

Advances in *in vitro* endpoints for inhaled drug delivery safety assessment

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Summary

While *in vivo* studies currently remain the regulatory standard for assessing the safety and efficacy of inhaled drug candidates, the role of *in vitro* methods is rapidly evolving for inhaled candidate screening. Traditional *in vitro* testing for inhaled drug candidates typically focuses on aerosol characterization, cytotoxicity assays, epithelial barrier integrity, and permeability studies using airway epithelial cell lines or primary human cells. While these models provide valuable early insights, they lack the physiological complexity needed to predict long-term toxicity, immune responses and persistence in the lung. Key areas of unmet need are particularly models for the pulmonary region and include models that better represent interactions with immune cells, immune cell and early inflammatory endpoints as well as surrogate endpoints for lung clearance. The aim of this study was to evaluate if novel endpoints including macrophage motility (surrogate marker for clearance) and detailed individual subcellular morphological feature characterisation could provide better mechanistic insights to the fate and safety of medicines in the lung. Human alveolar macrophages in monoculture and co-culture were exposed to a panel of 12 compounds including inhaled medicines, therapeutics with established off-target lung toxicities and mechanistic control substances. A motility assay was developed tracking the velocity of macrophages alongside high content image analysis of intracellular features and established techniques for assessing early inflammation (cytokine production, polarisation), cell stress and phagocytic activity. Concentration and time dependent effects could be discriminated using macrophage motility and morphology techniques and added to a battery of *in vitro* tests that was able to comprehensively evaluate the safety and fate of inhaled medicines.

Key Message

Integrating advanced *in vitro* assessments (morphology, inflammation, function and motility) early in preclinical testing of can support more robust earlier go or no-go decision making for the development of safer and more effective and quicker to market inhaled therapies.

Introduction

While *in vivo* studies currently remain the regulatory standard for assessing the safety and efficacy of inhaled drug candidates, the role of *in vitro* methods is rapidly evolving for inhaled candidate screening [1]. Advances in lung-on-chip models, 3D airway tissues, and improved aerosol characterization tools are enhancing the predictive value of *in vitro* testing. New microphysiological systems (MPS) are capable of mimicking key aspects of human lung biology and cutting edge new analytical approaches are able to yield new insights to predicting cellular responses [1,2]

Traditional *in vitro* testing for inhaled drug candidates typically focuses on aerosol characterization, cytotoxicity assays, epithelial barrier integrity, and permeability studies using airway epithelial cell lines or primary human cells. While these models provide valuable early insights, they lack the physiological complexity needed to predict long-term toxicity, immune responses and persistence in the lung [2,3]. Key areas of unmet need are particularly in the pulmonary region and include models that better represent interactions with immune cells, immune cell-driven early inflammatory endpoints as well as surrogate endpoints for lung clearance [3,4]. Gathering this type of human-relevant mechanistic safety information early in drug development could have a significant impact by identifying potential risks and reducing the chances of failure in later clinical stages [3-5].

A co-culture of epithelial and alveolar macrophage cells as a screening platform enables standard toxicity and permeability endpoints to be combined with more discriminatory inflammatory and clearance endpoints to support identification of immune-mediated adverse effects and improved the translatability of early-stage data to *in vivo* outcomes. The aim of this research was to demonstrate how a combination

of traditional and novel *in vitro* endpoints could be used to provide a more robust approach to inhaled candidate screening.

Materials and Methods

Cell culture and treatments:

Human alveolar macrophages (ImmuPHAGE™) were obtained from ImmuONE (Hertfordshire, UK). Cells were authenticated prior to experimentation and validated for macrophage biochemical and physical characteristics, cell surface markers and functionality (ImmuONE, Hatfield, Herts, UK). For experiments, cells were seeded in 96-, 24- or 6-well plates in a density of 5.6×10^5 cells/mL and treated with a panel of 12 compounds, including inhaled medicines and therapeutics with established off-target lung toxicities (0.001-100 μ M). Cells were assessed for health, cell stress, early inflammation (cytokine production, polarisation) and phagocytic activity alongside advanced endpoints of high content image analysis and motility.

Assessment of cell health, morphology and lipid content:

Population and individual cell responses were assessed using high-content image analysis to assess cell health (mitochondrial activity, membrane integrity) and morphological features (cell area, nuclear area, sphericity, number of vacuoles, extent of vacuolisation). Cells were fixed and treated with a cocktail of fluorescent dyes as previously described [4,5]. Neutral lipids or phospholipids content were assessed using high-content image analysis. For detection of phospholipids, cells were incubated with treatments and HCS LipidTOX Red Phospholipidosis fluorescent dye (Invitrogen, Paisley, Renfrewshire, UK) for 24 h. Cells then were washed once with PBS and stained with HCS LipidTOX Green Neutral Lipid fluorescent dye (Invitrogen, UK) for 30 min to detect neutral lipid. Images were captured using the InCell Analyser 6000 (GE Healthcare, UK) and analysed using In Cell Developer Toolbox v 1.9.2, Level 3 analysis.

