**In vitro assessment of alveolar macrophage responses to inhaled particulate medicines**

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**Summary**

Lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) are an increasing global health burden affecting hundreds of millions of people worldwide. Despite considerable investment to develop new inhaled drugs, in recent years there has been limited success, in part due to the observation of foamy macrophage responses in rat studies which questions their safety for use in humans. The aim of this work was to use *in vitro* cell culture models of rat and human alveolar macrophages to better characterise the biological response of these airway immune cells to particulate medicines. U937 (human) and NR8383 (rat) macrophage models were exposed for up to 24 h with 0.01 – 100 mg/ml of amiodarone, salbutamol or salmeterol in cell culture medium. Cells were assessed for a variety of responses including toxicity, induction of phospholipidosis, cytokine and chemokine production, cell signalling pathways and production of volatile organic compounds. No significant difference (p<0.05) was observed for viability between U937 and NR8383 for any of the compounds tested, however phospholipidosis was observed to a greater extent in the rat model in comparison with human cells. Immunological, cell signalling and headspace analysis revealed several potential markers of foamy macrophage induction that were raised in amiodarone treated cells in comparison with untreated controls. It is anticipated that these markers may have the potential to identify the foamy macrophage phenotype in drug screening via longitudinal and less invasive animal studies and gain a better understanding of the safety of airway macrophage responses to inhaled particulates.

**Introduction**

Over the past decade there has been considerable investment into the development of new inhaled drugs for the treatment of airway diseases. Despite these efforts, new medicines have been largely limited to combination treatments of previously marketed drugs. One of the key reasons for the attrition of novel inhaled drug treatments during pharmaceutical development is the induction of foamy macrophage responses observed in rat studies. This may be exacerbated by the low solubility of many new therapeutic molecules which can result in accumulation of undissolved particulate matter in the lung after repeated or high dosing. There is increasing evidence that inhaled medicines can be taken up by alveolar macrophages resident within the lung giving them a foamy, vacuolated appearance. The mechanism for induction of this ‘foamy’ alveolar macrophage response and their significance in relation to lung pathophysiology are currently poorly understood. These observations have affected new inhaled drugs reaching the market as regulatory bodies are unable to approve new medicines that appear to sensitise the immune system in animal models, despite not knowing fully if these observations are adverse in human patients.

The main objective of this work was to use *in vitro* cell culture models of rat and human alveolar macrophages to better characterise the biological response of these airway immune cells to particulate medicines. By using a combination of established assays and developing new techniques the aim of this research is to apply these techniques to establish a range of non-invasive methods to characterise inflammatory responses in the lung during short and long term administration of inhaled medicinal particles. The ultimate goal will be to apply the markers in longitudinal studies to provide an in depth understanding of the immune responses in the lung, thereby reducing the numbers of animal experiments required to achieve an accurate prediction of the safety of new inhaled medicines in humans.

**Materials and Methods**

*Cell culture:* The NR8383 rat macrophage and U937 human monocyte cells lines were purchased from the LCG Standards (Teddington, Middlesex, UK) and used between passage 2 and 20 from purchase. NR8383 cells were cultured in Kaighn’s modified Ham’s F12 (K-F12) medium with 15 %v/v fetal bovine serum (FBS) and supplemented with 1% v/v penicillin/streptomycin and 2 mM L-glutamine. U937 cells were cultured in RPMI with 10 %v/v FBS and supplemented with 1% v/v penicillin streptomycin and 2 mM L-glutamine. Cells were cultured in a humidified atmosphere at 37 °C with 5%v/v CO2 and cell number was maintained between 1 x 10^6 to 2 x 10^6 cells/ml. For experiments cells were seeded on to 96 well plates at a density of 1 x 10^4 cells/well in 100 µl of complete cell culture medium (NR8383) or medium supplemented with 50nM phorbol myristate acetate (PMA) (U937) for differentiation into an alveolar macrophage phenotype as previously described.

*Induction and characterisation of foamy macrophages:* After 72 hours, 0.01 – 100µg/ml suspensions of amiodarone (established inducer of foamy macrophages), salbutamol or salmeterol in complete cell culture media were added and cells incubated at 37 °C, 5 %v/v CO2 for 24 hours. The formation of lipid lamella bodies
indicating phospholipidosis was determined using oil red O, nile red and immunological staining techniques as previously described\(^6,7\).

**Immune response and signalling:** After 4 and 24 hour exposures to particulate drugs, the supernatant and remaining cells were harvested and assessed for the presence of inflammatory cytokines and cell signalling pathways. Rat and human multiplex cytokine/chemokine assays (Millipore, Watford, Herts, UK) were used to determine the presence of 27 rat cytokines and 30 human cytokines after exposure to amiodarone, salbutamol, salmeterol and lipopolysaccharides (LPS) from E. coli as a positive control. Intracellular signalling arrays (Cell Signaling Technology, Leiden, Netherlands) were used to probe the activation of 18 intracellular signalling pathways in both rat and human macrophage models. Macrophage cell models were also loaded with the Fura2 calcium-sensitive reporter to probe for a downstream read out from screened signalling pathways as outlined previously\(^8\).

