Alternative approaches for acute inhalation toxicity testing to address global regulatory and non-regulatory data requirements: An international workshop report

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Abstract

Inhalation toxicity testing, which provides the basis for hazard labeling and risk management of chemicals with potential exposure to the respiratory tract, has traditionally been conducted using animals. Significant research efforts have been directed at the development of mechanistically based, non-animal testing approaches that hold promise to provide human-relevant data and an enhanced understanding of toxicity mechanisms. A September 2016 workshop, "Alternative Approaches for Acute Inhalation Toxicity Testing to Address Global Regulatory and Non-Regulatory Data Requirements", explored current testing requirements and ongoing efforts to achieve global regulatory acceptance for non-animal testing approaches. The importance of using integrated approaches that combine existing data with *in vitro* and/or computational approaches to generate new data was discussed. Approaches were also proposed to develop a strategy for identifying and overcoming obstacles to replacing animal tests. Attendees noted the importance of dosimetry considerations and of understanding mechanisms of acute toxicity, which could be facilitated by the development of adverse outcome pathways. Recommendations were made to (1) develop a database of existing acute inhalation toxicity data; (2) prepare a state-of-the-science review of dosimetry determinants, mechanisms of toxicity, and existing approaches to assess acute inhalation toxicity; (3) identify and optimize *in silico* models; and (4) develop a decision tree/ testing strategy, considering physicochemical properties and dosimetry, and conduct proof-of-concept testing. Working groups have been established to implement these recommendations.

Keywords

1. Introduction

Inhalation is a major route of human exposures for substances such as particles, fibers, nanomaterials, gases, and volatile organic chemicals. As the respiratory tract serves as both target tissue and portal of entry (POE) from the external environment to the systemic circulation for these substances, it is important to characterize the hazards they may present. Historically, acute inhalation toxicity tests expose rodents in whole-body or nose-only systems for 24 h or less to identify substances that could cause toxicity after a short-term exposure. Several critical differences between the rodent and human respiratory tract have the potential to affect the precision with which the rodent test predicts the human dose and response. These differences include (1) respiratory physiology (*e.g.*, breathing mode and ventilation rates; metabolic rates), (2) anatomy (*e.g.*, airway architecture and branching pattern; cell types and composition within the regions of the respiratory tract), and (3) biochemistry (*e.g.*, composition and capacity of biotransformation enzymes) (Prytherch and BéruBé, 2014a; Parent, 2015). To explore approaches with the potential for a more accurate prediction of human response, recent research has focused on the development and application of human-relevant *in vitro* and *in silico* methods that can be used in testing approaches that facilitate a mechanistic understanding of the toxic effects of inhaled materials (Loizou et al., 2008; Fröhlich and Salar-Behzadi, 2014).

Recommendations from a 2015 workshop, "Alternative Approaches for Identifying Acute Systemic Toxicity: Moving from Research to Regulatory Testing," highlighted a need to identify and further develop approaches that could reduce or replace animal use for acute inhalation toxicity testing (Hamm et al., 2017). To address this need, an international group of experts convened at a workshop on September 22–23, 2016 to discuss progress and challenges associated with the development, validation, and implementation of alternatives. The product of this workshop was a defined strategy for further development and implementation of approaches to acute inhalation toxicity testing that would reduce or replace animal use for both regulatory and non-regulatory purposes. In advance of the workshop, a six-part webinar series was organized to review the state-of-the-science of non-animal approaches to acute inhalation toxicity testing (Table 1).

This paper aims to summarize the presentations and discussions that took place during the webinars and workshop. Specifically, it describes (1) the current regulatory and non-regulatory needs for acute inhalation toxicity data; (2) the information obtained from acute animal inhalation tests that is currently used by regulators to define hazard, in order to determine what information is needed from alternative approaches; (3) the mechanistic determinants of dosimetry and toxic effects as these will have a large bearing on the development and application of alternative approaches; (4) what alternative approaches are currently used by companies for in-house decisions and/or accepted for regulatory purposes; (5) the data gaps or other issues that are precluding the uptake and acceptance of alternative approaches; and (6) the final recommendations agreed on at the workshop.

2. Regulatory and non-regulatory needs for acute inhalation toxicity data

Acute inhalation toxicity data are used for both regulatory and non-regulatory purposes. For regulatory purposes, data are used in hazard identification as part of product or substance registration; classification and labeling; determining handling and shipping requirements; and providing information for safety data sheets. Non-regulatory purposes for which data may be used include development of risk assessments to determine short-term occupational exposure levels and emergency response values to inform first responders in cases of an unexpected release or accident. Other applications for which data may be used include product stewardship; filling data gaps by read-across (*i.e.*, applying data from one substance(s) to predict the same property or effect for a 'similar' substance); assessing the impact of alterations in the safety profile due to product reformulation; and determining when development of products should progress or be halted due to toxicity concerns.

In a global economy, achieving international harmonization is a significant challenge. Many regulatory authorities require acute inhalation toxicity data (Fig. 1). While there are differences in specific testing requirements across regional regulatory authorities, the general principles of the acute inhalation toxicity testing guidelines are discussed below.

2.1. Regulatory test guidelines

Data on the potential effects from airway exposures may be required if inhalation is likely, taking into account the various potential uses of the substance, the vapor pressure, and the

potential aerodynamic size and distribution of materials that are liquids or solids under standard conditions (Doiron, 2007).

Test guidelines (TGs) adopted by the Organization for Economic Co-operation and Development (OECD) (Table 2) may be used to fulfill specific requirements for acute inhalation data when an animal study is requested by a regulatory agency. OECD TG 403 (OECD, 2009a) recommends ways to minimize animal usage, including consideration of any information on the test substance use scenario or expected human exposure, as well as existing toxicological data (*i.e.*, *in vivo* human or animal, *in vitro*, or *in silico*) on the test substance or structurally similar substances. For example, one approach to reducing regulatory-required animal use when the test article is known or expected to be virtually non-toxic would be to conduct a limit test, in which a single dose group is exposed to the limit concentration (or maximum attainable concentration if a limit concentration cannot be attained), generally for 4 h. OECD TG 436 (OECD, 2009b) uses fewer animals than OECD TG 403; it follows a stepwise procedure of 4 h exposure to fixed concentrations, with the outcome of the previous step determining the subsequent step. Lethality is used as the main endpoint for both OECD TG 436 and 403. A third test guideline for acute inhalation toxicity, OECD TG 433 (OECD, 2017) is a refinement alternative that follows the fixed concentration approach used in OECD TG 436 but replaces lethality as an endpoint with the observation of evident clinical signs of toxicity. Evident toxicity is defined as clear signs of toxicity that indicate that exposure to the next highest concentration will cause severe toxicity or death in most animals (*e.g.*, irregular respiration or tremors) (Sewell et al., 2015). Ultimately, the choice of test guideline is driven by the regulatory requirement for the intended use of the inhaled material.

2.2. Regulatory efforts to advance alternative approaches for acute inhalation toxicity

The ICCVAM Authorization Act of 2000 (42 U.S.C. 285l-3) established the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). ICCVAM is comprised of 16 federal regulatory and research agencies that require, use, generate, or disseminate toxicological and safety testing information (NTP, 2017). Within ICCVAM, the Environmental Protection Agency (EPA) and the Department of Defense (DoD) sponsor an acute toxicity working group with additional members from the Consumer Product Safety Commission, the Department of Transportation, the Occupational Safety and Health Administration (OSHA), and the National Institute of Environmental Health Sciences (NIEHS). The working group also includes liaison representatives from the European Union Reference Laboratory for alternatives to animal testing and the Korean Center for the Validation of Alternative Methods. The aim of the ICCVAM working group is to evaluate existing *in vivo*, *in silico*, and *in vitro* tests for acute systemic toxicity and to contribute to an ICCVAM strategic roadmap[1] on using *in vitro* and *in silico* approaches to reduce or replace current *in vivo* acute systemic toxicity tests.

The ICCVAM working group's current activities are being informed in part by a recent National Research Council publication, "Application of Modern Toxicology Approaches for

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[1]https://ntp.niehs.nih.gov/pubhealth/evalatm/natl-strategy/index.html

Predicting Acute Toxicity for Chemical Defense," which was sponsored by DoD (National Research Council, 2015). The report reflects DoD's interest in advancing a mechanistic testing framework that first considers properties of the test substance to inform the need for additional evaluation using *in silico* or *in vitro* methods.

In addition to the DoD interests, EPA is actively pursuing implementation of alternatives to animal testing. The EPA Office of Pesticide Programs (OPP) has publicly committed to significantly reduce the number of animals used in the agrochemical registration process (US EPA, 2016a). To accomplish this goal, EPA OPP's strategy includes considering acceptance of a broader suite of *in silico* approaches and *in vitro* assays, waiving study requirements under certain circumstances (*e.g.*, when a data endpoint is not relevant to the chemical), and supporting an improved understanding of toxicity mechanisms to inform data needs and allow for the development of non-animal tests that better predict how exposures are related to adverse effects. EPA OPP is working in partnership with other governmental entities, industry, and non-governmental organizations to achieve the mutual goal of more efficient human-predictive testing that does not use animals.

EPA OPP has released several guidance documents to aid in reducing animal use including "Guiding Principles for Data Requirements" (US EPA, 2013) and "Process for Establishing and Implementing Alternative Approaches to Traditional *In Vivo* Acute Toxicity Studies" (US EPA, 2016c). Subsequent to an EPA OPP 2012 guidance (US EPA, 2012b), the OECD released a "Guidance Document for Waiving or Bridging Acute Toxicity Tests" (OECD, 2016a) (discussed further in Section 6.1).

EPA OPP has also initiated a voluntary pilot program where registrants may submit calculations of toxicity for agrochemical formulations using the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (GHS) additivity formula along with currently required *in vivo* acute oral and inhalation test data (US EPA, 2016b). The GHS additivity formula can be used to classify a mixture (such as an agrochemical formulation) based on acute toxicity values and concentrations of its ingredients, without requiring any additional testing of the final formulation (United Nations, 2015). The goal of the pilot program is to evaluate the ability of the GHS additivity formula to predict the acute toxicity categories for oral and inhalation routes (*i.e.*, compare the *in vivo* and calculated LD_{50}/LC_{50} values for each ingredient, and compare the EPA and GHS category based on the *in vivo* test and the calculated value). If the analysis shows the approaches to be comparable, EPA OPP will take steps towards waiving the *in vivo* test requirement for these substances, as appropriate.

EPA OPP is also working with the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to develop a database of curated acute toxicity data from agrochemical products, including acute inhalation toxicity data. NICEATM provides scientific and operational support for ICCVAM technical evaluations and related activities. The resulting database will be used to assess the variability within and across studies, to develop (Q)SAR and/or read-across approaches, and to compare with the results from alternative approaches, such as the GHS additivity formula or *in vitro* studies.

3. Inhalation dosimetry

Accurate dosimetry characterization requires determining the amount, rate, distribution, and form of a substance delivered to the target tissue of interest (Kuempel et al., 2015). Anatomical and physiological differences in the various species used in inhalation toxicological studies can result in different doses delivered both to analogous respiratory tract regions and systemically to other tissues. Therefore, an understanding of the comparative dosimetry of inhaled compounds in humans *versus* animals is critical both for translation of exposure-response relationships found in existing *in vivo* data and to inform the design of novel *in vitro* and *in silico* approaches to assess acute inhalation toxicity. Consideration of the concepts discussed in this section will be used in the development of a decision strategy to guide case studies.

