Need and Perspectives for the Implementation of Relevant *In-Vitro* Methods in the Field of Inhalation Toxicology

Niklas Möhle, Jessica Rach, Steffanie Scheffler, Dendy-Jessica Budde, Michaela Aufderheide
Cultex® Laboratories GmbH, Hannover, Germany

Cultex® Laboratories
The Airborne Exposure Experts

Abstract

The EU REACH legislation for chemicals of 2006 represents one of the largest challenges for toxicological testing, because 68.000 to 101.000 chemicals have to be investigated according to the newest data. In the field of acute toxicology, internationally accepted methods are available with regard to oral toxicity. But comparable validated approaches for inhalation toxicology are lacking, probably due to the difficulties in exposing cells of the respiratory tract directly to inhalable substances in a way comparable to the *in-vivo* situation. In the last ten years, the optimization of the biphasic cell culture and exposure techniques as well as the availability of human cell lines for such studies offer promising possibilities to integrate this type of *in-vitro* study into the research strategies for inhalable chemical compounds. Prevalidation studies in particular are under investigation for analysing the biological activity of gaseous and particulate matter using a special *in-vitro* exposure system, the CULTEX® RFS module, exposing cultivated cells at the air-liquid interface (ALI). Special attention is placed on issues like controlled generation, distribution and deposition of the test atmosphere, in order to optimize and standardize the *in-vitro* exposure of cells at the air-liquid interface.

Need

Although the incidence of lung disease is increasing (Barnes, 2010; Lopez and Murray, 1998) and is one of the major causes of lethality in many countries, studies in the field of inhalation toxicology today mainly concentrate on mechanisms of lung injury and repair after exposure to toxicants. The toxicological characteristics of inhalable substances are usually studied in animal experiments according to the OECD guidelines for testing acute and chronic toxicity. In 2005, ten billion Euros were spent and 100 million rodents were used in animal experiments throughout the world (Taylor et al., 2008) of which about 20 % were used for toxicological studies. These studies are mandatory for regulatory and legislative purposes, but current discussions point out limitations in the transferability of the results to humans with regard to positive or negative endpoints (Hartung, 2008), the costs and ethical concerns. For logistical and ethical reasons, the legislation demands the reduction and replacement of animal experiments and claims to develop, establish and validate alternative test methods as well as strategies for the toxicological evaluation of inhalable compounds. Although several *in-vitro* models with human lung cells are available, there is an urgent need for new relevant test strategies to study airborne material. Here, there are challenges concerning the cell model, the exposure procedure, the determination of dose-response relationships and relevant endpoints.

Information	REACH Annex	53.048 sub. ≥ 100 t/y Deadline 2013	47.858 sub. ≥ 1000 t/y Deadline 2010	Total
Acute oral tox	VIII	37.240	33.596	70.836
Acute inhalation toxicology	VIII	20.689	18.665	39.353

Table 1: Total number of tests required based on actual preregistration (estimated existing data & possible waiving; Rovida & Hartung, 2009).

Information	REACH Annex	Total no. of tests required	Animals/ test on average	Total animals used
Acute oral tox	VIII	70.836	8	566.688
Acute inhalation toxicology	VIII	39.353	20	787.067

Table 2: Total number of animals required based on actual preregistration considering both estimated existing data and possible waiving but no alternative methods (Rovida & Hartung, 2009).

Strategy

In-vitro Models

Limitation in using animals for inhalation and toxicological studies promotes the utilization of cell cultures or *in-vitro* tissue equivalents (BéruBé et al., 2009; Forbes and Ehrhardt, 2005; Forbes, 2000; Hartung, 2008). As yet, there is no standardized *in-vitro* airway model and especially human cell lines are desirable to take into account the species-specific characteristics.