**Volatile markers:** For the extraction of volatile gases, U937 and NR8383 cells were cultured in 100 ml culture vessels in 40 ml complete cell culture medium containing 1 x 10\(^5\) cells.ml\(^{-1}\), and differentiated as outlined above and used for experiments after 72 h. An air-sampling system was developed accommodating airflow at a rate of 50 mL.min\(^{-1}\) to collect the volatile gases emitted from the cultures. Discontinuous sampling and a bespoke metering microprocessor were used to capture evolved gases on a dual-bed sorbent. Samples were analysed using thermal desorption-gas chromatography-time of flight mass spectrometry.

**Results and Discussion**

Cell viability was decreased in the presence of amiodarone and salmeterol but not salbutamol at the highest concentration tested (100 mg/ml) (Figure 1). Similar concentration-dependent cellular responses were observed for rat and human macrophage models and no significant differences (\(p<0.05\)) were observed between the rat and human species cell lines with any of the compounds tested.

![Figure 1: Cell viability of U937 (A-C) and NR8383 (D-F) determined using MTS (blue diamonds) and LDH (red square) assays. Data are presented relative to control (untreated cells) and are displayed as mean ± SD (n = 3-4).](image)
Markers of phospholipidosis indicated amiodarone and salmeterol induced the formation of lipid lamella bodies in a concentration-dependent manner in both rat and human cell models (Table 1) and was in agreement with other published studies\(^9\). In contrast, the presence of lipid lamella bodies observed for U937 cells treated with salbutamol were no different in comparison with untreated cells (80-120%).

Table 1: Determination of phospholipidosis in U937 and NR8383 cells treated with amiodarone, salbutamol and salmeterol using oil red o, nile red and immunological cell staining techniques. Results are shown as relative to untreated cells. – indicates no change (80-120% of control); + indicates minor increase (120-200% of control); ++ indicates moderate increase (200-400% of control).

<table>
<thead>
<tr>
<th>Phospholipidosis Marker</th>
<th>Cytology (oil red o staining)</th>
<th>Immunological staining (PIP2/LAMP2)</th>
<th>Flow cytometry (Nile Red staining)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amiodarone concentration (mg/ml)</td>
<td>0.01 0.1 1 10 100</td>
<td>0.01 0.1 1 10 100</td>
<td>0.01 0.1 1 10 100</td>
</tr>
<tr>
<td>U937 (human)</td>
<td>- - + + ++</td>
<td>- - - + +</td>
<td>- - + ++ +</td>
</tr>
<tr>
<td>NR8383 (rat)</td>
<td>- ++ + ++ ++</td>
<td>- - + ++ +</td>
<td>- + ++ ++ +</td>
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<tr>
<td>Salbutamol concentration (mg/ml)</td>
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<td>0.01 0.1 1 10 100</td>
<td>0.01 0.1 1 10 100</td>
</tr>
<tr>
<td>U937 (human)</td>
<td>- - - - -</td>
<td>- - - - -</td>
<td>- - - - -</td>
</tr>
<tr>
<td>NR8383 (rat)</td>
<td>- - - - +</td>
<td>- - - - -</td>
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<tr>
<td>Salmeterol concentration (mg/ml)</td>
<td>0.01 0.1 1 10 100</td>
<td>0.01 0.1 1 10 100</td>
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<tr>
<td>U937 (human)</td>
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For both U937 and NR8383 macrophage models G-CSF, IL-1β, IL-6, IL-8, and IL-10 and additionally in rat models, IL-13, IL-17α and IL-18 were all raised 2-4 fold greater than control (medium alone). Similar observations for IL-6 and IL-8 have been reported for U937 cells exposed to pollutant particulate matter\(^10,11\). Increased calcium transient frequency was observed in the cells following the application of amiodarone in comparison with control indicating the presence of activated signalling pathways in the treated cells. Increased numbers of vacuoles were also observed for amiodarone-treated macrophage cells as early as 10 minutes after exposure to amiodarone (Figure 2) in line with the induction of phospholipidosis.

Figure 2: Increased vacuole appearance in NR8383 cells following application of 100 mg/ml amiodarone. A - C depict the same field of view before amiodarone perfusion (A), 10 minutes (B) and 30 minutes (C) after amiodarone perfusion. New vacuoles that were not present before amiodarone application are highlighted by the red arrowheads.

Several volatile compounds including pentanoid and pentenoid derivatives were identified from the culture headspace for macrophages incubated with amiodarone, but were absent in the untreated control samples. These compounds were measured at higher concentrations (p<0.05) for headspace samples analysed after 24 h of exposure to amiodarone in comparison with 4 h exposure. The compounds identified were in agreement with volatiles identified previously in other airway cell line headspace and exhaled breath studies\(^12,13\). It is anticipated that the compounds identified may act as markers for the presence of foamy macrophages.
Conclusion
This study indicates that there are observed differences in the foamy macrophage responses for rat and human in vitro models. Early indications suggest that the induction of foamy macrophage response is greater in the NR3838 rat model compared with the U937 human model. Whilst this study phospholipidosis is observed to a greater extent for the cells exposed to the more poorly soluble compound, salmeterol than in comparison to salbutamol, other studies have shown that fluticasone, one of the most poorly soluble inhaled drugs does not cause this effect. This indicates that physicochemical properties may be one of several factors responsible for the foamy response. The different markers identified in immunological, cell signalling and headspace analysis have the potential to identify the foamy macrophage phenotype in a longitudinal and less invasive manner in animal studies. It is anticipated that by characterising the foamy macrophage response from a number of biological readouts will provide a better understanding of the pathophysiology of airway immune responses to inhaled particulates and permit the development of new, safe inhaled therapeutics for airway disease to reach the market.

References