3.1. Factors controlling comparative disposition of inhaled agents

The adverse toxic effects that are considered in a risk assessment are more related to the quantitative pattern of deposition within the respiratory tract rather than to the exposure concentration (Brain and Mensah, 1983). The deposition pattern of an inhaled substance determines not only the initial dose in the portal of entry (POE) but also the specific pathways by which inhaled material is cleared and redistributed. This section briefly discusses the two major factors impacting comparative inhalation dosimetry: (1) respiratory anatomy and physiology and (2) the physicochemical characteristics of the inhaled toxicant. Although these factors are discussed as distinct entities, their influence on the disposition of an inhaled agent are dynamic and interactive, with the relative contribution different in each species and respiratory tract region. Thus, an accurate description of the disposition of inhaled substances requires integration best afforded by the models described in the next section. Further, these factors are influenced by exposure concentration, duration, and frequency, all of which are discussed in more detail elsewhere (Fiserova-Bergovera, 1983a; Fiserova-Bergovera, 1983b; International Commission on Radiological Protection (ICRP), 1994; US EPA, 1994; McClellan and Henderson, 1995; Miller, 1995; Phalen et al., 1995; Gardner et al., 1999; Harkema, 1999; Gardner, 2005; Harkema et al., 2006; Morris and Shusterman, 2010; Parent, 2015).

Disposition of inhaled agents encompasses the processes of initial deposition and absorption, distribution, metabolism, and elimination (ADME). The phrase "initial deposition" is used when referring to gases and particles because contact with the respiratory tract surface precedes absorption or uptake. Clearance mechanisms are defined herein to include processes such as dissolution, phagocytosis by macrophages, transport to the gastrointestinal tract *via* the mucociliary escalator or nasal outflow, translocation *via* the lymphatics, absorption into the blood, and metabolic transformation. Disposition varies across species and among the respiratory tract regions; for example, interspecies variations in cell type, morphology, number, distribution, and functional capacities contribute to variations in clearance of initially deposited doses (Bogdanffy and Jarabek, 1995). Retained dose in a given region is defined as deposition minus clearance; in other words, the actual amount of particles or gas found in the respiratory tract at any time is determined by the relative rates of deposition and clearance. The efficiencies of the deposition mechanisms are

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different in each respiratory tract region and species. The defense mechanisms and clearance rates for each of these regions are also different and ideally require quantitation to arrive at an accurate description of the dose in each species.

3.1.1. Respiratory tract anatomy and physiology—The respiratory tract in humans and animals can be divided into three major regions on the basis of structure, size, and function: (1) the extrathoracic region that extends from just posterior to the external nares to just anterior to the trachea; (2) the tracheobronchial region, defined as the trachea to the terminal bronchioles where proximal mucociliary transport begins; and (3) the pulmonary region, including the terminal bronchioles and alveolar sacs. The thoracic region is defined as the tracheobronchial and pulmonary regions combined. The anatomic structures included in each of these respiratory tract regions are depicted in Fig. 2 and listed in Table 3A.

The respiratory tract of humans and animals differ in anatomy and physiology in several quantitative and qualitative ways (Table 3B). These variations affect airflow pattern in the respective respiratory tract architecture, which affects in turn the deposition of the given inhaled agent as well as its clearance and retention. Human and animal respiratory tracts exhibit differences in, for example, gross anatomy, types and location of nasal epithelia, and the distribution of mucous secretory products. Interspecies differences in structure of the upper respiratory tract and resultant differences in airflow dynamics (Fig. 3) and lesion distribution have been shown to result in quantitative differences relevant to dose-response analysis and interspecies extrapolation (Kimbell et al., 1997; Kimbell et al., 2001a; Kimbell et al., 2001b; Overton et al., 2001). There are also clear differences in the anatomy and geometry of airways in the lower respiratory tract that influence deposition and uptake of inhaled substances (International Commission on Radiological Protection (ICRP), 1994; US EPA, 1994; Dahl, 1995). An obvious difference between rodents and humans is the branching pattern and angle of the bronchi and bronchioles in the lower respiratory tract. As shown in Fig. 3, the branching pattern in rodents is asymmetric or monopodial, which results in a relatively unimpeded flow, whereas the branching pattern in humans is symmetric that results in an airflow pattern more susceptible to deposition at its bifurcation points. Airway dimensions such as length and diameter, tissue volumes, cell types and their distribution, and mucous composition also differ across species (Parent, 2015). Differences in cell types for the pulmonary region are less dramatic than in other regions, with significant homogeneity in populations of epithelial, endothelial, interstitial, and macrophage cells as well as in the percentage of the alveolar surface area covered by Type I and Type II cells (Crapo et al., 1983; Parent, 2015). Consideration of these parameters can be used to guide the development of *in vitro* test systems, and to extrapolate results from *in vivo* tests and to target human exposure scenarios.

One of the most obvious and significant physiological differences between rodents and humans is breathing mode. Humans are oronasal breathers while rodents are obligate nose breathers. This difference has important ramifications for particle and gas deposition in the URT of humans as compared to rodents. For example, there is less filtering of particles and gases in oral breathing compared to nasal breathing, resulting in a greater delivery of material to the peripheral airways. Oral breathing increases with exertion (Niinimaa et al., 1981; International Commission on Radiological Protection (ICRP), 1994; US EPA, 1994),

thus the ventilation rate and activity pattern associated with various exposure scenarios (*e.g.*, occupational *versus* resting) is an important factor when constructing internal dose metrics for response analysis. Differences in ventilation rates affect the tidal volume and ventilation-to-perfusion ratios across species, and cardiac outputs also vary; all interact with the anatomical differences described above to result in dramatic differences in deposition and uptake across species. Another factor that differs among species is the chemotactic attraction of macrophages involved in clearance. Biochemical mechanisms of airway activation, detoxification, and response are other factors affecting internal dose, which need to be characterized to target development of *in vitro* test systems. Metabolic capabilities of critical enzymes are different across species (Csanady et al., 1992; Bogdanffy and Jarabek, 1995; Bond and Medinsky, 1995; Dahl, 1995; Fisher, 1995; Bogdanffy and Keller, 1999; Sarangapani et al., 2002a); for example, cytochrome P450 activities for a variety of substrates are metabolized less efficiently in microsomes from human nasal mucosa than in microsome preparations from rodents, whereas phase II enzymes such as epoxide hydrolase and glutathione S-transferase appear to be less active in rodents. Carboxylesterase activity is particularly prominent in the nasal tissues of rodents. In the lower respiratory tract, cytochrome P450 and glutathione S-transferase activities are lower in humans than in most species, and considerably lower in humans than in mice.

3.1.2.   Physicochemical properties—The physicochemical properties of an inhaled agent will influence the initial deposition and subsequent disposition within the respiratory tract, distribution to other tissues, and ultimately the toxic effect. Two general categories for inhalation dosimetry can be made: particles (including fibers and nanomaterials) and gases; within gases there are three major categories for dosimetry model selection (1, 2, and 3). Consideration of physicochemical properties according to these categories will be essential to the design of test systems and the evaluation and extrapolation of the effects of a given inhalation exposure. A number of anatomical and physiological factors that affect deposition and uptake of particles and gases in the respiratory tract, which are diagramed in Fig. 4 and further described in Sections 3.1.2.1 and 3.1.2.2.

3.1.2.1.   Particles.: Particle, fibre, and nanomaterial dosimetry has been evaluated in a number of reviews (Schlesinger, 1995; Snipes, 1995; Miller, 1999; Asgharian et al., 2005; Bernstein, 2005; Jarabek et al., 2005; Oberdorster et al., 2005; Warheit, 2005; Teeguarden et al., 2007; Morris et al., 2010). Factors affecting deposition of particles in the respiratory tract include mechanisms of impaction, sedimentation, interception, diffusion, and electrostatic precipitation (Fig. 4a) (US EPA, 1994; Jarabek et al., 2005). The density, size, and distribution of particles influence their aerodynamic behavior in the respiratory tract according to these mechanisms (International Commission on Radiological Protection (ICRP), 1994; US EPA, 1994; McClellan and Henderson, 1995). The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of a particle are used to characterize these properties in dosimetry modeling. Mass median aerodynamic diameter may be influenced by the hygroscopic nature of some particles and can be used to refine applied dosimetric adjustments. For fibers and nanomaterials, shape is also a critical determinant, which can be described by aspect ratios or bivariate distributions of length and width, coil length, and coil diameter (US EPA, 1994). Subsequent clearance of a deposited

dose is dependent on both the initial site of deposition and the solubility or biodurability of the particle. Time since deposition is another important consideration.

3.1.2.2. Gases.: Dosimetry of gases has also been considered in a number of reviews (Bond and Medinsky, 1995; Dahl, 1995; Fisher, 1995; Miller and Kimbell, 1995; Bogdanffy and Keller, 1999; Kimbell and Miller, 1999; Medinsky et al., 1999; Sarangapani et al., 2002a; Morris et al., 2010; Morris, 2012). Uptake of a gas requires that it move from the gas phase in the airway lumen to the tissue phase (*i.e.*, the surface-liquid lining layer, the tissue layers of the respiratory tract, the capillary endothelium or the blood. The major mechanisms of gas transport in the respiratory tract involve convection, diffusion, absorption, dissolution, and chemical reaction (Fig. 4b). Convection is comprised of advection (*i.e.*, horizontal movement of a mass of air relative to the airway wall) and eddy dispersion (*i.e.*, air mixing by turbulence) so that individual fluid elements transport the gas and generate a flux to the epithelial lining fluid and tissue. Molecular diffusion is superimposed at all times on convection due to local concentration gradients. Absorption removes gases from the lumen and affects concentration gradients. Chemical reactions in the respiratory tract tissue can increase absorption by acting as a sink to drive the concentration gradient. Systemic metabolism can also drive the concentration gradient for soluble gases that are removed from the respiratory tract primarily by perfusion. Thus, gas dosimetry is dictated by its mass transfer coefficient consisting of a gas-phase component and tissue-phase component (US EPA, 1994; McClellan and Henderson, 1995; Hanna et al., 2001; US EPA, 2012a; Kuempel et al., 2015).

3.2. Dosimetry modeling

Recent advances in the availability of mechanistic data and mathematical models that describe the behavior of inhaled particles and gases in the respiratory tract discussed above can serve as the basis for approaches that integrate critical determinants of ADME into testing strategies and risk assessment. "Dosimetry modeling" is a comprehensive term encompassing model structures that describe the inhaled disposition of non-volatile and irritant gases, particles, and fibers, including physiologically based pharmacokinetic (PBPK) models.

Selection of the dosimetry model to use in a particular risk assessment depends on the purpose of the predictions (*e.g.*, screening *versus* full characterization), the physicochemical properties of the inhaled agent, the degree of understanding of ADME and the mechanism of toxicity, and the level of detail and specificity of the available data. A general equation for a dosimetric adjustment factor (DAF) is shown below (US EPA, 1994; Jarabek, 1995a):

$$DAF_r = POD_{HEC} \div POD_{ADJ}.$$

where:

POD = the point of departure (*i.e.*, threshold for activity) of a given endpoint or key event relevant to the adverse outcome pathway;

HEC = human equivalent concentration, constructed using parameters such as ventilation rate for age and exertion level relevant for the human exposure scenario under consideration;

ADJ = if needed, adjustment for animal exposure level (*e.g.*, 6 h/day, 7 day/week);

DAFr = is either the regional deposited dose ratio or the regional gas dose ratio in the respiratory tract region associated with the observed toxicity, r, in the study being extrapolated.

The DAF approach can be used to adjust for interspecies differences or *in vitro* to *in vivo* extrapolation (IVIVE), and a similar strategy can be used to adjust to different target human exposure scenarios such as occupational exposures by adjusting the terms used to construct the DAF ratio (Kuempel et al., 2015).

