THE USE AND APPLICATIONS OF 3D TISSUE MODELS FOR RESPIRATORY TOXICOLOGY AND EFFICACY TESTING WITHIN THE CRO INDUSTRY

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DISCLAIMER

The opinions expressed in this presentation are those of the author, and do not represent the opinions, official policy or position of Charles River Laboratories, Inc.



Biosketch: Clive graduated with a toxicology PhD in *in vitro* dermal absorption and metabolism from Newcastle University. He joined Charles River in 1996 as a Research Officer to develop the *in vitro* skin penetration service. After serving in study director and scientific manager roles, he became Head, *In Vitro* Sciences in 2010 and Director, *In Vitro* Toxicology in 2020. His department currently performs in vitro skin absorption, in vitro safety pharmacology, investigational and mechanistic toxicology using advanced tissue models and in vitro respiratory toxicology. He has presented at many meetings and organized small meetings (Skin Metabolism) and large conferences (WC9). Clive has authored and co-authored many posters and abstracts as well as peer review papers. He is a peer reviewer for journals including TIV, Regulatory Pharmacology & Toxicology, Annals of Work Exposures, and Health and Skin Pharmacology and Physiology. He has been actively involved in advising regulatory agencies including NIH, SCCS, PMRA and EPA as well as industry bodies and has presented a webinar on in vitro toxicology with the FDA. He is also a member of the global Charles River 3Rs Working Group as well as being a founding member of the North American 3Rs Collaborative. In January 2021, Clive became a Member of the Board of the UK NC3Rs.



Abstract: 3D human tissue models have been used in regulatory toxicology for many years with test guidelines for many of the acute tests (e.g. skin irritation and corrosion and ocular irritation and severe damage). The availability of models has increased to include oral, gingival, vaginal and the lung. The airway is a particularly important route of administration of drugs as well as environmental pollutants, cosmetic (e.g. deodorant) ingredients, agrochemical sprays and pathogens. One of the fundamental questions is how relevant these models are in predicting toxicity or efficacy when our benchmark tests are using animals, rather than the patient/ consumer. Are the differences observed between the animal in vivo data and the human in vitro data to do with the species or the cellular model and if it is the former, is the latter a better model? How also can we protect animals, which are still required for regulatory testing? Therefore, a rat upper airway model has been created using the Charles River rat by MatTek. This presentation evaluates the different human upper and lower airway models and the rat upper airway model for predicting toxicity and also in evaluating efficacy in human disease derived tissue - the most important translation that we can assess is the human patient derived model. This presentation will also examine where the tests may be able to be further developed.

USES IN SAFETY ASSESSMENT

- 1. Regulatory: OECD Test Guideline Tests
 - Skin Irritation (OECD 439) EpiDerm™ or EpiSkin ™
 - Skin Corrosion (OECD 431) EpiDerm™ or EpiSkin™
 - Ocular Irritation (OECD 492) EpiOcular™ or HCE ™

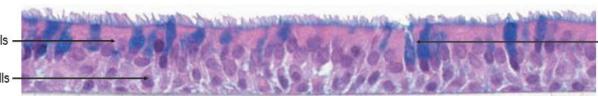


- Respiratory Toxicology
 - Upper Airway MucilAir™ or EpiAirway™ or SmallAir™
 - Lower Airway EpiAlveolar™
- Oral
 - Gingival EpiGingival™
 - Buccal EpiOral™
- Vaginal EpiVaginal™



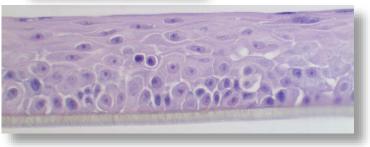


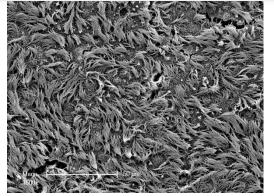






MatTek









Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit



REGULATORY TOXICOLOGY

Multi-laboratory validation of SkinEthic HCE test method for testing serious eye damage/eye irritation using liquid chemicals



N. Alépée ^{a,*}, V. Leblanc ^a, E. Adriaens ^b, M.H. Grandidier ^a, D. Lelièvre ^c, M. Meloni ^d, L. Nardelli ^a, C.S. Roper ^e, E. Santirocco ^d, F. Toner ^e, A. Van Rompay ^f, J. Vinall ^e, J. Cotovio ^a

- a L'Oréal Research & Innovation, Aulnay Sous Bois, France
- ^b Adriaens Consulting, Aalter, Belgium

18 June 2019

- ^c Episkin SA, Lvon, France
- d VitroScreen, Milano, Italy
- ^e Charles River Laboratories, Edinburgh, United Kingdom
- ^f VITO NV (Flemish Institute for Technological Research), Mol, Belgium

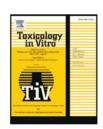


Contents lists available at ScienceDirect

Toxicology in Vitro 34 (2016) 55-70

Toxicology in Vitro

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OECD/OCDE

SkinEthicTM HCE and a related Cosmetics Europe study on HPLC/UPLCspectrophotometry as an alternative endpoint detection system for MTT-formazan. ESAC opinion No. 2014-03 of 17 November 2014; EUR 28173 EN; doi: 10.2787/043697. at:

11. Alépée, N., Leblane, V., Adriaens, E., Grandidier, M.H., Lelièvre, D, Meloni, M., Nardelli, L., Roper, C.S., Santirocco, E., Toner, F., Van Rompay, A., Vinall, J., Cotovio, J. (2016). Multi- laboratory validation of SkinEthic HCE test method for testing serious eye damage/eye irritation using liquid chemicals. Toxicol. In Vitro

12. Alépée, N., Adriaens, E., Grandidier, M.H., Meloni, M., Nardelli, L., Vinall, C.J., Toner, F., Roper, C.S., Van Rompay, A.R., Leblane, V., Cotovio, J. (2016). Multi-laboratory evaluation of SkinEthie HCE test method for testing serious eye damage/eye irritation using solid chemicals and overall performance of the test method with regard to solid and liquid chemicals testing. Toxiocal In Vitro 34, 55-

Multi-laboratory evaluation of SkinEthic HCE test method for testing serious eye damage/eye irritation using solid chemicals and overall performance of the test method with regard to solid and liquid chemicals testing



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REGULATORY TOXICOLOGY

Review

Pathway-based predictive approaches for non-animal assessment of acute inhalation toxicity

Amy J. Clippinger^{a,*}, David Allen^b, Holger Behrsing^c, Kelly A. BéruBé^d, Michael B. Bolger^e, Warren Casey^f, Michael DeLorme^g, Marianna Gaça^h, Sean C. Gehenⁱ, Kyle Glover^j, Patrick Hayden^k, Paul Hinderliter^l, Jon A. Hotchkiss^m, Anita Iskandarⁿ, Brian Keyser^o, Karsta Luettichⁿ, Lan Ma-Hock^p, Anna G. Maione^k, Patrudu Makena^o, Jodie Melbourne^a, Lawrence Milchak^g, Sheung P. Ng^q, Alicia Paini^r, Kathryn Page^s, Grace Patlewicz^t, Pilar Prieto^r, Hans Raabe^c, Emily N. Reinke^u, Clive Roper^v, Jane Rose^w, Monita Sharma^a, Wayne Spoo^o, Peter S. Thorne^x, Daniel M. Wilson^m, Annie M. Jarabek^y

PETA International Science Consortium Ltd., London N1 9RL, UK. bIntegrated Laboratory Systems, Contractor Supporting the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods, Research Triangle Park, NC, US. cInstitute for In Vitro Sciences, Gaithersburg, MD 20878, US. dCardiff School of Biosciences, CF10 3AX, Wales, UK. eSimulations Plus, Inc., Lancaster, CA 93534, US. fNIH/NIEHS/DNTP/NICEATM, Research Triangle Park, North Carolina 27709, US. g3M, St. Paul, MN 55144, US. hBritish American Tobacco plc, London WC2R 2PG, UK. Dow AgroSciences, Indianapolis, IN, US. Defense Threat Reduction Agency, Aberdeen Proving Ground, MD 21010, US. MatTek Corporation, Ashland, MA 01721, US. Syngenta, Greensboro, NC, US. The Dow Chemical Company, Midland, MI 48674, US. Philip Morris Products SA, Philip Morris International R&D, Neuchâtel, Switzerland. RAI Services Company, Winston-Salem, NC 27101, US. PBASF SE, 67056 Ludwigshafen am Rhein, Germany. GE.I. du Pont de Nemours and Company, DuPont Haskell Global Center for Health Sciences, Newark, DE 19714, US. European Commission, Joint Research Centre (JRC), Ispra, Italy. The Clorox Company, Pleasanton, CA 94588, US. US. Environmental Protection Agency, Office of Research and Development, National Center for Computational Toxicology, Research Triangle Park, NC, US. US. Army Public Health Center, ATTN: MCHB-PH-HEF Gunpowder, DO 21010-5403, US. CHAPES NIGHT Lealth, Iowa City, IA. US. Protect & Gamble Co, Cincinnati, OH 45241, US. Vuniversity of Iowa College of Public Health, Iowa City, IA. US. Protection Agency, Office of Research and Development, National Center for Environmental Protection Agency, Office of Research and Development, National Center for Environmental Protection Agency, Office of Research and Development, National Center for Environmental Assessment, Research Triangle Park, NC, US.

COMMERCIALLY AVAILABLE 3D MODELS: RESPIRATORY TRACT

Ciliated cells

Nasal/Laryngeal

- MucilAir (Epithelix)
- EpiAirway (MatTek)
- Human derived tissue
- Healthy/ smoker/ diseased



Small Airway / Bronchiole

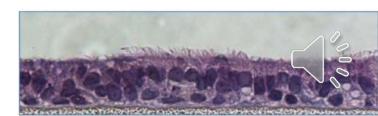
Goblet cells

• SmallAir (Epithelix)

Alveolar

EpiAlveolar (MatTek)

