Next Generation Risk Assessment approach for Inhalation

I. Muller[1], H. Behrsing[2], A. Bowden[1], S. Cable[1], S. Constant[3], G. Fitton[1], A. Middleton[1], M. Theiventhran[1*], J. Wallace[4], and M. Baltazar[1].

[1]Unilever, Sharnbrook, United Kingdom; [2]Institute for In Vitro Sciences Inc., Gaithersburg, MD; [3]Epithelix, Geneva, Switzerland; and [4]Charles River, Edinburgh, United Kingdom

Introduction

The exposure-led, hypothesis-driven approach in Next Generation Risk Assessment (NGRA) integrates new approach methodologies (NAMs) to assure human safety without the use of animal data. While there are *in silico* and *in vitro* methodology available for lung toxicity testing following pulmonary exposure, there is still a need to generate case studies to evaluate their applicability for risk assessment for decision making. As part of evaluating the NGRA approach for inhalation risk assessment on the lower airways, the 3D cell model EpiAlveolar™ was exposed to 2 case study polymers and 8 benchmark substances. The *in vitro* model system EpiAlveolar™ is a co-culture model of the air-blood barrier mimicking the human-relevant biological response of the lower airways (Fig.1). Lung fibrosis/ inflammation and lung surfactant inhibition were identified as relevant endpoints to evaluate the benchmark substances with known pulmonary effects following exposure. To investigate the inflammatory or fibrotic response of the treatments for lower airways, collected samples were analysed for cytokine/chemokine release for potential biomarkers.

The compounds Polyhexamethyleneguanudine phosphate (PHMG) and Amiodarone which are known to cause pulmonary inflammation were tested as benchmark compounds to evaluate the suitability of the EpiAlveolar cell models. In vitro doses were selected based on predicted realistic human exposures. For PHMG, consumer exposure was based on its use as antimicrobial compound previously used in humidifiers. For this study, an airborne concentration of 0.95 mg/m^3 and a worst-case exposure duration of 11hrs per day was used to estimate the equivalent deposited mass per surface area (µg/cm^2) in the lung (MPPDv2.8) per day, per week and per month in the pulmonary region (0.0007; 0.0049 and 0.021 µg/cm^2, respectively)[6]. For amiodarone, the *in vitro* concentrations were selected to mimic the therapeutic plasma levels in patients (1-4 µM) following oral administration of 400mg/day. The top concentration was selected based on cytotoxicity studies[1].

Experimental design: chronic repeated exposure of compounds in 3D models

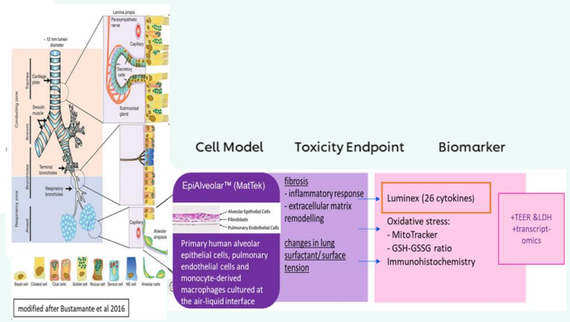

Fig. 1: Experimental design. Upon identifying the relevant toxicity endpoints, EpiAlveolar™ cell model and potential biomarkers of toxicity measurements were selected that represents the most prominent *in vivo* functionality of the lower airways. Cells were exposed for up to 12 days at the apical side with nebulised (neb.) test substances using the Vitrocell cloud chamber or via liquid if nebulisation was not possible. To investigate on an inflammatory or fibrotic response of the treatment, collected media from days 0,1,4,8 and 12 were analysed for up to 26 cytokines using Luminex multiplexing assay.

Data Analysis: Time and concentration-dependent effects were modelled using a nonlinear state space model. Here, the various endpoints measured in the data were represented in the model as different states that can dynamically evolve over time in response to treatment. Gaussian processes were used to the nonlinear dependencies between these states and the chemical treatment. The data were fitted to the model using Bayesian inference, which is a probabilistic approach that allows for quantification of uncertainties in the model predictions. For further information see [5].

Luminex assay principle

The cytokine expression following treatment were evaluated using the Luminex multiplex assay. The assay involves a bead-based assay in which cytokines of interest are recognised through antibodies that are conjugated to magnetic beads. Followed by analyte specific biotinylated antibodies that are recognised by streptavidin-phycoerythin (streptavidin-PE) conjugate (Fig.2). The samples were anaylsed by the Luminex 200™ which uses two light emitting diodes to excite both the microparticle and the attached PE antibodies. The excited microparticle identifies the bound cytokine and the streptavidin-PE conjugate fluorescence intensity provides quantitative value of the cytokine.

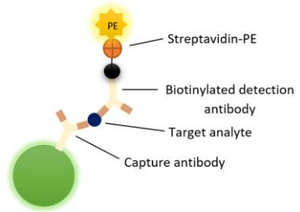

Fig 2. Analyte of interest form an antibody-antigen sandwich with antigen specific biotinylated antibody and fluorescent capture antibodies. Adapted from (Luminex Assay Principle, 2021)[7]