A two-tiered hierarchy of model structures that can be used as the DAF is shown in Table 4. Analysis of more mature and sophisticated models can be used to construct reduced forms that are conceptually consistent with the more detailed model structures (Jarabek, 1995a). In the first tier, the default structure relies on categorical descriptions of key processes and parameters; in the second tier, the incorporation and integration of the critical mechanistic determinants allows more elucidation of the exposure-dose-response continuum and may represent a range of model structures preferred to the default. Depending on the knowledge of model parameters and fidelity to the biological system, a comprehensive model structure can be constructed that provides a more accurate characterization of the pathogenic process.

For particle dosimetry, the freely available Multiple-Path Particle Dosimetry (MPPD) computational model (Applied Research Associates Inc., 2017) is widely used, including by EPA and the National Institute for Occupational Safety and Health (NIOSH), which is working to expand the model to describe nanoparticles. EPA also supports a semi-empiric model, the regional deposited dose ratio model, for animal species not yet available in MPPD (US EPA, 1994). The same mechanisms responsible for inhaled particle deposition in the respiratory tract are operative in *in vitro* systems. The *in vitro* sedimentation, diffusion, and dosimetry (ISDD) model was developed to describe *in vitro* kinetic mechanisms of particles to better describe the "cellular dose," or dose delivered to the cell (Hinderliter et al., 2010). These models can be used to target test concentrations to be used in *in vitro* systems, and provide context for inferences and support integration across *in vitro* and *in vivo* tests.

Numerous dosimetry models are also available to describe gas uptake and disposition in the respiratory tract. Since inhaled gases can cover such a large range of potential physicochemical properties, the EPA gas category scheme describes three different model structures that can be used to arrive at dose estimates (Table 5; (US EPA, 1994; Hanna et al., 2001). The scheme considers properties such as water solubility and reactivity, which includes the propensity for dissociation of the parent gas in tissue (*e.g.*, hydrolysis) and its ability to react either spontaneously or *via* enzymatic reactions in the respiratory tract (US EPA, 1994). The goal of the EPA gas category scheme is to guide approaches to dosimetry adjustment that are commensurate with the available data on physicochemical properties, the nature and location of the toxicity, and the level of detail regarding the mechanism (US EPA, 1994; Jarabek, 1995a; US EPA, 2012a). However, it should be recognized that the gas

category scheme represents a way to select specific model components from a continuum and that the same comprehensive model structure could be applied to all categories (Kuempel et al., 2015). The scheme does not apply to stable gases that exert effects by reversible physical interactions of gas molecules with biomolecules (*e.g.*, displacement asphyxiants).

In the case of Category 1 gases, description of dose delivered to the tissue in a given region must account for the scrubbing of the gas out of the convective airstream as it travels through the nose (proximal) to pulmonary (distal) airways. Scrubbing is caused by uptake into the tissue, for example, by dissolution or reactions such as metabolism in the tissue. If the reaction rate is fast, then the gas-phase component dictates the overall mass transfer and would thus be the basis of default modeling algorithms (US EPA, 1994; US EPA, 2012a). Thus, for Category 1 gases, computational fluid dynamic (CFD) models (Kimbell et al., 2001a; Kimbell et al., 2001b; Corley et al., 2015) or single-path mass transfer models (Hanna et al., 2001; Overton, 2001) more accurately describe the species-specific anatomical influences on airflow delivery (mass transfer) and PBPK models then employed to describe tissue reaction kinetics. In fact, hybrid CFD-PBPK model structures are used to best capture behavior of gas uptake and disposition because they consider the combined influences of airway architecture and tissue metabolism (Frederick et al., 2002; Schroeter et al., 2006; Schroeter et al., 2010; Asgharian et al., 2011).

Such hybrid models represent a more comprehensive structure suitable for modeling of Category 2 gases, as well as those that are intermediate in reactivity and water solubility. Category 2 gases may have local effects in the POE, accumulate in the blood to cause systemic effects, or may deliver the toxicant back to airway tissues from the endothelial side of the respiratory/circulatory tissues where they may react with respiratory tract or be exhaled (Jarabek, 1995a; Kuempel et al., 2015).

Gases in Category 3, such as volatile organic solvents, have limited reactivity in the respiratory epithelium and are generally insoluble in water. These gases are not scrubbed out in the upper respiratory tract or conducting airways but instead readily penetrate to the pulmonary region where they are available to be absorbed into the systemic circulation. Toxic effects from these gases typically occur in systemic target tissues, although some metabolism in airways can lead to POE effects (Kuempel et al., 2015). The underlying model structure for gases in Category 3 is a ventilation:perfusion model in which the blood:gas partition coefficient is used to modulate the rate of transfer from the pulmonary region to the blood. For these gases, the human-to-animal ratio of blood:air partition coefficients is used as a default for the regional gas-dose ratio (Kuempel et al., 2015).

Typically, PBPK models used to describe systemic distribution of Category 3 gases represent the lung as a single homogenous tissue compartment in equilibrium with arterial blood for simulating inhalation exposure. These simple models do not reflect respiratory tract dosimetry as target tissue. In addition, they do not capture air-phase delivery of the inhaled substance to the target cells in the respiratory tract; species differences in the metabolic constants for formation and clearance of metabolites by club cells, the primary detoxifying cells that also produce secretory proteins to protect the bronchiolar epithelium; or regional

club cell density. In contrast, PBPK models with regional compartments in the respiratory tract can improve the estimation of dosimetry at the target site after inhalation exposure, capturing both POE and systemic dosimetry (Sarangapani et al., 2002b; Campbell et al., 2014; Campbell et al., 2015).

Selection of the appropriate dose metric (*i.e.*, the measure of the dose) is another important consideration for the design of *in vitro* test systems and interpretation of results. The best dose metrics are those that are most closely associated with the mechanism determining the adverse response in the target tissue. Calculation of a DAF for interspecies extrapolation for particles typically involves construction of dose metrics based on fractional deposition of mass normalized by factors related to the mechanism (Jarabek et al., 2005). PBPK models to characterize tissue reactions and systemic delivery can be used in combination with particle models like the MPPD as an approach for development of a DAF based on tissue concentrations (Ramoju et al., 2017).

The selection of a relevant dose metric also depends on whether a disease or adverse outcome is better described by an acute or chronic pathogenesis process. The challenge is to select a dose metric that is mechanistically associated with or experimentally closely correlated to the biological response (Jarabek, 1995b). Internal dose may be accurately described by particle deposition alone if the particles exert their primary action on the epithelial surface tissues (Dahl, 1990). When different types of particles are compared, inhaled dose may be more appropriately expressed as particle volume, particle surface area, or number of particles rather than mass, depending on the toxic effect being evaluated (Oberdorster et al., 1994). For gases, consideration of the mechanism and available data are also used to motivate selection of the dose metric. Descriptions of internal dose can be based on the parent compound or metabolite in various tissues. Dose metrics must take into account the frequency, duration, and magnitude of the exposure as well as the toxicity mechanism to be characterized (Jarabek, 1995b; Kuempel et al., 2015). For example, a given toxicity may be described by the peak concentration or the area under the blood or tissue concentration and time (AUC).

The cellular dose-response is modified by fractional deposition, local metabolism, and sensitivity of cell populations in specific regions of the respiratory tract. The major challenge in investigating respiratory tract metabolism is the non-homogenous distribution of metabolic enzymes in the respiratory tract. Metabolic enzymes are differentially expressed in various regions of the respiratory tract. For example, CYP450 metabolism only occurs in certain pulmonary epithelial cells, such as Type I, Type II, and club cells, with club cells being responsible for most of the metabolic activity (Plopper et al., 1980; Plopper et al., 1992). In addition, there are species differences in respiratory tract metabolism. For example, in mice, club cells are found throughout the respiratory tract, but they are only found in the transitional airway of rats and humans immediately before the alveolar regions of the lung (Plopper et al., 1992; Mercer et al., 1994).

Dosimetry modeling approaches are used during product development to aid in toxicology study designs based on predicted systemic exposures, as well as to understand how differences in toxicity between exposure routes, life stages, genders, and/or species correlate

with differences in internal dose. However, to facilitate adoption of dosimetry modeling tools to support non-animal testing, there is a need to standardize modeling approaches with common tools and a large user base. In developing a suite of standardized toxicokinetic models, a number of modeling criteria are important to consider.

- The models should be predictive of internal target site exposure (*i.e.*, either in the respiratory tract or to systemic target tissues) after acute, episodic, or steady-state exposures.

- The models should incorporate critical (quantitative) structure-activity relationships ((Q)SARs) for key ADME parameters, including absorption rates and amounts, metabolic clearance and metabolite structure prediction, plasma protein binding, and disposition in both the respiratory tract and systemic tissues.

- The suite of models should be flexible so that the components are tractable across different structures and can be adapted for use with either compartmental PK or more complex physiologically based structures (*i.e.*, dosimetry or PBPK models) depending on the needs of the application, and provide predictions for either parent compound only or parent and metabolite(s).

- The models should provide outputs in a user-friendly format and be easy to communicate with regulatory agencies.

To increase use of dosimetry/PBPK modeling in support of alternatives to animal testing for safety evaluations of chemicals and also to improve efficiency by accepting more high throughput testing results, novel *in vitro* and *in silico* approaches will be required to provide the necessary ADME parameters for dosimetry/PBPK models. Numerous *in silico* tools exist for prediction of one or more parameters involved in PBPK modeling of oral exposures and systemic toxicity (*i.e.*, solubility, absorption, metabolism, or tissue distribution) (Bessems et al., 2014); however, these tools were primarily developed for use with pharmaceuticals. In order for these tools to support non-animal approaches to acute inhalation toxicity testing, additional work will be needed to develop *in vitro* test systems that address the physicochemical properties across the range of inhaled substances, including nanomaterials, fibers, and gases (*e.g.*, volatile organics and reactive gases). A cross-cutting need is defining metabolic rate terms for the various cell types and capacities for various enzymes in the different respiratory tract regions (*e.g.*, carboxylesterases). Key to the use of predictive data from *in silico* expert systems is an understanding of the relevant adverse outcome pathways and associated mechanistic key events that result in acute inhalation toxicity, either in the POE or in systemic tissues. In follow-up to the workshop, a decision strategy is under development that will assess the likelihood of adverse outcomes in either the POE or systemic delivery, based on physicochemical properties and guided by the gas category scheme described above. Approaches to describe and extrapolate different dose metrics for target site exposures, at a minimum in the respiratory tract *versus* systemic delivery, will be developed and explored.

3.3. In vitro to in vivo extrapolation

In silico high-throughput PBPK models that incorporate IVIVE are becoming more widely used to prioritize chemicals for testing in large safety evaluation programs (Wambaugh et

al., 2015; Wetmore et al., 2015). IVIVE can build on current understanding of biological and physicochemical mechanisms and thereby aid in the comparison and translation of results across exposure conditions, between species, and across exposure or use scenarios. IVIVE may also facilitate comparisons of regional to local estimates of different doses (either to the respiratory tract or systemic distribution), provide insight on mechanism, and refine risk assessment predictions when developing alternatives for acute inhalation testing.

Models for IVIVE that are developed to estimate inhalation exposures should use as inputs cellular exposure concentrations corresponding to the appropriate dose metric in the target respiratory tract region under *in vivo* conditions (*e.g.*, for comparison to animal studies) and incorporate parameters based on likely human exposure to predict estimates that characterize target exposure scenarios. A challenge for the application of IVIVE to inhalation is that current IVIVE practices and approaches are largely based on the experience with hepatic metabolism of select pharmaceuticals after oral dosing, for which specific dosimetry within a tissue, such as the respiratory tract as the POE, is not an issue (Basketter et al., 2012; Yoon et al., 2012). Therefore, developing *in vitro* respiratory tract models that can represent target dosimetry, cellular components, and toxicity mechanisms in specific regions of the respiratory tract *in vivo* and which characterize *in vitro* dosimetry of the test material will be critical to developing a strategy for IVIVE for inhalation testing alternative methods.

