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Review

Pathway-based predictive approaches for non-animal assessment of acute inhalation toxicity

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ABSTRACT

New approaches are needed to assess the effects of inhaled substances on human health. These approaches will be based on mechanisms of toxicity, an understanding of dosimetry, and the use of *in silico* modeling and *in vitro* test methods. In order to accelerate wider implementation of such approaches, development of adverse outcome

Abbreviations: ADME, absorption distribution metabolism and elimination; AEP, aggregate exposure pathway; AOP, adverse outcome pathway; BAL, bronchoalveolar lavage; BEAS-2B, adenovirus-12 SV40 hybrid transformed, non-tumorigenic human bronchial epithelial cells; CFD model, computational fluid dynamics model; CxT, concentration x time exposure; d_{ae}, aerodynamic diameter; DAF, dosimetric adjustment factor; HBE cells, human bronchial epithelial cells; HEC, human equivalent concentration; IATA, integrated approach to testing and assessment; IVIVE, *in vitro* to *in vivo* extrapolation; KE, key event; LC_{50}, lethal concentration 50%; MMAD, mass median aerodynamic diameter; MIE, molecular initiating event; MPPD model, Multiple-Path Particle Dosimetry model; NICEATM, NTP Interagency Center for the Evaluation of Alternative Toxicological Methods; NSAIDs, nonsteroidal anti-inflammatory drugs; NTP, National Toxicology Program; OECD, Organisation for Economic Co-operation and Development; PBPK model, physiologically based pharmacokinetic model; POE, portal of entry; PCLS, precision-cut lung slices; QSAR, quantitative structure-activity relationship; RDDR, regional deposited dose ratio; REACH, Registration, Evaluation, Authorisation and Restriction of Chemicals; RGDR, regional gas dose ratio; RRDR, regional retained dose ratio; SAEC, small airway epithelial cells; TG, test guideline; TSE, target site exposure

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In silico
Ex vivo
Quantitative structure-activity relationships (QSAR)
Adverse outcome pathway
Aggregate exposure pathway
Dosimetry
Integrated approach to testing and assessment (IATA)
Risk assessment

pathways (AOPs) can help identify and address gaps in our understanding of relevant parameters for model input and mechanisms, and optimize non-animal approaches that can be used to investigate key events of toxicity. This paper describes the AOPs and the toolbox of *in vitro* and *in silico* models that can be used to assess the key events leading to toxicity following inhalation exposure. Because the optimal testing strategy will vary depending on the substance of interest, here we present a decision tree approach to identify an appropriate non-animal integrated testing strategy that incorporates consideration of a substance's physicochemical properties, relevant mechanisms of toxicity, and available *in silico* models and *in vitro* test methods. This decision tree can facilitate standardization of the testing approaches. Case study examples are presented to provide a basis for proof-of-concept testing to illustrate the utility of non-animal approaches to inform hazard identification and risk assessment of humans exposed to inhaled substances.

1. Introduction

Acute inhalation toxicity testing is conducted to characterize potential portal-of-entry (POE) effects (those that directly affect the respiratory system) and systemic toxicity hazards of substances that can be inhaled, including gases, vapors, and liquid (mist) or solid (dust) aerosols. Both the decision to conduct acute inhalation toxicity testing and the design of appropriate test systems are informed by an evaluation of a substance's physicochemical properties and other available information, which will indicate whether inhalation is a likely route of human exposure and the potential target tissues. Data from acute inhalation toxicity tests may be used to identify intrinsic hazard properties of chemicals or end-use products, hazard classification and labelling, or to inform risk management decisions. Depending on the approach used, these data may also help elucidate the mechanism through which a chemical causes toxicity or to select exposure levels for subsequent subacute and subchronic inhalation tests. Other applications of acute inhalation toxicity data include development of emergency response guidance levels to inform evacuation or re-entry decisions, setting short-term occupational exposure levels, and informing operational decisions of military personal facing chemical warfare threats (Jarabek, 1995a; US EPA, 2009).

Acute inhalation toxicity is defined according to the Organisation for Economic Co-operation and Development (OECD) as the totality of adverse effects caused by a test substance following a single, uninterrupted exposure over a period of < 24 h (OECD, 2009a). Acute inhalation toxicity data have historically been generated by exposing animals to single or multiple inhaled concentrations of a substance in a short period of time ($\leq$ 24, usually 4–6 h) and assessing the adverse effects. OECD and other authorities have issued various test guidelines describing methods to assess inhalation toxicity (US EPA, 1998; 40 CFR 799.9130, 2002; OECD, 2009b; OECD, 2009c; OECD, 2017b). OECD Test Guideline (TG) 403 (OECD, 2009b) and OECD TG 436 (OECD, 2009c) consider lethality as the primary endpoint, whereas evident toxicity is the primary endpoint in OECD TG 433 (OECD, 2017b). For these tests, acute inhalation toxicity may be expressed as a point estimate of the median lethal concentration (LC_{50}; the concentration that would be expected to cause death in 50% of animals during a 14-day observation period) (US EPA, 1998; OECD, 2009b); a probit analysis of exposure-response data based on multiple concentrations and exposure durations (concentration x time; CxT) (OECD, 2009b); a benchmark dose analysis (Vincent, 1995; Kulkarni et al., 2011); or a hazard-based classification into categories based on exposure to predetermined fixed concentrations (OECD, 2009c; OECD, 2017b). LC_{50} data generated from these tests are used to categorize and rank test substances based on lethality, often with little or no elucidation of the site or underlying mechanism of toxicity. Other acute assessment derivations currently based on *in vivo* data consider exposure durations spanning a range from 10 min to 24 h, designate various non-lethal severity categories, and consider clinical measures or endpoints (*e.g.*, developmental, reproductive) in addition to LC_{50} values (Vincent, 1995; OECD, 2016b; National Research Council, 2017; Hofmann et al., 2018). Developing non-animal approaches that leverage pathway-based mechanistic information will not only provide a predictive tool for establishing potential hazard, but will likely provide more information to the risk assessor than an LC_{50} or other *in vivo* observations.

Extrapolating animal data to predict human health consequences presents numerous challenges due to physiological, anatomical, and metabolic differences across species (*e.g.* dissimilar airway dichotomies, types and composition of cells, different bio-transforming enzymes, and physiological variations in breathing patterns and metabolic rates) (BéruBé, 2013). Data generated in these acute toxicity studies may not be appropriate or sufficient to predict and manage potential adverse effects in humans (Zbinden and Flury-Roversi, 1981; Balls, 1991; Chapman et al., 2010; Seidle et al., 2010). As various adverse outcome pathways (AOPs) following inhalation exposures are elucidated, the opportunity arises to develop human cell-based *in vitro* and *in silico* approaches to evaluate endpoints relevant to those AOPs. An AOP is a conceptual framework that organizes existing mechanistic evidence by connecting—*via* key event relationships—a defined molecular initiating event (MIE) on the cellular or subcellular level to subsequently occurring key events (KEs) at the tissue and organ levels that lead to an adverse outcome at the organism or population level (Villeneuve et al., 2014b; Villeneuve et al., 2014a). AOPs describe a series of essential, measurable events culminating in toxicity, and can be useful in delineating endpoints that can be assessed *in vitro*. These AOP-motivated *in vitro* approaches can then be used to inform interspecies extrapolation, assess target organ effects, and support a better understanding of how specific substances cause toxicity in humans (*i.e.*, providing mechanistic insight that goes beyond what can be gleaned from an LC_{50} value). While these approaches are yet to be accepted by global regulatory agencies, they represent a promising and emerging area of research.

The implementation of alternative approaches for the assessment of acute inhalation toxicity was the focus of a 2016 workshop co-organized by the PETA International Science Consortium Ltd. and the U.S. National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) (Clippinger et al., 2018). This workshop was attended by government agencies, industry, academics, and non-governmental organisations interested in developing approaches that can replace or reduce the use of animals for acute inhalation toxicity testing. Experts in attendance at the workshop were tasked with developing a strategy to establish confidence in these approaches.

Working groups were formed to fulfill each of the workshop recommendations. One of these recommendations was to publish the current paper, a state-of-the-science review to:

1) Detail the mechanisms of acute inhalation toxicity of inhaled substances (gases, vapors, and dust/mist aerosols), and define relevant AOPs that could be used to inform the appropriate integrated testing and assessment approach;
2) discuss the influence of physicochemical properties (*e.g.*, pH, low volatility, gas category, and particle size) on the relevance of inhalation as a route of exposure or on the ability to generate a test atmosphere;
3) summarize factors influencing dosimetry as well as the potential for

POE effects, including physicochemical characteristics, airway architecture, ventilation rate, blood flow, and metabolism;
4) catalog the currently available and emerging non-animal (non-testing and *in vitro* testing) approaches relevant to these mechanisms and define their usefulness and limitations; and
5) using information from the points above, develop a decision tree and discuss the steps necessary to implement an integrated approach to testing and assessment(s) (IATA) that will reduce and replace the use of animals for acute inhalation toxicity testing

The following sections discuss each of these areas, concluding with a definition of activities needed to develop IATAs that will succeed in replacing animal use for assessing inhalation hazards. Such IATAs are likely to include a combination of any existing evidence (including epidemiological and toxicological data), exposure information, physicochemical properties of the test material, non-testing approaches (*e.g.*, QSARs, read-across, or computational dosimetry models), and *in vitro* testing. The optimal approach will likely continue to evolve as more data and approaches become available, and as our understanding of the mechanisms of toxicity advances. Because the LC_{50} is commonly used as a metric for regulatory needs (*e.g.*, hazard classification), there is an interest in developing non-animal approaches to predict either point estimates of LC_{50} or ranges of LC_{50} values that could be used for hazard classification. Ultimately, however, these non-animal approaches may provide mechanistic information on how inhaled substances cause systemic toxicity or POE effects in humans, thereby providing a better characterization of their potential hazards than existing *in vivo* methods. While this paper focuses on acute inhalation toxicity testing, many of the cell systems and concepts discussed herein could be adapted and applied to designing longer-term repeat dose inhalation studies.