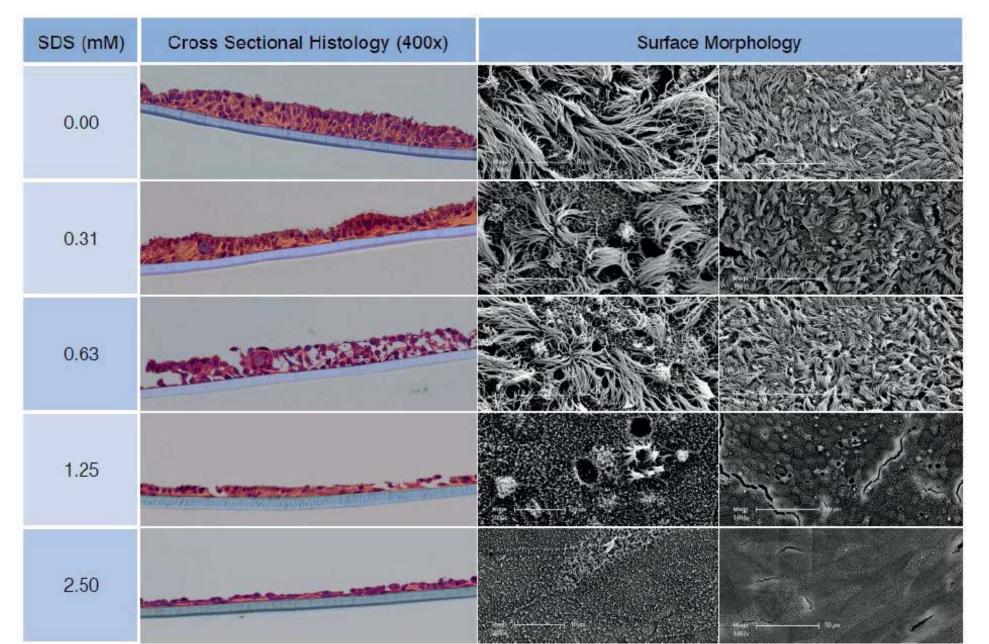




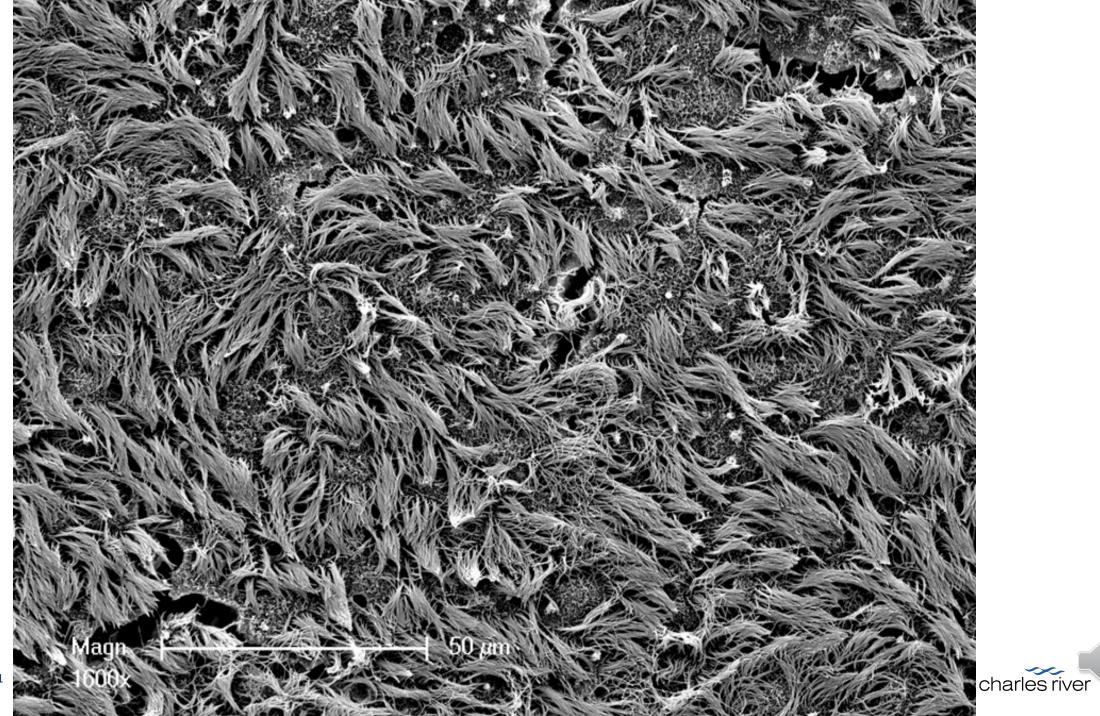
CAN THE 3D MODELS EFFECTIVELY IDENTIFY RESPIRATORY TOXICANTS?



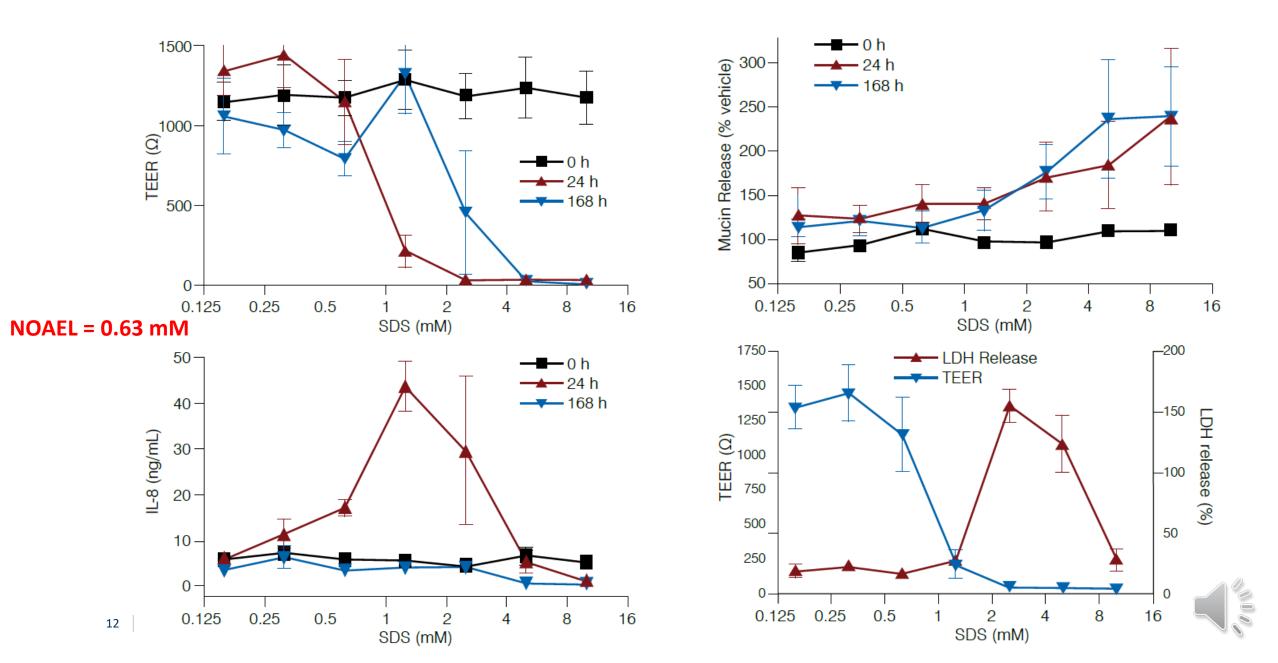
EFFECT OF SDS ON MUCILAIR™: HISTOLOGY AND MORPHOLOGY







EFFECT OF SDS ON MUCILAIR™: BIOMARKERS



HOW CAN WE PROTECT ANIMALS WHICH ARE STILL REQUIRED FOR REGULATORY TESTING?



Determining a No Effect Level for Selection of a Point of Departure for Risk Assessment of an Irritant Aerosol Using a 3D in vitro Assay

A. J. Charlton¹, B. Parr-Dobranski¹, S. Flack², T. Ramanarayanan², P. Hinderliter², and D. C. Wolf² Syngenta Crop Protection, 1 Bracknell, United Kingdom and 2 Greensboro, NC, USA

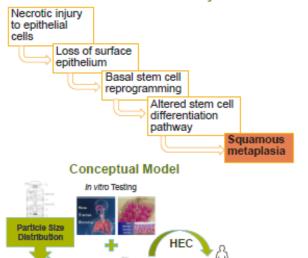
Poster Board: 101

Background

Chlorothaionii (CTN) is a broad spectrum fungicide that acts as a respiratory irritant when inhaled. A CTN containing formulation (Bravo 720*) was assessed for respiratory toxicity in a repeat dose inhalation toxicíty study. This study showed á dose dependent increase in squamous metaplasia of the larvnx at all doses tested. Additional animal studies to determine a no effect level would be unnecessary if a scientifically validated in vitro approach could be identified.

Syngenta developed such an alternative approach based upon an understanding of the mode of action that leads to squamous metaplasia of the larynx. A 3D in vitro model of respiratory epithelium was used to define the dose-response including a no effect level of the initial key

Adverse Outcome Pathway

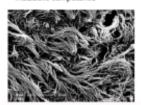


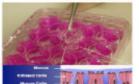
| | Cell-cell Interactions? | Representative tissue organisation? | Correlation with In vivo findings? |
|----------------------------------|----------------------------|---|---------------------------------------|
| immortalised cell monoculture | | No | |
| Primary cell monoculture | | No | No |
| 2D/3D cellular co- culture | Yes | Yes | No |
| Lung-on-a-Chip | Yes (Limited cell types) | No | No |
| MucliAir | Yes | Yes | Yes |

MucilAir

MuclAir™ is a 3D model of the human airway epithelium formed from differentiated primary human cells. Syngenta has shown good concordance between MucliAir data and in vivo study outcomes. Measures of membrane and cell damage as markers of irritation selected were:

- Trans-epithelial electrical resistance (TEER) a decrease indicates a decrease in the integrity of tight junctions between cells in the
- Lactate dehyrogenase (LDH) increased release indicates cellular cytotoxic membrane damage
- Resazurin metabolism Resazurin is metabolized to a fluorescent product in viable cells, decreased fluorescence indicates a decrease in





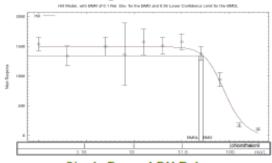


Study Design

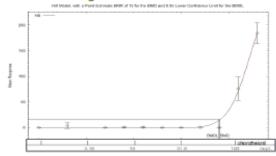
- Endpoints: TEER, LDH, and resazurin metabolism
- Tissues from 5 separate donors
- 24 hour topical exposure
- Chlorothalonii applied as Bravo 720 formulation
- 10 concentrations / donor
- 6 replicates / concentration / donor
- Concentration range 2 200 mg chlorothalonII/L

Data from each donor for each endpoint was fitted to a Hill model and benchmark dose analysis was conducted to derive benchmark dose lower 95% confidence Interval (BMDL) values.

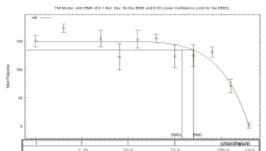
Single Donor TEER



Single Donor LDH Release



Single Donor Resazurin Metabolism



| | | BMDL (| mg/cm²) | |
|---------|---------|---------|-----------|---------|
| | TEER | LDH | Resazurin | Mean |
| Donor 1 | 0.00430 | 0.00703 | 0.00344 | 0.00470 |
| Donor 2 | 0.00317 | 0.00963 | 0.00224 | 0.00409 |
| Donor 3 | 0.00822 | 0.00878 | 0.00400 | 0.00661 |
| Donor 4 | 0.00937 | 0.00998 | 0.00066 | 0.00395 |
| Donor 5 | 0.00909 | 0.00875 | 0.00674 | 0.00813 |
| Mean | 0.00625 | 0.00877 | 0.00267 | 0.00527 |

MucliAir assay data showed very low inter-donor variability with all 5 donors exhibiting similar BMD and BMDL values. Additionally, a good concordance between TEER, LDH release and resazurin metabolism endpoints was observed. Given the low inter-donor and inter-endpoint variability BMDL values were combined to derive an overall in vitro BMDL of 0.00527 mg chlorothalonll/cm2 of the respiratory tract.

Conclusions

- Necrotic injury to the luminal surface of the respiratory epithelium is a key event in squamous metaplasia of the larynx in Chlorothalonii treated rats
- . This initial irritant response can be assessed in MucliAir, a sophisticated 3D in vitro model of the human respiratory epithelium
- MucliAir tissues from 5 separate donors were exposed to 10 concentrations of Bravo 720 for a period of 24 hours
- TEER, LDH, and resazurin metabolism were measured following treatment and these results were interrogated to determine BMD and BMDL values
- BMDL values were in the 30 100 mg chlorothalonii /L range, translating to mass per unit surface area concentrations of 0.0027 - 0.0088 mg/cm2 (in vitro mean BMDL = 0.00527 mg/cm2)

Please see the application of this in vitro PoD on our companion poster #2379 (board 103).

charles river

For detailed Information on study designs, please contact, the authors: Alex.Chariton@syngenta.com

Risk

Characterization



https://www.epa.gov/sap/meeting-materials-december-4-7-2018-scientific-advisory-panel-0

Exposure

An Alternative Approach For Evaluating The Human Health Risk From Exposure To An Irritant Aerosol

P. M. Hinderliter¹, R. Corlev², S. Kabilan², A. Kuprat², S. Suffield², S. Flack¹, T. Ramanarayanan¹, A. J. Charlton³, B. Parr-Dobrzanski³, D. C. Wolf¹

1Syngenta Crop Protection, Greensboro, NC, ²Pacific Northwest National Laboratory, Richland, WA, ³Syngenta Crop Protection, Bracknell, United Kingdom

Abstract Number: 2379 Poster Board: 103

Introduction

Modernizing strategies for evaluating human health risk from pesticide exposure requires new approaches that increase our ability to conduct an integrated evaluation of exposure and hazard. Accurate and relevant risk evaluation based on actual inhalation exposure scenarios and target site-specific respiratory surface concentrations is one such strategy to describe human health risks. Exposure data that includes measured aerosol characteristics of non-volatile pesticide formulations provides an improved input to exposure models. Coupling an airflow model with a 30 in vitro model of respiratory epithelium was used to determine a Human Equivalent Concentration. The in vitro assay was used to define the dose-response and a no effect level of the initial key event (see <u>Abstract 2377</u>, board 101 of this session for details of the *in vitro* assay).

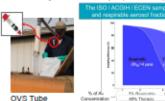
Computational Fluid Dynamic models can be used to describe target site-specific dosimetry for the rat and human and can calculate surface concentrations of deposited aerosol formulations in discrete regions of the respirationy tract. This approach can provide a more accurate reflection of the deposition necessary to initiate the cascade of events that result in an initiant mediated response in the upper respiratory tract.