- Primary airway epithelial cells: ready-to-use commercially available cell culture systems (EpiAirway™, MatTek Corporation and MucilAir™, Epithelix Sàrl) and undifferentiated primary cells
- Permanent airway epithelial cell lines: a) conducting airways: 16HBE14o-, Calu-3, BEAS-2, NuLi-1, CuFi-1, b) alveoli: A549, TT1
- 3D tissue equivalents (co-culturing of different cell types)

Test Strategy

The basic considerations for such a direct exposure strategy mimicking the inhalation cycle *in-vivo* are: a) **relevant cell models** of the respiratory tract, b) cultivation of the cell systems at the **air-liquid interface (ALI)**, c) **direct contact** between the cells and airborne material without interfering medium and d) **uniform dynamic exposure** of the entire cell layer. The requirements are fulfilled by the **CULTEX® RFS exposure module**, a sophisticated experimental setup guaranteeing a homogeneous and reproducible exposure process, meaning a continuous and guided transport of the test atmosphere to the surface of the cells, a reproducible deposition of particles, and the continuous removal of the atmosphere.

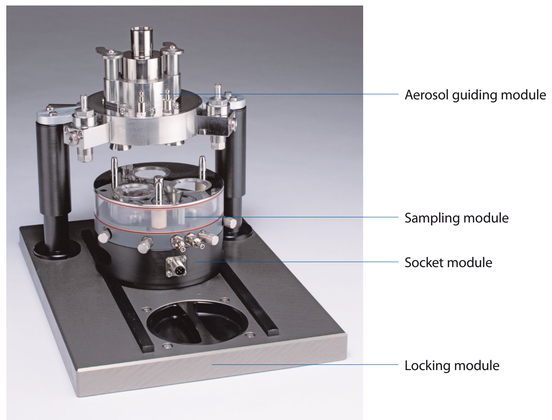

Figure 1: The modularly designed Cultex® **R**adial **F**low **S**ystem (RFS) is composed of 4 basic components: the aerosol guiding module, the sampling module, the socket and locking module. The construction allows comfortable handling and loading of the system with the cell culture inserts. The insertion of adapters for transwell inserts of different sizes and manufacturers make the system very flexible.

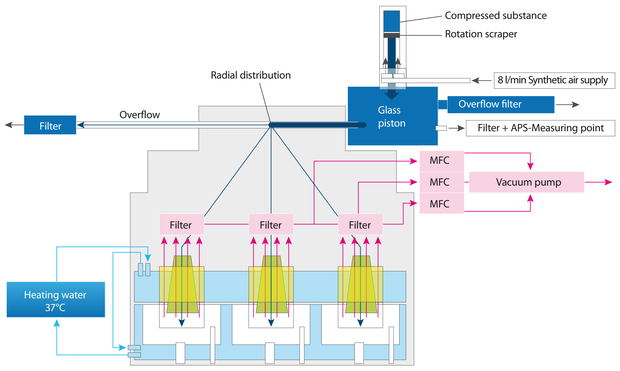

Figure 2: Schematic drawing of an experimental set-up including the Cultex® RFS module. The module houses 3 insert positions connected via a uniform radial tubing system with the aerosol inlet tube running through the module. Particles are generated by means of a particle generator according to Wright and transported by compressed air into a mixing chamber (glass) for homogeneous distribution and dilution of the aerosol. The generated and adjusted test atmosphere is sucked via negative pressure through the module and above the cells located in the 3 transwell insert positions.