2. Biological pathways of inhalation toxicity

Designing an appropriate IATA for inhalation toxicity requires considering both dosimetry and the underlying biological processes that link exposure to non-lethal and lethal outcomes. Key to development of such an IATA is the identification and evaluation of relevant AOPs. A framework that parallels AOPs and applies to exposure science is the aggregate exposure pathway (AEP), which organizes exposure data and predictions to link the introduction of a stressor from sources, transport, and transformation through environmental media, patterns of external exposure, and biokinetic processes leading to target site exposures (TSEs). The TSE is the point of integration between the AEP and AOP, representing a specialized KE characterizing the concentration at the biological location of the MIE in an AOP (Cheng and Moss, 1995), and a critical consideration for dose-response analysis in risk assessment. The TSE is measured at the level of organisation corresponding to a defined protection goal; for example, the TSE could be a cellular concentration or an air concentration, depending on the assessment objective. The AEP framework allows for integration of exposure science with AOPs and dose-response data, linking exposure and hazard. Together, AEPs and AOPs create a flexible framework that enables risk-based, hazard-based, or exposure-based decision making (Fig. 1) (Teeguarden et al., 2016). Here we focus on the biological (anatomical and physiological) factors involved in describing the TSE and KEs of an AOP for an inhaled agent.

Following inhalation exposure, toxicity may be induced *via* multiple mechanisms. Methods that focus solely on the generation of LC_{50} values are limited in their ability to generate data that support a better understanding of these mechanisms (Hamm et al., 2017). However, progress has been made in recent years to use information from mechanistic approaches to develop AOPs. AOPs provide a framework to better understand the mechanisms that lead to adverse outcomes (local POE toxicity and systemic toxicity) following inhalation exposures, and substantial work is underway to further develop AOPs related to inhalation exposure (Table 1). Table 1 shows specific AOPs with potential relevance to acute inhalation toxicity based on the general KEs illustrated in Fig. 2 (https://aopwiki.org/aops; accessed 22 January 2018). These AOPs describe adverse outcomes (acute or otherwise) that are likely to occur in the respiratory tract, or encompass MIEs, KEs, or adverse outcomes that are relevant to the mechanisms in Fig. 2. Any stressor that evokes the MIE in question may potentially lead to the adverse outcome in humans and one substance may perturb multiple AOPs (Allen et al., 2014). Specific adverse outcomes may take different timeframes to be expressed, and also depend on the exposure concentration, frequency, and duration.

AOPs can be used to help identify suitable non-animal tests to assess specific KEs. As additional *in vitro* data are collected, they can be used to further develop AOPs (Ankley et al., 2010; Tollefsen et al., 2014). The iterative nature of the AOP development process means that, as knowledge gaps are filled, pathways and testing strategies can be continually refined, guiding the *in vitro* and non-testing approaches required to support regulatory decision-making (Tollefsen et al., 2014). Thus, AOP-informed IATA development can drive the evolution of *in vitro* or non-testing approaches that contribute to a mechanistic understanding of acute inhalation toxicity and improved hazard assessment for potential human exposure scenarios. Ideally, the integration of all information will also lead to the development of quantitative AOPs that can be used for dose-response analyses (Wittwehr et al., 2017), and iteratively, inform refinements of the next generation of mechanistic IATAs.

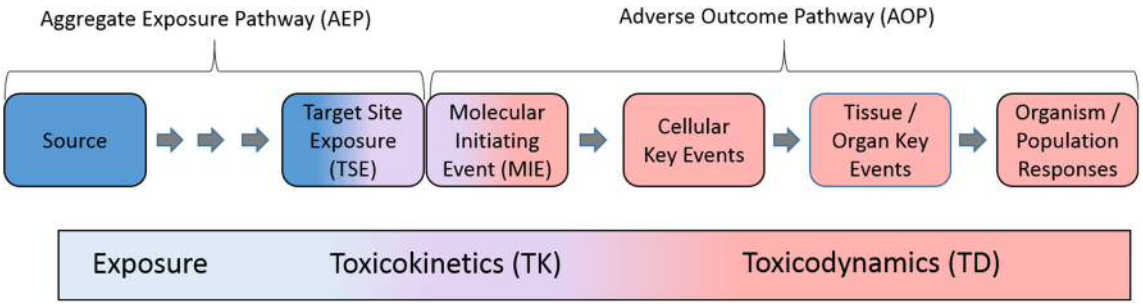

Fig. 1. Integration of exposure information into an aggregate exposure pathway:adverse outcome pathway (AEP:AOP) framework. Key events (KE) in the AOP are indicated by the pink-colored boxes. The arrows between the environmental air contaminant source to the target site exposure (TSE) denote key exposure states (*e.g.*, in different media) representing a measurable change in a chemical state and concentration that describes the movement of a chemical from a source to the TSE in the AEP. The TSE in the respiratory tract that corresponds to the MIE (*e.g.*, dose to a given region or cell type in the portal of entry or delivered to a systemic target tissue) links the AEP and AOP frameworks for inhaled agents. Toxicokinetics (TK) refers to the processes of absorption, distribution, metabolism, and elimination (ADME) of a toxicant while toxicodynamics (TD) refers to the processes of a toxicant within an organism at the organ, tissue, cellular, and molecular level (Faustman and Omenn, 2013).

Table 1
Key events with potential relevance to toxicity following acute or longer-term inhalation exposures and associated AOPs (hyperlinks direct to specific AOP in the AOPWiki).

MIE/key events	Example stressor	Adverse outcome	Reference
- Acetylcholinesterase inhibition - Accumulation of acetylcholine in synapses - Increased atrioventricular block and bradycardia - Increased respiratory distress - Induction of ataxia, paralysis, or hyperactivity	Organophosphorous compounds	Acute mortality (AOP 16)	(Russom et al., 2014)
- Axonal sodium channel inhibition - Prolonged depolarization of neuronal membrane - Neurotransmitter release - Muscle contraction - Induction of ataxia, paralysis, or hyperactivity	Volatile anaesthetics	Acute mortality (AOP 96)	N/A
- Cell injury - Narcosis (membrane disruption) - Decompartmentalization - Direct mitochondrial inhibition		Cytotoxicity (AOP 205)	(Vinken and Blaauboer, 2017)
- Narcosis (membrane disruption) - Decreased cell respiration and metabolism - Decreased respiration	Volatile organic compounds (VOC) and solvents	Respiratory failure (AOP 35)	(Perkins et al., 2015)
- Oxidative Stress - ILC2 modulation		Respiratory epithelial remodeling (AOP 239)	(Jarabek and Harkema, in preparation)
- EGFR activation (phosphorylation) - Transdifferentiation of ciliated epithelial cells - Goblet cell metaplasia - Hyperplasia of goblet cells - Proliferation of goblet cells - Apoptosis of ciliated epithelial cells - Transcription factor (SP1) modulation - Increased mucus production	Cigarette smoke	Decreased lung function (AOP 148)	(Luettich et al., 2017)
- Oxidative stress-mediated perturbation of endothelial nitric oxide bioavailability - Glutathione oxidation - S-Glutathionylation, eNOS - Decrease in GTPCH-1 - Decrease in tetrahydrobiopterin - Uncoupling of eNOS - Depletion of nitric oxide - Impaired vasodilation - Increase in vascular resistance - Decrease in Akt/eNOS activity	Cigarette smoke	Hypertension (AOP 149)	(Lowe et al., 2017)
- Glucocorticoid receptor activation - Inhibition of NF-κB - Suppression of inflammatory cytokines - Decreased lymphocytes - Induction on IκB - Suppression of the immune system	Corticosteroids	Increased disease susceptibility (AOP 14)	N/A
- Inhibition of vitamin K epoxide reductase (inhibition of vitamin K cycle) - Depletion of functional clotting factors - Failure to form clot - Prevention of vascular repair	Anticoagulant rodenticide	Coagulopathy and haemorrhage (AOP 187)	(Rattner et al., 2014)
- DNA alkylation - Failure of DNA repair - Mutation	Cyclophosphamide	Cancer (AOP 139)	N/A
- Protein alkylation - Cell injury/death - Activation and recruitment of Kupffer cells - Upregulation of TGFbeta1 expression - Activation of stellate cells - Collagen accumulation	2-Iodoacetamide	Liver fibrosis (AOP 38)	(Horvat et al., 2017)
- Organic anion transporter (OAT1) inhibition - Increase in uric acid concentration in the blood - Renal proximal tubular necrosis - Increased blood potassium concentration - Increased tophi (urate) deposition - Increased occurrence of cardiac arrhythmia - Increased oxidative stress	NSAIDs	Renal failure and mortality (AOP 138)	N/A
- Nav1.1 channel inhibition - Decreased sodium conductance - Reduced swimming speed and feeding in fish, leading to increased predation	Volatile anaesthetics	Reduced survival (AOP 95)	(Fay et al., 2017)
- Chronic cytotoxicity of the serous membrane - Persistent cytotoxicity - Increased inflammation - Increased oxidative stress - Increased secretion of local growth factors - Increased cell proliferation	Asbestos	Mesothelioma (AOP 171)	N/A