Post-Analysis Cell Phenotyping:

A phenotypic analysis approach was adopted using three descriptors of cell morphology (cell area, number of vacuoles and total area of vacuolation) and one for cell stress (mitochondrial activity). These descriptors were selected to enable separation of the cell population and identify different mechanisms of cellular response. Each descriptor was assigned a classification level (low < 10th centile; moderate 10th – 90th centile; high > 90th centile) resulting in 81 potential phenotypes. Cells were sorted into these 81 phenotypes and data expressed as the percentage of the population with the given phenotype.

Assessment of macrophage motility:

Cell motility was measured over 24 h using a PHIO Cellwatcher M and PHIOme Data Management and Analysis Platform Software (PHIO scientific GmbH, Munich, Germany), with untreated cells as controls. Motility data was integrated with existing high-content image analysis datasets covering cellular activation and morphological changes.

Statistical Analysis:

A two-way ANOVA analysis with Dunnett's multiple comparison post-hoc tests were used to assess the statistical significance between the different cell treatments. Statistical significance was evaluated at a 99.9% confidence level ($p < 0.001$). All statistical tests were performed using GraphPad InStat® version 9.0.0 (GraphPad Software, San Diego, CA).

Results and discussion

Twelve reference compounds including inhaled therapeutics with established safety profiles and compounds with established adverse lung macrophage responses were screened using six assessment techniques related to cell health and stress, morphology (cell/nuclear size, sphericity, vacuolation characteristics), lipid profiles, motility and inflammation (**Table 1**).

Table 1 Summary of alveolar macrophage assays. ↑, ↓ and - indicate reduction, increase or no change in comparison with control (untreated cells).

Test compound	Motility	Cell health	Cell stress	Inflammation	Foamy morphology	Phospho-lipidosis
Fluticasone	↑	-	-	-	↑	-
Salmeterol	↑	-	-	-	↑	↑
Salbutamol	↑	-	↑	-	-	-
Budesonide	↑	-	-	-	-	-
Amiodarone	↑	-	↑	-	↑	↑
Imipramine	↑	-	↑	-	↑	↑
Trastuzumab-deruxetecan	-	-	↑	↑	↑	-
Deruxetan	↓	↓	↓	↑	↓	-
Silica DQ12	↓	↓	↑	↓	↑	-
Staurosporine	↓	↓	↓	↑	↓	↑
Campothecin	↓	↓	↓	↑	↓	↑
LPS	↑	-	↑	↑	-	-

Significant changes in macrophage motility were observed in a concentration dependent manner for the majority of test items. A reduction in motility was observed at the same or lower concentrations of known toxicants (deruxetan, silica DQ12, staurosporin and camptothecin, but increased for marketed inhaled therapeutics and phospholipidosis control, amiodarone (**Table 1, Figure 1**). Specifically, a dose-dependent increase in motility was observed for all concentrations of fluticasone tested (**Figure 1B**). Similarly, salmeterol a commonly used bronchodilator demonstrated a dose-dependent increase in motility at concentrations above 10 μM (**Figure 1A**).

Both fluticasone and salmeterol showed no toxicity or adverse cell phenotypes in high-content profiling (**Figures 1D and 1E**), with motility deviations remaining within safe limits of 80 to 120% of baseline, consistent with their known safety profiles. While amiodarone showed an increase in macrophage motility to 170% of control at 10 μM , likely reflecting phospholipidosis, while at 50 μM motility sharply decreased to 30% of control, indicative of cell death (**Figure 1C and 1F**). High-content image analysis revealed that loss of motility precedes complete immobility, highlighting motility reduction as a potential early, sensitive indicator of toxicity, cellular damage, and macrophage mechanistic response.

Individual cell data analysis followed by single-cell profiling identified linked cellular responses which would not be reported with population averaged measurements. For example, amiodarone-induced phospholipidosis was linked with an increased subpopulation of cells with elevated mitochondrial activity and cellular area. While fluticasone and salmeterol showed alternation in cell morphology by increased number and area of vacuoles without changes to mitochondrial activity (**Figure 2**).