Ultimately, user-friendly IVIVE programs that do not require computational expertise will be needed for routine regulatory application. Open source tools are being developed that allow modelers to incorporate computational workflows for IVIVE. For example, an open source "httk" package is available in R, a software platform for statistical computing and graphics (https://www.r-project.org/), that provides a set of tools for IVIVE using high throughput screening data (*e.g.*, ToxCast) to estimate real-world exposures. Resources being developed by EPA (*e.g.*, the EPA Chemistry Dashboard available at https://comptox.epa.gov/dashboard) and NICEATM (Integrated Chemical Environment) will include QSAR model predictions for hepatic clearance and protein binding that can be applied for IVIVE.

4. Mechanisms of toxicity and adverse outcome pathways

An understanding of mechanisms that lead to toxicity can help in devising relevant non-animal testing approaches, and it must be kept in mind that these mechanisms are highly dependent on the physicochemical properties of the inhaled agent. Basal cytotoxicity assays address many of these mechanisms, which include reactivity, nonspecific lipid membrane disruption, chelation, mitotic spindle poison by binding tubulin, disruption of energy production, vitamin interference, protein synthesis inhibition, and nucleotide synthesis inhibition (Vinken and Blaauboer, 2017). *In vitro* testing can be conducted using assays targeted to one of more of these mechanisms, for example, assessing mitochondrial membrane potential depolarization or dopamine receptor binding. In addition to basal cytotoxicity assays, tests may be used to assess more specific mechanisms, such as those acting through G-protein coupled receptors or Cys-loop ligand gated ion channels.

An adverse outcome pathway (AOP) is a framework used to organize data across a series of casually linked key events, beginning with a molecular initiating event and ending with an

adverse outcome (Ankley et al., 2010; Villeneuve et al., 2014b; Villeneuve et al., 2014a). The molecular initiating event and adverse outcome are linked by key events, which are measurable and essential to the progression of one or more defined biological perturbations leading to the adverse outcome. AOPs can be built using existing *in vivo*, *in vitro*, *in chemico*, and *in silico* data from the published literature. AOPs can facilitate the study of potential effects in the respiratory tract subsequent to inhalation exposures. Construction of AOPs can also help identify research gaps and with design of non-animal testing strategies (Wittwehr et al., 2017). The confidence in an AOP determines its potential use, with more quantitative AOPs being useful for risk assessment and less developed AOPs useful for prioritization or hazard identification (Vinken, 2013; Becker et al., 2015; Patlewicz et al., 2015; OECD, 2016b). The general mechanisms listed above may be used as a starting point for the development of AOPs that characterize key events of pathogenesis for diseases and toxic effects in either the respiratory tract or systemic tissues following inhalation exposure. These AOPs can then be used to select *in vitro* assays that assess specific mechanisms of toxicity.

5. *In vitro* testing approaches

Numerous reviews on *in vitro* inhalation toxicity testing models have been published (BéruBé et al., 2009; BéruBé et al., 2010a; BéruBé et al., 2010b; Gordon et al., 2015; Wiemann et al., 2016). This review focuses on three specific examples presented during the 2016 webinar series: (1) *in vitro* cell cultures, (2) lung-on-a-chip models, and (3) *ex vivo* human precision cut lung slices.

5.1. In vitro cell culture

In vitro test systems can range in complexity from relatively simple submerged mono- or co-culture systems to co-culture systems incorporating human respiratory tract cells at the air-liquid interface (ALI). The overlying medium in submerged cell cultures can interfere with the maintenance of a normal epithelial phenotype, specific gene expression, and sedimentation and aggregation of particles, as well as being a diffusion barrier for gases (Aufderheide, 2005; Xie et al., 2012; Rach et al., 2014). Therefore, to study the effects of airborne substances in a more human-relevant manner, systems have been developed that allow direct exposure of the cells of the respiratory tract at the ALI. There are many types of 'laboratory-based' or commercially available *in vitro* ALI exposure systems, each of which has unique design features that may pose advantages or disadvantages for different exposure scenarios focusing on specific particle sizes, types, physicochemistries, and concentrations (Polk et al., 2016).

Many human cell-based *in vitro* systems were developed with the intention of best mimicking human respiratory biology. Co-culture models have been developed that incorporate embryonic stem cells, tumor-derived cell lines (*e.g.*, NIH-H292 cells), immortalized cell lines (*e.g.*, BEAS-2B cells), and primary cells (*e.g.*, normal human bronchial epithelial cells). There are also three-dimensional organotypic models in which cells are used in physiologically-relevant ratios and arranged in a way that mimics certain regions of the human respiratory tract (Diabaté et al., 2008; Kirkpatrick et al., 2008;

Lehmann et al., 2011; Prytherch et al., 2011; Kuehn et al., 2015). These tissues are generated using human-derived cells cultured at the ALI, allowing them to mimic the biology of the *in vivo* respiratory tract by modeling barrier function (*e.g.*, trans-epithelial electrical resistance and functionality of tight junctions), mucous production, and cilia function, and can be used to study infection and acute or long-term toxicity (Mathis et al., 2013; Neilson et al., 2015; Essaidi-Laziosi et al., 2017). There are a number of three-dimensional organotypic respiratory tract models available that can be used to study inhalation toxicity (*e.g.*, MucilAir™ or SmallAir™ [Epithelix Sàrl]; EpiAirway™ or EpiAlveolar™ [MatTek Corporation]; and Micro-Lung™ and Metabo-Lung™ [Cardiff University]), and each of these models have different properties which make them best suited for specific studies (Table 6). For example, these models all contain primary human cells, but vary in the specific cell types included (*e.g.*, nasal, tracheal/bronchial epithelial cells, fibroblasts, goblet cells, or alveolar cells from healthy or diseased donors). The field of three-dimensional tissue model development is rapidly evolving with new and improved systems continuing to enter the market. In the webinar series, two examples (the Micro-Lung™ and Metabo-Lung™, Cardiff University) that are not yet commercially available, were used to illustrate the state-of-the-science. They are described in brief in the following section.

5.1.1. Three-dimensional in vitro lung models—Examples of three-dimensional *in vitro* lung models include the Micro-Lung™ and Metabo- Lung™, both developed at Cardiff University. The Micro-Lung model uses normal human bronchial epithelial (NHBE) cells, isolated from surgical patients and post-mortem donors (Prytherch et al., 2011; BéruBé, 2013; Prytherch and BéruBé, 2014b; Prytherch and BéruBé, 2015). After basal epithelial cells are removed from isolated NHBE cells, they differentiate into a mucociliary phenotype when cultured at the ALI. After exposure, the cells can be used to assess a wide range of histopathology, toxicogenomics, and proteomics endpoints. The apical wash (*i.e.*, fluid collected from apical surface wash after dosing) can also be analyzed for proteins or inflammatory mediators, and the basal media can be studied for metabolic processes using high performance liquid chromatography (HPLC).

Such models can reproduce features of intact physiological anatomy, such as the formation of tight and adherens junctions, desmosomes, cilia, and microvilli (Prytherch, 2010). Prytherch (2010) has demonstrated the appropriate morphological organization of these features using light and electron microscopies. The mature tissue expresses cytokines, secretes mucin (*i.e.*, mucous glycoproteins), and contains non-cancerous or transfected genes. Transepithelial electrical resistance can be measured to quantify the integrity of the epithelial barrier, with high resistance indicative of a healthy barrier function and *vice versa*. Transepithelial electrical resistance readings can be supported by conventional toxicological assays to assess changes in histology, cell secretions, viability, and cell death.

Prytherch (2010) investigated the irritation potential of a range of compounds using the methods described above with 77% concordance to *in vivo* irritancy (Prytherch, 2010). The NHBE model was co-cultured with human primary hepatocytes to create a metabolizing bronchial model (Metabo-Lung). Cells in the respiratory tract can biotransform compounds, either reducing their toxicity or contributing to the *in situ* activation of inhaled toxins and leading to adverse reactions. Cells grown in isolation do not permit *in situ* metabolism to

take place, but primary human bronchial cells that are co-cultured at the ALI with primary human hepatocytes achieve metabolic activity comparable to that found *in vivo* (BéruBé, 2011a). NHBE cells co-cultured with donor-matched hepatocytes have been used to detect acetaminophen toxicity after 24-h exposure to phenacetin and dextromethorphan (BéruBé, 2011b; Prytherch and BéruBé, 2014b).

5.2. Lung-on-a-chip

Lung-on-a-chip systems provide an *in vitro* platform to model and predict physiological responses of human lung tissue to environmental materials. Typically, these *in vitro* models are created in microfabricated polymeric devices consisting of multiple layers of cell culture chambers to mimic three-dimensional micro-architecture of the tracheobronchial airways and alveoli (Huh et al., 2010; Huh et al., 2012; Esch et al., 2015). Lung-on-a-chip devices offer unique capabilities to recapitulate the dynamic microenvironment of native lung tissue produced by cyclic breathing and hemodynamic flow that play a crucial role in organ structure and function. Moreover, the ability of these systems to co-culture multiple cell types makes it possible to mimic complex tissue-tissue interactions and resultant integrated physiological responses at the organ level.

A breathing lung-on-a-chip model has been designed to mimic the alveolar-capillary unit of the human lung (Huh et al., 2010). This microdevice consists of two parallel cell culture chambers separated by a thin, flexible, porous membrane (Fig. 5). These elements are all fabricated from an optically transparent and gas permeable elastomer. The device architecture allows for co-culture of human alveolar epithelial cells and pulmonary microvascular endothelial cells on the opposite sides of the membrane to recreate the structural organization of the air-blood interface. By using computer-controlled cyclic vacuum suction applied to the hollow chambers adjacent to the cell culture channels, this system can also mimic physiological breathing motions and con-comitant tissue deformation. Importantly, this lung-on-a-chip system has been used to successfully model lung infection and nanoparticle transport across the *in vitro* alveolar-capillary barrier (Huh et al., 2010).

5.3. Ex vivo lung slices

Ex vivo precision-cut lung slices can be produced from a variety of species, including humans, and offer the advantage of maintaining the spatial orientation and structural microenvironment of all the cell types present in the lung, including those associated with the immune response (*i.e.*, dendritic cells, macrophages, and mast cells) and intercellular communication (Fisher et al., 1994). Additionally, precision-cut lung slices may be maintained at the ALI for weeks or more and can be created from healthy or diseased human donor lungs. However, because there is no standardized production method, variations in thickness of the tissue slice are inevitable, which impacts comparative functionality. Studies using precision-cut lung slices have shown that high doses of cigarette smoke result in a substantial loss of alveolar epithelium (Lin et al., 2012).

5.4. In vitro testing conclusion

There are many *in vitro* tools that can be used to evaluate acute inhalation toxicity, and each tool should be evaluated for its potential use as a component of an integrated approach. Table 6 describes a number of currently available models along with their advantages and limitations.

Important considerations for selecting an *in vitro* model include cost, complexity, reproducibility, accessibility, endpoints modeled, and the amenability to high-throughput testing; the most appropriate *in vitro* system will depend on the specific data needs of the study and ultimately the accurate prediction of acute human toxicity.

While numerous systems that model specific regions of the respiratory tract exist, none is currently accepted by regulatory agencies as a standalone replacement for the animal test. Because substances may cause toxicity through differing mechanisms, a single *in vitro* approach is unlikely to provide sufficient coverage of the chemical universe. Regulatory use also requires consideration of the varying classification systems used in different jurisdictions, complicating the development of sufficiently predictive decision criteria.