3. Developing an IATA for inhalation testing

Integrated approaches to testing and assessment (IATA) are pragmatic, science-based approaches for chemical hazard or risk characterization that rely on an integrated analysis of existing information in a weight of evidence assessment coupled with the generation of new information using testing strategies (OECD, 2016c). IATA follow an iterative approach to answer a defined question in a specific regulatory context, taking into account the acceptable level of uncertainty associated with the decision context, and thus afford the flexibility required to address the various applications identified herein for inhalation testing and translation. Development of successful IATA has been demonstrated to evaluate both skin irritation or sensitization and eye irritation (OECD, 2014; OECD, 2016b; OECD, 2017a) but there is a need for a systematic framework to characterize the individual biological and toxicological relevance of *in vitro* methods for predicting toxicological endpoints from inhalation exposures, notably those challenges are inherent in assessing toxicity to the respiratory tract.

The respiratory tract is composed of > 40 types of cells that are localized to specific regions based on their function (International Commission on Radiological Protection (ICRP), 1994; Parent, 2015). For example, the upper respiratory tract includes the respiratory, transitional, and olfactory epithelium; including sustentacular cells, ciliated cells, and basal, goblet, serous, and brush cells. The alveolar region is comprised of alveolar type I and type II (surfactant-producing) cells, serous cells, and Club cells (Crapo et al., 1982; Crapo et al., 1983; Parent, 2015). Immune cells (*e.g.*, dendritic cells and macrophages) exist in different locations throughout all respiratory regions (Holt, 2005; Brain, 2011).. These differences in populations of cell types as the airways move from being conducting to respiratory in function (*e.g.* the replacement of goblet with Club cells; which begins in the bronchioles), make studying the respiratory tract a unique challenge, and the choice of a representative test system is paramount to obtaining relevant results. Creating one *in vitro* test system containing all of these cell types is currently not technically feasible. However, systems containing cells critical to a given pathogenesis (alone or in co-culture) could be useful to predict the effects of inhaled substances in specific regions of the respiratory tract.

The complexity of the respiratory tract underscores the importance of using an AOP framework as a mechanistic scaffold to aid in the design of intelligent *in vitro* and *in silico* testing approaches to inhalation exposures. By applying AOP-directed knowledge and understanding, the following components are likely to contribute, combined with other tools and methods, to the development of defined approaches (OECD, 2016a) and IATA on guidance for testing and interpretation of inhalation exposures.

a. Physicochemical property information

Development of an IATA starts with problem formulation, an approach to toxicological risk assessment that links the exposure scenario of interest to a substance's potential adverse health outcomes in humans. Problem formulation considers factors such as the purpose of the assessment, physicochemical properties of the test substance and its potential dosimetry or ADME processes, likely exposure concentration and route of exposure, exposed populations, exposure scenario (*e.g.*, consumer *versus* occupational exposure), existing information, which methods to use for the generation of new data, and the relevant endpoints to assess (US EPA, 2014; Borgert et al., 2015; National Research Council, 2017).

If exposure to a test substance is either known to be minimal or predicted to be minimal based on physiochemical property information, certain regulatory requirements for testing could be reconsidered. For example, a large particle size (aerodynamic diameter > 100 μm), low

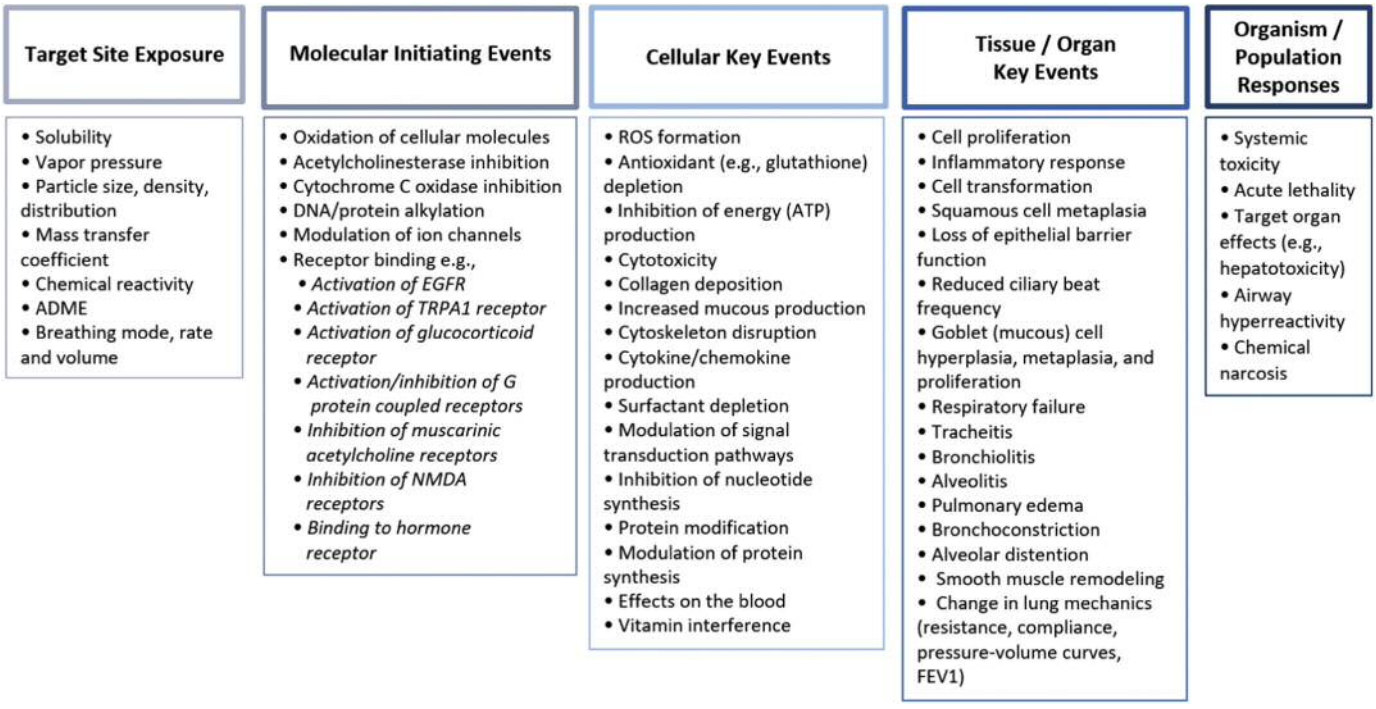

Fig. 2. Selected key events and adverse outcomes that may occur systemically or in the respiratory tract as the portal of entry following acute exposures to an inhaled material. The TSE is a function of dosimetry including absorption, distribution, elimination and metabolism (ADME) processes that dictate disposition of the parent chemical or its metabolite(s). Chronic lung effects may occur as a result of acute toxicity that does not cause death or with repeated acute exposures notably when either the chemical or its damage persists; exposure concentration, frequency, and duration also impact the trajectories of different pathways. Tissue remodeling would not be evident after a single acute exposure, and protective cellular effects that could mitigate the listed adverse responses are not listed here. Additionally, while respiratory sensitization may occur following repeated acute exposure to an inhaled substance, this endpoint is not included as it is not generally considered in the current acute systemic toxicity animal tests.

volatility, and high viscosity would suggest that the substance is not inhalable by humans without some form of physical manipulation (*e.g.*, low volatility substances sprayed into the air). Thus, this information is important to consider before generating new data and can provide context for the interpretation and translation of existing information for both the substance of interest and analogous substances. However, caution must be used with this approach as there may be situations where a chemical not expected to be inhaled is incorporated into a product that is delivered as an aerosol or some other manner that increases inhalability.

Once access to the respiratory tract is established as a likely route of inhalation exposure, physicochemical property information can also then be used to determine whether it is technically feasible to test a substance and to inform a testing strategy. For instance, the vapor pressure of a gas or the partial pressure of the components in a mixture can provide an indication of its volatility, informing whether it will be possible to generate a test atmosphere. Test material density and size distribution are determinants of aerosol physics that provide information on the technical feasibility of testing.

Test material density and size distribution also determine the local dosimetry of a substance in the airways or to remote systemic sites and thus help inform considerations for characterizing the TSE and requisite features for design of an *in vitro* test system (Clippinger et al., 2018). Particle size affects inhalability and how deeply into the respiratory tract a substance would penetrate (Heyder et al., 1986; Patton and Byron, 2007), which will in turn determine whether an *in vitro* model of the upper and/or lower respiratory tract is more appropriate. For example, small particles, such as nanomaterials, are able to deposit in all regions of the respiratory tract, including penetrating deeply into the lung to the alveoli. Alveolar macrophages have been shown to be important in clearing the alveoli from particles; therefore, alveolar macrophages may present a useful test system to assess the effects of nanomaterials and other small particles (Clippinger et al., 2016; Wiemann et al., 2016). In addition, nanoparticles have been demonstrated to deposit in the nasal region and subsequently translocate to the brain (Oberdorster et al., 2004; Oberdorster et al., 2009).