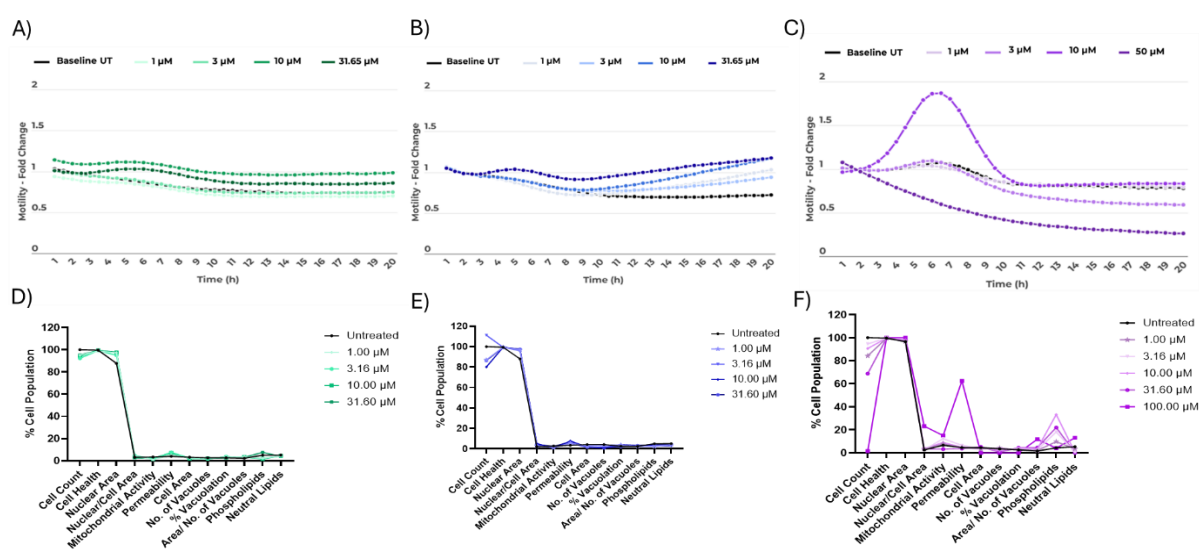


Figure 1 ImmuPHAGE™ responses to salmeterol (A and D), fluticasone (B and E) and amiodarone (C and F) treatment for 24 h presented as cell motility assessed using a PHIO Cellwatcher M. Data is normalised to established baseline and expressed as a fold change to untreated cells (A,B,C) and multiparameter profiles obtained from high content analysis screen (D,E,F).

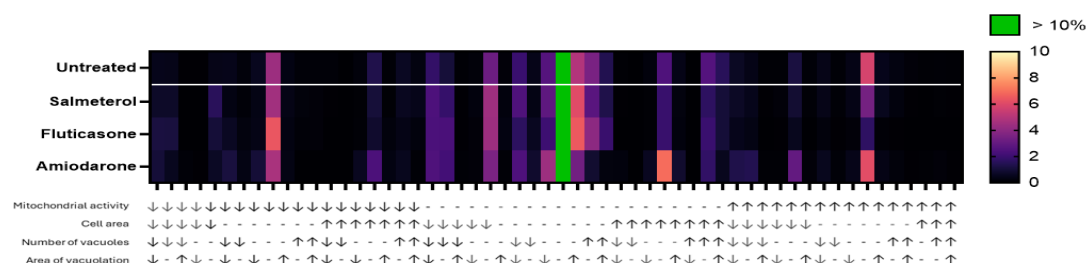


Figure 2. Phenotypes derived from individual cell analysis. High content image analysis was used to assess detailed morphological (cellular and nuclear area, vacuoles numbers and size) and health (mitochondrial activity, membrane permeability and cell count) descriptors of individual alveolar macrophage cells. Phenotypes above 2% were selected and presented as heatmaps. Each square represents the % of the cell population with that given phenotype in one experiment (n=3). The colour gradient sets the lowest value for each given parameter in the heat map (black 0%), highest value (green above 15%) and mid-range values (yellow 15%) with a corresponding gradient between these extremes.

Discussion and conclusion

Whilst traditional *in vitro* assays based on epithelial cells can provide an early indication of broad toxicity (live/dead), they are typically not sufficiently sensitive or complex to distinguish between lower grade adaptive/adverse responses and provide limited mechanistic context. The inclusion of macrophages in these models, permits a mechanism-driven assessment approach to elucidate the potential adversity of early inflammatory, morphological and functional changes which more robustly differentiates and supports categorisation of response of test compounds. The study demonstrates how the advanced endpoints are supporting the evolution of inhaled safety assessment which can be applied during early preclinical screening, adding value in supporting earlier go or no-go decision making and developing safer, more effective inhaled therapies.

References

- [1] Prideaux, B., Kumar, A., Singh, P., Walker, R., Patel, V. *In vitro* models to study inhaled drug delivery and respiratory toxicity: current status and future perspectives. *Journal of Aerosol Medicine and Pulmonary Drug Delivery*. 2018; 31(1), 1-14.
- [2] Bedford, R., Perkins, E., Clements, J., Hollings, M. Recent advancements and application of *in vitro* models for predicting inhalation toxicity in humans. *Toxicology in Vitro*. 2022; 79, 105299.
- [3] Stolper, R., Green, T., Carlson, M., Diaz, F., Nguyen, H. Advanced co-culture models for inhalation toxicity testing: a focus on immune-epithelial crosstalk and mechanistic endpoints. *Frontiers in Toxicology*. 2021; 3, 650994.
- [4] Hoffman E., Patel A., Ball D., Klapwijk J., Millar V., Kumar A., Martin A., Mahendran R., Dailey L.A., Forbes B., Hutter, V. Morphometric Characterization of Rat and Human Alveolar Macrophage Cell Models and their Response to Amiodarone using High Content Image Analysis. *Pharmaceutical Research*. 2017; 34:2466–2476.
- [5] Hoffman, E., Urbano, L., Martin, A., Mahendran, R., Patel, A., Murnane, D., Page, C., Dailey, L.A., Forbes, B., Hutter, V. Profiling alveolar macrophage responses to inhaled compounds using *in vitro* high content image analysis. *Toxicology and Applied Pharmacology*. 2023; 474, 116608.