Results: PHMG

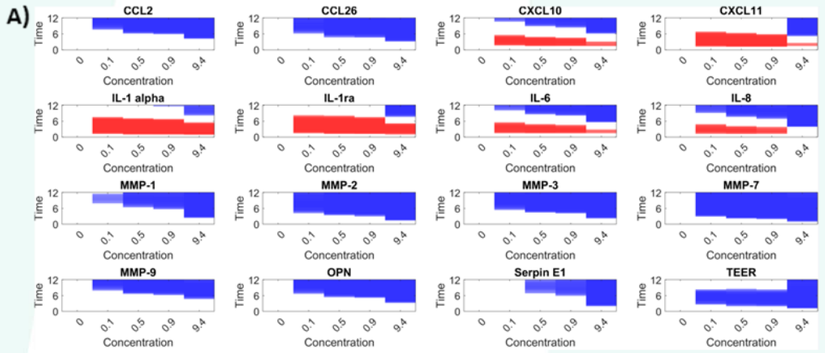

Fig 3. Preliminary results showing time and dose dependent response of PHMG treatment on EpiAlveolar cell model at two independent laboratories (A and B respectively). Concentration dependency score (CDS) plots summarising if a particular biomarker change detected by the state space model as increasing (red) or decreasing (blue) at a given time point/dose. White indicates no changes compared to control. Different dose ranges of PHMG were tested with the highest dose of 0.2 µg/cm^2 in lab A) and up to 9.4 µg/cm^2 in lab B). PHMG were nebulised using a cloud chamber onto the air-liquid interface of EpiAveolar cell model.

Amiodarone

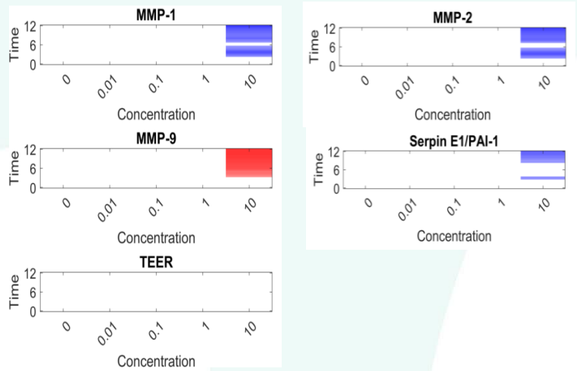

Fig 4. Preliminary results of CDS plots representing time and dose dependent response of Amiodarone treatment on EpiAlveolar cell model. Cells were treated with Amiodarone at the indicated doses (µM) at the apical side of the air-liquid interface via liquid.

Conclusion

The antimicrobial compound PHMG has been previously used in humidifiers and is well known to cause acute interstitial pneumonia and pulmonary fibrosis following pulmonary exposure in humans[4]. Daily exposure of PHMG at the lowest dose range of 0.1 -0.2 µg/cm^2 resulted in an efflux (red) of cytokine markers (See Figure 3B). However, increasing dose resulted in downregulation (blue) of cytokine markers at later timepoints (See figure 3A). This might be due to the cytotoxicity of PHMG to the cells represented by the down regulations of TEER measurements (Figure 3).

Amiodarone an additional benchmark substance is a therapeutic drug that has the potential to cause amiodarone-induced pulmonary toxicity; (AIPT)[3]. Following treatment, slight changes within 4 of the 26 cytokines measured at the highest doses were detected (Fig 4). Both PHMG and Amiodarone are known pro-fibrotic compounds with a larger changes in PHMG detected overall in the preliminary data. This maybe due to within consumers, exposure of PHMG is through inhaling but exposure of Amiodarone is via the bloodstream. Therefore, it is possible that due to the lack of vasculature within the cell model, the response of Amiodarone is not as sensitive as PHMG.

Overall, both PHMG and Amiodarone preliminary data detected changes in cytokine expressions in a time and dose dependent manner. Out of the 8 benchmark substances, 2 are shown that were found to be some of the most responsive compounds following chronic repeated exposure within the EpiAlveolar™ cell models. Full analysis of the ongoing study with all the 16 benchmark substances and 2 case study chemicals in both the upper (MucilAir™) and Lower (EpiAlveolar™) cell models and the toxicity endpoints with the relevant biomarkers will help to investigate the suitability of the most appropriate models in filling the gaps within the proposed NGRA framework.

Reference

1) Andreasen, F., H. Agerbæk, P. Bjerregaard, and H. Gøtzsche. 1981. 'Pharmacokinetics of amiodarone after intravenous and oral administration', *European Journal of Clinical Pharmacology*, 19: 293-99

2) Bustamante-Marin, X. M., Ostrowski, L. E. Cilia and mucociliary clearance. Cold Spring Harb. Perspect. Biol. 9, a028241 (2017).

3) Schwaiblmair, Martin, Thomas Berghaus, Thomas Haeckel, Theodor Wagner, and Wolfgang von Scheidt. 2010. 'Amiodarone-induced pulmonary toxicity: an under-recognized and severe adverse effect?', *Clinical Research in Cardiology*, 99: 693-700.

4) Song, J., W. Kim, Y. B. Kim, B. Kim and K. Lee (2018). "Time course of polyhexamethyleneguanidine phosphate-induced lung inflammation and fibrosis in mice." Toxicol Appl Pharmacol 345: 94-102

5) Svensson, A., & Schön, T. B. (2017). A flexible state–space model for learning nonlinear dynamical systems. Automatica, 80, 189-199.

6) Park, D., Friesen, M., Roh, H., Choi, Y., Ahn, J., Lim, H., Kim, S., Koh, D., Jung, H., Lee, J., Cheong, H., Lim, S., Leem, J., Kim, Y. and Paek, D., 2015. Estimating retrospective exposure of household humidifier disinfectants. *Indoor Air*, 25(6), pp.631-640

7) Www.rndsystems.com. 2021. *Luminex Assay Principle*. [online] Available at: <https://www.rndsystems.com/resources/technical/luminex-assay-principle> [Accessed 25 April 2021].