to MucilAir™ cultures (Kooter et al., 2017). In the current study, five inserts from three different batches of MucilAir™ inserts were used. These came from three different donors with different history and genetics. As might be expected, the baseline values varied between batches even prior to exposure to SDS. Therefore, for statistical purposes each batch was considered individually and the effects of SDS on a particular insert, compared to the pre dose value for the batch. A graphical comparison between batches was possible by normalising post application responses to the pre-exposure measurements for each batch.

In a working day, workers can be expected to inhale a potentially harmful chemical for 6–8 h. Time to clear the chemical from the lung can be variable. Therefore, toxicity was examined after 24 h to represent a 'worst case' for risk assessment and represents an acute exposure time of interest to the chemical industry.

TEER is an indicator of tight -junction and barrier integrity (Srinivasan et al., 2015). It is reduced or lost in response to cellular damage and is, therefore, a sensitive indicator of cytotoxicity. Prior to SDS exposure, the measured TEER provided functional evidence that the

Table 5
Mucin release (% of 0 h control for each batch) for MucilAir™ inserts from three batches (1, 2 and 3) at 0 h, 24 h post dose exposure and 168 h post dose.

Concentration of SDS (mM)	Mucin release (% of 0 h control for each batch)														
	0 h					24 h					168 h				
Batch number	1	2	2	3	3	1	2	2	3	3	1	2	2	3	3
0.00	85.0	96.3	94.4	89.8	67.9	132	92.1	94.4	173	299	163	107	112	86.6	89.2
0.16	96.6	67.2	92.5	123	85.0	191	92.8	96.6	387	197	134	103	104	110	114
0.31	90.5	92.8	96.9	84	79.6	154	113	103	420	449	155	106	104	98.9	106
0.63	108	115	117	82	97.8	183	111	126	306	270	148	108	86.4	118	102
1.25	102	98.9	93.1	101	81.2	175	119	126	225	231	179	111	109	124	146
2.50	95.6	94.4	103	90.3	127	248	135	131	176	195	237	139	154	111	141
5.00	106	117	107	177	97.8	284	133	135	333	284	368	148	193	100	103
10.00	116	102	112	104	112	393	160	164	236	228	350	197	170	93.0	85.5

Table 6
Effect of SDS on metabolic competence of individual MucilAir™ inserts from three batches (1, 2 and 3) by determination of Resazurin reduction (% of vehicle control for each batch) at 168 h post dose.

Concentration of SDS (mM)	Resazurin metabolism (% of vehicle control for batch)				
Batch number	1	2	2	3	3
0.00	100	81.8	118.2	93.4	107
0.16	96.4	93.2	264	112	95.0
0.31	99.7	366	280	81.6	111
0.63	135	56.8	200	101	94.1
1.25	185	52.3	47.7	77.6	90.8
2.50	151	20.5	27.3	44.1	44.3
5.00	32.1	2.3	−11.4	16.8	13.8
10.00	11.7	−20.5	−4.5	−0.1	−0.1

MucilAir™ cell structure contained tight, high integrity, junctions. The values were similar to, or higher than, those seen in the literature (Balogh Sivars et al., 2018; Huang et al., 2019; Cervena et al., 2019). The TEER of the cells decreased at 24 h following applications ≥1.25 mM SDS. Loss of barrier function can result from an effect on the tight junctions or damage to/loss of the cells.