Source to Outcome Approach

- Source Evaluate the particle size distribution of pesticide application and mixing/loading activities.
- Exposure Investigate the impact of spray quality and activity on inhalation exposure.
- . Dosimetry Compare deposition in human versus rat airways.
- Ourcome Predict human Inhaiation toxicity incorporating relevant particle size distributions.

Pesticide Exposure Data

- Exposure data is commonly collected from agricultural workers using an OSHA Versatile Sampler (OVS) tube. Typically, OVS tube data is only reported as total concentration, without consideration of particle size.
- Studies of spray particle size were undertaken at Syngenta to compare OVS tube with standard sizing methods. Side-by-side air sampling was conducted using OVS tube and Respicon for various agricultural nozzles/spray qualities.
- Air concentration of inhalable particles varies with spray quality, but particle size distributions are similar. Thus, can use same distribution for different spray applications.
- Identified particle size distribution of 35 µm (GSD=1.5) for applicator & residential bystander and 13 µm (GSD = 1.5) for mixer/loader

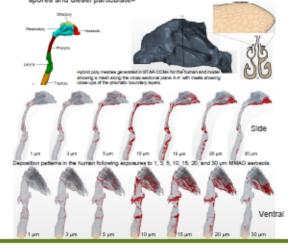


Particle Size Distribution Inhalation Exposure HEC Risk Characterization Risk Characterization Risk Characterization

Conceptual Model

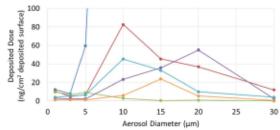
Computational Fluid Dynamics (CFD) Modeling

- · Simulates the fluid dynamics of the airflow in the respiratory tract
- · Surfaces are meshes of polygons, each of which can be monitored
- Commonly used in simulation of smaller particulates like bacteria spores and diesel particulates



Deposition from the CFD model –





-- Vestibule -- Respiratory -- Olfactory -- Pharynx -- Larynx -- Trachea

| Aerosol | | C | Deposition | (mg/cm2) | | |
|---------------|-----------|-------------|------------|----------|----------|----------|
| Diameter (µm) | Vestibule | Respiratory | Olfactory | Pharynx | Larynx | Trachea |
| 1 | 4.29E-06 | 3.04E-06 | 5.22E-06 | 1.71E-06 | 2.16E-06 | 7.34E-07 |
| 3 | 3.39E-06 | 2.43E-06 | 4.55E-06 | 1.26E-06 | 2.48E-06 | 7.69E-07 |
| 5 | 5.73E-06 | 2.86E-06 | 1.26E-05 | 1.48E-06 | 3.08E-06 | 6.36E-07 |
| 10 | 1.62E-04 | 4.50E-06 | 1.77E-06 | 5.41E-06 | 1.40E-05 | 1.30E-06 |
| 15 | 2.91E-04 | 2.90E-06 | 9.73E-07 | 3.49E-06 | 8.46E-06 | 1.39E-06 |
| 20 | 2.76E-04 | 2.27E-06 | 6.50E-07 | 1.85E-06 | 2.67E-06 | 5.57E-07 |
| 30 | 1.50E-04 | 1.89E-06 | - | 5.61E-07 | 1.03E-06 | 2.13E-07 |

Calculation of Human Equivalent Concentration - Applicators

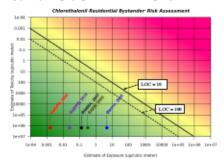
- Aerosol: 35 (1.5) µm polydisperse
- Breathing rate: 16.4 breaths/minute or 7872 breaths/8hr
- Dilution Factor 0.438 lb CTN/lb diluted formulation
- In vitro BMDL 5.27x10⁻³ mg CTN/cm²

| | Respiratory | Olfactory | Pharynx | Larynx | Trachea |
|---|-------------|-----------|----------|----------|----------|
| Deposition (mg/cm²/breath) | 2.00E-06 | 1.41E-07 | 8.82E-07 | 1.61E-06 | 3.12E-07 |
| Deposition (mg/am ² /8hr) | 6.89E-04 | 4.85E-05 | 3.04E-04 | 5.54E-04 | 1.08E-04 |
| HEC (mg/L) | 0.34 | 4.8 | 0.76 | 0.42 | 2.1 |

 The exposure concentration is the air concentration necessary to match the in vivo 8 hour deposition with the in vitro BMDL

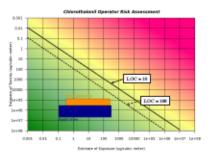
Residential Bystander Risk Assessment

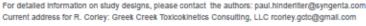
- Max Average Exposure Range = 0.002 4.86 µg/m³
- Toxicity (HEC) Range (mg/L converted to mg/m³) = 112 1,590 mg/m³



Operator Risk Assessment

- Exposure Ranges:
- Applicators = 0.11 313 μg/m³; Mixer/Loader = 0.33 259 μg/m³
- Toxicity (HEC) Ranges (mg/L converted to mg/m³):
- Applicator = 335 4,760 mg/m³; Mixer/Loader = 77 616 mg/m³







OCCUPATIONAL TOXICOLOGY USING MUCILAIR™

Calculation of Human Equivalent Concentration - Applicators

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| HEC (mg/L) | 0.34 | 4.8 | 0.76 | 0.42 | 2.1 |



 The exposure concentration is the air concentration necessary to match the in vivo 8 hour deposition with the in vitro BMDL



HOW DOES THE RAT 3D MODEL COMPARE WITH THE HUMAN 3D MODEL AND THE *IN VIVO* DATA?



TEST PANEL OF 14 CATEGORISED RESPIRATORY IRRITANT/ NON IRRITANTS WITH HUMAN AND RAT EPIAIRWAY™

Human and rat EpiAirway™ units were treated with 14 test chemicals formulated in either corn oil or ultrapure water at each of 4 different concentrations with vehicle and positive (formaldehyde) controls.

Experimental conditions and study design were similar except the tissues were rinsed with PBS at 3 h post dose and allowed to recover for 21 h.

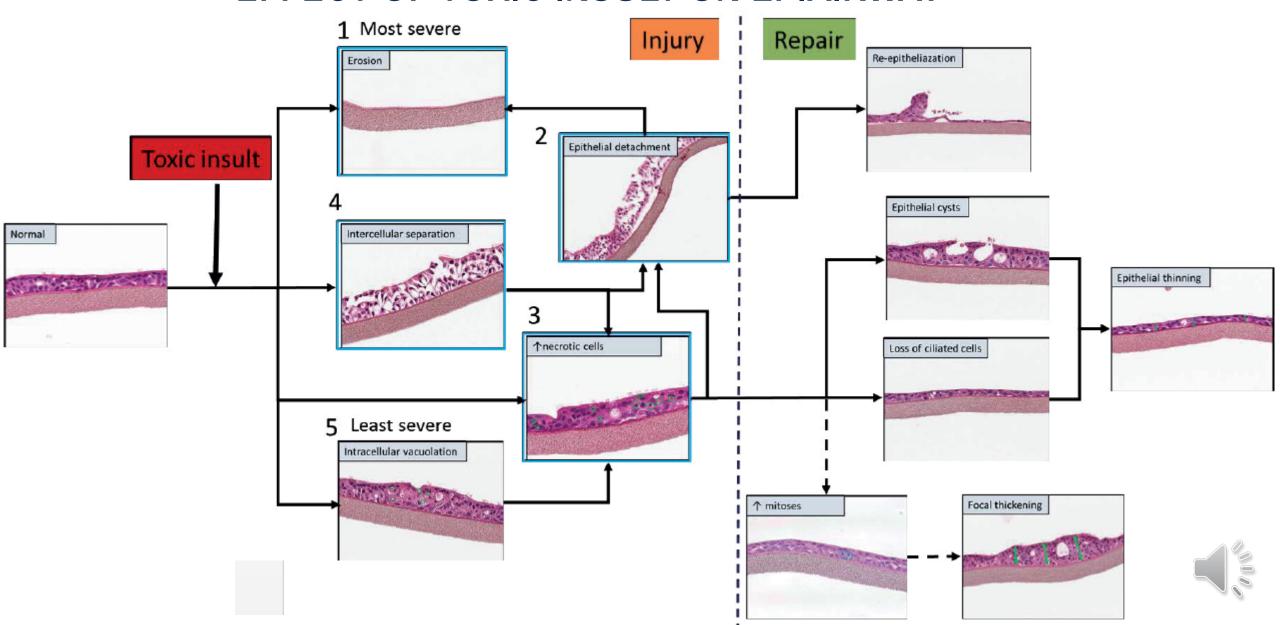
TEER, MTT and histopathology were the endpoints.

2 Laboratories

- MatTek Corp., Ashland, MA, USA
- Charles River, Edinburgh, UK



ADVERSE OUTCOME PATHWAY EFFECT OF TOXIC INSULT ON EPIAIRWAY™



TEST PANEL OF 14 CATEGORISED RESPIRATORY IRRITANT/ NON IRRITANTS WITH HUMAN AND RAT EPIAIRWAYTM (IC₇₅)

| | Char | les River | Edinburg | h | | M | atTek | | | | |
|-----------------------|-----------|-----------|----------|------|-------|------|-------|------|--------------|----------------|-----------------------------|
| | MT | Т | TEEF | ₹ | MT | MTT | | ER . | Known 1 | oxicity Inform | mation** |
| Test Chemical | Human | Rat | Human | Rat | Human | Rat | Human | Rat | GHS Category | Known RI* | Skin/Eye Irritant |
| Acrolein | 0.05 | 0.06 | 0.04 | 0.05 | 0.04 | 0.03 | 0.03 | 0.03 | 1 | Υ | Υ |
| Formaldehyde | 0.64 | 0.81 | 0.81 | 0.32 | 0.17 | 0.13 | 0.20 | 0.18 | 3 | Υ | Υ |
| NaOH | 1.11 | 1.15 | 1.63 | 1.10 | 0.73 | 0.73 | 0.88 | 0.31 | No data | Υ | Υ |
| Butyl Amine | 1.34 4.56 | | 1.39 | 3.16 | 0.92 | 1.07 | 0.89 | 0.51 | 3 | Υ | Υ |
| Oxalic Acid | 1.22 | 1.16 | 1.39 | 0.48 | 1.16 | 0.67 | 0.88 | 0.27 | No data | Υ | Υ |
| Morpholine | 7.90 | 18.3 | 9.13 | 19.0 | 10.6 | 16.2 | 11.8 | 16.2 | 3 | Υ | Υ |
| Vinyl Acetate | 56.2 | 47.2 | 52.4 | 33.0 | 21.3 | 18.8 | 16.1 | 10.1 | 4 | Υ | N |
| Ethyl Formate | 190 | 199 | 152 | 161 | 109 | 115 | 63.2 | 62.4 | 4 | Υ | N |
| 2-Ethoxyethyl Acetate | 174 | 134 | 143 | 75.7 | 117 | 72.6 | 57.5 | 57.3 | 4 | N | N |
| Methyl Methacrylate | 146 | 41.3 | 126 | 28.9 | 132 | 29.9 | 130 | 14.9 | 5 | Υ | N |
| Dimethyl acetamide | 265 | 286 | 271 | 303 | 266 | 221 | 263 | 147 | 4 | N | N |
| N,N-Dimethylformamide | 269 | 281 | 304 | 291 | 311 | 285 | 309 | 229 | 4 | N | N |
| Ethyl Alcohol | 304 | 242 | 338 | 142 | 320 | 217 | 306 | 140 | 5 | Υ | M Color |
| p-Dichlorobenzene | 251 | 221 | 242 | 233 | 346 | 240 | 358 | 235 | 5 | N | $N \supset S_{\mathcal{O}}$ |