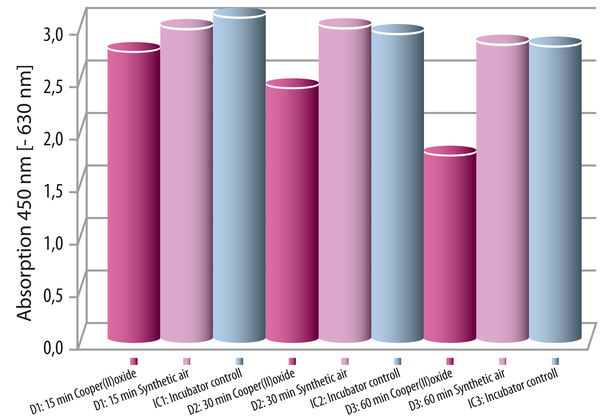

Figure 3: WST-1 results after exposure of A549 cells in a CULTEX® RFS module to different concentrations of copper(II) oxide. Generation via a dust feeder according to Wright; feed rate: 4 mm/h, 800 rph. By increasing particle concentration with the exposure time (60, 30 and 15 min), cell vitality decreased in a dose-dependent manner.

Perspectives

- Development of ***in-vitro* test systems** for toxicological screenings of gases, volatile compounds and particles comprising acute cytotoxic or genotoxic effects. Although *in-vitro* inhalation tests cannot replace animal testing completely at present, the availability of such test systems may significantly reduce the number of animal tests.
- The possibility to test such harmful atmospheres is a promising domain for the **screening of chemicals, industrial and pharmaceutical products as well as complex atmospheres like tobacco smoke, diesel exhaust** and **environmental aerosols** (biological monitoring). Several places of employment, especially those of employees who are exposed to harmful substances like welding dust, respirable dust or other harmful substances are evaluated concerning their emissions with physical and chemical methods. Extension of the routine testing methods by a variety of biological parameters can give detailed information about biological activities.
- Equally, the measurement of atmospheric pollution, particularly with regard to respirable dust in cities, may be an important area of application. Furthermore, harmful substances can be studied mechanistically concerning their mode of action in biological systems using *in-vitro* models. With the *in-vitro* data obtained, it is possible to classify harmful substances related to their toxicity. Such a ranking can help to provide information about the risk assessment of the tested substances.
- In summary, the application of direct exposure methods to analyse the biological effects of inhalable substances offers new ways to analyse dose-response relationships of such substances by simulating the *in-vivo* situation. The challenge of such studies is the multidisciplinary approach by combining biological and technical as well as aerosol physical aspects in such a cell-based approach.

References

Barnes PJ. (2010) New therapies for chronic obstructive pulmonary disease. Med Princ Pract 19:330-8. DOI: 000316368 [pii]10.1159/000316368.

BéruBé K, Aufderheide M, Breheny D, Clothier R, Combes R, Duffin R, Forbes B, Gaca M, Gray A, Hall I, Kelly M, Lethem M, Liebsch M, Merolla L, Morin JP, Seagrave J, Swartz MA, Tetley TD, Umachandran M. (2009) In vitro models of inhalation toxicity and disease. The report of a FRAME workshop. Altern Lab Anim 37:89-141.

Forbes B (2000) Human airway epithelial cell lines for in vitro drug transport and metabolism studies. Pharm Sci Technolo Today 3:18-27. DOI: S146153479900231X [pii].

Forbes B, Ehrhardt C. (2005) Human respiratory epithelial cell culture for drug delivery applications. Eur J Pharm Biopharm 60:193-205. DOI: S0939-6411(05)00073-1 [pii]10.1016/j.ejpb.2005.02.010.

Hartung T. (2008) Thoughts on limitations of animal models. Parkinsonism Relat Disord 14 Suppl 2:S81-3. DOI: S1353-8020(08)00116-8 [pii]10.1016/j.parkreldis.2008.04.003.

Lopez AD, Murray CC. (1998) The global burden of disease, 1990-2020. Nat Med 4:1241-3. DOI: 10.1038/3218.

Taylor K, Gordon N, Langley G, Higgins W (2008) Estimates for worldwide laboratory animal use in 2005. Altern Lab Anim 36:327-42

Rovida C, Hartung T (2009) Re-evaluation of animal numbers and costs for in vivo tests to accomplish REACH legislation requirements for chemicals – a report by the transatlantic think tank for toxicology (t(4)). ALTEX. 2009;26(3):187-208.