Damage to cell membranes can be detected by measuring the release of the cytosolic enzyme, LDH (Korzeniewski and Callewaert, 1983; Decker and Lohmann-Matthes, 1988). SDS increased the release of LDH from the cells at concentrations of 2.5 mM and above indicating leakage through damaged cell membranes and cell death. Comparing this with the effect of SDS on TEER, it would seem likely that 1.25 mM SDS affected the tight junctions and that concentrations greater than this affect both tight junctional proteins and cell membranes. Tight junctions are composed of multiple proteins and associated with the underlying apical actomyosin ring. Tight junction proteins include transmembrane proteins (occludin and claudin) and cytoplasmic plaque proteins e.g., zonula occludens-1 (ZO-1) and E-cadherin. In a study of the effect of SDS on the cell membrane and tight junction permeability of a human colonic cell line (Caco-2) cultured on permeable supports, 0.4 mM SDS caused actin disbandment, disorganisation of the terminal web and structural separation of tight junctions (Anderberg and Artursson, 1993). A similar study, also using Caco-2 cells cultured on permeable supports, reported 0.2 mM SDS to cause structural changes to tight junctions with a change in the distribution of the proteins, ZO-1, claudin-1, occludin and E-cadherin from the circumference of the cells to the cytoplasm (Yu et al., 2013). These concentrations are lower than those observed to affect tight junctions in the current study and suggest an increased sensitivity of Caco–2 cells to SDS possibly due to a lack of protective mucus or reflecting a difference between 2D and 3D cell models. Caco-2 cells grow as a monolayer on permeable supports and therefore differ in their organisation when compared to 3D MucilAir™ tissue which is stratified in appearance. With cell monolayers, a higher number of cells for a given area of culture are in direct contact with the exposure medium potentially increasing their sensitivity. Anionic surfactants, including SDS, are known to reduce the barrier function of keratinocytes at nontoxic concentrations (as measured by a lack of LDH release) (Xian et al., 2016). This was explained by the ability of charged surfactants to bind to and denature proteins (Otzen, 2011) and an effect on tight junction protein expression.

Unexpectedly, 10 mM SDS did not appear to increase LDH release from MucilAir™. This was most likely due to immediate, extensive damage to the cells and cell death on dosing at this concentration. This would prevent continued release of LDH over the incubation period. The half-life of LDH in culture medium is *ca* 9 h (Riss et al., 2004), thus less than 20 % of initially released LDH will remain for detection at 24 h.

The airway epithelium synthesises and releases cytokines that are involved in the initiation and maintenance of inflammatory responses. Cytokine release, particularly IL-6 and IL-8, is widely used to determine the pro-inflammatory activity of inhaled substances in studies of irritancy/toxicity (Balogh Sivars et al., 2018; Balharry et al., 2008; Chow et al., 2010; Dankers et al., 2018). In common with at least one other study (Ritter, 2018), the release of IL-8 from the cells was more sensitive to the application of SDS than other endpoints of toxicity as an increase in release was seen at 0.63 mM SDS. The lack of a significant increase in IL-8 release from tissues exposed to 5 and 10 mM SDS levels is likely to be related to the extensive cytotoxicity in these cultures.

SDS resulted in no significant effect on IL-6 release. However, it should be noted that the greatest variability between batches was observed with this endpoint. In addition, several data points were above the limit of reliable measurement (LoRM) of 300 pg/mL of the assay. Due to the variability in data and some values above the LoRM, the data were not considered to be reliable despite a suggestion that 1.25 and 2.5 mM increased release of IL-6, with a decrease at higher concentrations that could be related to the extensive cytotoxicity and cell loss. This endpoint should be explored further following optimisation of the assay.

The concentration of mucus pre dosing was highest for batch 2 and lowest for batch 1, which might have been expected to translate into greater protection from the effects of SDS for batch 2. However, mucus was sampled immediately prior to dosing with SDS, which would have reduced the amount of available mucus in all cultures reducing any potentially protective effect. Mucus was also actively removed from MucilAir™ inserts before testing in the study of Sauer et al. (2013). On average, mucin secretion measured in the 24 h before dosing was less than that measured at 24 h. However, the increase in mucin secretion at 24 h was only significant in response to concentrations of 1.25 mM SDS and above indicating a defensive response to the toxic insult. In contrast to the decrease in LDH and IL-8 release observed at 10 mM SDS, mucin secretion was high. Mucin released from the damaged cells is unlikely to be degraded over the 24 h incubation period in the way LDH is. Even if the overall size of the glycoprotein was reduced, the glycosylated regions are still likely to be able to interact with the lectin of the ELLA.