TEST PANEL OF 14 CATEGORISED RESPIRATORY IRRITANT/ NON IRRITANTS WITH HUMAN AND RAT EPIAIRWAYTM (IC₇₅)

| | Char | les River | Edinburg | h | | Ma | ıtTek | | | | |
|---------------------|-------|-----------|----------|--------------|------|------|-------|------|--------------|-----------------|-------------------|
| | MT | Т | TEEF | 3 | MT | Т | TEE | R | Known | Toxicity Inforr | mation** |
| Test Chemical | Human | Dat | | 2 | | | Uuman | Rat | GHS Category | Known RI* | Skin/Eye Irritant |
| Acrolein | 0.05 | 0.06 | 0.04 | 0.05 | 0.04 | 0.03 | 0.03 | 0.05 | 1 | Υ | Υ |
| Fraidehyde | 0.64 | 0.81 | 0.81 | 0.32 | 0.17 | 0.13 | 0.20 | 0.18 | 3 | Υ | Υ |
| NaOH | 1.11 | 1.15 | 1.63 | 1.10 | 0.73 | 0.73 | 0.88 | 0.31 | No data | Υ | Υ |
| ıtyl Amine | 1.34 | 4.56 | 1.39 | 3.16 | 0.92 | 1.07 | 0.89 | 0.51 | 3 | Υ | Υ |
| Oxan | 1.22 | 1.16 | 1.39 | 0.48 | 1.16 | 0.67 | 0.88 | 0.27 | N arta | Υ | Υ |
| Morpholine | 7.90 | 18.3 | 9.13 | 19.0 | 10.6 | 16.2 | 11. | -16 | 3 | Υ | Υ |
| Vinyl Acetate | 56.2 | 47.2 | 52.4 | 33. U | 21.3 | 18.8 | 16.1 | 10.1 | 4 | Υ | N |
| Ethyl Formate | | 199 | 152 | 101 | 109 | 110 | | 62.4 | 4 | Υ | N |
| 2-Ethor Acetate | 174 | 134 | 143 | 75.7 | 117 | 72.6 | 57.5 | 57.3 | | N | N |
| ethyl Methacrylate | 146 | 41.3 | 126 | 28.9 | 132 | 29.9 | 130 | 14.9 | 5 | Υ | N |
| Dimethyl acetamide | 265 | 286 | 271 | 303 | 266 | 221 | 263 | 147 | 4 | N | N |
| - Dimethylformamide | 269 | 281 | 304 | 291 | 311 | 285 | 309 | 229 | 4 | N | N |
| Ethyl Are | 304 | 242 | 338 | 142 | 320 | 217 | 306 | 140 | | Υ | M S |
| p-Dichlorobenzene | | 221 | 2/12 | 222 | 2/16 | 240 | | 200 | 5 | N | $N \cap S$ |

TEST PANEL OF 14 CATEGORISED RESPIRATORY IRRITANT/ NON IRRITANTS WITH HUMAN AND RAT EPIAIRWAYTM (IC₇₅)

| | Char | les River | Edinburg | h | | M | atTek | | | | |
|-----------------------|--------------|-----------|-----------------|------|-------|------|-------|------|--------------|-----------------|----------------------|
| | MT | Т | TEER | | MT | MTT | | ER | Known | Toxicity Inform | mation** |
| Test Chemical | Human Rat Hu | | Human | Rat | Human | Rat | Human | Rat | GHS Category | Known RI* | Skin/Eye Irritant |
| Acrolein | 0.05 | 0.06 | 0.04 | 0.05 | 0.04 | 0.03 | 0.03 | 0.03 | 1 | Υ | Υ |
| Formaldehyde | 0.64 | 0.81 | 0.81 | 0.32 | 0.17 | 0.13 | 0.20 | 0.18 | 3 | Υ | Υ |
| NaOH | 1.11 | 1.15 | 1.63 | 1.10 | 0.73 | 0.73 | 0.88 | 0.31 | No data | Y | Υ |
| Butyl Amine | 1.34 | 4.56 | 1.39 | 3.16 | 0.92 | 1.07 | 0.89 | 0.51 | 3 | Υ | Υ |
| Oxalic Acid | 1.22 | 1.16 | 1.39 | 0.48 | 1.16 | 0.67 | 0.88 | 0.27 | No data | Υ | Υ |
| Morpholine | 7.90 | 18.3 | 9.13 | 19.0 | 10.6 | 16.2 | 11.8 | 16.2 | 3 | Υ | Υ |
| Vinyl Acetate | 56.2 | 47.2 | 52.4 | 33.0 | 21.3 | 18.8 | 16.1 | 10.1 | 4 | Υ | N |
| Ethyl Formate | 190 | 199 | 152 | 161 | 109 | 115 | 63.2 | 62.4 | 4 | Υ | N |
| 2-Ethoxyethyl Acetate | 174 | 134 | 143 | 75.7 | 117 | 72.6 | 57.5 | 57.3 | 4 | N | N |
| Methyl Methacrylate | 146 | 41.3 | 126 | 28.9 | 132 | 29.9 | 130 | 14.9 | 5 | (Y) | N |
| Dimethyl acetamide | 265 | 286 | 271 | 303 | 266 | 221 | 263 | 147 | 4 | N | N |
| N,N-Dimethylformamide | 269 | 281 | 304 | 291 | 311 | 285 | 309 | 229 | 4 | N | N |
| Ethyl Alcohol | 304 | 242 | 338 | 142 | 320 | 217 | 306 | 140 | 5 | Υ | M S |
| p-Dichlorobenzene | 251 | 221 | 242 | 233 | 346 | 240 | 358 | 235 | 5 | N | $N \cap \widehat{o}$ |

Evaluation and Comparison of *In Vitro* Rat and Human Airway™ Epithelial Models for Inhalation Toxicity Testing

H. Simpson, J. Bally, C. Roper and J. Vinali Charles River Laboratories Edinburgh Ltd, Tranent, EH33 2NE, UK





Acute sirvey training is en important consideration during the development of phermaceutosis, chemicals, cosmetics and agrochemicals. As part of our integrated toxicology and SFs testing programs, there is a requirement to assess in with SD human and animal derived models to generally information on buildly. This date can be used for screening, in support of dose range finding for in vivo leading or as an early prediction for translation from eximal to human

Human Epi-Anway^{as} (Malfel: Corporator) is a commercially available functional model of the human airway epifestum derived from sells collected from the streets of healthy denote and collected at the strings of high interface. Red Epi-Anway^{as} (MetTek Cooperators) uses at large epi-terial cells collected from Chartes River sels and is created similarly to the human model. The objective of this study was to evaluate and compare the responses of the two sirvey models.



METHODS

On enhall of Charles River, rat (WR-190-R) and burson (WR-100-DM20) EpiAnway* were equilibrated in culture for 5 days prior to beling in a humidited incubelor set to maintain 37°C, 5% CO₄. Media was changed at 3.0 day intervals On the letting day, because were sinced with phosphale bulliered soline (PDS) to remove the muous and branchesed to Sech media. Transpillhelid (Decirical Resistence (TESP) was measured in the sir liquid interface-controls (ALI controls) prior to desire. 14 test there were form tailed in cosmoil or ultrapure water at each of 4 different comprehelions and legisd in parallel in appropriate vehicle and positive (formaldehyde, 147 mg/ml.) controls. ALI controls were breated exactly the same as lest item breated its uses with the exception that they were not do set

The stude probabilis summerized in Figure 1



All insubations were conducted in a humidified insubation set to maintain SPO, 596-00, with the exception of the foreignen extraption which was conducted at room temperature. During the exposure-period, Millioni caps were placed on top of each fissue to prevent emporation of soletile leaf items (Figure 2). After all his groupe, the figures were stosed with FGG to remove the institlents and bandlemed to first pre-warmed media for a 21th



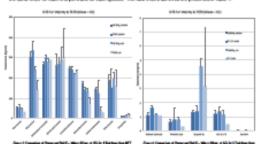
Following recovery, post dose TEER was measured in all lists as. Prior is TEER measurements, the natificatives were rhand with FBS to remove mutus. After TEER was measured, fiscure were bandward to MTT solution and insubsted for 1.5 h then formacian extracted in extraction solution for 2 h on exhating platform. The extract solution from the top and bollom of the Souse were combined and mixed prior to being duplicate allquots from each sample and analysed using a MultiSkan Go Specing between at 570 nm with correction at 650 nm. At leaf items were feeled on at least 3 independent occasions. On one occasion, a fourth rat and human tissue were dosed with each lest item and processed as described above with the exception that following TEER resourcement they were function of the parents make in yet. embedded in parallin then abined with HBE before cross sectioning. No NET assay was conducted on these fasses. From the MTT and TEER date, on Council or the concentration required to reduce the sistility to 70% of the appropriate

RESULTS AND DISCUSSION

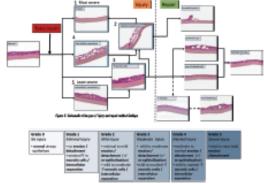
The delayers presented in Figures 3 and 4. For MET, there were 3 or 4 acceptable runs of leating (i.e. where a doce regionse from the MTT assey was observed under KC₁₄ could be calculated; except for viry lacetate in the human EpiAmey**. For TEER, exceptible data was achieved for all samples except for NN-directly formanide in human. EpiA/may**. For rat TEER, stable measurements were not always possible or dose responses were not apparent resulting in a TBER IC₆₀ for only? of the 14 leaf items on 3 or more occasions.

Overall, the data from the MTT and TIER assays were in good agreement predicting stimler ID, values for each test liers. These was stoogs of into sun reproductbility in the predicted ID, values showing considercy between batches

For most of the bod items, the Ki_W values calculated from the human and reliciouses were very similar, although the set issues were particularly sensitive to methyl methycrytets and ethyl alcohol. Strough, the 14 feet lives were natived in the same order for each and point and till each species. The same orders achieved are consented in Table 1.



Human and reliainway Status morphology correlated well with its alto primate and reliainway muccoses. Airway Statuss softbilled lower numbers of olisied and mucous cells and increased numbers of degenerate cells. A moderate increase in mogificities variability was seen in nellificitiows (** compared to the furner. The spectrum of microscopic changes stressed following exposure to known bulbants was similar as seen in an inal model studies. The kee injury related findings (Figure 5) were equal on, epithelial detachment, intercellular separation and an increase in necrolic cells. Here repair related findings were bas of cliabel cells, epithelial thinning, re-epithelialisation, cost formation, increased micros, spitelial trinning and focal trickening. Using the key injury related findings, a composite scoring system was constructed to easign a single grade to each sample (Figure II). Samples were asset in a blinded fastion and both burroun and and airways were found to respond to instruction a similar manner with clear doze-eviational/dox widerst



| Park III | | | | | | | | | | | | | | ** | |
|----------|-------|---------|-----------------|--------------------|---------------------------|----------------|------------|--------------------|------------------|-------------------------|--------------|------------------|---------------------------|-----------------------|-----------------------------|
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| | | | | | Ordinanti-baylanks follow | | | | | | | | | | |
| - | | | | | | | | | | | | | | Michaely females | |
| - | TIO | Action | Pomelódy (e.) | OwelloActd | Social Systematic | Bright Service | Morpholina | Methyl Melharykini | Virgilitaties | Ehospahylikalate | Distributed. | Ethyl formalis | \$4066estavan | Millionally females | PROTECTION OF THE PROPERTY. |

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CONCLUSION

In conclusion, the penel of fill lect items were auccessfully lected on burson and rel Epi-Knewy^{NA} Source. The TEER and MET visibility results for both the reliand human Epikirway^{as} fasses were highly reproducible. The results from the pathology observations support and complement the TEICR and MTT results for each test item. The nat and human Epi-kirway^{an} Dasses were generally similar, with the notable exception of methyl methacrytets and ethyl alcohol, for which the nell boxes were considerably more consider. However, the results produced across each led our will in each species were swy completent.

ACKNOWLEDGMENTS

The authors would like to thenk Poblick Heyden (MarTek Corporation, 200 Homer Avenue, Ashland, Massachuselfs 01721, USA) and his fearn for their polishosation, expentise and advice-over the course of this project.

This work was generalally supported by the Charles River immunions Rund.

Evaluation of In Vitro Models of the Rat and **Human Airway Epithelium for Assessment** of Acute Toxicity



H. Simpson¹, P. Hayden², GJ. Jackson J², J. Vinali¹, E. Storey¹, M. Debetts², A. Malone², C. Roper¹ and M. Kleusner² *Charles River Laboratories, Edinburgh Ltd, Edinburgh, UK, *MelTek Corporation, Auhland, MA., USA

Introduction

Acute sirvey toxicity is an important consideration for the development of pharmage alignic, obersignic, controlled and safe-use of products, procedures for accidental exposure and dose range selection for in who studies. Currently validated melhods require the use of animals settli is widely

acknowledged that these methods may notibe appropriate for accurate prediction of human sists. To comball this, and as part of a SK's shaboy (before, reduce, replace), in alto models are being developed as an alternative diralogy for assessment of intrafettos levicity.

