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In vivo–in vitro comparison of acute respiratory tract toxicity using human 3D airway epithelial models and human A549 and murine 3T3 monolayer cell systems

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ABSTRACT

The usefulness of *in vitro* systems to predict acute inhalation toxicity was investigated. Nineteen substances were tested in three-dimensional human airway epithelial models, EpiAirway™ and MucilAir™, and in A549 and 3T3 monolayer cell cultures. IC_{50} values were compared to rat four-hour LC_{50} values classified according to EPA and GHS hazard categories. Best results were achieved with a prediction model distinguishing toxic from non-toxic substances, with satisfactory specificities and sensitivities. Using a self-made four-level prediction model to classify substances into four *in vitro* hazard categories, *in vivo–in vitro* concordance was mediocre, but could be improved by excluding substances causing pulmonary edema and emphysema *in vivo*. None of the test systems was outstanding, and there was no evidence that tissue or monolayer systems using respiratory tract cells provide an added value. However, the test systems only reflected bronchiole epithelia and alveolar cells and investigated cytotoxicity. Effects occurring in other cells by other mechanisms could not be recognised. Further work should optimise test protocols and expand the set of substances tested to define applicability domains. *In vivo* respiratory toxicity data for *in vitro* comparisons should distinguish different modes of action, and their relevance for human health effects should be ensured.

1. Introduction

Substance-induced acute inhalation toxicity can develop by a variety of complex pathological processes. Aerosols that are inhaled can enter the nasopharyngeal, tracheobronchial or pulmonary regions of the airway tract, with particles penetrating more deeply into the airway tract with decreasing size (Hayes and Bakand, 2010). Once deposited onto the respiratory wall, airborne particles can exert toxic effects if they are able to overcome its barrier components. Preventing foreign material from gaining access to the lung, these barrier components include the surfactant film, the mucociliary system, phagocytic airway macrophages, and the epithelium with its tight junctions (Rothen-Rutishauser et al., 2005). Aerosol particles that reach the alveolar regions of the lung can be taken up into the pulmonary blood supply system.

For gases and vapours, target organs and tissues as well as absorption within the respiratory tract depend upon their water solubility and reactivity. Gases and vapours that reach the lung can diffuse into its cells and capillaries (Bakand et al., 2005).

Toxic effects upon inhalation can occur locally and range from irritation to destruction of the respiratory epithelium. Depending on the region of the airway tract that the substances reach, more pronounced damage of the respiratory system can arise, such as pulmonary edema or emphysema. If taken up into the blood, inhaled substances can exert systemic effects, also in organs remote from the respiratory system (Hayes and Bakand, 2010).

To prevent human health effects from arising upon respiratory exposure to man-made substances and products, European Union (EU) legislation requires information on acute inhalation toxicity for all non-corrosive chemicals manufactured or imported in quantities of 10 tonnes per year or more if inhalation is the relevant

Abbreviations: 3T3-LDH/3T3-WST-1, test using 3T3 mouse fibroblasts and determining LDH release/WST-1 reduction; A549-LDH/A549-WST-1, test using A549 human alveolar epithelial cells and determining LDH release/WST-1 reduction; CLP, Classification Labelling and Packaging; CSR, Chemical Safety Report; Dnp, determination not possible (for technical reasons); EPA, Environmental Protection Agency; EpiAirway™-MTT, test using EpiAirway™ tissue and determining MTT reduction; Fn, false negative; Fp, false positive; GHS, Globally Harmonised System; MSDS, Material Safety Data Sheet; MucilAir™-LDH/MucilAir™-MTT/MucilAir™-TEER, test using MucilAir™ tissue and determining LDH release/MTT reduction/changes in TEER; mwb-IC_{50}, molecular weight-based IC_{50} value; nad, nothing adverse detected; OD, optical density; RTECS, Registry of Toxic Effects of Chemical Substances; SIDS, Screening information Data Sets; tn, true negative; tp, true positive; wb-IC_{50}, weight-based IC_{50} value.

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secondary route of exposure (EU REACH regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals: Anon., 2006) as well as for agrochemicals, regardless of tonnage, if there is likelihood of inhalative exposure (EU Commission regulation on data requirements for plant protection products: Anon., 2011). Acute inhalation toxicity is expressed in terms of LC_{50} values, the test substance concentration that leads to death of 50 percent of the animals within a two-week observation period after four hours inhalation exposure. Animals that die have succumbed to one of the broad spectrum of modes of toxicological action upon exposure to airborne particles.

With regard to animal distress, the EU Directive on the protection of animals used for scientific purposes assigns toxicity studies *"where death is the endpoint or fatalities are to be expected"* to the highest severity category (Anon., 2010). It further implements the 3Rs principle of replacing, reducing and refining animal testing (Russell and Burch, 1959). Whereas a reduction and refinement alternative to the traditional *in vivo* acute inhalation toxicity study (OECD Test Guideline 403: OECD, 2009a) was adopted in 2009 (OECD Test Guideline 436: OECD, 2009b), non-animal testing methods for this endpoint remain to be developed.

To make a contribution towards addressing this challenge, the present study aimed at investigating the usefulness of different *in vitro* test systems in predicting acute inhalation toxicity. Selected to be applicable for routine testing while at the same time possessing physiologically relevant features of the airway tract, the following test systems were included in the study: Two commercially available three-dimensional (3D) *in vitro* human airway epithelial tissue models, EpiAirway™ (MatTek Corporation, Ashland, MA, USA) and MucilAir™ (Epithelix Sàrl, Geneva, Switzerland), a monolayer cell culture system making use of human A549 adenocarcinomic alveolar type II basal epithelial cells and a monolayer cell culture system using 3T3 mouse embryonic fibroblasts as a non-specific control for respiratory tract tissues.