Other cell models have been investigated to screen for irritation and toxicity to the respiratory mucosa. MucilAir™, EpiAirway™, A549 and 3T3 cells were compared with each other and *in vivo* data in a single study that concluded that the models were of similar value in their predictive ability (Sauer et al., 2013). The effect of SDS was studied using a mixture of assays (MTT, LDH, TEER *etc.*). The concentration of

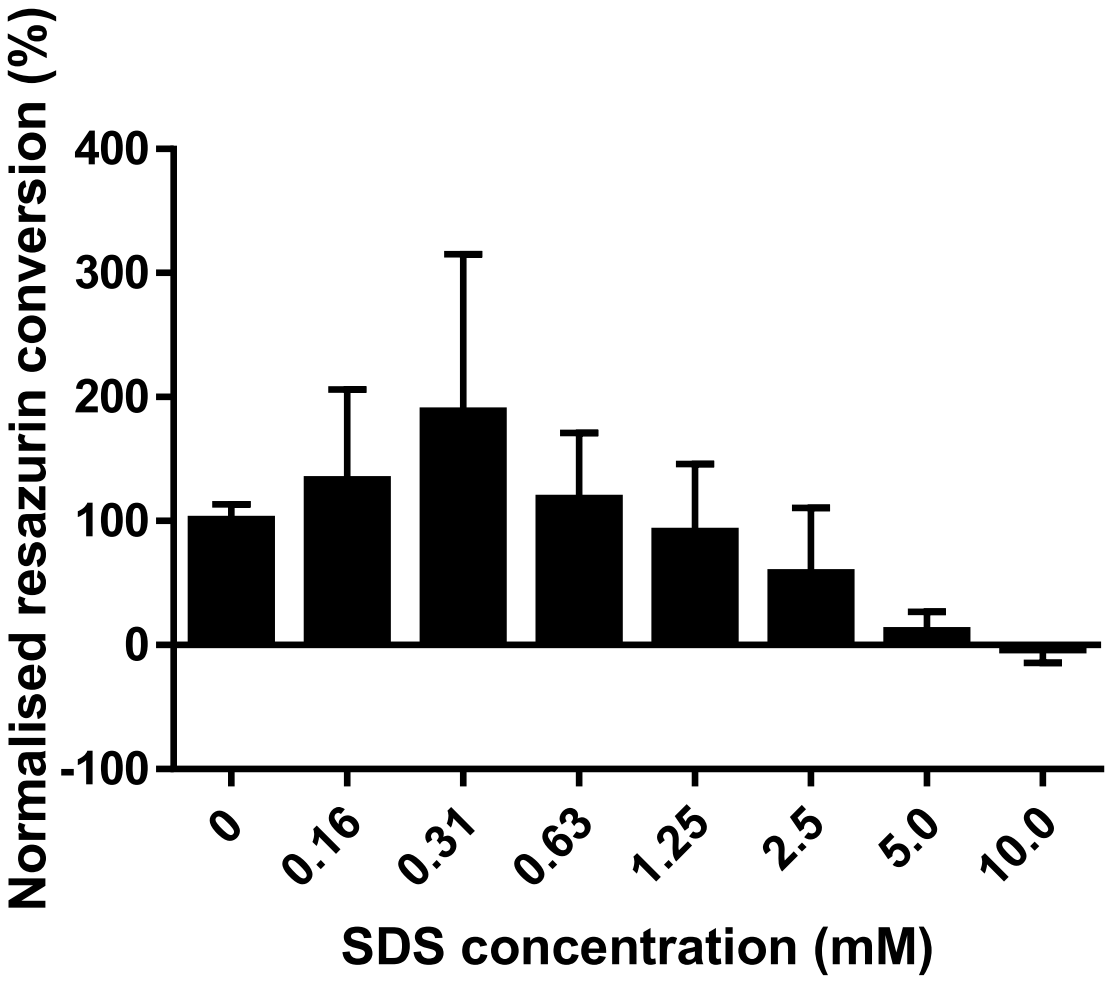

Fig. 6. Effect of SDS on metabolic competence of MucilAir™ by determination of resazurin reduction (% of pre dose control for each batch). Mean ± SD; n = 5.