One such commercially available sirvery model is EpoNewsyth (Histlink Corp., Ashland, MA, USA) constructed from primary human sirvery epithelial cells. All present frees is no means to constall the responses of these fissues to human cultumes as available in vivo data has been produced in notects. To address this information pap, an in vitro sirvey model has been generated in a similar manner to the turnantigs Airway ** model using cells from the sirvey epithelium of Charles River rats. In this study the rat and human models were compared on three independent experiencing two laboratories (Charles Florer and MalTel) using 14 leaf chemicals



Methods

RESULTS

Hussen EpitArray*: The human model was prepared by seeding roomal human branchial spithelial sells on a Hillipore colleges coaled memberse insert. Until a confused soundates was timed, the inserts were callured. subsected. When a confluent monstager had developed. He inserts were further callused at the similarity interface for up to 21 days. A fully of fiversiated airway model was formed (Figure 2).

Pat Baliforne * Reven with did note individual from the conduction already of the each old male. On talk (Charles River Laboratories, NA, USA) and seeded onto Milipore memberne inserts. These cultures were cultivated at the airiquid interface for up to 27 days (Figure 2).

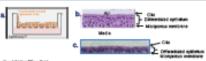
Histological Evaluation: Human and relicultures were fixed, embedded, sectioned and stained in HIEE and exemined



Fire-dose TBEP measurements from air liquid interface IALB though were found to be high indicating the suppressful production of a robust barrier in both human and sal Epikineay**. Haddogical evaluation of the Epikineay** models. revenied a new chalastified exitted und much statistically functionally

The MTT and TEER date from Chares River and MatTelcare presented in Table 1. King values from the MTT data are presented in Figure 5 and Figure 6. In each species, a clear dose response was observed in the MTT data. There were relances in both laboratories where it was not possible to obtain stable TEER measurements or see a clear class response in the TEER data. At Charles River, this was most pronounced with the ratifigi-Airway ** where an IC₁₄ was calculated on 0 or more exceptions for only? chemicals. At Matter, instances of this were depended where human and of Estatemp?

| | - | The same | | | С | - | | | | |
|---------|-------|---------------------------|------------------------------------|-----------------|----------------------|---|---|--|--------|---|
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| ilumen. | ** | home | - | firme | | in. | Ret. | GIS Laboury | Down W | Chilly by |
| 100 | 0.06 | 9.06 | 0.00 | 600 | 961 | 640 | 640 | | - | Y |
| 6.68 | 6.86 | 0.84 | 440 | 662 | 0.00 | 640 | 644 | - | - 1 | - Y |
| 144 | 144 | 1.60 | 140 | 4/8 | 634 | 648 | 844 | Inches. | | Ŷ |
| 1.00 | 415 | 1.00 | 818 | 640 | 1409 | 6.00 | 684 | | | Y |
| 6.50 | 11.64 | 1.36 | 64 | 6.00 | 0.61 | 1.00 | 839 | Distance. | | - Y |
| 380 | 184 | 540 | 880 | 95.6 | 160 | 10.0 | 1864 | | | - 4 |
| 560 | 402 | 504 | 880 | 21.8 | 154 | 1064 | 184 | | - 1 | |
| 181 | 200 | 80 | 160 | 100 | MA. | 482 | 634 | | - | |
| 636 | 100 | 140 | 183 | 142 | 704 | 676 | 674 | | - | |
| 944 | 48.0 | 636 | 04.6 | 180 | 2004 | 100 | 18.8 | | | |
| 166 | OM. | 160 | 100 | 100 | Dis. | 260 | 140 | | - | |
| 260 | 260 | 600 | 284 | 10.0 | 200 | 604 | 500 | | | |
| 900 | 940 | 100 | 180 | 160 | 20 | 106 | 140 | | - | |
| 266 | 200 | 963 | 200 | 96 | 20 | 904 | 200 | | | |
| | None: | MY lunes to cor cor | MY TO lamen to fume 02 00 00 | term to turn to | tioner has been con- | tioner had from the from take and now now one con non- | 100 100 | Wilson W | | Mart Mart |

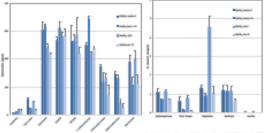


fact Chamical Selection: Assert of Michanicals was askeded to include arrange of SHG dead Scalars and chemical chasses acids, bases, colduris, aldebucies, kelbose, esters, alcohols, amines, bylosensied enumalius, shensi and positive derivatives. A dose range finding experiment was conducted to determine appropriate text concentrations.

inflation. Assay: Culture: were allowed to equilibrate for 19-24 h (MatTei) or up to 50 h (Charles Rived in 1 ni. EpiFrway ** assay medium. Prior to desing, muous was removed from the apical listue-studies and disparted. Each test chemical, formulated at a concentrations, was applied to refland human Epikinese¹⁰⁰ columns. in triplicate. Each insert was 'oppost' to prevent cross contemination and evaporation of volatile compounds. After all the operare period (in a humidiled incubator set to maintain SPC, SN-CQL, because were sinced then allowed to recover oversight. Visibility was assessed by MTT assay and barrier function by transepthetial electrical resistance (TEER), ID_a values (the consentration required for reduce stability or TEER to 19% of the vehicle control) were calculated. Appropriate vehicle and positive controls were included. This procedure wa pediamed on Discussions by each laboratory. Auchematic of the protocol is presented in Figure 3:







Court Courties of December 1965, West, Marco of Style Chairman for March 1966, No. 1974, State 1966.

Overall, there was broad agreement between MTT and TIBER data and the results were considerabetween leating occupions. A plantar response is each led chemical was observed between species with C₁₂ unknowledge of the same order of magnificial. The exceptions is this were methyl methanologic and ethyl about it is with not fighthere, ³⁴ yet more sensitive than human Epikhreng. ³⁴ (in both laboratories). Additionally, EpiAirway^{AA} (both species) lested at MatTek was more sensitive to these compounds than when feeled at Charles Flowr Between laboratories, the test items were generally ranked in the same order, in terms of potency, highlighting the consistency and leastfeability of the Epi-Krwey** models and both locus. The rank orders of chemicals based on IRET data from human and set Epi-Krwey** each laboratory are presented in Table 2 showing as insign pattern both between laboratories and species. In each instance, the TEER dafa

| | ior (das Dig (e11) | | | • | | | | | | | • | | | | |
|-------|-----------------------|------------|-------------------------|-------------------|--------------------|----------------|-------------|--------------------|------------------------|---|-----------------------|----------------------------|--|--|--------------------------|
| | 66. | Simple in | Participation (Company) | folion/sylvation | Ordered | Bud bridge | Moghaire | Name Assessed | Water Matter of the | 3 Elloyetyl Avenue | Dhyl Formale | pikiskingberena | IN DESCRIPTION OF THE | 100 Smally Formande | (MylAlekel |
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| 20.0 | 66. | ander. | Formal Setyle: | folken instructor | Ordered | DOM: NAME OF | Meghalise | Water Mathematical | Weyl Asserts | 3 El horystryl Austrian | Dhyl formale | pikiskingisensora | DAY KINKS | Introductive Formatide | Withouthylaconthic |
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CONCLUSION

in conduction, the genet of 14 test from were successfully tested on human and set Epi-Wavey^{ave} Sources. The TEER and MET visibility results for both the rail and human lightway. A fiscure were highly reproducible. The responses of the set and human Epi-Armay¹⁴ feaces were generally similar, with the notable exception of methyl method state and ethol alcohol. for which the set former were considerably more seculive

However, the results produced across each leaf run within each species were very consistent. In addition, the leakwas caused out by two independent laboratories and the two delaureb were in general agreement. Combation of These results with in vivo results will be important for showing the predictiveness of these models for building screening and



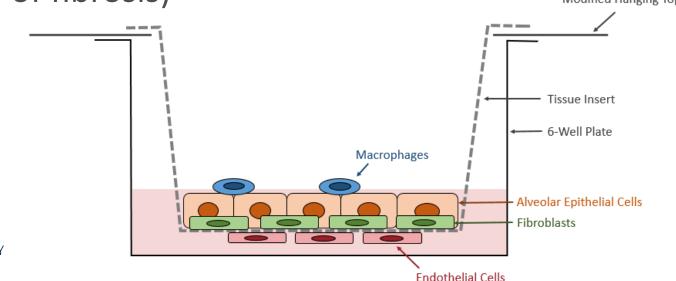
The continue funded in part by training help by a discrepance of the discrepance of the ANSE COLD COLD and by the Courte Sizer Innovatives Fund.

HOW CAN WE USE THE ALVEOLAR MODELS?



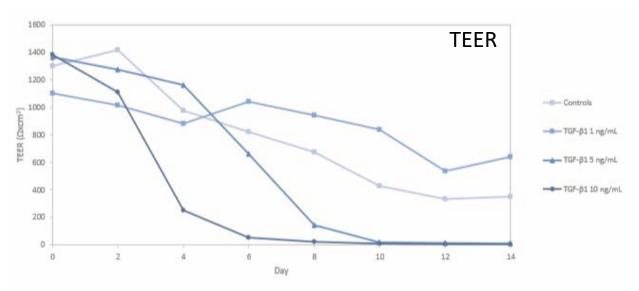
MODEL RESPIRATORY FIBROTIC AGENT: TGF-β1

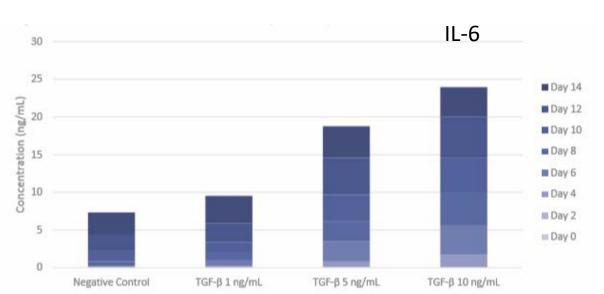
- TGF-β1 dosed at the liquid interface at 1, 5 and 10 ng/mL
- Media containing TGF-β1 was changed every 2 days, exposure to 14 days
- TEER measured every 2 days prior to media change
- On Days 2, 6, 10 and 14, tissues were processed for histopathology
- The spent media analyzed by ELISA for fibronectin, IL-6 and Pro-Collagen I $\alpha 1$ (biomarkers of fibrosis)