Alternative methods have historically been evaluated based on direct comparison to the *in vivo* test method, despite the recognized inherent variability of *in vivo* methods and the interspecies uncertainty of predicting human responses based on an animal result. To maximize the utility of *in vitro* methods for regulatory decision-making, it will be critical to consider potential human exposure during the interpretation of *in vitro* results and how they relate to potential human effects. Application of IVIVE will be useful in this context.

Additional case study examples, such as those presented below, will be important in showing the regulatory utility of *in vitro* approaches. Regardless of the *in vitro* system used, an understanding of *in vitro* and *in vivo* dosimetry is critical. In addition, *in vitro* results may be improved using computer models that take into account bioavailability and metabolic clearance, although these models need to be further developed to reliably predict toxicity from inhalation exposure.

6. Non-testing approaches

Non-testing approaches use existing information to make predictions about the likelihood of a substance to cause toxicity without running tests to generate new data. Non-testing approaches for substances may include (Q)SARs, expert systems, grouping and read-across, and for mixtures, bridging principles and the theory of additivity (United Nations, 2015). These approaches, as shown in the following examples, can substantially reduce the number of animals used for acute inhalation toxicity assessment.

6.1. General opportunities for waivers

Non-testing approaches can be used to waive acute inhalation toxicity testing requirements (OECD, 2016a) based on:

- Little or no risk of human inhalation exposure

- • The theory of additivity (the GHS mixtures equation)

- • Read-across from a substantially similar, well-characterized mixture or formulated product

- • Read-across using data from another route of entry, in a weight-of-evidence approach (*i.e.*, using acute oral toxicity data to predict acute inhalation toxicity).

- • Severe local irritation and corrosivity

- • Low volatility

- • Inability to safely generate a toxic concentration (*e.g.*, for a gas or vapor that is explosive or an asphyxiant at the concentrations needed for testing)

- • Non-inhalable aerosol particle size (if > 99% of particles are > 100 μm aerodynamic diameter and are resistant to mechanical size reduction by attrition)

The above criteria are included in OECD (2016a) and EPA OPP (US EPA, 2012b) guidance documents on waiving or bridging existing acute toxicity data from one chemical or product formulation to a similar substance instead of conducting additional tests. The European Union also provides for waiving acute inhalation tests based on a lack of exposure (Annex VIII, 8.5.2) and general adaptations noted under Annex XI of REACH; for example, use of existing data; weight-of-evidence; grouping and read-across; QSARs; *in vitro* approaches; or if testing is not technically possible (*e.g.*, the vapor pressure is too low resulting in the inability to generate an atmosphere suitable for testing). For certain industry sectors (*e.g.*, agrochemicals) in the European Union, acute inhalation testing is not a registration requirement unless specific conditions are met. For example, requirements for inhalation testing for agrochemical end-use products are triggered only if the physical state and properties of the product make it likely for the substance to be inhaled (European Commission, 2013).

6.2. Read-across using data from oral route of exposure

Data from one route of exposure can, in certain circumstances, be used to predict the toxicity of the same substance exposed by a different route. Use of acute oral toxicity data, while not definitively establishing toxicity specific to the airway epithelium, may provide a conservative means to categorize acute inhalation toxicity when used in a weight-of-evidence approach, particularly for the most toxic hazard categories. A review of *in vivo* oral *versus* inhalation data suggests that, for 30 agrochemical formulations, those chemicals classified as GHS Category I by the oral route are expected to be Category I *via* the inhalation route and those classified as Category II by the oral route are expected to be classified as Category I or II *via* the inhalation route (Wilson et al., unpublished data). Therefore, when evaluated using a weight-of-evidence approach with physicochemical properties and other information, agrochemicals that are GHS Category I or II by the oral route may be classified as GHS Category I for the inhalation route. Another study demonstrated that orally non-toxic substances are unlikely to be toxic *via* the inhalation route (Corvaro et al., 2016). More specifically, 96% (95 out of 98) of products with an oral LD_{50} > 2000 mg/kg (*i.e.*, GHS not classified) were Not Classified for acute inhalation (LC_{50} equal to or higher than 5.0 mg/L air). In the remaining case of formulations with an oral

$LD_{50} > 2000$ mg/kg, 18 out of 25 had LC_{50} equal or higher than 5.0 mg/L air. Further comparison of curated data from archived acute oral and inhalation toxicity studies across different product types is needed to show how or when oral data may be used in a regulatory context to waive inhalation testing.

6.3. (Q)SAR models for inhalation toxicity

QSAR models can be used to predict a biological effect based on the structure of a chemical. Only a handful of QSAR regression models have been developed for predicting inhalation toxicity. These models are typically only applicable to semi-volatile substances, with parameters such as vapor pressure and boiling point used as descriptors to predict the LC_{50} value. These QSAR models for inhalation toxicity assume that toxicity occurs by the non-specific mechanism of narcosis and that the LC_{50} data used as inputs for the models are from tests in which a steady-state concentration has been reached in the blood. An example of one of these QSAR models is the baseline model derived by Veith et al., which relates vapor pressure to the 4-h molar $LogLC_{50}$ using data from inhalation studies conducted in rats and mice (Veith et al., 2009).

Veith and Wallace (2006a, 2006b) determined that vapor pressure is not a good predictor of LC_{50} for chemicals that are reactive as electrophiles, in that the inhalation toxicity of such chemicals is underestimated by the baseline model. They were able to establish a QSAR for electrophilic chemicals (such as acrylates) where reactivity was quantified by the RC_{50} value, the concentration of test compound that produced a 50% reaction of glutathione thiol groups in 120 min in a glutathione depletion assay (Schultz et al., 2005; Veith and Wallace, 2006a, 2006b).

In silico models, such as the OECD QSAR Toolbox, contain profilers, or rules based on structural alerts, that can be useful for creating chemical categories for acute inhalation toxicity. For example, profilers based on the mechanism for acute aquatic toxicity can provide evidence for potential inhalation toxicity by identifying substances acting as non-specific narcotics, electrophilic substances, or substances acting through a specific mechanism. The OECD QSAR Toolbox also contains experimental data on acute inhalation toxicity from ECHA Chem, the Rodent Inhalation Toxicity database, and the Toxicity Japan MHLW.

TOPKAT (or Toxicity Prediction from Komputer Assisted Technology) contains a model based on rat LC_{50} data with five submodels related to different chemical classes, including (1) single benzenes, (2) heteroaromatics and multiple benzenes, (3) alicyclics, and (4) acyclics with or without halogens (Accelrys Inc., 2004). This model is underpinned by *in vivo* data from rat studies with exposure times in the range of 0.5–14 h. To normalize the data to adjust for different durations of exposure, it was assumed that toxicity was proportional to duration, per Haber's Law ($C^n \times t = k$, where $n = 1$). This normalization ignores the possibility that the slope at the observed time may not be the unit slope (*i.e.*, n does not equal 1), but the approach was a pragmatic one to ensure the broadest coverage. Data presented during the workshop indicated poor sensitivity of the TOPKAT model for GHS class 1–2 compounds when it was challenged using broad chemical categories, suggesting a need to optimize the model and/or better define its applicability domain.

Regardless of the model, there is a need for a better understanding of the mechanisms of acute inhalation toxicity in order to further develop and optimize *in silico* approaches to predict acute inhalation toxicity potential for a broad spectrum of chemicals.

7. Case studies

This section presents three examples of alternative approaches for predicting toxicity following inhalation exposure: (1) a mathematical approach to justify study waivers for agrochemical formulations; (2) a combined approach using computational dosimetry modeling in conjunction with an *in vitro* airway epithelium to evaluate an agrochemical formulation; and (3) an integrated *in vitro* approach to testing next generation tobacco products. These examples illustrate the utility of *in silico* and *in vitro* models to predict human toxicity and the potential of these models to reduce and replace animal use.

7.1. Using the GHS additivity approach for classification of agrochemical mixtures

An additivity approach to estimating the toxicity of a chemical mixture assumes that at high single doses, as is the case in an acute systemic toxicity test, toxicity of the components of the mixture is additive. This approach applies a mathematical model to predict the toxicity of a mixture based on the relative contribution (concentration and potency) of each component (Finney, 1952; Pozzani et al., 1959; Smyth et al., 1969). Accordingly, the potential toxicity of a formulation could be estimated and used to apply for study waivers for the formulation without any additional testing.

The GHS additivity formula is essentially a harmonic mean calculation:

$$\frac{100}{\text{ATE}_{mix}} = \sum_n \frac{C_i}{\text{ATE}_i}$$

where C_i = concentration of ingredient i (%w/w or v/v); i = the individual ingredient from 1 to n; n = the number of ingredients; and ATE_i = acute toxicity estimate of ingredient i.

The acute toxicity estimate for classification of a substance in an inhaled mixture is derived using an LC_{50} where available; however, a classification category or LC_{50} range can also be used (United Nations, 2015). From a regulatory use perspective, the theory of additivity approach can be used as a stand-alone replacement for animal tests according to GHS (United Nations, 2015), in the EU Classification, Labeling, and Packaging (CLP) Regulation (European Union, 2008), the New Zealand and Australian regulations on agrochemical formulations (New Zealand Government, 2012; APVMA, 2015), and in global transport regulations (United Nations, 2011).

In this case study of a non-testing approach, 225 agrochemical formulations were retrospectively reviewed and the resulting GHS additivity calculations compared against existing *in vivo* data (Corvaro et al., 2016). This dataset included 123 acute inhalation studies (122 liquid/dust aerosol and one vapor) with the vast majority of the substances tested having low toxicity (*e.g.*, > 90% were GHS Not Classified based on animal tests). The predicted classifications using the GHS additivity formula were 94.3% accurate for

GHS/CLP (3.3% underestimated; 2.4% overestimated) and 96.7% accurate for EPA and the Brazilian National Health Surveillance Agency classifications (1.6% over and underestimated). While this data set included a broad range of different pesticide formulations including herbicides, insecticides, and fungicides, the low number of toxic substances included necessitates the retrospective analysis of additional products using this approach.

Considering this information, the authors proposed an approach to significantly reduce the testing conducted for agrochemical formulations (Corvaro et al., 2016). The approach first considers the need for inhalation data and potential to waive data requirements (OECD, 2016a). If a regulatory agency determines that quantitative hazard characterization is needed, the GHS additivity calculation can be performed to predict acute inhalation (and oral) toxicity. Concentrations of 1.0, 2.0 or 5.0 mg/L would result in human exposures of 5000, 10,000 and 25,000 mg, respectively. According to the classification proposed by Hoffmann and colleagues, 1000, 2000 and 5000 mg/m^3 corresponds to the concentration of PM_{10} dust particle that would be expected, respectively, in a normal, strong, or severe dust storm (ISO, 1995; Hoffmann et al., 2008). As such, the authors propose that when testing is justified, a concentration of 1.0 mg/L air represents a worst-case scenario for agrochemical formulations, since those are tested in concentrated forms and diluted remarkably with water before the actual use in the field. Therefore, if the LC_{50} is > 1.0 mg/L air, the results may be considered as negative. If the LC_{50} is < 1.0 mg/L (which is expected in only 2.4% of cases), the weight-of-evidence and physicochemical characteristics should be evaluated to determine whether there is sufficient information to require additional personal protective equipment as opposed to requiring additional testing. Currently, this proposed approach can be used internally within a company (*e.g.*, for formulation design), to meet certain regulatory requirements (*e.g.*, for any EU-only products), and as a tool to predict a starting dose level when animal testing is required by a government agency.

7.2. Evaluating the human health risk following exposure to an irritant aerosol

By default, inhalation dosimetry typically assumes that all of the material in the air is available for inhalation exposure. For non-volatiles, however, material is suspended as an aerosol or particulate, and thus the dosimetry approach used to estimate internal exposure will be different from that used for a volatile gas. The inhalation risk of a non-volatile substance can be assessed using a source-to-outcome approach including the evaluation of the exposure and internal particle size distribution, dosimetry considerations, and *in vitro* test data.