SDS decreasing the measured endpoint by 50 % (IC_{50}) in MucilAir™ after an exposure of 24 h was 5.0 mM (LDH), 2.0 mM (TEER) and 0.2 mM (MTT). As a comparison, the IC_{50} for SDS in A549 cells, an adenocarcinomic human alveolar epithelial cell line which serves as a model for human type II pneumocytes, found in the alveolar region of the lung, was 0.1 mM (LDH). This placed SDS in a high category for *in vitro* cytotoxicity despite it being in a low acute toxicity hazard class *in vivo* (GSH/EPA category 3/III) (Sauer et al., 2013). This compares to an IC_{50} value between 1.25 and 2.5 mM (LDH) and between 0.63 and 1.25 mM (TEER) for MucilAir™ in the current study.

The airway cell line, Calu-3, is well characterised forming a polarised epithelium with tight junctions and capable of secreting mucus. The cells are readily accessible and are widely used in studies of drug deposition, metabolism, and absorption (Forbes and Ehrhardt, 2005; Sporty et al., 2008; Macdonald et al., 2013). Cytotoxicity of SDS to Calu-3 cells was studied using an MTT assay. 0.2 % (w/v) (6.9 mM) SDS decreased the percentage viability of the cells to 4.3 % after 60 min which supports the high level of toxicity observed in the present study (Ihekwereme et al., 2014).

Most MucilAir™ tissues, although cultured at an air liquid interface, have been exposed to the substance of interest in liquid form, as in the current study. However, some studies have used aerosols (Kooter et al., 2017) and gases (Bisig et al., 2018; Rossner et al., 2019), which, while better reflecting *in vivo* delivery is more technically demanding requiring specialised equipment.

When cultured at air liquid interface, A549 cells were used to assess the effect of SDS delivered as dry particle aerosols. Cells were exposed to SDS for 1 h and toxicity assessed after 1 or 24 h using the WST-1 assay and release of IL-8. SDS (0.17–700 mg/m^3) caused dose dependent toxicity starting at 30 mg/m^3 (equivalent to 3 $\mu g/cm^2$) (Ritter, 2018). Comparing this to the current study, cytotoxicity (LDH) was observed at between 1.25 and 2.5 mM (calculated to be equivalent to 33–66 $\mu g/cm^2$ or 330–660 mg/m^3). Considering the difference in cell types, assay used and delivery method, these could be considered to represent good concordance. However, these data indicate that MucilAir™ tissues are more robust than A549 cells or that application in aerosol form results in greater toxicity. It should be noted that different assays are recognised to yield different IC_{50} values even within a single study (Sauer et al., 2013). IL-8 release increased at the lowest aerosol dose tested (0.15 mg/m^3, equivalent to 0.015 $\mu g/cm^2$) but then, similar to the current study, decreased at higher cytotoxic SDS concentrations ($\geq$60 mg/m^3, equivalent to 6 $\mu g/cm^2$) (Ritter, 2018). This compares to an increase in IL-8 release in the current study at 16 $\mu g/cm^2$ (0.63 mM) and then a decrease at 131 $\mu g/cm.^2$ These results, using the same endpoint, also suggest that A549 cells are more sensitive to SDS than MucilAir™ (perhaps due to a lack of mucus layer) or that application of a dry aerosol is more damaging than application in liquid form to MucilAir™ tissues, perhaps due to high localised concentrations. The volume of liquid applied in the current study was 30 µL which was in line with the suppliers' protocol. Such a small volume dispersed over an area of 0.33 cm^2 effectively maintained the MucilAir™ tissue at an air liquid interface and provided an even distribution of SDS dose.

The cytotoxicity (MTT) and inflammatory potential (IL-8) of a solution of SDS (0.0025–0.04 mg/mL, *i.e.*, 8.6–139 µM) following a 24 h exposure time was assessed using A549 cells. The IC_{50} was approximately 0.01 mg/mL (35 µM) SDS, which increased the release of IL-8