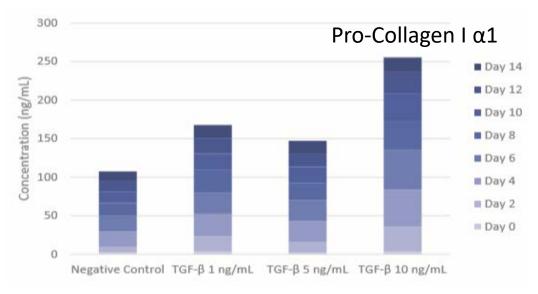


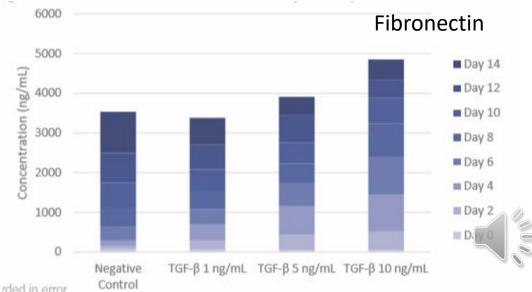


MODEL RESPIRATORY FIBROTIC AGENT: TGF-β1



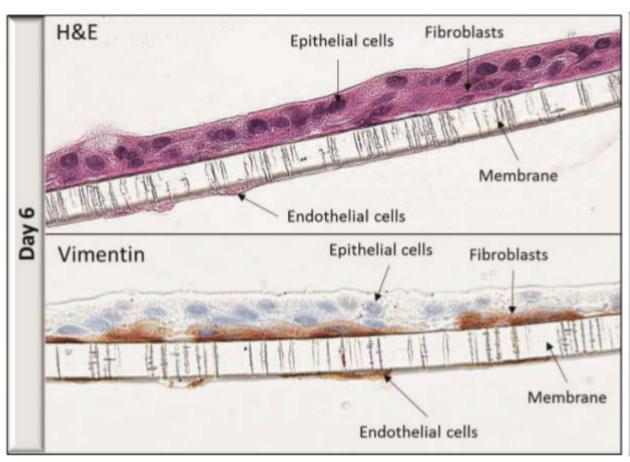


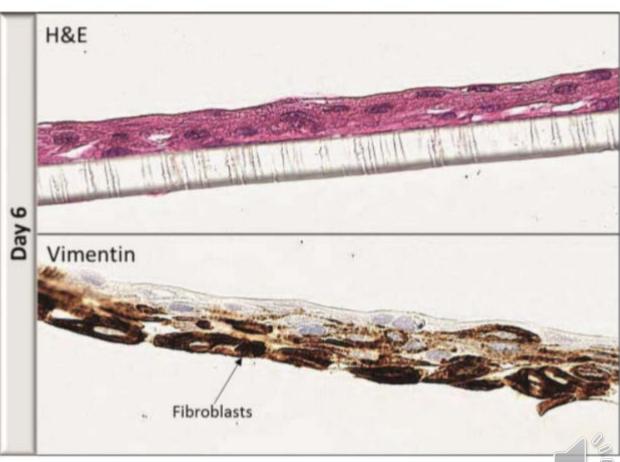




MODEL RESPIRATORY FIBROTIC AGENT: TGF-β1

Representative Images of Untreated and TGF-β1 Treated Samples on Day 6

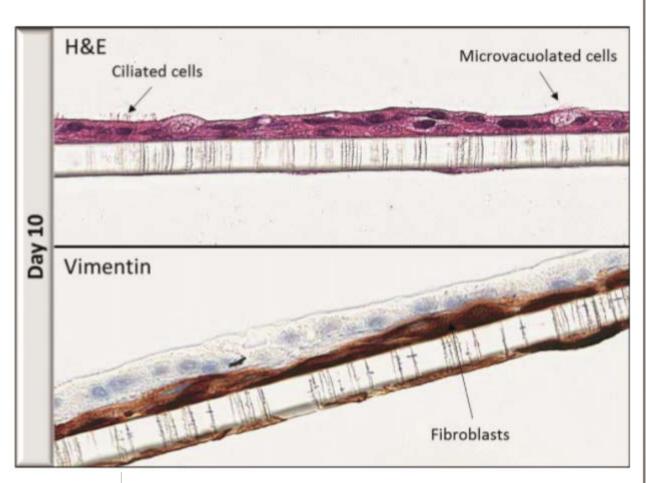


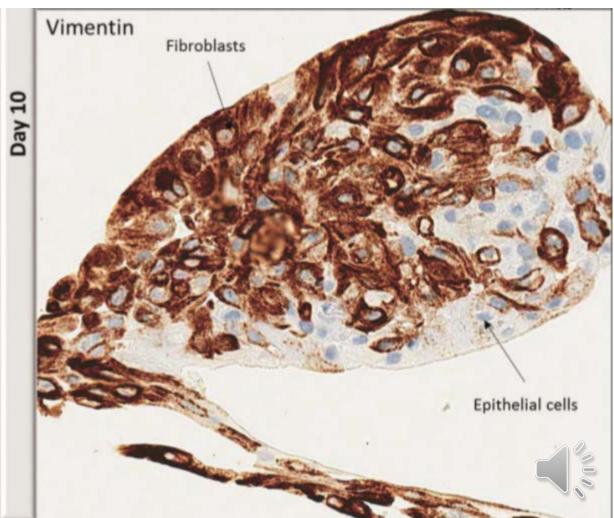


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MODEL RESPIRATORY FIBROTIC AGENT: TGF-β1

Representative Images of Untreated and TGF-β1 Treated Samples on Day 10





Assessment of Alveolar Toxicity and Fibrotic Potential In Vitro using the MatTek EpiAlveolar™ Model

CS Roper, JE Baily, HJ Simpson and JL Vinall Charles River Laboratories, Edinburgh, UK





INTRODUCTION

Pulmonary fibrosis (PF) is a debilitating, typically fatal, condition caused by a variety of factors. including environmental or occupational exposures, drugs, radiation and genetic predisposition. No current in vitro (immortalized cell lines), ex vivo, in silico or in vivo models of PF fully recapitulate all salient features of the human disease.

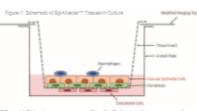
In this study, a novel in vitro organotypic 3D airway model from primary human cells (EpiAlveolar**) from MatTek Corporation) with macrophages was examined to evaluate alveolar toxicity and the capacity of the model as a method to assess fibrotic potential in vitro. Previously, this model has been shown to behave in a manner reflecting the parent fissue and under some experimental conditions can develop aspects of PF seen in the lower respiratory tract^{NI}.

The study design was based on the protocol provided by MatTek⁽³⁾. EpiAlveolar™ tissues were challenged to develop aspects of PF, by exposure to known PF causing agents; bleomycin (12, 0.12 or 0.0012 μg/mL) or TGF-β1 (10, 5 or 1 μg/mL) in the culture media, or to repeated serosol applications of silice (50 nm perticle size, ce 10 or 1 µg/cm²), for up to a 14 day period. Tissue viability was assessed by transepithelial electrical resistance (TEER) and lactate dehydrogenase (LDH) release every second day. Tissue samples and spent culture media were collected throughout the time course. for analysis by pathology, immunohistochemistry (IHC) and ELISA to further characterise the healthy model and to identify aspects of PF developing over time.



MATERIALS AND METHODS

EpiAlveolar™ is a novel commercially available functional model of human alveoli, derived from primary human alveolar epithelial cells, pulmonary endothelial cells, fibroblasts and monocyte-derived macrophages (THP-1s), cultured at the air-liquid interface (Figure 1).



Tissue viability was assessed by TEER and LDH release assays on Day 0. Following this, a group of untreated tissues were processed for histology/ pathology. In addition, 11 tissues per treatment group were treated on Day 0 (either aerosol exposure then incubation in blank media for 2 days or incubation in media containing doses for 2 days). The following groups were tested:

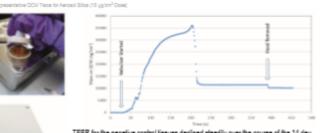
- Sílica nanospheres (50 nm) aerosol application at ca 1 µg/cm² and ca 10 µg/cm² doses
- Bleomyoin sulfate (BLM) at 12 (0.1 Unit), 0.12 (1 mU) and 0.0012 µg/mL (0.01 mU).
- TGF-81 at 10, 5 and 1 no/mL
- Controls: vehicle control for aerosol and negative control (control for BLM /TGF-\$1). Aerosol exposure was conducted using a saline vehicle in the Vitrocell Cloud 12 exposure chamber with associated quartz crystal microbalance (QCM) for assessment of applied dose (Figure 2).

Dosing was repeated every 2 days. The TEER and LDH measurements were repeated after every 2 day incubation. On Day 2, Day 6 and Day 10, 2 tissues per group were processed for histology. pathology. Five tissues per group underwent the full 14 day regime, at which point all remaining tissues were processed for histology/ pathology. Spent culture media at each time point was retained for ELISA analysis (2 tissues that underwent 14 day treatment per group) for the following markers: fibronectin, IL-6 (kits obtained from R&D Systems) and Pro-Collegen I a1 (Abcam).



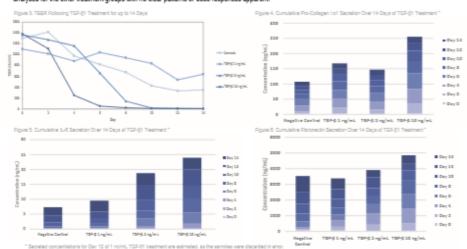
RESULTS





TEER for the negative control tissues declined steadily over the course of the 14 day experimental period from ca 1300 to ca 350 Ωxom¹. This may relate to contraction of the fissues away from the insert walls, and has been observed previously. TEER results from the serosol vehicle group, silica groups and BLM groups were similar to this. A clear dose response was observed in the TEER data from TGF-\$1 treated tissues (Figure 3).

Compared to the untreated control, the 5 and 10 ng/mLTGF-61 treated groups TEER dropped to close to zero by Day 10 and Day 6, respectively. LDH release each day was compared to an independent set of Triton X-100 lysed tissues prepared delity and calculated as a percentage of that value. Generally, compared to the lysed controls, the levels of LDH released from all samples were negligible. However, on Day 6 and Day 8, a small increase in released LDH was measured in all TGF-81 groups, silica (1 µg/cm²) and the top BLM dose group (data not shown). These data indicate that the TGF-\$1 treated tissues, in particular, are being put under stress, manifesting as a reduction in tight junction integrity (TEER) and leaking of some cell contents, peaking between 6 and 8 days into the treatment regime. However, the LDH released did not approach lysed cell values, indicating that the majority of the cells remained alive. A clear dose response was measured for secreted extracellular matrix proteins. Pro-Collegen I a1 and Fibronectin and the inflammatory marker, IL-6, from the TGF-β1 treated groups over time (Figures 4-6). Pro-Collegen I a1 secretion was also analysed for the other treatment groups with no clear patterns or dose responses apparent.





PATHOLOGY AND IMMUNOHISTOCHEMISTRY

Model Characterisation (Figure 7)

Up to Day 6, untreated controls displayed consistent morphology with a single deep layer of vimentin (VM) positive spindle shaped fibroblasts covered by a layer of 2 to 3 cells thick of polygonal VM negative epithelial cells. Rare ciliated cells and microvacuolated cells were observed in the surface layer. Degenerate cells were also rare and occasional VM positive endothelial cells were observed on the underside of the

membrane. From Day 10, there was overall thinning of the epithelial cell layer with increased numbers of VM positive cells and surface layer ciliated and microvecuclated cells. This suggests a change in phenotype between Day 6 and Day 10 characterised by proliferation of the fibroblast subpopulation and proliferation/ preferential survival of a subset of

ainway epithelial cells included in the initial population.

TGF- 81 Treatment (Figure 8)

By Day 6 of TGF- \$1 (10 ng/mL) treatment, the overall thickness of the cell layer was similar to untreated controls, however, the majority of the cell population expressed a spindle shaped VM positive fibroblast phenotype. From Day 10, samples exhibited multifocal thickening and, in places, protuberant foci composed of multiple levers of VM positive fibroblasts mixed with lesser-VM negative epithelial cells. Increased numbers of degenerate cells were observed in these samples.







DISCUSSION AND CONCLUSION

Silica and BLM treatments at the selected dose levels did not show clear patterns in their responses and were generally similar to their respective controls, indicating that the 14 day challenge with these materials did not result in toxicity or induce markers of PF in the EpiAlveolar ** tissue model. However, LDH release (cytoloxicity) increased slightly after approximately 6 days of treatment for all TGF-81 dose levels. TGF-81 treated fissues also displayed a dose response reduction in TEER. (tight junction integrity) around the same time point and increased secreted Pro-Collegen I at over time (precursor of collegen type 1, for which production and accumulation is the hallmark of PFI. This data correlates well with the histopathology observations. Lower TEER values and increased Pro-Collagen I of are likely due to the change from a predominantly epithelial to fibroblast phenotype whilst increased LDH is likely a result of increased numbers of degenerate cells observed from Day 6 of TGF-& treatment.

These data suggests that repeat dosing of EpiAlveolar *** via both apical (serosol) and basel routes can be successfully performed over a 14 day period and that this would be a valuable model to identify elveolar toxicological and coints. Furthermore, these data indicate that while TGF-61 treatment for 14 days has not caused large scale toxicity, it has induced a number of markers of PF in the still viable cells. Therefore, the model has the potential be used to identify PF causing agents and new PF treatments whilst reducing the need for in vivo testing and for improving translation to human outcomes.

ACKNOWLEDGEMENTS

The suffers would like to express their grafflude for provision of the lissue model and advice regarding study design to Patriot Hayden, Arms Malone and the learn at MatTek Corporation, 200 Homer Avenue, Ashland, Massachusetts 01721, USA.

The sultions rould like to thenk it's Muller, Marie Sellacar and Anthony Sources (SSAC). Uniterer Research, Shambrook, Secfordahire, WK44 1UC, UK) for their innovative ideas on

The sultion would like to thank Stepner Stoney Joy Millions Kernl Countroyest, Nicl Smith and Marie Alchison (Charles River) for their lachnical assistance in curring the experiment

- 1. Majore AG, Jackson GR, O'Cornell C, Walteley J. Charmer I. Poster 47535, MatTek Comoration, NA, USA,
- Methol (2015). Delphanter's (\$151,100,00,000) and AUL/1996 TANACAS (2) Use Protocol, Protocol No. 106-24-251-2105. MetTek Corporation, NA, USA.