Exposure-based risk assessment takes into account the expected human exposure level to a substance. Agrochemical exposure in agriculture workers can be monitored using wearable OSHA Versatile Sampler (OVS) tubes. OVS tube data is typically reported only as the total concentration without consideration of the particle size; however, studies have been conducted to compare the concentration determined with use of an OVS tube and standard methods to derive particle size distributions (using internationally accepted sampling conventions for the inhalable, thoracic, and respirable aerosol fractions) (Hewitt, 1995; Brown et al., 2013). Particle size varies with spray nozzle type, with fine sprays being more

inhalable than coarse particles. Regardless of the nozzle type, the fraction of particles available for systemic exposure (as defined by thoracic or respirable fractions) is approximately 40% of the total air concentration levels. Thus, the OVS data can be considered as the external exposure, which can be used to calculate the particle amount deposited at the target site.

The particle size distribution in a typical rodent inhalation toxicity study does not accurately represent human exposure to aerosols (Table 7). Human *versus* rat deposition in the respiratory tract can be estimated using the MPPD model (Anjilvel and Asgharian, 1995; Asgharian and Anjilvel, 1998; Asgharian et al., 2001). The MPPD model calculates the deposited dose of monodisperse and polydisperse aerosols in the respiratory tracts of rats and humans. Within each airway, deposition is calculated using theoretically derived efficiencies for the mechanisms of diffusion, sedimentation, and impaction within the airways or airway bifurcation (Table 8). Inhalability and filtration of aerosols by the nose and mouth is also determined using empirical efficiency functions. Clearance can also be calculated in the MPPD model but is not used in this assessment as data are not available for validation. The lack of clearance is also health protective as it then over-estimates total exposure. For chemicals with known modes of action, dosimetry may be needed at more exact locations in the respiratory tract. CFD models can be used to simulate the deposition of a large number of individual particles at very fine resolutions in the respiratory tract (Corley et al., 2015). Additionally, pharmacokinetic models can be coupled to dosimetry models if enough data are available for quantification within tissues.

The exposure and deposition calculations provide a concentration for comparison with toxicology data. *In vitro* testing can provide such endpoints for inhalation toxicity. For example, commercially-available three-dimensional *in vitro* reconstructed human tissue models of the upper or lower respiratory tract can be used to assess point-of-contact toxicity following inhalation exposure. Multiple markers of membrane/cell damage and functional competence, such as trans-epithelial electrical resistance and lactate dehydrogenase leakage, can be evaluated. An *in vitro* POE can be calculated from the *in vitro* tissue models by using MPPD or CFD to calculate either (1) the equivalent inhalation exposure conditions necessary to give the same surface concentrations in rats or (2) the equivalent inhalation exposure conditions for predicted human exposures.

7.3. In vitro assessment of tobacco and nicotine products

DNA damage and oxidative stress have emerged as important endpoints to consider when assessing the potential toxicity of next-generation nicotine products. Given this emphasis, an opportunity exists to apply a new approach to testing based on mechanistic, human-relevant *in vitro* assays that could provide necessary regulatory information without using animals. Haswell et al. used NHBE cells cultured at the ALI to assess the impact of tobacco smoke on cell function as a model of goblet cell hyperplasia (Haswell et al., 2010). Repeated exposures over 28 days to cigarette smoke total particulate matter increased the number of mucous-secreting cells as determined by examination of cell morphology by transmission electron microscopy and decreased transepithelial electrical resistance (Haswell et al., 2010). In another study, NCI-H292 human pulmonary epithelial cells were exposed to total

cigarette smoke aerosol for 30 min at the ALI. An assessment of oxidative DNA damage using the comet assay showed that cigarette smoke increased DNA damage in a concentration-dependent manner (Thorne et al., 2016). In addition, ALI systems have been used to assess the cytotoxicity of e-cigarettes aerosols compared to reference cigarettes. These systems were used to demonstrate that e-cigarette aerosols were less cytotoxic than cigarette smoke in NCI-H292 cells using a neutral red uptake assay (Azzopardi et al., 2016). *E*-cigarette aerosols were demonstrated to be less mutagenic than the reference cigarette (Thorne et al., 2016) and caused less DNA damage (Thorne et al., 2017). Three-dimensional reconstructed human airway tissues have also been used to study cytotoxicity *in vitro* following exposure to e-cigarette aerosol (Neilson et al., 2015; Banerjee et al., 2017). Cytotoxicity assay results showed that while cigarette smoke reduced cell viability in a time-dependent manner, e-cigarette smoke did not (Neilson et al., 2015; Banerjee et al., 2017; Haswell et al., 2017). In addition, studies have demonstrated the metabolic competency of the tissues, in particular, MucilAir™ has been shown to express CYP1A1/1B1 and activity of CYP2A6/2A13 for several months in culture (Baxter et al., 2015). This case study demonstrates the utility of human cells or reconstructed tissue models grown at the ALI to assess various toxicity endpoints, and their use in an integrated approach could prove useful for replacing animal tests.

8. Workshop recommendations

The need for more high-throughput and human-relevant methods that don't use animals has led to the development of multiple *in vitro* and *in silico* approaches to assess acute inhalation toxicity. When designing *in vitro* or *in silico* methods to assess acute inhalation toxicity, researchers must consider several factors, including (1) which region of the respiratory tract the test substance will reach and at what dose; (2) whether acute toxicity may include POE or systemic effects involving other organs or organ systems; (3) that mechanisms of exposure generation, respiratory tract delivery, and location of toxicity will vary with the physicochemical properties of the test materials; and (4) that toxicokinetics of inhaled *versus* orally administered test materials may differ because of POE effects and for systemic effects because there is no first-pass hepatic metabolism associated with test materials absorbed by the upper and lower respiratory tract.

There is growing acceptance of the use of predictive *in silico* and *in vitro* models for hazard assessment. In order to be accepted, the models must be robust, reproducible, accurate, and transparent in how the data are generated. Currently, the greatest impact on reducing animal use can be achieved by using the *in silico* and *in vitro* models early in the development cycle to flag materials of high hazard, or to identify existing data that are informative of the toxicity of the substance of interest. Although many regulatory agencies still require *in vivo* testing in a number of contexts, animal-free testing approaches can be incorporated into current testing strategies as a means to validate and optimize the non-animal tests. Additionally, data from non-animal tests can be incorporated into read-across models, further helping to build confidence in the predictive power of the non-animal models and allowing regulatory authorities to see how the methods can be used. Simplification and standardization of non-animal models will facilitate regulatory acceptance. Ultimately, there is a need for government, industry, academics, and non-governmental organizations to work

together to advance the implementation and global regulatory acceptance of non-animal approaches.

The purposes of the September 2016 workshop were to bring experts together to discuss experiences using alternative approaches for acute inhalation toxicity testing and how to build upon those approaches to develop strategies that regulatory agencies will accept. Because acute inhalation toxicity data are used for a variety of regulatory and non-regulatory purposes, participants agreed that a key first step in designing any testing strategy is defining the question to be addressed and establishing the specific informational needs for decision-making. Participants also agreed that alternative approaches could be developed to identify POE risks to prioritize compounds that require further assessment or to justify waiving the inhalation toxicity testing requirement altogether.

Ultimately, the workshop discussions were refined into four recommendations, which are listed below. Working groups were established and tasked with follow-up actions to implement each of these recommendations.

1. **Develop a database of existing acute systemic toxicity data**. Central to implementing alternative approaches for acute inhalation toxicity testing will be consolidating existing databases and obtaining additional data that have not yet been shared publicly. This working group will catalog the many existing acute toxicity databases and determine the most efficient and user-friendly way to consolidate information relevant to acute inhalation toxicity assessment.

2. **Prepare a state-of-the-science review on mechanisms and available *in vitro/ in silico* models for acute inhalation toxicity**. This working group will catalog the available alternative approaches—*in vitro*, *in chemico*, and *in silico*—and define their usefulness and limitations, including the specific applicability domain for each approach. Additionally, the group will detail the numerous mechanisms of action associated with acute inhalation toxicity, with careful consideration of the physicochemical properties of the inhaled agents and the impact of dosimetry, including metabolism and the potential for POE effects. Central to these efforts will be defining relevant adverse outcome pathways that can be used to inform the appropriate integrated testing approach. The resulting review article (in preparation) will discuss mechanisms of acute systemic toxicity in general, as well as focus on specific POE considerations and the influence of physicochemical properties.

3. **Identify and optimize *in silico* approaches**. This working group will identify *in silico* models that can be used to predict toxicity that may result from general acute toxicity mechanisms (*e.g.*, reactivity) or mechanisms specific to the inhalation route and to predict whether exposure *via* the inhalation route is feasible based on a substance's physicochemical properties. This work will directly inform the work of Working Groups 2 and 4.

4. **Develop a decision tree/testing strategy and conduct an *in silico* and *in vitro* proof-of-concept study**. This working group will design a testing approach to show the utility of *in vitro* and *in silico* methods in assessing acute inhalation

toxicity. Building off the research of the other three working groups, this group will (1) develop a list of reference chemicals based on curated *in vivo* data (rat, human, and/or other species) that can be used to interrogate available alternative approaches; (2) develop a decision tree that will inform the need to conduct testing following *in silico* modeling; (3) select the relevant *in vitro* assays to include in an integrated approach based on mechanisms of acute inhalation toxicity in humans; and (4) optimize the *in vitro* and *in silico* assays and develop standardized protocols that can be used across laboratories. The resulting tiered testing strategy will use both *in silico* and *in vitro* models.

Coordination of the working groups and progress on established milestones and timelines is being coordinated by the workshop co-sponsors.

Acknowledgements

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Fig. 1.

Agencies that require or use acute inhalation data. Abbreviations:

ACICM = Chinese Association of International Chemical Manufacturers;

ANVISA = Brazilian Agência Nacional de Vigilância Sanitária (The National Health Surveillance Agency);

APVMA = Australian Pesticides and Veterinary Medicines Authority;

CIBRC = Indian Central Insecticide Board & Registration Committee,

DOD = Department of Defense;

ECHA = European Chemicals Agency;

EFSA = European Food Safety Authority;

EPA OPP = United States Environmental Protection Agency Office of Pesticide Programs;

EPA OPPT = United States Environmental Protection Agency Office of Pollution Prevention and Toxics;

FDA CBER = United States Food and Drug Administration Center for Biologics Evaluation and Research;

FDA CDER = United States Food and Drug Administration Center for Drug Evaluation and Research;

FDA CTP = United States Food and Drug Administration Center for Tobacco Products;

FDA CVM = United States Food and Drug Administration Center for Veterinary Medicine;

HECSB = Canadian Healthy Environments and Consumer Safety Branch;

ICAMA = Chinese Institute for the Control of Agrochemicals, Ministry of Agriculture;

MAFF = Japanese Ministry of Agriculture, Forestry and Fisheries;

MAFRA = South Korean Ministry of Agriculture, Food and Rural Affairs;

MEP = Chinese Ministry of Environmental Protection;

MHRA = United Kingdom Medicines & Healthcare Products Regulatory Agency;

MOE = South Korean Ministry of Environment;

OSHA – United States Occupational Safety and Health Administration;

PMRA = Canadian Pest Management Regulatory Agency;