While the EpiAirway™ tissue model consists of primary human tracheal or bronchial epithelial cells (Balharry et al., 2008), the MucilAir™ system is composed of primary human nasal or bronchial epithelial cells isolated from biopsies (Huang et al., 2008). These reconstructed tissue models are grown on porous membranes at the air–liquid interface with nutrients supplied basely through the porous membrane and test substances applied onto the superficial apical side of the cells (Aufderheide et al., 2003; Lenz et al., 2009).

Both 3D models reflect normal human bronchiole histology and form differentiated pseudo-stratified cell layers resembling the epithelial tissue of the respiratory tract. They possess microvilli and cilia on the apical surface of the cultures capable of mucin secretion as well as tight junctions formed between the cells. Active ion transport of the airway epithelia is maintained (Hirsh et al., 2008; Huang et al., 2011). Whereas the EpiAirway™ system has already been used extensively for a variety of investigations (e.g. Sharma et al., 2010; Ren and Daines, 2011; Sexton et al., 2011), peer-review articles mentioning the MucilAir™ system could not be found.

To determine if 3D tissue models have an added value for *in vitro* respiratory toxicity testing, the EpiAirway™ and MucilAir™ test systems were compared to two *in vitro* monolayer cell culture systems. The first made use of human A549 alveolar epithelial cells, a cell line that is widely applied to investigate respiratory effects *in vitro* (Roggen et al., 2006; Kim et al., 2011). The second cell culture system used 3T3 mouse fibroblasts, thereby enabling comparing airway epithelial-related *in vitro* test systems to an *in vitro* assay determining unspecific cytotoxicity (ICCVAM, 2006). In contrast to the 3D tissue models cultured at the air–liquid interface, the cell monolayer systems were cultured completely submerged in culture medium.

Taking into account the respective manufacturers' recommendations, the selected endpoint detection methods were reduction of the tetrazolium salt MTT for the EpiAirway™ test system (MatTek Corporation EpiAirway (AIR-100) toxicity test protocol; available from the supplier), release of lactate dehydrogenase (LDH), MTT reduction and changes in transepithelial electrical resistance (TEER) for the MucilAir™ system (Epithelix's protocols; available from the supplier) and, for the A549 and 3T3 monolayer cell culture systems, LDH release and reduction of water-soluble tetrazolium (WST-1).

Thus combinations of test systems and endpoint detection methods resulted in a total of eight different tests: Substance-induced changes in mitochondrial activity (MTT or WST-1 reduction) were determined in all test systems (EpiAirway™-MTT, MucilAir™-MTT, A549-WST-1, and 3T3-WST-1). Cell membrane damage resulting in release of the intracellular enzyme LDH was measured in the MucilAir™ and in the two monolayer cell culture systems (MucilAir™-LDH, A549-LDH, 3T3-LDH). As an additional parameter, TEER, measuring tight junction and cell layer integrity, was determined in the MucilAir™ system (MucilAir™-TEER).

A further important aspect for consideration when compiling *in vitro* systems for acute inhalation toxicity testing relates to the form of substance application. Ideally, *in vitro* dosimetry should reflect *in vivo* dosimetry and enable calculating test results in terms of particles deposited on the surface area of the cell cultures (Lenz et al., 2009). In the test systems used in the present study, however, test substances were applied in solution or suspension, and not in the aerosolised or vapourised form, and test substance concentrations were expressed in mass units per volume solution. Being aware that *in vitro* dosimetry therefore did not reflect the *in vivo* situation of acute inhalation toxicity studies, it was nevertheless selected to determine the influence of a simplified form of substance application on the outcome of *in vitro* respiratory tract toxicity testing.

For *in vitro* testing, 19 substances were selected for having known toxicological effects via inhalation, i.e. known modes of toxicological action (Andersen and Dennison, 2001), and their LC_{50} value upon four-hour exposure in rats was recorded. In the regulatory context, LC_{50} data are most frequently used to satisfy hazard classification and labelling requirements (OECD, 2009c). Based upon the LC_{50} data, the 19 test substances were therefore sorted into one of the five acute inhalation toxicity hazard categories of the United Nation's Globally Harmonised System (GHS) of classification and labelling of chemicals (United Nations, 2011) which has been implemented in the EU classification, labelling and packaging (CLP) regulation (Anon., 2008), and further into the four hazard levels of the US Environmental Protection Agency's (EPA) acute inhalation toxicity categories (EPA, undated).

For an *in vitro* test method to become applicable for regulatory toxicity testing, a meaningful prediction model has to be established, and it is generally required to relate the results of the *in vitro* test to *in vivo* effects obtained in animal tests. Based upon these considerations, for all tests IC_{50} values (i.e. the test substance concentrations leading to a 50% reduction in cell viability in comparison to the untreated control) were calculated and compared to available acute inhalation rat LC_{50} data. For each *in vitro* test system attempts were made to find IC_{50} threshold values that would enable distinguishing non-toxic from toxic substances or ruling out substances of high concern.

To further investigate whether it might be possible to relate the IC_{50} values to a four-level hazard categorisation scheme, for each test system an *in vitro* inhalation toxicity categorisation scheme was designed. The IC_{50} values were classified accordingly, and the level of concordance with the GHS and EPA hazard categories was determined.