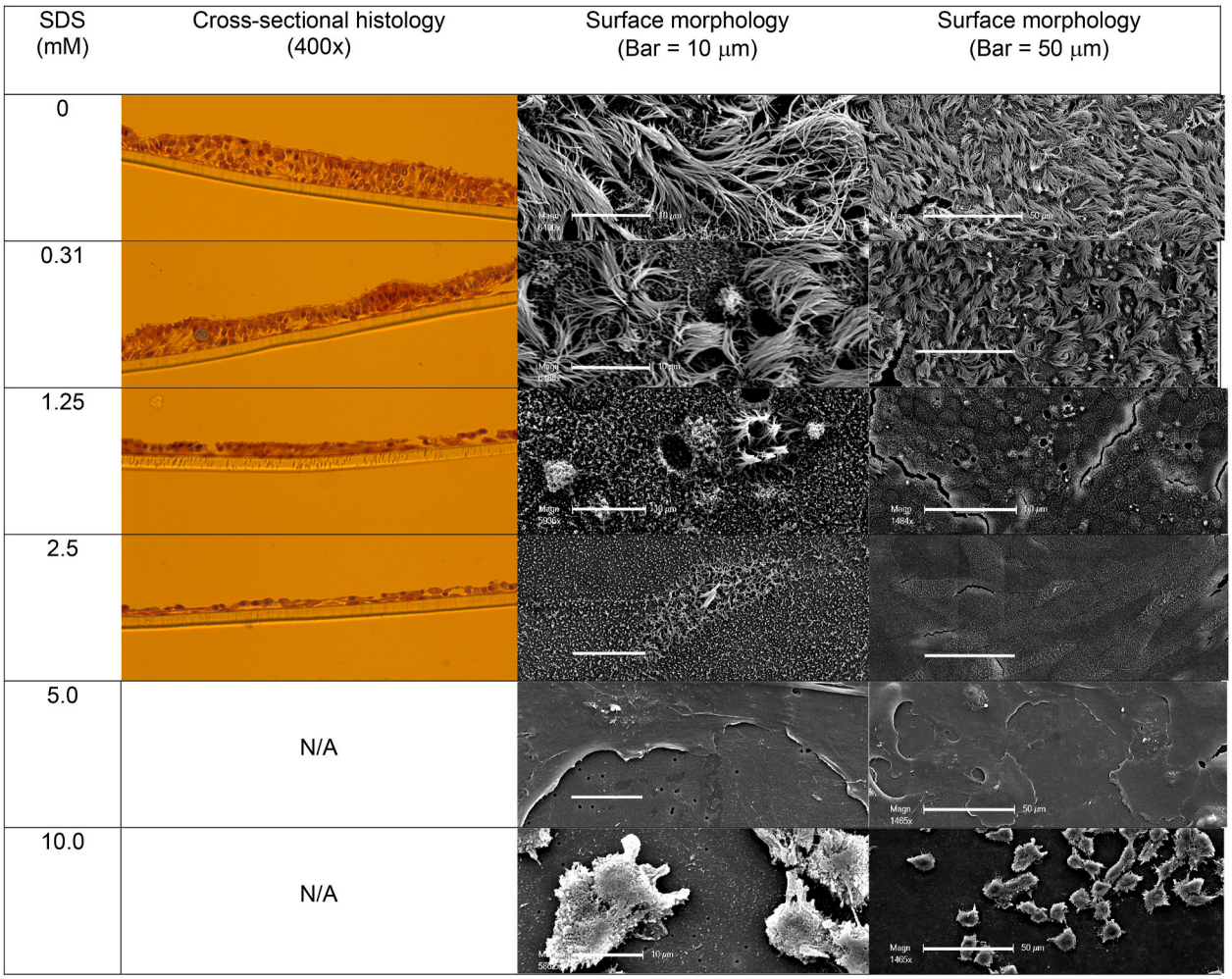

Figure 7

Fig. 7. Effect of SDS on MucilAir™ morphology assessed by histology (Batch 1) and Scanning Electron Microscopy (Batch 3) at 168 h. It was not possible to prepare histology sections of tissues exposed to SDS concentrations of 5 mM and 10 mM due to the extensive damage caused by this treatment.

secretion *ca* two-fold (Xu et al., 2003). These concentrations are lower than those used in the current study and support the suggestion that A549 cells are more sensitive to SDS that MucilAir™.