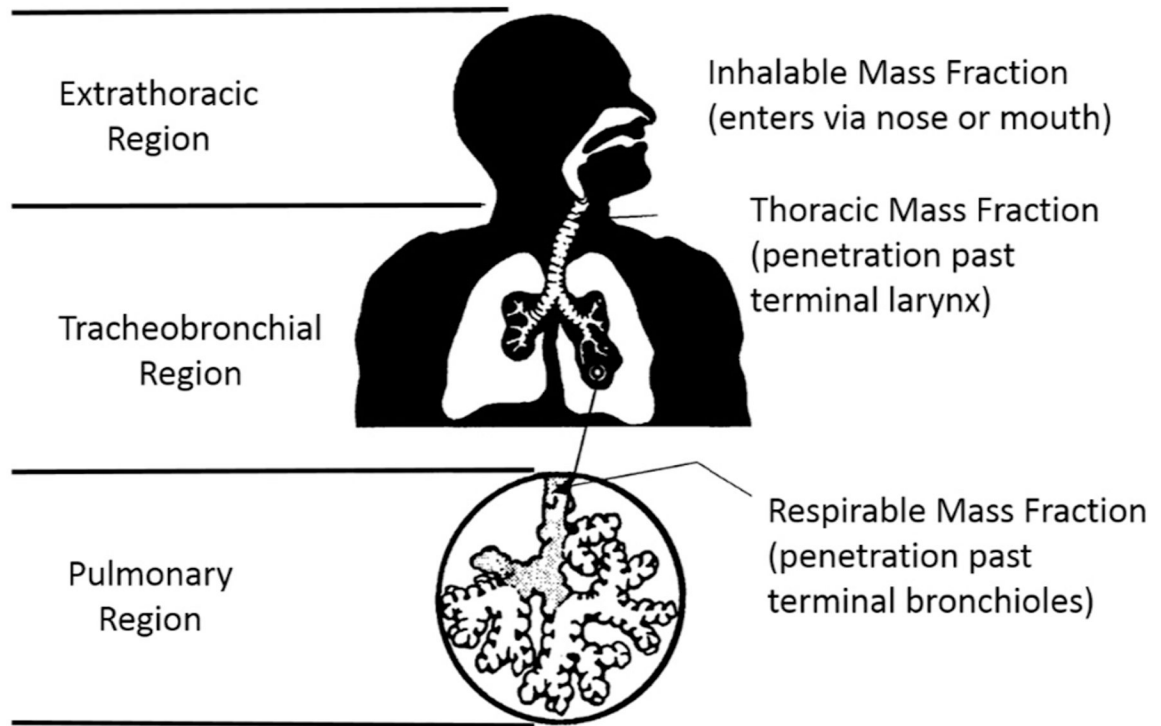SAWS = Chinese State Administration of Work Safety.

Extrathoracic
Region

Inhalable Mass Fraction
(enters via nose or mouth)

Thoracic Mass Fraction
(penetration past
terminal larynx)

Tracheobronchial
Region

Respirable Mass Fraction
(penetration past
terminal bronchioles)

Pulmonary
Region

Fig. 2.
Three respiratory tract regions, with definitions of mass fractions for particle inhalation and exposure sampling (US EPA, 1994).
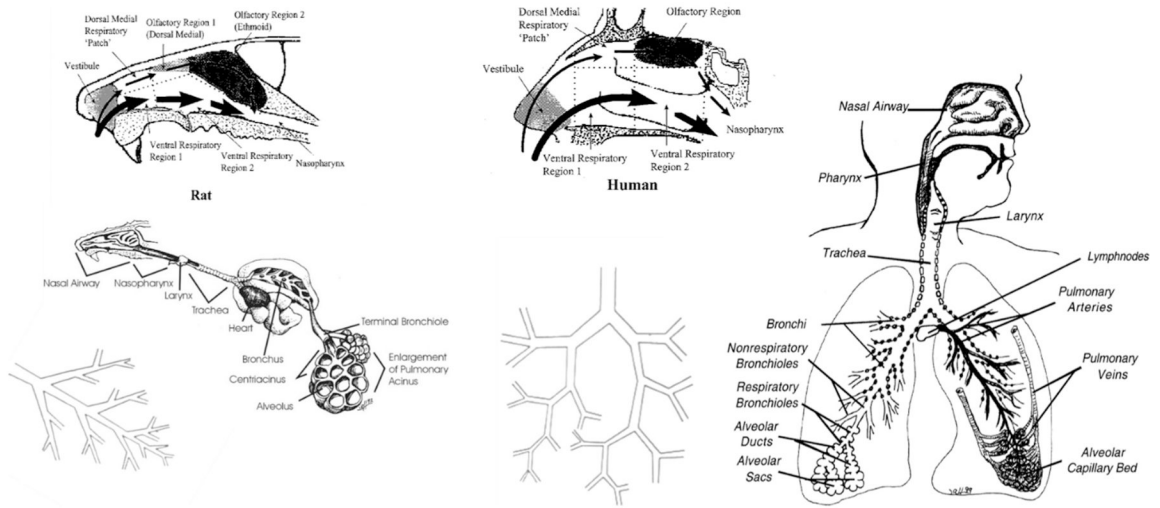
Fig. 3.
Comparative respiratory tract anatomy and airflow in the upper respiratory tract. Illustrations courtesy of Dr. Jack R. Harkema, Professor of Comparative Pathology, Michigan State University.
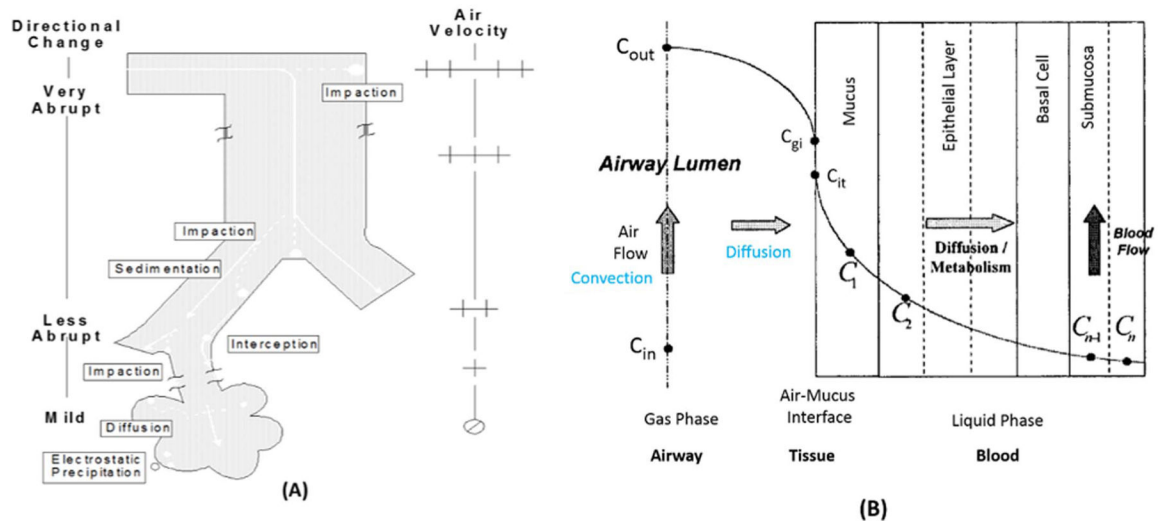
Fig. 4.

Selected anatomical or physiological parameters and mechanisms of inhaled particle deposition **(a)** and gas uptake **(b)** in the respiratory tract. Particle clearance is not illustrated. Directional change and air velocity refer to airflow direction and rate, respectively, as it travels through the respiratory tract. As shown in (a), airflow in the extrathoracic region is distinguished by abrupt directional changes and high velocity; deposition here is principally through impaction. As the airways bifurcate, airway volume increases with increasing cross-sectional area thereby decreasing air velocity and allowing more gradual directional airflow changes such that sedimentation can also occur. (b) provides a schematic of the uptake and metabolism of an inhaled chemical from the air phase into the tissue sub-compartments. C_{in} is the concentration of inspired gas entering an airway region and C_{out} is the concentration exiting the same airway by convection if no reactions occur. C_{gi} is the gas concentration at the interface of the airway and epithelial lining fluid or mucus layer, and due to molecular diffusivity C_{it} is the concentration at the epithelium interface. C_1 and C_2 illustrate concentrations in mucus and epithelial layers, and C_{n-1} and C_n are concentrations in the submucosa and blood due to perfusion. Adapted from (US EPA, 1994; Bogdanffy et al., 1999).
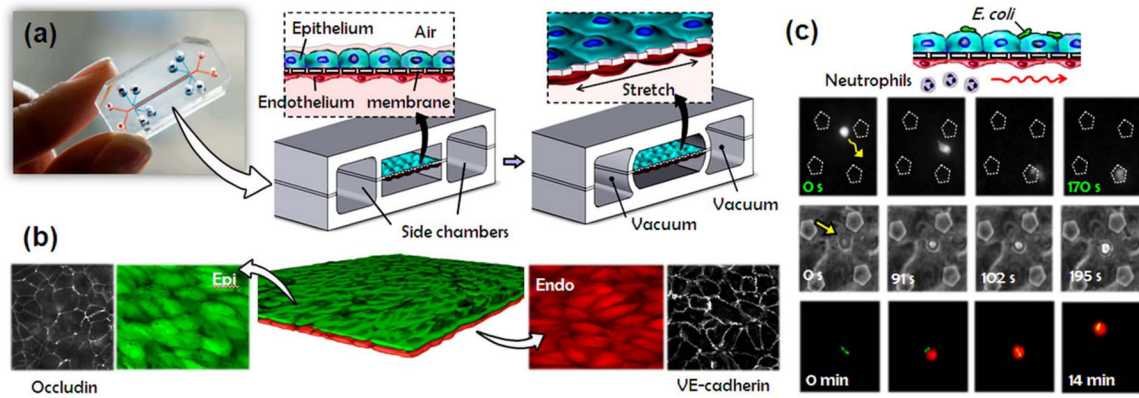
Fig. 5.
A human breathing lung-on-a-chip. (a) The microfabricated lung mimic device recreates physiological breathing movements by applying vacuum to the side chambers and causing mechanical stretching of the PDMS membrane forming the alveolar-capillary barrier. (b) Long-term microfluidic co-culture produces a tissue-tissue interface consisting of a single layer of the alveolar epithelium (Epi; green) closely apposed to a monolayer of the microvascular endothelium (Endo; red), both of which express intercellular junctional structures such as occludin or VE-cadherin. (c) Neutrophils flowing in the lower vascular channel adhere to the endothelium activated by *E. coli* in the alveolar chamber, transmigrate (top row), emigrate into the alveolar space (middle row), and engulf the bacteria (bottom row).

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Table 1

List of webinar and workshop presentations. Presentation slides and webinar recordings can be found online at www.piscltd.org.uk/acute-inhalation-toxicity.