Other physicochemical properties of a substance (*e.g.*, chemical reactivity, water, and lipid solubility) will additionally affect whether its potential toxicity will predominantly manifest as local effects in the respiratory tract or systemic effects and thus aid in determining the most relevant *in vitro* test system (Kriebel et al., 2007; Grimm et al., 2016; Stanton and Kruszewski, 2016). For example, a strong acid or base or a substance that contains structural features indicative of electrophilicity or reactivity in tissues (such as epoxides or α/β unsaturated aldehydes) may have the potential to cause local POE effects such as respiratory irritation or corrosion. In particular, non-lung cell systems will be needed to assess substances with properties that make them likely to be absorbed through the lung tissue and cause organ-specific toxicity in locations other than the respiratory tract (*e.g.*, heart, liver, or nervous system). Consideration must also be given to metabolites that may possess different physical properties than the parent compound, potentially altering distribution and other target organ exposures. Human lung samples and three-dimensional reconstructed human tissues models have been characterized for their expression of key metabolic enzymes (Castell et al., 2005; Bernauer et al., 2006; Willoughby, 2015).

b. Non-testing approaches

Several different types of non-testing approaches can help predict the toxicity of substances after inhalation exposures and can be used in a “fit for purpose” fashion to address various assessment needs that span from prioritization, hazard identification, quantitative risk assessment, and national exposure standard setting (Bell et al., 2018). For the purposes of this manuscript, the phrase “non-testing approaches” describes (1) empirical grouping approaches; (2) quantitative structure-activity relationship (QSAR) analyses; (3) expert systems; and (4) mechanistic computational dosimetry models. These approaches, to varying degrees, are based on the underlying principle that similar substances are expected to exhibit similar biological activities.

i. Computational models based on historical data or chemical structure

Grouping approaches, such as read-across (*i.e.*, applying data from one substance(s) to predict the same property or effect for a structurally ‘similar’ substance), use data mining approaches to fill data gaps and delineate chemical categories. Grouping—which encompasses category and analog approaches and data gap-filling techniques—generally uses a structure-based approach to define categories of substances; these categories can then be used to generate hypotheses about toxicity and mechanisms of toxicity of untested chemicals.

QSARs are theoretical models that can be used to predict the physicochemical, biological, and environmental fate properties of substances based on a knowledge of their chemical structure in a qualitative or quantitative manner (ECHA, 2008). Guiding principles exist to facilitate QSAR use for regulatory purposes. OECD has described five principles that collectively characterize the scientific validity of a specific QSAR (OECD, 2004; OECD, 2007; Patlewicz et al., 2016). One principle describes the importance of defining an applicability domain for the QSAR model itself so that the adequacy of the prediction for a given substance can be assessed. The applicability domain is extracted from the training set of chemicals used to derive the QSAR model. Expert systems are software tools that use QSARs to predict toxicity based on chemical structure; expert systems are usually categorized as knowledge-based, statistics-based, or hybrids (Patlewicz et al., 2007; Worth et al., 2007).

The availability of non-testing approaches to predict toxicity after acute inhalation exposures is limited. The commercial expert system TOPKAT is a structure-based global QSAR model that specifically aims to predict an LC_{50} value that would be generated in a rat four-hour inhalation test (https://omictools.com/toxicity-prediction-by-komputer-assisted-technology-tool). Because the model, data, and training set used are proprietary, and it has not been described in the peer-reviewed literature, a definitive evaluation of its utility remains a challenge. Local QSARs for prediction of inhalation toxicity include one developed by Veith et al., which uses log vapor pressure as a predictor of LC_{50} (expressed as the log molar equivalent) for volatile neutral organic substances assumed to cause toxicity *via* narcosis (Veith et al., 2009); however, the applicability domain does not extend coverage to other physicochemical properties or endpoints.

Information submitted under the European Union Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) is used in toxicity prediction tools such as OECD's QSAR Toolbox (https://www.qsartoolbox.org/), and the OECD's eChemPortal (www.echemportal.org) provides information on chemical properties, exposure, and toxicity. These data could be further exploited to facilitate development of new QSAR models for predicting inhalation toxicity. In fact, the REACH*Across*™ software (www.ulreachacross.com/REACH) was developed using these REACH data. MultiCASE has also recently released a QSAR model to predict acute inhalation toxicity.

Other sources of inhalation data exist in the U.S. Centers for Disease Control's Registry of Toxic Effects of Chemical Substances (www.cdc.gov/niosh/rtecs/default.html); this data set is available under license from Accelrys (www.accelrys.com) and value-added resellers such as Leadscope Inc. (www.leadscope.com). The OECD QSAR Toolbox also encodes profiling tools called SAR rule bases that help group substances into toxicological categories on the basis of a presumed mode of action (*e.g.*, unspecific reactivity, polar narcosis, baseline narcosis, or receptor-mediated toxicity) and structural similarity. This information is key to identifying relevant source analogs for read-across predictions and determining whether additional *in vitro* testing should be conducted.

Additional work is needed to develop and optimize QSARs and non-testing approaches for predicting the potential toxicity of inhaled materials.

ii. Mechanistic computational dosimetry models

In general, dosimetry involves determining the amount, uptake rate, and distribution of a substance in the body and various models can be used to predict these factors (Kuempel et al., 2015). Disposition of inhaled agents in the respiratory tract encompasses the processes of initial inhalability, airflow convection and diffusion, and ADME processes. The major determinants of disposition, which include the physicochemical properties of an inhaled agent and the anatomy and physiology of the respiratory tract, determine the initial deposition and subsequent disposition within the respiratory tract, distribution to other systemic tissues, and ultimately the toxic effect. These determinants have been described and previously reviewed (Clippinger et al., 2018).

Studies that mimic realistic exposure conditions (such as exposure concentration, duration, and in the case of aerosols, particle density, size, and distribution) will be most likely to generate results that are predictive of human outcomes. Development of risk assessments require dose-response information with a range of doses relevant to human exposure.

There are two general categories for inhalation dosimetry: aerosols (including fibers and nanomaterials) and gases (including vapors); within gases there are three major categories for dosimetry model selection (gas Category 1, 2, and 3; see Table 5 in (Clippinger et al., 2018)). Consideration of physicochemical properties according to these gas categories will be essential to the selection of the test system and the evaluation and extrapolation of the effects to a given target human equivalent. Different mathematical models can be applied to the three different categories of inhaled agents and span a range from default algorithms to sophisticated computational models with detailed structures to address all anatomical, physiological, and physiochemical determinants and their interactions, as previously summarized (Clippinger et al., 2018). The choice of model structure depends on the available data and the dose metric desired, for example, the regional uptake or a localized dose to a specific cell type (US EPA, 1994; Jarabek, 1995b; Kuempel et al., 2015).

An example of a sophisticated model routinely used to estimate particle dose *in vivo* (humans and animals) is the Multiple-Path Particle Dosimetry (MPPD) model (www.ara.com). The MPPD model can be used for interspecies extrapolation and to describe the inhaled dose of various exposure scenarios. Tools to help set and translate *in vitro* doses include the *in vitro* sedimentation, diffusion, and dosimetry model, which describes the dose delivered to the cell (Hinderliter et al., 2010), and its extension, the *in vitro* sedimentation, diffusion, dissolution, and dosimetry model or ISD3 model, which addresses soluble particokinetics *in vitro* (Thomas et al., 2018). Models for reactive gases include computational fluid dynamics (CFD) models and typical-path mass transfer descriptions or hybrid CFD-PBPK models (Kimbell et al., 2001; Overton et al., 2001; Schroeter et al., 2006; Schroeter et al., 2010; Corley et al., 2012; Kolanjiyil and Kleinstreuer, 2017). Models for solids, liquids, and gases with pulmonary absorption linked to systemic distribution and clearance include physiologically-based pharmacokinetic (PBPK) models (Backman et al., 2014; Borghardt et al., 2015; Backman et al., 2018). Development of approaches linking dosimetry models describing *in vivo* dose metrics associated with different exposure scenarios for particles or gases with *in vitro* systems will help translation and application of *in vitro* data.

c. *In vitro* and *ex vivo* testing methods

In vitro to *in vivo* extrapolation (IVIVE) methods are a key resource for correlating bioactive chemical concentrations in *in vitro* assays to plasma and tissue dose levels that lead to adverse effects *in vivo*. An accurate estimate of the toxicological effects of a substance depends on the input factors used for the IVIVE calculation (*i.e.*, dosimetry and ADME). To perform an accurate IVIVE, the cellular concentration is a more reliable dose metric to serve as the basis for translation and alignment of exposure and test systems compared to the nominal exposure concentration (Hinds, 1999; National Research Council, 2017). *In silico* fate models to describe free *in vitro* concentration have been developed (Kramer, 2010; Armitage et al., 2014; Comenges et al., 2017; Fischer et al., 2017). These models describe the *in vitro* test system as a set of mathematical equations and simulate the chemical partitioning between proteins, plastics, and lipids in the cell culture medium and within the cell. Models currently include a lung cell line (Zaldívar et al., 2012) and the 3 T3 Balb/c fibroblast cell line (Kramer et al., 2012; Comenges et al., 2017; Proença et al., 2017).