SDS is known to cause acute respiratory toxicity and there are few *in vivo* studies reporting its toxicity. The irritancy of inhaled SDS *in vivo* was assessed by monitoring the cough reflex of guinea pigs. Particulate SDS (mass median diameter 3.3 μm) was inhaled in aerosol form over 30 min and a dose dependent effect on cough was observed at all concentrations tested (17.3, 28.9 and 48.6 mg/m^3). This supported a previous study in mice (Ciuchta and Dodd, 1978) and was believed to result from stimulation of nerve endings in the tracheobronchial region *via* an unknown mechanism (Zelenak et al., 1982). Comparing this dose to aerosol delivery of SDS to A549 cells, a toxic effect was reported over a similar concentration range (Ritter, 2018). The LC_{50} of SDS in rat by acute inhalation is > 3900 mg/m^3/hour. SDS (0.1 %, w/v, *i.e.*, 3.5 mM) instilled into rat lungs for 24, 48 and 72 h significantly increased total protein concentration and extracellular LDH in bronchoalveolar lavage (BAL) fluids. Its irritant effect on the lungs of rats was indicated by an immediate increase in most BAL parameters. Increased permeability of the alveolar-capillary barrier was indicated by elevated total protein content, which may be in part due to epithelial cell lysis. Increased LDH indicated cell damage and lysis (Garcia-Contreras et al., 2001). These observations demonstrate a predictive in vitro-*in vivo* correlation of the MucilAir™ model to the animal even though the species are different, and the end point measurements are different, an irritation response can be inferred from both *in vivo* and *in vitro* measurements.

4.1. Recovery/effect seven days after exposure

In contrast to many cell -line models of the airway, MucilAir™ cultures lend themselves to long term study (Cervena et al., 2019). However, in-house observations suggest that multiple manipulations (handling, dosing, rinsing) can reduce the duration of viability. Therefore, a time point of seven days from the application of SDS was selected to assess any delayed response as well as recovery from effects observed after 24 h. This was suggested to permit sufficient time to allow cell turnover to repair any damage.

For cultures where no toxic effect was observed at 24 h, recovery was unnecessary, and no delayed response was observed. Where an effect was observed at 24 h, this was partially or completely reversible at intermediate concentrations of SDS (1.25 and 2.5 mM) and recovery occurred. At higher concentrations of SDS (5 and 10 mM) the effects measured at 24 h were usually irreversible. On occasions, the results obtained for individual assays at 10 mM SDS were unexpected but can be readily interpreted when considering the entire matrix of assays and particularly when observing the morphological data e.g., loss of cells

from inserts in the response to 10 mM SDS. This emphasises the importance of conducting morphological studies in mechanistic toxicity testing (Epa, 2018).

The morphological studies were conducted on two different batches of cells after 168 h. Histological studies were carried out on batch 1. This batch was represented by a single insert exposed to each SDS concentration; therefore, a different batch (batch 3) was used for SEM studies.

After 6 days of recovery, the effect of SDS on TEER was partially or completely reversible for SDS concentrations of 1.25 and 2.5 mM but not for higher concentrations. An exception was the single insert from batch 1 exposed to 0.63 mM SDS where the measured TEER value was less than the pre dose TEER value. This is of note as this tissue was used for histology and the cells appeared to be damaged (not shown). A similar pattern was observed for LDH release; at 5 and 10 mM LDH, release was lower than pre dose values almost certainly because of complete cell death early on at these elevated SDS concentrations. Comparison of the LDH release and TEER data at 24 h allows some insight into the mechanisms behind this reduction: in the intermediate dose groups (1.25–2.5 mM) this was probably due to repair, whereas in the highest dose groups (5 and 10 mM), this was probably due to cell death. This is supported by the morphological studies, which indicate that at 1.25 and 2.5 mM SDS, recovery was observed, suggesting that sufficient cells are present after exposure to the intermediate concentrations to repopulate the surface of the insert and to form tight junctions even if a fully pseudostratified culture cannot be achieved within 6 days. However, at higher concentrations extensive erosion and loss of cells was observed indicating no recovery. IL-8 release also returned to pre dose values for all concentrations of SDS except 10 mM, supporting this explanation.