Speaker	Affiliation	Title
Webinars		
Ian Indans	Health and Safety Executive, UK	Acute Inhalation Toxicity Testing: The 3Rs, Current Needs and Future Prospects.
Jon Hotchkiss	The Dow Chemical Company	A Primer on Acute Inhalation Toxicity Testing: Where do Alternative Methods Fit?
Marianna Gaca	British American Tobacco	Acute Inhalation Toxicity: *In Vitro* and *Ex Vivo* Systems.
Annie Jarabek	U.S. EPA, National Center for Environmental Assessment, ORD	What a Difference the Dose Makes: Dosimetry Approaches to Aid Experimental Design, Evidence Integration, and Inferences for Risk Assessment.
Grace Patlewicz	U.S. EPA, National Center for Computational Toxicology, ORD	State-of-the-Science and Practical Application of *In Silico* Methods.
Daniel Wilson	The Dow Chemical Company	*In Silico* Approaches for Acute Inhalation Toxicity.
Marco Corvaro	Dow AgroSciences	GHS Additivity Approach to Classify Mixtures based on Ingredient Toxicity. *A* Case Example: Agrochemical Formulations.
Mathieu Vinken	Free University of Brussels	Adverse Outcome Pathways as Tools to Assess Chemical-Induced Toxicity.
Barbara Buckley	U.S. EPA, National Center for Environmental Assessment, ORD	A Conceptual Model for Assessing Criteria Air Pollutants in a Multipollutant Context: A Modified Adverse Outcome Pathway Approach.
Kelly BéruBé	School of Biosciences, Cardiff University	The NHBE and Metabo-Lung™ Models: Normal and Metabolizing *In Vitro* Alternatives for Lung Research.
Dongeun Huh	University of Pennsylvania	Microengineered Physiological Bio-mimicry: Human Organs-on-Chips.
Workshop presentations		
Jon Hotchkiss	The Dow Chemical Company	The Case for an Integrated Approach to Acute Inhalation Toxicity Testing and Assessment
Dan Wilson	The Dow Chemical Company	An Alternative Framework for Acute toxicity using Mechanistic *In Silico* and *In Vitro* Approaches
Paul Hinderliter	Syngenta	An Alternative Approach for Evaluating the Human Health Risk from Exposure to an Irritant Aerosol
Michael Bartels	ToxMetrics.com, LLC	Toxicokinetics in Risk Assessment: Evaluation of *In Silico* Approaches
Miyoung Yoon	ScitoVation, LLC	Assessing Bioavailability and Systemic Delivery of Inhaled Compounds: Current Status and Future Directions
Anna Lowit	U.S. EPA Office of Pesticide Programs	U.S. EPA OPP Regulatory Perspective on Acute Inhalation Toxicity Testing
Iris Camacho	U.S. EPA Office of Pollution Prevention and Toxics	U.S. EPA OPPT Regulatory Perspective on Acute Inhalation Toxicity Testing
Grace Patlewicz	U.S. EPA, National Center for Computational Toxicology, ORD	ICCVAM's Vision and Strategy for Acute Toxicity Testing

Table 2

Comparison of select features of OECD acute inhalation TGs.

	OECD TG 403: acute inhalation toxicity	OECD TG 436: acute inhalation toxicity - acute toxic class method	OECD TG 433: acute inhalation toxicity - fixed concentration procedure
Use	Used for quantitative risk assessments where a concentration-response relationship is required, as well as for classification and labeling purposes	Used mainly for classification and labeling purposes	Used mainly for classification and labeling purposes
Number of animals (generally rats)	*Traditional protocol[1]:* *Limit test (single concentration tested):* 10 animals/limit concentration (5 M/5 F); total 10 animals. *Main Study (at least 3 concentrations tested):* 10 animals/concentration (5 M/5 F); total at least 30 animals. *Concentration × time protocol (C × t):* 8–10 animals/concentration (1 M/1 F at 4–5 exposure durations); 4–5 exposure concentrations; total 40–50 animals	*Main study:* 6 animals (3 animals per sex, 1 or more concentrations)	*Main study:* 5 animals (5 animals, 1 or more concentrations)
Limit test concentration	5000 ppm (gas) 20 mg/L (vapor) 5 mg/L (aerosol)	5000 ppm (gas) 20 mg/L (vapor) 5 mg/L (aerosol)	5000 ppm (gas) 20 mg/L (vapor) 5 mg/L (aerosol)
Exposure duration	4 h (other durations permitted with justification)	4 h (other durations permitted with justification)	4 h (other durations permitted with justification)
Endpoint	Lethality	Lethality	Evident toxicity
Lethality estimate[2]	Point estimate	Range estimate	Range estimate
Relevance to the 3Rs (replace, reduce, and refine animal use)	Careful selection of initial exposure concentration can minimize animal use	Decreased group size and fixed concentration testing result in reduction in animal use compared to OECD TG 403	Reduction and refinement to OECD TG 403. Based on evident toxicity.

[1] OECD TG 403 includes two study types: (1) a traditional protocol and (2) a concentration × time (C × t) protocol. The traditional protocol provides concentration-response data used to derive a LC50 and establish the exposure-response slope. A C × t study can be used as an alternative to the traditional protocol when there is a regulatory requirement to test over multiple time durations (*e.g.*, emergency response planning).

[2] A point estimate is a single lethality estimate (*e.g.*, LC50 = 5 mg/L) calculated from the study results. A range estimate is a range of lethality estimates (*e.g.*, 0.5 mg/L < LC50 ≤ 2 mg/L) determined based on stepwise exposures to fixed concentrations.

Table 3A

Respiratory tract regions. Adapted from (US EPA, 1994).

	Anatomic structure	Other terminology
Region		
Extrathoracic (ET)	Nose	Head airways region
	Mouth	Nasopharynx (NP)
	Nasopharynx	Upper respiratory tract (URT)
	Oropharynx	
	Laryngopharynx	
	Larynx	
Tracheobronchial (TB)	Trachea	Conducting airways
	Bronchi	
	Bronchioles (to terminal bronchioles)	
Pulmonary (PU)	Respiratory bronchioles	Gas exchange region
	Alveolar ducts	Alveolar region
	Alveolar sacs	
	Alveoli	

Table 3B

Key aspects of the structure and function of the respiratory tract of rats and humans. Adapted from (International Commission on Radiological Protection (ICRP), 1994; Miller, 1999).
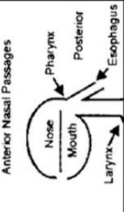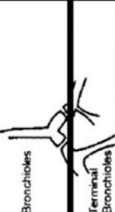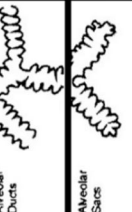
Zones (Air)	Region	Functions	Cytology (Epithelium)	Histology (Walls)	Anatomy	Comments
Conduction	Extrathoracic	Air conditioning; Temperature and Humidity; and Cleaning; Fast Particle Clearance, Air Conduction	Epithelium Types: Squamous, Transitional, Respiratory, Olfactory. Cell Types: Ciliated Cells, Nonciliated Cells: Goblet Cells, Mucous (Secretory) Cells, Serous Cells, Brush Cells, Endocrine Cells, Basal Cells, Intermediate Cells	Mucous Membrane, Respiratory Epithelium (Pseudostratified, Ciliated, Mucous), Glands. Mucous Membrane, Respiratory or Stratified Epithelium, Glands	Anterior Nasal Passages (Nose, Mouth, Pharynx, Larynx, Posterior, Esophagus)	Rats are obligate nasal breathers. Adult humans switch to oronasal breathing when minute ventilation exceeds about 35 L/min (Ninimaa et al, 1981)
	Thoracic / Tracheobronchial			Mucous Membrane, Respiratory Epithelium Cartilage Rings, Glands	Trachea	
				Mucous Membrane, Respiratory Epithelium, Cartilage plates, Smooth Muscle Layer, Glands	Main Bronchi / Bronchi	Rat lung has predominantly a monopodial branching system (Crapo et al, 1990)
Gas Exchange Transitory	Alveolar	Air Conduction; Gas Exchange, Slow Particle Clearance	Respiratory Epithelium With Clara Cells (No Goblet Cells). Cell Types: Ciliated Cells, Nonciliated Cells: Clara (Secretory) Cells	Mucous Membrane, Respiratory Epithelium, No Cartilage, No Glands, Smooth Muscle Layer. Mucous Membrane, Single-Layer Respiratory Epithelium, Less Ciliated, Smooth Muscle Layer	Bronchioles / Terminal Bronchioles	
			Respiratory Epithelium Consisting Mainly of Clara Cells (Secretory) and Few Ciliated Cells	Mucous Membrane, Single-Layer Respiratory Epithelium of Cuboidal Cells, Smooth Muscle Layer	Respiratory Bronchioles	Rats do not have respiratory bronchioles. From the bronchiolar-alveolar duct junction in a rat, alveolar sacs are reached after anywhere from 3 to 13 branchings (Mercer & Crapo, 1987)
		Gas Exchange; Very Slow Particle Clearance	Squamous Alveolar Epithelial Cells (Type I), Covering 94% of Alveolar Surface Areas. Cuboidal Alveolar Epithelial Cells (Type II, Surfactant-Producing), Covering 6% of Alveolar Surface Area. Alveolar Macrophages	Wall Consists of Alveolar Entrance Rings, Squamous Epithelial Layer, Surfactant. Interalveolar Septa Covered by Squamous Epithelium Containing Capillaries, Surfactant	Alveolar Ducts / Alveolar Sacs / Lymphatics	

Table 4

Hierarchy of model structures for dosimetry and extrapolation (US EPA, 1994).

Hierarchy of model structures for dosimetry and extrapolation
"Optimal" or preferred model structure
• Structure describes all significant mechanistic determinants of chemical disposition, toxicant-target interaction, and tissue response
• Uses chemical-and species-specific parameters
• Dose metric(s) are described at level of detail commensurate to toxicity data
Default model structure
• Limited or default description of mechanistic determinants of chemical disposition, toxicant-target interaction, and tissue response
• Uses categorical or default values for chemical and species parameters
• Dose metric(s) are described at generic or categorical level of detail

Table 5

Gas category scheme and characteristics (US EPA, 1994; Hanna et al., 2001; Kuempel et al., 2015).

Gas category	Water solubility	Reactivity and tissue uptake	Examples
1	High[a]	• Rapidly reactive in tissues (including metabolism)[a] • Primarily scrubbed out in extrathoracic region, causing local tissue effects • Not absorbed into systemic circulation	Chlorine, formaldehyde, hydrogen fluoride, vinyl acetate
2	Moderate	• Rapidly reactive or moderately to slowly metabolized in respiratory tissue • Can penetrate beyond the extrathoracic region into the bronchi and pulmonary regions • Some absorption into blood (perfusion limited)	Ozone, sulfur dioxide, xylene, propanol
3	Insoluble	• Nonreactive in the extrathoracic and tracheobronchial tissues • Penetrates to pulmonary region and potential for endothelial delivery • Can be absorbed into systemic circulation and metabolically activated	Chloroform, styrene, trichloroethelene

[a]Gases with either of these characteristics are included in Category 1.

Table 6

Characteristics relevant to regulatory applicability of the *in vitro* models presented in the webinar series. PCLS, precision-cut lung slices.

	MucilAir (Epithelix)	EpiAirway (MatTek)	Micro-Lung	Metabo-Lung	Lung-on-a-chip	PCLS
Uses normal human cells	×	×	×	×	×	×
Can be maintained at the ALI	×	×	×	×	×	×
Mimics aspects of human lung morphology and physiology	×	×	×	×	×	×
Cilia beating	×	×	×	×	×	×
Production of mucous	×	×	×	×	×	×
Potential for metabolic activity	×	×	–	×	×	×
Suitable for long-term culture	×	×	×	×	×	×
Incorporates cyclic breathing and hemodynamic flow	–	–	–	–	×	–
Includes immune cells	×	×	–	–	×	×
Commercially available	×	×	–	–	–	–

Table 7

Exposure distributions for aerosol exposure mass in size-selected sampling of human use case and rat inhalation studies.

Droplet size cutoff (mm)	Fraction of total mass below cutoff (%)
Fine/medium reference nozzle	
2.5	0.77
10	1.03
30	5.5
100	13.8
150	22.4
200	37.3
Rat inhalation study	
0.37	16.5
0.90	22.8
1.6	28.4
3.7	40.4
5.6	70.6
8.0	94.1

Table 8

Fraction of total mass deposited in respiratory tract regions of rats and humans. Human exposures are approximately an order of magnitude lower than rodents; conducting and alveolar ratios are many orders of magnitude different.

Particle type	Exposure	Fraction of total mass			
		Head	**Conducting**	**Alveolar**	**Total**
Polydisperse MMAD(GSD)	Rat – 2.33 (4) μm	0.331	0.01	0.035	0.376
	Human – 100 (1.5) μm	0.035	$2.3 \times 10{-}6$	$3.1 \times 10{-}10$	0.035
	Rat:Human Ratio	9.5			10.7