NEXT STEPS BUILD AND EVALUATE AN EFFICACY MODEL FOR LUNG FIBROSIS

We are looking for partners for these and any innovations you may need

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Early De-risking Approaches for Identification of Anti-Fibrotics for Idiopathic Pulmonary Fibrosis (IPF) Treatment



M. Wagstaff¹, A. Young², C. Roper³, M. McElroy³, J. Aarbiou⁴ and A. Zuurmond⁴

Charles River Laboratories Wilmington, MA, USA, ² Charles River Laboratories, Saffron Walden, UK, ³ Charles River Laboratories, Edinburgh, UK, ⁴ Charles River Laboratories, Leiden, The Netherlands

1 INTRODUCTION

Figure 2. High Content Imaging of oSMA and Nuclei

1.25 ng/ml TGF-β1

1 uM SB525334

Idiopathic Pulmonary Fibrosis (IPF) is a chronic lung disease leading to a progressive and irreversible decline in lung function caused by repetitive micro-injuries to the alweolar epithelium. To date, only nintedanib and pireinidone have been licensed to treat IPF, therefore, there is an increased effort to discover new therapies. We have created a testing strategy for identifying new and improved therapies for IPF using in vitro and in vivo models. The strategy starts with the in vitro high throughput screening model, in vitro FMT Assay, followed by the low throughput 3D, EpiAlweolar model (which needs further optimization as it was developed for assessment of lung toxicity), and finally testing in the well established in vivo bleomycin model. This testing strategy provides an integrated (human in vitro and animal in vivo) testing program with 3Rs benefits.

IN VITRO FMT ASSAY

METHOD: Lung-derived primary human bronchial fibroblasts were seeded and subsequently refreshed in preparation for addition of small molecule compounds and the fibrotic trigger TGF-51 (Figure 1). After 3 days, cells were fixed and stained for dSMA and DAPI and imaged by high-content analysis (Figure 2).

Figure 1. Schematic Overview of TGF-\$1-Mediated Trans-differentiation of Fibroblasts to Myofibroblasts (FMT) and Modulation by Small Molecules



RESULTS: Exposure to TGFβ-1 demonstrated a clear, concentration dependant increase in αSMA levels (Figure 3). This was fully inhibited by treatment with the ALV-5 inhibitor (BSE25334) and initedanib. IC_{SS} values for initedanib are consistent across donors and strong Sociatria rank correlation denotes consistency between biological reflicites.

Figure 3, NEEDS A TITLE

Numbers (DAPI Stained) with an INCell Analyzer 2200

FMT trigger reference compound

TOR- \$1 (l.cg mg/ml) controls

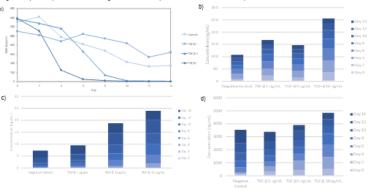
Coverlay

Overlay

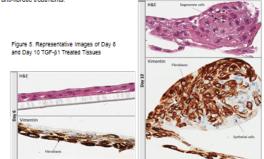
3 IN VITRO EPIALVEOLAR™ MODEL

METHOD: A novel in vitro organotypic 3D aiway model from primary human cells (EpiAlveolar™ from MatTek Corporation) with macrophages was examined to evaluate alveolar toxicity and the capacity of the model as a method to assess fibrotic potential in vitro. EpiAlveolar™ issues were challenged to develop aspects of pulmonary fibrosis, by exposure to known TGF-β1 (1, 5 and 10 µg/mL). Doses were replenished every second day over a 14 day period. Tissue viability was examined by TEER and LDH every second day and tissues were terminated at various intervals throughout the 14 day period to allow examination by histopathology throughout the period. Additionally, spent media was assayed by ELISA for fibronectin, IL-6 and Procollagen I of 1.

Figure 4. a) TEER b) Cumulative Pro-Collagen I of Secretion* c) Cumulative IL-6 Secretion* and d) Cumulative Fibronectin Secretion*



RESULTS: A dose response reduction in TEER was observed (Figure 4a) but no cell death measured in LDH release (data not shown). There were dose response increases in the fibrotic makers of Pro-Collagen, IL-6 and Fibronectin (Figures 4a, b and c). By Day 6 of TGF- β1 (10 ng/mL) treatment, the overall thickness of the cell layer was similar to untreated controls, however, the majority of the cell population expressed a spindle shaped VM positive fibroblast phenotype (Figure 5). From Day 10, samples exhibited multifocal thickening and, in places, protuberant foci composed of multiple layers of VM positive fibroblasts mixed with lesser VM negative epithelial cells. Increased numbers of degenerate cells were observed in these samples. These data show that it is possible to trigger a fibrotic phenotype in the EpiAlveolam model indicating it may be a useful tool to investigate the efficacy of novel anti-fibrotic treatments.

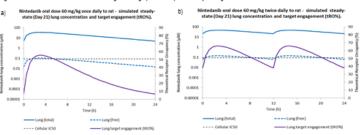


* Secreted concentrations for Day 12 of 1 ng/mL TGF-\$1 treatment are estimated, as the samples were discarded in error.

4 IN VIVO BLEOMYCIN MODEL

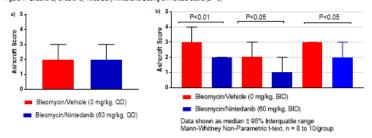
METHOD: Studies were performed in male SD rats (250-300 g). Lung exposure was modelled using integrated pharmacokinetic data, estimates of free nintedanib lung concentrations and receptor affinity data. Pharmacokinetic (PK) parameters were determined following administration of nintedanib at 60 mg/kg (QD or BID) and free drug concentrations were determined using rat lung tissue and plasma protein binding data. The repetitive fibrosis model was established by direct administration of bleomycin (1.66 units/kg, Days 1, 2, 3, 6 and 7); initedanib or vehicle were dosed orally from Day -1 to Day 27. On Day 28, the right lung was inflation-fixed and several for fibrosis using the modified Ashcroft scale (blind assessment on 6 lung sections per right lung was retained for hydroxyproline determination using an LCMS assay (n = 8 to 10 rats/group).

Figure 6. Nintedanib Pharmacokinetic Modelling Following a) Once or b) Twice Daily Dosing in the Rat



RESULTS: PK modelling of unbound exposure, relative to cellular potency, demonstrated that 60 mg/kg nintedanib (BID) gave 40-70% target coverage between dose occasions (Figure 7b) compared to 60 mg/kg (QD) which gave only 10% target coverage at C₁₀₀₀ (Figure 7a). These data suggest twice daily dosing will be more efficacious compared to once daily dosing at 60 mg/kg. Nintedanib (60 mg/kg, BID) significantly reduced fibrosis in three independent studies (Figure 7B). Nintedanib (60 mg/kg, QD) was not consistently antifibrotic in three independent studies (data from one study presented) (Figure 7A).

Figure 7. Effect of a) Once or b) Twice Daily Nintenanib Dosing on Fibrosis Score (a + b)



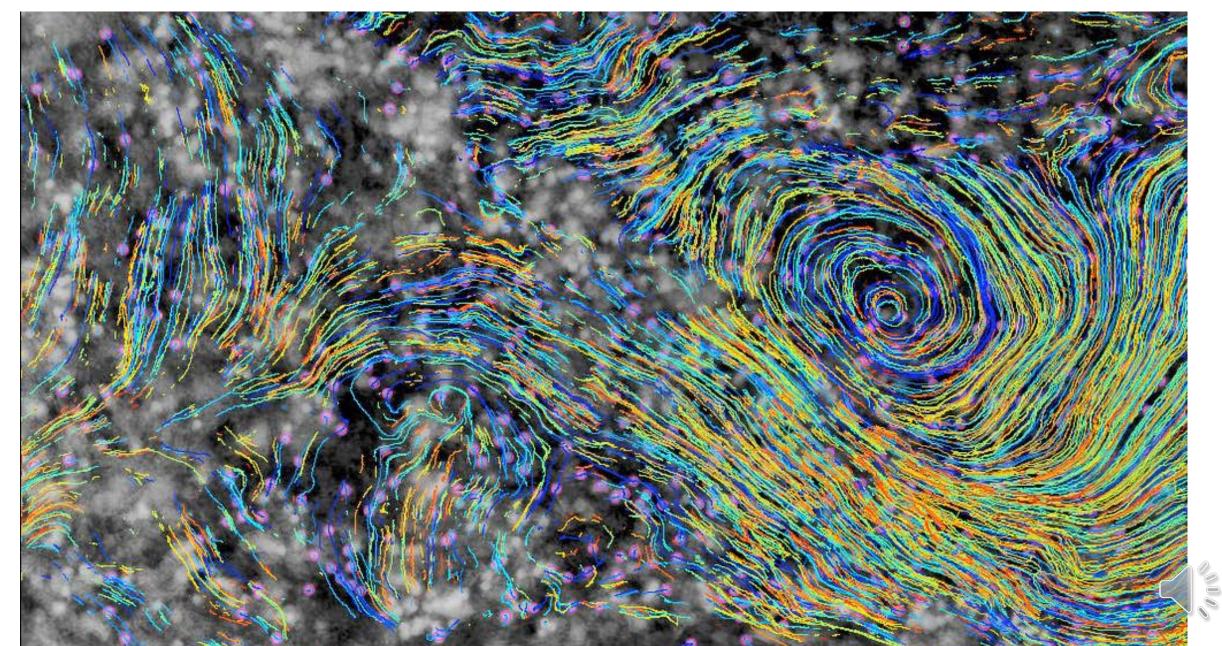
CONCLUSION

In conclusion, human-derived in vitro models can be used to assess potency and toxicity of novel fibrotic compounds are relevant assays (e.g. FMT). Only compounds with the optimum potency and low toxicity will move forward into in vivo assay. In assess exposure and efficacy. Integration of in vitro and in vivo data will improve with screening and selection for novel therapeutic anti-fibrotic drugs.

TRANSLATION AND EFFICACY



MUCOCILIARY CLEARANCE FROM CYSTIC FIBROSIS DERIVED MUCILAIR™



Assessment of Mucociliary Clearance and Cilia Beating Frequency in MucilAir™-Cystic Fibrosis *In Vitro*



C. S. Roper, E. Storey, H. Paulo, and J. Wallace Charles River Laboratories, Edinburgh, UK



INTRODUCTION

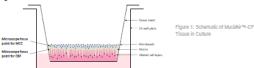
The lungs are cleared of particles and kept sterile by cilia beating the mucus resulting in mucociliary clearance (MCC) of these materials. In cystic fibrosis patients, the mucus produced is thicker and, therefore, the MCC system does not function effectively. MucilAir™-Cystic Fibrosis (MucilAir™-CF) tissues are a functional model of the human airway epithelium derived from cystic fibrosis patients' cells (homozygote $\Delta F503$ mutation) cultured at the air-liquid interface resulting in a similar morphology to the patient, with functioning cilia, but defective MCC. Efficacy testing for novel cystic fibrosis treatments requires assessment of cilia beating frequency (CBF) and MCC. Therefore, this proof of concept study was performed to asses the effects of a known surfactant, Tyloxapol, in MucilAir™-CF on % active cilia, CBF and MCC, with cell viability assessed by Transepithelial Electrical Resistance (TEER). MucilAir™-CF was chosen as the test system for this study as this is an in vitro model for the respiratory airways known to reflect the disease conditions of cystic fibrosis patients.

The aim of the study was to measure the effects of the surfactant drug, Tyloxapol, β-Lactose (negative control) both in physiological saline, and physiological saline on the critical end-points for cystic fibrosis drug efficacy testing; % active cilia, CBF and MCC.

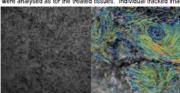


MATERIALS AND METHODS

A dose range finding test (data not presented) was performed to select the concentrations of Tyloxapol in saline for the main test. For the main experiment, triplicate MucilAir™-CF were dosed (30 µL) with Tyloxapol (Low Dose; 0.0005%, v/v and High Dose; 0.1%, v/v) in physiological saline. The tissues were exposed for 1, 10 and 30 min and 1, 2 and 24 h. Triplicate MucilAir™-CF tissues were also dosed with the vehicle control (30 µL: physiological saline) or the negative control (30 µL: ß-Lactose; 75 µM in physiological saline) for 24 h only. Figure 1 shows a schematic of the cells in culture.