ii. In vitro models of the respiratory tract

In vitro cellular systems differ not only with respect to the types of cells included, but also the format in which they are grown (*e.g.*, submerged or grown at the air-liquid interface), which influences important transport and transformation mechanisms. Each system has its advantages and limitations and the choice of which system to use will depend on the specific purpose of the testing. While submerged monolayer cultures have been used extensively to study basic human airway biology and to elucidate mechanisms of toxicity, co-culture systems comprised of epithelial and other cell types (*e.g.*, lung residence immune cells) and structured to incorporate an air-liquid interface can be used to more closely recapitulate physiological conditions and study cell-cell interactions. Studies showed that functional and phenotypic characteristics, such as beating cilia and active mucus secretion, can be successfully reproduced in an air-liquid interface system. Systems including commercially available three-dimensional reconstructed tissue models and biomimetic models such as the breathing human lung-on-a-chip can be exposed to a test substance at the air-liquid interface. In addition to considering the biological system for evaluation, the method for exposing the test chemical is also important; for example, whether a vapor or aerosol will be generated to expose cells, or whether a liquid will be applied directly to a tissue or cell culture. The properties of the test chemical will impact the exposure method decision.

The use of co-culturing to create a more *in vivo*-like situation can take three forms: 1) the addition of multiple cell types within or closely related to the organ in question; 2) the inclusion of immune cells; or 3) the use of cells from other organs to generate a multi-organ-based approach (Prytherch and BéruBé, 2014). The first scenario might involve the co-culture of bronchial or alveolar cells, fibroblasts, and endothelial cells. An immune cell type(s) may be added to the airway epithelium alone or to a more complex co-culture model of the epithelium. Multi-organ-based co-cultures attempt to recreate the potential downstream fate of inhalable compounds. For instance, many potential therapeutics fail due to cardiotoxic or hepatotoxic effects, making the use of a connected system of respiratory, liver, and/or heart cells attractive.

Careful characterization of the mechanistic competencies (*e.g.*, antioxidant capacities, metabolic profiles) as well as the structure, barrier properties, and spatial arrangement of the cells in the test system is important when selecting the system. Human primary cells (*e.g.*, human bronchial epithelial (HBE) cells or small airway epithelial cells (SAEC)) or human-derived cell lines (*e.g.*, BEAS-2B, Calu-3, A549, or NCI-H292) have been widely used to better understand how a substance is likely to affect humans. In particular, there is much interest in the use of primary human cells due to their biologic-relevance, such as the ability to maintain expression of important markers and functions seen *in vivo*.

iii. Ex vivo models of the respiratory tract

Ex vivo human precision-cut lung slices (PCLS) are relevant to study airway biology and the adverse effects of inhaled toxicants. Human

PCLS are created from organs donated, but not used, or from surgically removed tissue. As with reconstructed human airway models generated from donor cells, either healthy or diseased tissue may be used in toxicological studies, depending on the goal of the study. Years of published research has demonstrated PCLS metabolic competence (Vickers et al., 1997; De Kanter et al., 2002a; de Kanter et al., 2002b) and sensitivity to known pulmonary toxicants (Fisher et al., 1994; Switalla et al., 2010). While this system lacks recruited immune cells, PCLS contain resident immune cells that allow for immune responsiveness (Sewald and Braun, 2013; Lauenstein et al., 2014) (Hess et al., 2016). Several characteristics unique to PCLS include native architecture of the lower respiratory tract and a full complement of cell types (including macrophages and dendritic cells) in the tissue at the time of slice creation. PCLS can be particularly useful for applications that require maintenance of cultures for several weeks (Morin et al., 2013; Westra et al., 2013). The small size of PCLS confines slice creation to the periphery of the lung and includes the respiratory parenchyma (alveolar space) and small airways, the latter allowing for functional studies involving airway contractility.

iv. Selection of in vitro / ex vivo test systems

Reviews of exposure generation and characterization (Cheng and Moss, 1995; Kennedy et al., 1995; Moss and Cheng, 1995; Vincent, 1995; Wong, 1995; Hinds, 1999; Kulkarni et al., 2011; Polk et al., 2016) and *in vitro* and *ex vivo* systems (BéruBé et al., 2009; BéruBé et al., 2010; Gordon et al., 2015; National Academies of Sciences and Medicine, 2015; Wiemann et al., 2016) that can be used to assess the toxicity of inhaled substances were published previously. As for *in vivo* exposure systems, generation and characterization of exposures both in the system and at the cell interface, analytical considerations such as appropriate methods for detection of a specific chemical, and determination of proper operating conditions (*e.g.*, temperature, relative humidity, flow rates) are critical to ensure consistent results from *in vitro* systems. Additionally, the differences in culture systems (*e.g.*, three-dimensional or monolayer) and cell types (*e.g.*, metabolic competencies and anti-oxidant capacities) must be considered when selecting a test system (Sauer et al., 2013; Zavala et al., 2016).

Supplementary Table 1 provides an updated summary of the cell types, biomarkers, and test substances that have been used in the published literature. While the table does not represent an exhaustive list of every cell type that could be used, the existing information illustrates current capabilities and may help inform selection of a cell-based system to test a new substance.

Selection of an *in vitro* system will depend on the substance being tested and the objective of the study (*e.g.*, prioritization/screening or hazard classification *versus* quantitative risk assessment). In general, analysis of the substance's properties can be used to predict whether it is likely to be reactive; if so, a preliminary screen for overt toxicity could be conducted in a simpler monoculture test system. While a respiratory cell model may be critical to assess respiratory-cell-specific mechanisms, it is likely that other (non-respiratory tract) cell types may be sufficient to assess general cytotoxicity (Sauer et al., 2013). Use of a monoculture test system comprised of mammalian epithelial cells may also be sufficient if the primary purpose of testing is to rank the toxicity of several compounds to determine which to prioritize for further testing. A monoculture system could also be used to examine acute endpoints, but a model with longer viability would be needed if the goal of the study is to examine repeated exposures or longer-term endpoints.

The appropriate cell system can be selected after determining

Table 2
Considerations and tools useful to the development of an IATA to assess effects after inhalation exposures.

Exposure use case	• Problem formulation is first step of targeted testing or assessment approaches. Obtaining human exposure data under real-world conditions (*e.g.*, monitoring for air concentrations and particle size distributions in the human breathing zone in a field study) may provide more robust evidence of human exposure potential *versus* laboratory data or default assumptions (National Research Council, 2009). • Population demographics (*e.g.*, age, sex, and race) • Exposure regimen (*e.g.*, frequency) • Exposure duration • Inhaled agent (*e.g.*, particle or gas) • Concentration (mg/m^3)
Existing information	• Human *e.g.*, case reports from accidental or intentional exposures, studies of bronchoalveolar lavage (BAL) fluid. • *In vivo* toxicity • *In vitro* toxicity • Information related to the likelihood of exposure • Information on the intended use or target of the chemical
Physicochemical properties	• Physical form *e.g.*, gas, liquid, or solid • Viscosity • Volatility *e.g.*, what is the substance's vapor pressure? • Particle density, size, and distribution *e.g.*, is the substance inhalable or likely to enter the bloodstream? • Gas category *e.g.*, reactive substances (gas Category 1) or volatile organic compounds (gas Category 3) • Irritation/corrosivity as predicted by pKa (*e.g.*, is the substance a strong acid or base?) • Solubility in airway surface liquid, phagolysosomal fluid, or mucus
Chemical properties	• Reactivity *e.g.*, is the test substance—parent compound or metabolite—electrophilic or likely to react *via* one of the reaction mechanistic pathways: ● Hydrolysis ● Oxidation ● Acylation ● Alkylation ● Nitrosylation ● Michael addition reaction ● Schiff base formation
Dosimetry modeling	• To predict dose metric in specific respiratory tract region or delivered to systemic target tissues
Non-testing methods	• QSARs, grouping, read-across, and computational dosimetry models • Threshold of toxicological concern • For mixtures: theory of additivity
Weight-of-evidence analysis	• Review existing information, physicochemical properties, and non-testing methods to decide if additional testing is needed.
In vitro testing	• The information in the above rows can be used to inform selection of an *in vitro* test system(s) to interrogate POE and systemic effects following inhalation exposures. See Supplementary Table 1 for examples of *in vitro* systems of interest to assess toxicity following exposure to inhaled substances.

whether the inhaled substance is likely to cause local POE effects, systemic toxicity, or both. If the substance is expected to cause systemic toxicity, PBPK models or default algorithms to predict systemic dose at target organ could be used to evaluate the ADME of the chemical in the body and its toxicity. These models can be combined with data from a general cytotoxicity assay, such as the 3 T3 neutral red uptake assay (ECVAM, 2013; Prieto et al., 2013), or data from other *in vitro* assays. An understanding of the dosimetry determinants and mechanism of toxicity or relevant AOP will help identify potential systemic toxicity sites and therefore, cell types to include in the test system. Conversely, if a substance is expected to cause local POE effects, such as respiratory irritation or sensitization, *in vitro* systems using appropriate respiratory cell types should be used. For example, an adverse outcome pathway and testing approaches have been proposed for sensitization of the respiratory tract (Kimber et al., 2014; North et al., 2016; Sullivan et al., 2017).