The mucin secretion measured at 168 h from tissues exposed to SDS concentrations of 5 and 10 mM was unexpected as the exposed area was effectively devoid of cells killed by the toxicity of SDS. However, there were viable cells observed remaining around the periphery of the well, and it was postulated that these cells produced extremely high levels of mucin as a defence response to toxic insult.

The metabolic competence of cells at the end of the recovery period was assessed by measuring the reduction of resazurin to resorufin by the MucilAir™ cells (O'brien et al., 2000). As resazurin is itself a potential airway irritant, this assessment was performed only at the 168 h time point to avoid it interfering with the recovery process. It did not appear to result in any observable tissue damage in the histology and morphology assessment of the control cells. There was noticeable inter-batch variability in the metabolic activity of the cells upon exposure to SDS with batch 1 appearing to be less sensitive to higher concentrations of SDS. The metabolic activity of the cells in batches 2 and 3 tended to decrease at higher concentrations of SDS indicating a lack of full recovery of the cells and supporting the findings of the other assays.

In their review, Singer and Tjeerdema stated that SDS causes biochemical and physical effects on cells principally *via* an effect on the cell membrane. The effects are concentration dependent and range from loss of barrier function and increased permeability to complete cell lysis (Singer and Tjeerdema, 1993). This is supported by the observations of the current study.

The critical micelle concentration (CMC) of SDS in water is 8.1 mM (0.23 %, w/v). This is decreased to 1.2 mM (0.03 %, w/v) in normal saline (0.15 M NaCl) as used in the current study (Williams et al., 1955). The effect of SDS was studied over the concentration range 0.16–10 mM, to include concentrations above and below the CMC. Below the CMC, SDS exists as monomers. Above the CMC, monomers of SDS self-assemble to form micelles yielding a mixture of monomers and micelles in dynamic equilibrium. The hydrophobic portion of the SDS monomer allows it to partition into the polar lipid bilayer of the cell. Below the CMC (0.16–0.63 mM SDS), this is likely to cause some perturbation of the bilayer which will increase as the number of monomers increases. It is likely that this, or an effect caused by adsorption to membrane proteins initiates the release of IL-8 observed at 0.63 mM SDS. At 1.25 mM SDS, barrier integrity (TEER) is compromised in the absence of a significant increase in LDH release. A thinning of the epithelium and loss of cilia and microvilli accompany this. 1.25 mM SDS is close to the CMC and is likely to be affecting the proteins of the tight junctions. It also stimulates the release of mucus, which could be *via* several mechanisms (ATP release, release of intracellular calcium ions, etc.). As the concentration of SDS increases, the monomers in the cell membrane destabilize the bilayer to yield mixed lipid -surfactant fragments. These can interact with surfactant micelles and eventually the addition of higher concentrations of surfactant leads to dissolution of the bilayer and solubilisation of proteins (le Maire et al., 2000; Almgren, 2000; Imokawa, 1980). This leads to the release of LDH and ultimately destruction of the cells.

In conclusion, data were successfully collected from multiple *in vitro* assays to assess the effects of SDS on MucilAir™ tissues. Dose–dependent responses were observed with cytotoxicity visualised by histology and SEM at 1.25 mM SDS and measured chemically at $\geq$0.63 mM SDS. IL-8 release was the most sensitive indicator of toxicity, followed by mucin release and TEER with LDH release the least sensitive to the toxic effects of SDS (IL-8 > TEER = mucin secretion > LDH release). This supports the use of human tissue -derived *in vitro* models as biomarkers reveal damage before an overt pathological response is present. After 6 days of recovery, histology demonstrated that although the pseudo-stratified morphology did not recover following exposure to SDS (1.25 and 2.5 mM), basic cellular functions did show recovery after moderate levels of damage. Together, these data support a No Observable Effect Level of SDS in MucilAir™ of 0.31 mM. The utility of MucilAir™ to identify potential toxic liabilities was evaluated in detail by Balogh Sivers et al. (Balogh Sivars et al., 2018). They showed that MucilAir™ could distinguish between drug substances that were demonstrated to be safe in the clinic and identify those that were picked up as unsafe at various points in the drug discovery and development phase. The current data also support the use of MucilAir™ as a relevant model for airway toxicity studies.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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