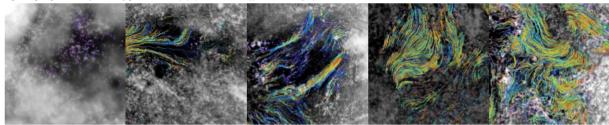
At each exposure timepoint, CBF and % active cilia (active cilia were considered to have a CBF > 3.5 Hz) were assessed immediately using CiliaX software (Epithelix, Switzerland). Microbeads (10 µm, dark blue) were then applied to the surface of each tissue and ca 30 s videos were captured. The videos were analysed using the TrackMate plugin on ImageJ (NIH). This software tracks the beads as they move and determines the rate at which they move (Figure 2). TEER is a sensitive and reliable method to confirm the integrity of the monolayer and was assessed in all tissues following MCC analysis. Six untreated tissues were analysed as for the treated tissues. Individual tracked images are presented in Figure 3.



icrobeads on Tissue With & Without



RESULTS: MONOLAYER INTEGRITY, % ACTIVE CILIA, CBF & MCC

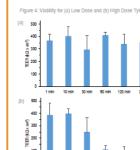


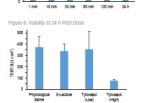
All values are given as mean ± SD. Undosed results demonstrated that MucilAir™-CF recapitulates the disease (% active cilia. CBF and MCC (displacement) were 82 ± 16%. 4.3 ± 0.4 Hz and 3.5 ± 7.0 um)

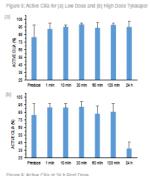
Cell viability was unaffected by the Low Dose Tyloxapol treatment (Figure 4a). The % active cilia (Figure 5a), CBF (Figure 6a) and MCC (Figure 7a) all increased immediately (1 min) to 93 ± 8%, 6.5 ± 1.4 Hz and 20.8 ± 26.3 µm, respectively. % active cilia and CBF were sustained to 24 h. MCC was sustained to 60 min, increased to 61.9 ± 34.0 µm at 2 h and remained at this high level to 24 h (62.1 ± 31.9 µm). These results are summarised in Table 1

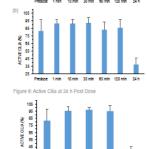
Conversely, following the High Dose treatment of Tyloxapol, cell viability was reduced from 30 min onwards (Figure 4b). However % active cilia (Figure 5b) and CBF (Figure 6b) were not reduced until 24 h. MCC increased up to 2 h (68.3 ± 54.1 µm), but fell by 24 h (7.3 ± 7.4 µm), presumably due to Tyloxapol toxicity.

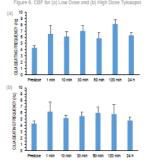
At 24 h. viability (Figure 8), % active cilia (Figure 9) and CBF (Figure 10) were unaffected by physiological saline, B-lactose or Low Dose Tyloxapol treatment. MCC (Figure 11) was improved for physiological saline (36.2 ± 20.8 µm), C (52.9 ± 40.7 µm) and Low Dose Tyloxapol (62.1 ± 32.0 µm) treatment compared to High Dose Tyloxapol that was reduced (7.3 ± 7.4 µm). The results are summarised in Table 2.

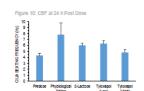


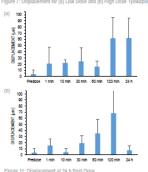


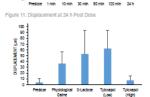












DISCUSSION

This study demonstrated that efficacy can be measured and changes observed in MucilAir™-CF using % active cilia. CBF and MCC. Toxicity can also be observed in parallel using TEER.

The study design clearly demonstrated efficacy and toxic effects of the treatments. The Low Dose Tyloxapol was both efficacious and non toxic. Conversely, the High Dose Tyloxapol was efficacious only over a short period but resulted in reduced MCC at 24 h.

Both the vehicle control (physiological saline) and negative control (B-Lactose) resulted in efficacy without causing toxicity to the MucilAir™-CF cells. Efficacy was, therefore, at least partly due to the mucus thinning effect of physiological saline used for all treatments. The surfactant properties of Tyloxapol increased MCC further at 24 h than physiological saline or ß-Lactose.

Table 1: Summary of CRE (Hz), % Active Citia, MCC and TEER for Low Dose Tyloxanol Treatment

| | CBF | (Hz) | % Activ | re Cilia | MCC | (µm) | TE | ER |
|---------|------|------|---------|----------|------|------|------|-----|
| Time | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Undosed | 4.3 | 0.4 | 81.6 | 15.7 | 3.5 | 7.0 | - | - |
| 1 min | 6.5 | 1.4 | 92.3 | 7.6 | 20.8 | 26.3 | 368 | 50 |
| 10 min | 6.1 | 0.8 | 95.3 | 2.2 | 22.1 | 5.0 | 403 | 77 |
| 30 min | 7.0 | 0.9 | 98.4 | 1.4 | 24.2 | 22.6 | 295 | 116 |
| 60 min | 5.8 | 1.0 | 93.9 | 7.4 | 15.9 | 8.9 | 411 | 24 |
| 120 min | 8.1 | 0.7 | 97.8 | 2.6 | 61.9 | 34.0 | 339 | 82 |
| 24 h | 6.3 | 0.5 | 95.0 | 7.9 | 62.1 | 32.0 | 354 | 162 |
| | | | | | | | | |

Table 2: Summary of CBF (Hz), % Active Cilia, MCC and TEER at 24 h Post Treatment

| | CBF (Hz) | | % Active Cilia | | MCC (µm) | | TEER | |
|----------------------|----------|-----|----------------|------|----------|------|------|-----|
| Time | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Predose | 4.3 | 0.4 | 81.6 | 15.7 | 3.5 | 7.0 | - | - |
| Physiological Saline | 7.8 | 2.0 | 95.4 | 6.0 | 36.2 | 20.8 | 374 | 97 |
| ß-Lactose | 6.0 | 0.4 | 97.0 | 3.4 | 52.9 | 40.7 | 338 | 66 |
| Tyloxapol (Low) | 6.3 | 0.5 | 95.0 | 7.9 | 62.1 | 32.0 | 354 | 162 |
| Tyloxapol (High) | 4.8 | 0.5 | 37.2 | 8.5 | 7.3 | 7.4 | 76 | 13 |



CONCLUSION

In conclusion, this study demonstrated that % active cilia, CBF and MCC can be measured and changes observed in MucilAir™-CF. Therefore, it is proposed to use these endpoints in this test system to identify improved and novel treatments for patients with cystic fibrosis.



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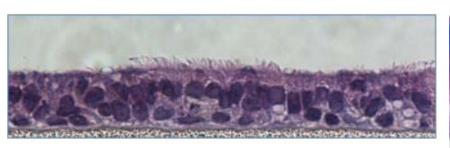
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- Beubler E., Fischer R., Untersteiner G. and Strohmaier W. (2016). Influence of the Surfactant Tyloxapol o. Mucociliary Clearance in Human Respiratory Cystic Fibrosis Cells. Pharmacology: 98: 1-3.

TRANSLATION - STUDY DESIGN

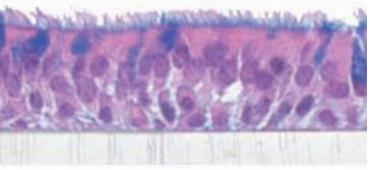
RAT VERSUS HEALTHY HUMAN VERSUS DISEASED HUMAN



Rat EpiAirway™



Healthy human MucilAir™





Diseased human OncocilAir™





IS THIS EASY TO DESIGN AND RUN?



KEY POINTS FOR STUDY DESIGN

WHAT SHOULD AN IN VITRO RESPIRATORY STUDY LOOK LIKE?

- Tissues arrive
- Returned to culture
- Recovery overnight or longer, model dependant
- Dose response curve
- Typically 4-6 replicates at each of 4-6 concentrations
 - Half log dilutions?

Vehicle Controls?

- Numerous possible measures
- Cell viability (LDH, MTT, resazurin)
- Pathology, ELISA, PCR......
- Replicate virtually anything possible in vivo
- Others: TEER, MCC, CBF

Tissue Receipt & Recovery

Dose Applied Apically Liquid or Aerosol

Exposure Duration Rinse Single or Repeat Dosing

Recovery

Measurements?

- Rinse with saline or PBS
- Very gently to avoid perturbing system

Measurements?

ALI and/or Untreated Control?

Positive Control? What Effect are we Assessing?



CONCLUSIONS



CONCLUSIONS

- In vitro models can be used to effectively screen out toxicants prior to in vivo toxicology assessment or identify potential candidates for drug development following efficacy testing
- In vitro models can be used to generate human equivalent concentrations in occupational toxicology as well as identify no effect levels in toxicology. From these data, formulations or active ingredients can be screened out. For test articles chosen for in vivo testing, this data can be used in support of identifying DRF starting doses
- Using the rat, healthy human and diseased human models in parallel provides translational information that often could not be considered until after first in man or later. Therefore, these data can be used in support of clinical decision making



CONCLUSIONS

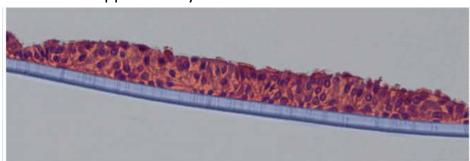
- As new microfluidic/ on-a-chip technologies and tissue models become available, so this science will rapidly expand to answer more complex questions such as chronic diseases as the current research has focussed on the acute effects
- These tests will have tangible 3Rs and animal welfare benefits
- Integration of these tests into testing strategies should reduce the costs of bringing products to market

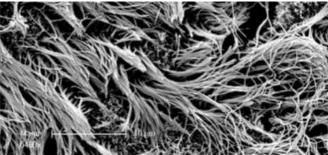
 And we didn't even discuss applications of these technologies for COVID-19 research!!!

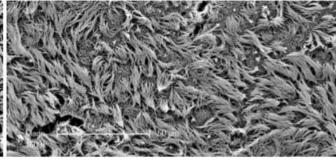


ORGANOTYPIC 3D TISSUE MODELS FOR RESPIRATORY SAFETY AND EFFICACY TESTING

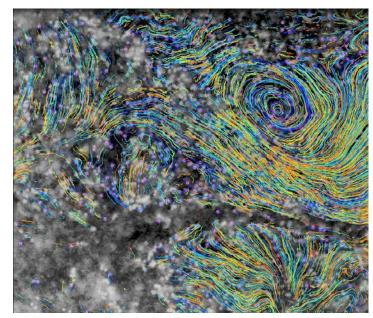
MucilAir™ Upper Airway Cross Section and SEM.



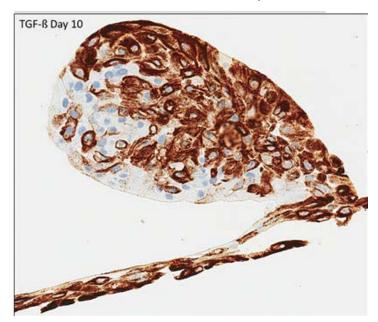




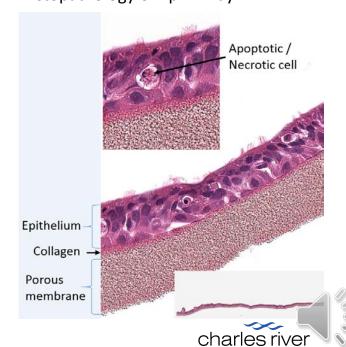
Mucociliary Clearance from CF MucilAir™



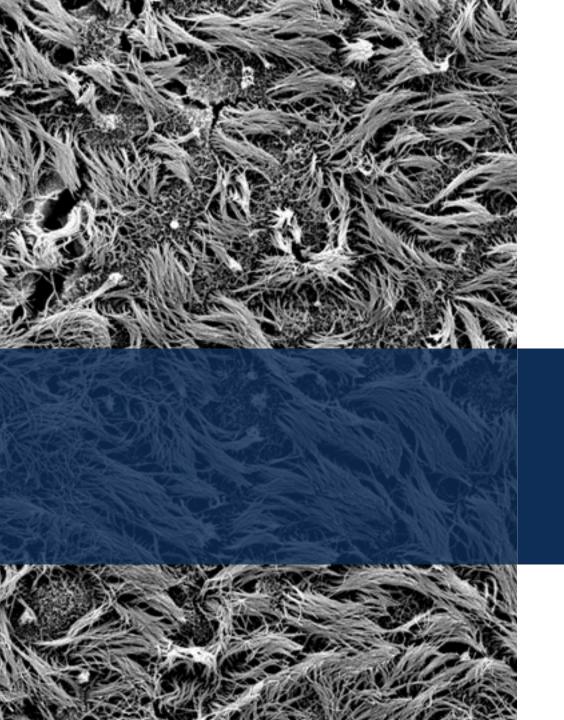
Fibrotic Protuberant Foci from EpiAlveolar™



Histopathology of EpiAirway™



EVERY STEP OF THE WAY



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