Identifying which *in vitro* endpoints (*e.g.*, apoptosis, barrier integrity, cytotoxicity, ER stress, inflammation, oxidative stress, or genomic/proteomic endpoints) are most predictive of whether an adverse outcome will manifest in humans is critical. Consideration should be given to how a perturbation in a human tissue model informs the risk assessment process. New tools and approaches should be developed and evaluated considering that many real-world human exposures are mixtures of multiple substances. There is also a need to analyze data produced using standardized protocols evaluating various endpoints to define the applicability domain of each test system. An example of a decision process to identify the appropriate test system is provided in the following section.

4. Decision tree and case studies

Adverse outcomes related to inhalation exposure can be predicted through the use of IATAs, which may consider existing information, likely exposure levels, physicochemical properties of the test substance, dosimetric determinants, mechanistic insights, and data from *in silico* and/or *in vitro* models (Table 2). The IATA will vary depending on the properties of the substance being tested and the purpose of the study.

4.1. Decision tree

The following decision tree is proposed to help guide consideration of the exposure parameters and design of an integrated strategy for inhalation testing (Fig. 3). Because the desired testing strategy varies depending on various factors, a decision tree that can be used to inform what testing is most appropriate is important. For example, current acute inhalation toxicity test guidelines used for calculating an LC_{50} allow waiving testing when the chemical has certain properties (*e.g.*, Fig. 3, steps 3 and 4); characterization of acute toxicity for other risk assessment applications (*i.e.*, other than determining an LC_{50}) may require additional considerations. Such considerations include the likely nature of the acute exposure (*e.g.*, its frequency and duration) and characteristics of the exposed population (*e.g.*, occupational or environmental). Careful evaluation of whether the chemical or its damage will persist is also necessary. Thus, the first step of problem formulation is to evaluate purpose of the assessment (*e.g.*, emergency response), the target exposure scenario, and characteristics of the inhaled agent. The target scenario includes parameters that characterize specific population considerations (*e.g.*, occupational, general population, adults, or children), exposure duration (*e.g.*, hours per day) and exposure regimen (*e.g.*, number of days per week or a more routine, intermittent pattern of exposures).

The human equivalent concentration (HEC) for these scenarios is calculated with different models for particles or gases. The general equation is provided here for interspecies extrapolation with considerations for application to *in vitro* systems:

$HEC_{POD} = POD_{ADJ} * DAF$.

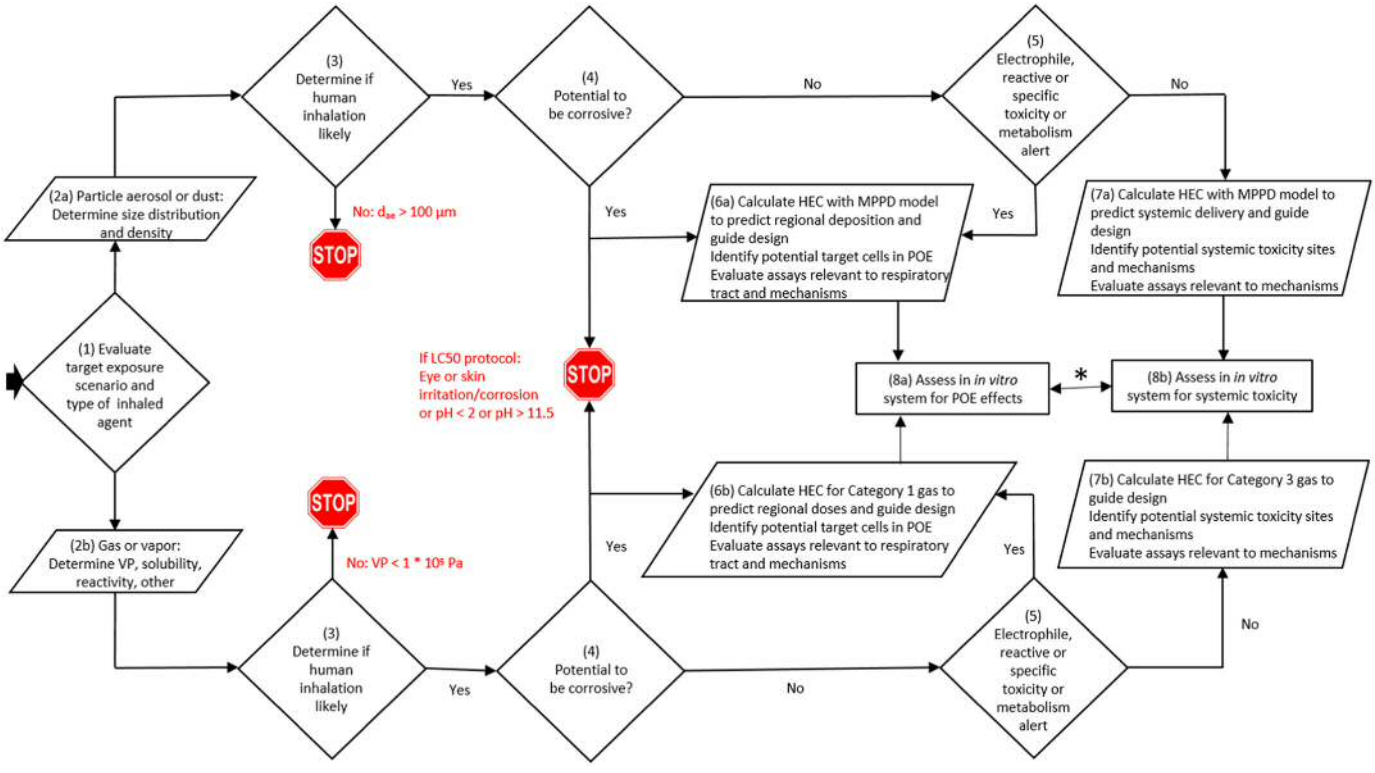

Fig. 3. Decision tree that can be used to guide consideration of the exposure parameters and design of an integrated strategy for inhalation testing. Results of proof-of-concept testing based on this decision tree, the AOPs in Table 1, and the considerations in Table 2 can be evaluated to develop guidance on classification and other inhalation toxicity applications. The decision tree does not supplant expert judgement but is intended as conceptual structure to help identify considerations for experimental design.

Where:

The HEC is the human equivalent concentration corresponding to the POD of the experimental system (*e.g.*, either the *in vivo* study for interspecies extrapolation or an *in vitro* system). The point of departure is derived from dose-response analysis such as benchmark dose (BMD) modeling (US EPA, 2012b) to describe a relevant response measure representing a key event or adverse outcome; the ADJ represents an adjustment used to align the experimental exposure regimen with the objective human exposure scenario.

The dosimetric adjustment factor (DAF) for particles is either the regional deposited dose ratio (RDDR) or the regional retained dose ratio (RRDR) and constructed using factors such as the particle size distribution, exposure-specific ventilation rate and experimental flow rate; and resultant deposition fractions in the region of predicted or observed toxicity in the human or animal respiratory tract and the *in vitro* system. Additional normalizing factors used to construct the dose metric should capture relevant mechanistic processes, for example, using a normalizing factor to express the deposited fraction relative to the surface area of the respiratory tract associated with the toxicity or specific effector cells (Jarabek et al., 2005; Kuempel et al., 2015). The DAF for gases is the regional gas dose ratio (RGDR) and is constructed similarly by considering determinants of ADME and normalizing for mechanistic factors related to the dose metric associated with the pathobiology in either the POE or systemic tissues.

The choice of dosimetry model to predict either the particle or gas DAF used to derive the HEC ranges from default to sophisticated model structures as described (US EPA, 1994; Jarabek, 1995b; US EPA, 2012a; Kuempel et al., 2015; Clippinger et al., 2018). Construction of the RDDR or the RGDR depends on the level of detail and specificity of the available data, the degree of understanding of ADME and the mechanism of toxicity, and should be commensurate with the biological level of organisation of the key event or the TSE. Duration adjustment across exposure times may be obviated if a dosimetry model is used to simulate the exposure scenarios and describe dose metrics; otherwise the default “C x t” adjustment and similar approaches, or more specific adjustments based on mechanisms (*e.g.*, use of peak concentration or area under the curve as the internal dose metric) is used (ten Berge et al., 1986; US EPA, 1994; Jarabek, 1995a; Rhomberg, 2009). The confidence in the type of dosimetry model used will affect confidence in the translation of resultant *in vitro* data to the HEC and resultant risk estimate. For example, the uncertainty factor applied to the HEC to derive an estimate would be larger when using a default algorithm than when using a sophisticated structure that addresses critical parameters mechanistically (US EPA, 1994; Kuempel et al., 2015). Uncertainty factors may also be applied to account for intra-human variability (*e.g.*, the general population includes both adults and children).

