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Transitioning Acute *In Vitro* Inhalation Toxicology Testing to Chronic and Repeat Dose Testing; the Challenge of Mucus Depletion in Upper Airway Test Systems and Use of Sputum Mimics

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Abstract

Acute *in vitro* inhalation toxicology has developed rapidly, due to advances in the availability of *in vitro* toxicology test systems, assay development and regulatory requirements. Chronic *in vitro* inhalation toxicology has progressed more slowly, due to effects of the experiment on the cells during repeat dosing. This opinion identifies a potential resolution to this using sputum mimics.

Keywords: *Sputum mimics*; Repeat dose; Mucus depletion; Toxicology testing

Introduction

Acute *in vitro* inhalation toxicology has developed rapidly, due to advances in the availability of *in vitro* toxicology test systems, assay development and regulatory requirements. Test systems include 2D cellular systems such as BEAS-2B, HBE, SAEC, Calu-3, A549, and NCI-H292 cells, 3D upper airway cellular systems, including Epithelix MucilAir™, SmallAir™ and MatTek EpiAirway™ and lower airway cellular systems such as Epithelix AlveolAir™, MatTek EpiAlveolar™, Invitrolize ALISens™ and ImmuONE ImmuLUNG™. These are reviewed in more detail in [1,2]. Most progress has been made in acute *in vitro* inhalation toxicity tests with the complex *in vitro* 3D Transwell culture models, where impacts have been felt across the pharmaceutical [3], crop protection [4,5] and tobacco [6] industries.

The relevance of any proposed test system is important in developing a study plan. Indeed, both an upper airway and an alveolar test system may be required, depending on where an inhaled test item or items (i.e., a formulation of a drug or chemical or the chemical alone) may reach into the airways result in toxicity to those cells. Progress into repeat dose *in vitro* inhalation toxicity studies has been slow due to considerations on clearance of exposed test chemicals.

Exposure of Test Items

There are broadly two methods of exposure to the test system. The simplest is to apply a small finite volume of the chemical in a liquid to the surface of the 3D culture model [5,7,8], described as "liquid" dosing. The more complex method is to use a cloud system [9,10] and this is described as "cloud" dosing. There are advantages and disadvantages to both dosing systems. For example, (a) it is simpler to quantify the dose exposed in liquid dosing than cloud dosing and (b) a cloud exposure is more likely to have the correct sized particles inhaled. Importantly, they both mimic the inhalation process of a patient, consumer or operator inhaling a formulation of a drug or chemical or the chemical alone. Justification of the exposure system is important in developing any test study plan.

Mucociliary Clearance and Exhalation

Once a drug or chemical has been exposed to the test system, it may have a toxic or efficacious effect on the cells. In the upper airway tract (e.g., MucilAir™ or EpiAirway™), the inhaled test item must first mix with the mucus layer. The cilia beat together in these model test systems and result in a mixing of the test

item into the mucus. Mucociliary clearance is measured using high speed cameras [10,11] which track movement across the surface of the mucus. Additionally, cilia beating frequency and percentage of active cilia can also be identified using these tools or inferred from grey-scale intensity fluctuations during phase-contrast imaging. Since the 3D tissue test systems are mounted on the Transwell plate, they are bound by the walls of the plate, so clearance away from the tissue cannot occur, resulting in only mixing of the mucus and no clearance of the test item. Simply put, there is no exhalation (even as limited as this may be) or test item clearance, and so a crucial aspect of inhaled drug delivery is not captured by existing model systems.

Chronic *In Vitro* Toxicology Considerations

Since there is no clearance or exhalation of inhaled test item, there is no change in the amount of test item available to cause toxicity to the cells at the air liquid interface of the Transwell test systems. Without removal of the mucus and test item, repeat dosing will be additive, resulting in higher exposures than would be expected *in vivo*. There are ways to remove this exposed test item from the test system. The simplest is to rinse off the mucus using a pipette containing physiological saline. The goblet cells retain a small reservoir of mucus, and these cells release mucus. However, it takes time for these cells to replenish this mucus stock. Therefore, in repeated exposures and tissue rinsing, there may be a resultant depletion of mucus with test item exposed directly to the cells or to a reduced volume of mucus. This does not occur *in vivo*, where there is continuous removal of mucus *via* the mucociliary escalator, resulting in mucus being swallowed or expelled from the nose. Replenishment of mucus takes place consistently across the upper airway tract. Since replenishment may be slower in the *in vitro* test systems, exposure directly to cells may occur, without the protection that the mucus gives to those cells. In turn, repeated dose exposures could result in higher levels of toxicity than would be anticipated *in vivo*.

Sputum mimics

One potential solution to the problem of mucus depletion during repeat *in vitro* dosing would be to supplement endogenous mucin production by manual application of purified mucin proteins at the air-liquid interface. Porcine mucins are cheap and readily available from commercial suppliers but have some important structural and functional differences with human mucins [12]. Effective protocols for preparation of human mucins have been developed, but extraction procedures can be time consuming and costly, yields are typically low, and the methods are often better suited to isolation of digestive tract mucins than to those of the airways [13].

An alternative approach is the use of cellular air-liquid interface test systems combined with chemically defined media designed to reflect the chemical and physical properties of airway liquid. Sputum mimics were originally developed for the study of the chronic bacterial lung infections that affect those with cystic fibrosis (CF) [14,15]. They were designed to capture key features of the composition of CF sputum and have been used extensively to reveal novel pathogen biology [16], for the study of inter-species interactions [17] and for antimicrobial susceptibility testing [18]. More recent iterations of these media have refined the chemical composition to be more reflective of airway conditions [19,20] or to produce alternative formulations representing different airway compartments, both in CF and healthy individuals [21]. The high mucin content of these media and the similarity of their biophysical properties to that of sputum makes them well suited for chronic or repeat dose toxicity testing. Crucially, airway epithelial cells can be cultured in the presence of sputum mimics at their apical surface [20].

For chronic or repeat dose *in vitro* inhalation toxicity testing, sputum mimics could be applied to the test system immediately after rinsing and prior to test item exposures in the upper airway 3D Transwell test system models, ensuring a consistent level of mucin in an airway-relevant environmental context.

The availability of CF test systems (e.g., Epithelix MucilAir-CF) and CF-specific sputum mimics, offers the opportunity for more rapid development of treatments for CF by using these models in combination, both for drug screens and for toxicology studies.

Conclusion

In conclusion, sputum mimics could be used to replenish the depleted mucus in the upper airway test system resulting in a more *in vivo*-like repeat dosing exposure scenario.

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