Fig. 3 legend

(1) *Evaluate target exposure scenario and type of inhaled agent:* Target scenario includes parameters that characterize specific population considerations (*e.g.*, general population, children, or occupational), regimen (*e.g.*, number of days per week), and duration (*e.g.*, hours per day and work week or lifetime). Evaluating the type of inhaled agent requires assessment of exposure generation and characterization. Target scenario may also include consideration of problem formulation or objective of the testing, including the information requirement (*e.g.*, identification of intrinsic hazard properties of chemicals or end-use products; hazard communication (*i.e.*, classification; product labeling statements/signal words); industrial hygiene (*e.g.*, short-term exposure levels or time-weighted averages); and emergency response (*e.g.*, egress or re-entry levels).

(2) *Determine particle size distribution and density:* a) Conversion of diameter to aerodynamic diameter (d_{ae}) or mass median aerodynamic diameter (MMAD) may be required (US EPA, 1994). b) Additional physicochemical properties will help determine likely dosimetry, such as molecular diffusivity; blood:air partition coefficients; reactivity including hydrolysis or to serve as an enzymatic substrate for ADME in respiratory tract tissue; or contains structural alerts indicative of inherent reactivity (*e.g.*, if the chemical contains an electrophilic center such as a carbonyl carbon in an aldehyde).

(3) *Determine if human inhalation is likely:* Human is presumed target population; if evaluating or translating an *in vivo* animal study would require consideration of that species' anatomy and ventilation parameters. It may be possible to avoid testing if the aerodynamic diameter of the particle is > 100 μM (Vincent, 2005).

(4) *Potential to be corrosive:* All available existing information that may be informative should be evaluated, including QSAR, read-across, and data from *in vitro* assays. Evidence of skin or eye irritation or severe pH could be used to justify waiving an LC_{50} study (OECD, 2014; OECD, 2017a); however, this generalization does not apply to all chemicals as some agents with a high/low pH are not corrosive, and not all corrosive agents will possess a high acute toxicity.

(5) *Electrophile, reactive or specific toxicity or metabolism alert:* If molecular structure (*e.g.*, chemical contains an electrophilic center such as the case with an activated ester or aldehyde) indicates likelihood of specific reactions such as hydrolysis or specific metabolism by cells in respiratory tract, then characterization of POE toxicity is necessary in addition to systemic toxicity.

(6) *Calculate HEC for effects in portal of entry (POE):* Human equivalent concentration (HEC) is calculated with different models for particles and gases; and for respiratory *versus* remote or systemic effects. The choice of model also depends on data availability and ranges from default to sophisticated model structures. The HEC calculations should use input parameters relevant to target exposure scenario and potential mode of action in the respiratory tract. a) For particles, the MPPD model is recommended and can predict regional (extrathoracic, tracheobronchial, or pulmonary) deposition to aid study design. b) For gases likely to react with tissues in the respiratory tract, these are designated as gas Category 1 (described in section 4b above and Table 5 of (Clippinger et al., 2018)) and default algorithms are applied or models such as CFD or hybrid PBPK-CFD models can refine dose predictions. As indicated by the asterisk (*), if potential for both POE and systemic toxicity exists (*e.g.*, systemic distribution of particles or components and gases in Category 2) both types of toxicity must be addressed (See Steps 8a and 8b). Guidance on the choice of model structure and necessary data are provided in (US EPA, 1994; US EPA, 2012a; Clippinger et al., 2018).

(7) *Calculate HEC for systemic toxicity:* Human equivalent concentration (HEC) is calculated with different models for particles and gases; and for respiratory *versus* remote or systemic effects. The choice of model also depends on data availability and ranges from default to sophisticated model structures. The HEC calculations should use input parameters relevant to target exposure scenario and potential mode of action for systemic toxicity, or possibly both POE and systemic toxicity. a) For particles, the MPPD model is recommended and can predict regional (extrathoracic, tracheobronchial, or pulmonary) deposition to aid study design. b) For gases likely to cause systemic toxicity, these are designated as gas Category 3 and default algorithms are applied, or PBPK-CFD models can refine dose predictions. As indicated by the asterisk (*), if potential for both POE and systemic toxicity exists (*e.g.*, systemic distribution of particles or components and gases in Category 2), both types of toxicity must be addressed (See Steps 8a and 8b). Guidance on the choice of model structure and necessary data are provided in (US EPA, 1994; US EPA, 2012a; Clippinger et al., 2018).

(8) *Assess in in vitro system for POE effects or systemic toxicity*: a) Assess in *in vitro* system, such as a three-dimensional human reconstructed human lung tissue model, for POE effects including respiratory irritation. See Supplementary Table 1 for potential cell systems of interest. b) Assess in *in vitro* system, such as cardiac, liver, or neuronal cells, for systemic toxicity. As indicated by the asterisk (*),

consideration of dosimetry and mechanisms may require testing in both types of systems.

Results of proof-of-concept testing based on this decision tree, the AOPs in Table 1, and the considerations in Table 2, can be used to inform the development of guidance for acute inhalation toxicity classification and other applications. Case study examples will be useful to further the development and optimization of testing approaches that will be fit for regulatory decision-making (*e.g.*, hazard classification and risk assessment). The following hypothetical case studies exemplify how the above decision tree could be applied to outline a testing strategy to assess acute inhalation toxicity. These case studies demonstrate the utility of the decision tree for evaluating relatively simple, well-defined scenarios. However, as noted above, many real-world human exposures are to mixtures of multiple substances and thus hypothetical cases should also consider complex mixtures such as cigarette smoke, cosmetic aerosols, fragrances, or air pollution. Such substances may require additional assessment that addresses the whole aerosol as well as individual components. For consumer products, further detailed information on tissue dose, cellular damage, and systemic effects would better inform risk assessments of potential effects from relatively low level cumulative exposures. Some specific considerations for the development of case studies to address different exposure scenarios and various inhaled agents are provided below.

a. Pesticide applicator (occupational exposure to particle)
   (1) *Evaluate target exposure scenario and type of inhaled agent* → Pesticide applicator in agricultural field setting exposed to a particulate aerosol
   (2a) *Determine particle size distribution and density* → MMAD and geometric standard deviation (GSD), density (ρ)
   (3) *Determine if human inhalation is likely* → Yes, the aerodynamic diameter of the particle is < 100 μm.
   (4) *Potential to be corrosive* → No
   (5) *Electrophile, reactive or specific toxicity or metabolism alert* → No
   (7a) *Calculate HEC with MPPD model to predict dose for systemic delivery and guide design* → The MPPD simulation should use the duration of exposure corresponding to an occupational scenario (*versus* parameter for the general population – see below) including the following:
      Anatomy and physiology: Adult male anatomy with ventilatory activity pattern that includes oro-nasal breathing mode at high ventilation rate due to exertion (see (International Commission on Radiological Protection (ICRP), 1994) and (US EPA, 1994) for activity patterns and rates). The ventilation rate and breathing mode differences will affect the deposition pattern in the respiratory tract, and thereby change the predicted deposition pattern and internal systemic dose.
      Exposure duration: Use a scenario specific to applicators (*e.g.*, 12 h spraying operation); otherwise an occupational work day default is 8 h per day (US EPA, 1994).
      Exposure regimen: Frequency of operation would dictate the regimen (*e.g.*, 12 h spraying operation, 5 days/week for a growing season; otherwise, the default for a "working lifetime" is 40 years (US EPA, 1994)) and can be considered as a conservative approach.
      Dose metric: Dose delivered to respiratory tract or specific regional surface area normalized to body weight.
   (8b) *Assess in in vitro system for systemic toxicity* → Evaluate assays that query KEs relevant to the substance's mechanism of toxicity in systemic target tissues. For example, hepatotoxicity, *in vitro* basal cytotoxicity for non-neural targets, or acetyl cholinesterase assays for neurotoxicity of a specific chemical class.

b. Reactive aldehyde gas
   (1) *Evaluate target exposure scenario and type of inhaled agent* → General population and gas category 1.
   (2b) *Determine particle size distribution and density* → Calculate molecular diffusivity using chemical structure as input to species-specific CFD model to predict mass transfer, estimate a mass transfer coefficient, or use a default extraction (US EPA, 1994; Hanna et al., 2001)
   (3) *Determine if human inhalation is likely* → *Yes*
   (4) *Potential to be corrosive* → *No*
   (5) *Electrophile, reactive or specific toxicity or metabolism alert* → Yes
   (6b) *Calculate HEC for extrathoracic, tracheobronchial, or pulmonary region effects of gas category 1* → Use CFD or hybrid PBPK model or default algorithm to estimate flux fraction to specific region and normalize that to surface area of region as the dose metric for the HEC calculation. CFD and PBPK models can predict more localized delivery to specific cell types. The simulation or default algorithm for HEC calculation should address the following:
      Anatomy and physiology: Adults with general population ventilation rate.
      Exposure duration: Specify the duration based on the acute exposure assessment need. Emergency response derivations range from 10 min to 24 h (Vincent, 1995); an acute reference concentration (ARFC) is derived for 1, 4, 8, and 24 h of exposure (Vincent, 1995); 8 h is the default for occupational exposures (US EPA, 1994) and the US EPA Risk and Technology Review (RTR) program uses 1 h (US EPA, 2009).
      Exposure regimen: Depends on the anticipated frequency of exposure.
      Dose metric: Dose delivered normalized to regional surface area (extrathoracic, tracheobronchial, or pulmonary regions) or specific cell type.
   (8a) *Assess in in vitro system for POE effects* → Evaluate *in vitro* assays relevant to mechanisms in the respiratory tract; for example, three-dimensional reconstructed human tissue models can be used to assess respiratory irritation (see Supplementary Table 1).

c. VOC gas
   (1) *Evaluate target exposure scenario and type of inhaled agent* → General population and gas Category 3.
   (2b) *Determine particle size distribution and density* → Determine blood:air partition coefficient and absorption fraction using PBPK model or use default algorithm to predict absorbed fraction (US EPA, 1994)
   (3) *Determine if human inhalation is likely* → Yes
   (4) *Potential to be corrosive* → No
   (5) *Electrophile, reactive or specific toxicity or metabolism alert* → No
   (7b) *Calculate HEC for systemic effects of gas Category 3* → Use PBPK model or default algorithm to estimate either tissue delivery normalized to tissue weight or systemic extraction normalized to body weight as the dose metric for the HEC calculation. PBPK models can predict more specific tissue doses such as those that address metabolism. The simulation or default algorithm for HEC calculation should address the following:
      Anatomy and physiology: Adults with general population ventilation rate.
      Exposure duration: Specify the duration based on the acute exposure assessment need. Emergency response derivations range from 10 min to 24 h (Vincent, 1995). An acute reference concentration (ARFC) is derived for 1, 4, 8, and 24 h of exposure (Vincent, 1995); 8 h is the default for occupational exposures (US EPA, 1994) and the US EPA RTR program uses 1 h (US EPA, 2009).
      Exposure regimen: Depends on the anticipated frequency of exposure
      Dose metric: Systemic blood concentration or dose delivered to remote target tissue
   (8b) *Assess in in vitro system for systemic toxicity* → Evaluate assays that query key events relevant to the substance's mechanism of

toxicity in systemic target tissues. For example, use organ-specific cell lines or primary cells, organ-chips, or a general cytotoxicity assays (*e.g.*, the 3 T3 neutral red uptake assay) in a weight-of-evidence approach. The purpose of testing (*e.g.*, regulatory requirement or in-house screening) may influence the choice of cell system and endpoints assessed.

5. Discussion and recommendations

This review summarizes the current state-of-the-science regarding mechanisms of and assays available to assess acute inhalation toxicity, including how our existing knowledge can be used to design effective non-animal testing approaches. This review was produced in response to a recommendation from a 2016 workshop on "Alternatives Approaches for Acute Inhalation Toxicity Testing to Address Global Regulatory and Non-regulatory Data Requirements" (Clippinger et al., 2018). Another recommendation of the workshop was to use the information from this review to design a proof-of-concept study to further define the underlying mechanisms of toxicity and elucidate how the assays may be used in an integrated approach. Results from the study will be used to refine the strategy and will be useful in continuing to build AOPs for mechanisms of inhalation toxicity.

Moving forward, the following gaps and challenges should be addressed:

- Curate data in a user-friendly database. Data from animal and non-animal tests have been made freely available by NICEATM in its Integrated Chemical Environment (ICE; https://ice.ntp.niehs.nih.gov/). ICE provides access to data for thousands of chemicals for several acute toxicity endpoints including systemic lethality subsequent to inhalation exposure. Often, publicly accessible databases only contain LC_{50} data; however, some stakeholders may collect additional information, such as the physical state of the test material, time to death, histopathology, clinical pathology, and detailed clinical observations. Therefore, NICEATM is working with stakeholders from government, industry, and academia to collate relevant information into ICE, which can be used to build *in silico* models and develop confidence in *in vitro* approaches.
- Evaluate existing and develop new QSAR models. Few QSAR models are currently available that can be applied to predicting inhalation toxicity. Modellers should interrogate the applicability domain of any QSAR (e.g., TOPKAT, REACH *Across*, and MultiCASE) to identify whether the model might be optimized for predicting inhalation toxicity. This could be accomplished *via* a partnership between modellers and companies that can provide test compounds. Such an approach could be used to identify advantages and gaps of existing models and clarify which models could be used for specific applicability domains. Additionally, data collected in ICE and other sources could be used to develop new models. The U.S. Environmental Protection Agency National Center for Computational Toxicology's Chemistry Dashboard (https://comptox.epa.gov/dashboard/) could serve as a convenient platform to house available models and their predictions. Importantly, government funding must be allocated for *in silico* model development and evaluation. It will also be important to ensure the variability associated with the *in vivo* data being relied upon for new model development is carefully evaluated so that the uncertainties associated with the model predictions can be quantified. A public-private partnership involving > 50 participants is currently developing best practices protocols for *in silico* method development relevant to a wide range of endpoints (Myatt et al., 2018).
- Advance mechanistic dosimetry models for IVIVE. Advancing model structures to address the range of physicochemical characteristics and compiling reliable input data on critical parameters (*e.g.*, exposure profiles, physicochemical properties, ventilation activity patterns, cell characteristics, and ADME) will facilitate quantitative IVIVE to support decision-making and assessment purposes. Mechanistic models are necessary to describe inhalation dosimetry to predict target human exposures as well as kinetic characteristics for different test systems. Such models should be available as open access to facilitate their application. Data to support evaluation of dose metrics to describe the TSE at various levels of observation (*e.g.*, subcellular) are necessary to characterize MIE and KE in various AOPs.
- Develop and share AOPs. There are numerous AOPs relevant to inhalation exposures (Table 1). However, these AOPs need to be further developed and additional AOPs must be added to specifically address outcomes that are likely following inhalation exposures. Identification of key events can aid the development of AOPs and is critical to construction of IATAs. AOPs can be used to identify appropriate *in silico* and *in vitro* tests to be used in an IATA to assess a substance's likelihood of causing a specific adverse outcome and build confidence in the use of *in vitro* assays to characterize the key events. Researchers should collaborate on the development of AOPs; for example, through the use of the AOP Wiki, which was created to provide an interactive and virtual platform for AOP development and to promote international consensus on the developed AOPs. It is particularly important that subject matter experts connect with AOP experts so that these pathways include relevant and necessary information in the proper format. Funding must also be made available to researchers developing AOPs and conducting *in vitro* testing to fill in knowledge gaps.
- Optimize *in vitro* test systems. Numerous systems can be used to assess the toxicity of inhaled test substances (Supplementary Table 1); however, optimization of the systems is needed. For example, although not unique to the inhalation route of exposure, there is a need to characterize the ability of cell-based systems to metabolize compounds, because metabolism can influence toxicity. Although *in vitro* test systems lacking metabolic capacity can effectively screen for biological effects of the parent chemical, the pharmacokinetic relationship between exposure and concentration at a target site needs to be evaluated. In particular, the metabolic activity of three-dimensional tissue and lung-on-a-chip models should be characterized. These models are considered to represent human biology, but cannot be uniformly representative unless they can account for metabolic activity. Standardized test protocols must also be developed to promote consistency across laboratories. Experimental designs should consider dosimetry and human-relevant exposure conditions. Inter-disciplinary collaboration, for example between experts in exposure science and tissue culture, should be incorporated into activities surrounding protocol development. In addition to the acute inhalation toxicity workshop discussed in this paper (Clippinger et al., 2018), an example of cross-sector collaboration is the recent workshops hosted by the Institute for *In Vitro* Sciences, specific to inhaled tobacco products. These workshops have included industry, academic, government, and non-governmental organisations to discuss the development, standardization, and harmonization of *in vitro* methods for next generation tobacco product and e-cigarette testing (Behrsing et al., 2016; Behrsing et al., 2017).
- Design and test integrated approaches. Use of the above information—existing data, AOPs, non-testing approaches, and *in vitro* assays—will be needed to design a comprehensive testing approach for acute inhalation toxicity. To ensure all needs are met and to facilitate implementation, experts with diverse expertise (*e.g.*, *in vitro* and *in vivo* inhalation toxicology, computational modeling, exposure science) from different sectors (regulatory, non-regulatory governmental organisations, industry, academic, and non-governmental organisations) should collaborate on the design of these approaches. A key step in this process will be proof-of-concept testing focused on a specific chemical space, such as agrochemicals, air toxics, tobacco, or pharmaceuticals, coupled with elucidating

key events of the pathogenesis for a specific AOP, which will be useful both to obtain toxicity data and to demonstrate the validity of this approach. The knowledge gained from this effort can then be applied to the design of testing strategies for other substances, as there will be similarities and differences in the approaches used for different chemistries. A list of reference chemicals can be developed based on the available *in vivo* data (rat, human, and/or other species) so that non-animal approaches can be retrospectively validated, while keeping in mind that non-human test results often do not reflect what happens in humans. Results from the proof-of-concept testing can be used to standardize test protocols and further develop AOPs.

The development, implementation, and global regulatory acceptance of non-animal approaches for acute inhalation toxicity testing is an ambitious but attainable goal, with success necessitating collaboration among diverse stakeholders. The PETA International Science Consortium and NICEATM are coordinating working groups to accomplish the tasks above and researchers who wish to become involved in these activities are encouraged to participate. Success in this area will produce models and test systems capable of predicting both acute lethality and local effects caused by inhalation exposures. These new approaches have the potential to better protect human health by using 21st century science rooted in contemporary understanding of human mechanisms of toxicity without using animals.

Disclaimer

The views expressed in this article are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency, the Defense Threat Reduction Agency, or the US Army. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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Appendix A. Supplementary data

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