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Respiratory bioavailability and comparative toxicology following inhalation of pesticide aerosols.

Background

An aerosol may be defined as a suspension of particles, which may be either solid or liquid, whilst dispersed in air or gas. Whilst aerosols may be of natural origin, there is a large focus on anthropogenic aerosols particularly concerning their; pharmaceutical applications for drug delivery, negative effects as environmental pollutants and also risks associated with occupational exposure. With regards to the risk of aerosols in an occupational exposure setting, the study of their physicochemical properties and biological effects once aerosols are inhaled is a critical field of research, particularly with regards to various industrial processes. It is estimated that over 10,000 different chemicals are in commercial production (Timbrell, 1999, Lee and Kacew, 2012), whilst only a modest percentage of these chemicals will be aerosolised, from a toxicological perspective the number of respirable xenobiotic aerosols produced remains vast. Within the agricultural industry many of the aerosols produced contain plant protection products (PPPs), which are pesticides used to protect crops or other valuable plants. PPPs contain at least one active substance (synthetic chemical, plant extract or micro-organism), that will protect the plant before or after harvest, or destroy undesired or harmful organisms e.g. weeds, fungi, insects, mites or molluscs. It has been estimated that within the EU approximately 400,000 tonnes of PPPs were sold in 2014 (Rani and Shanker, 2017), with a considerable fraction of these likely to have been applied as aerosols. Whilst the use of aerosols allows the efficient dispersion of PPPs, it also increases the risk of unintentional inhalation in an occupational setting by exposed workers as well as bystanders.

Current risk assessments required by the European Food Safety Authority (EFSA) for the inhalation of pesticide particles in an occupational/bystander setting, must assume that 100% of the inhaled dose is absorbed into the systemic circulation (European Food Safety, 2014). This conservative assumption is likely to be an overestimate and is not driven based on scientific evidence due to the current lack of biological *in vitro* or predictive *in silico* models for respiratory transepithelial bioavailability accepted by regulatory bodies such as EFSA. Despite this the assumption that 100% of inhaled pesticides are absorbed into systemic circulation is unlikely due to the potential for; exhalation of inhaled pesticides, non-absorptive clearance, local metabolism and a variety of other protective processes.

Whilst the alveolar region of the respiratory tract is highly efficient at the absorption of compounds due to its large surface area, thin layers and highly perfused tissues, the majority of the larger aerosol droplets/particles are likely to deposit in the upper respiratory tract.

It has been suggested that with exception of highly volatile pesticides such as those used as fumigants, a significant proportion of pesticides inhaled as aerosols do not reach the alveolar region and are instead deposited in the upper respiratory tract, swallowed via mucociliary clearance and contribute to oral absorption (Ye et al., 2013, Aprea, 2012). Pesticides deposited in the upper respiratory tract are likely to have a much lower bioavailability due to the relatively low surface area and protective mucociliary clearance in this region. Furthermore, whilst pesticides cleared by this mechanism may contribute to oral absorption, it is generally well accepted that oral absorption is often less than 100% (Dean and Ma, 2007, Woollen, 1993), with regulatory bodies such as EFSA more readily accepting oral absorption values derived from *in vivo* and *in vitro* toxicology data (European Food Safety, 2014). In contrast, within the pharmaceutical industry from the perspective of drug delivery, it is well established that most actively inhaled aerosols generated by Dry Powder Inhalers (DPI's) and Metered Dose Inhalers (MDI's) do not lead to 100% systemic absorption (Forbes et al., 2011, Tronde et al., 2003). Furthermore, there is greater value placed on characterising the physicochemical properties of the particular aerosol, especially those that will affect deposition in the lungs, and subsequent dissolution and absorption, e.g. aerodynamic diameter, drug lipophilicity and solubility.

Similarly, in the field of environmental toxicology it is also accepted that whilst air pollutants do have widespread health effects through both acute and chronic exposure, the extent of deposition in the lower respiratory tract and systemic absorption following transepithelial permeation, is in some part related to the size of the particulate matter (PM) and the physicochemical properties of the pollutant, rather than just estimating 100% systemic absorption of inhaled particles (Kelly and Fussell, 2015, Samoli et al., 2016, Dreij et al., 2017). Subsequently, with environmental toxicology studies there is also significant emphasis on the development and characterisation of appropriately sensitive lung cell lines so as to model and accurately predict toxicity *in vivo*, as researchers seek to accurately assess the effects that anthropogenic aerosols and various air pollutants will have within the lungs (Jarvis et al., 2018, Klein et al., 2017).

Despite the variations in different fields of respiratory research, there is a need for a reliable *in vitro* model that may aid the characterisation of respiratory absorption of aerosols. In addition to the focus on respiratory transepithelial bioavailability of these pesticide aerosols, another focus is the comparative toxicology within different regions of the respiratory tract, which must be considered for human exposure assessments.

Furthermore, the data obtained from this project may be used alongside complementary modelling approaches, to aid the development of a predictive *in silico* model (including the use of computational fluid dynamics), to predict not only particle deposition but also key clearance mechanisms and *in situ* absorption in regions of the respiratory tract selected to be most relevant to occupational exposures, as part of a physiologically-based pharmacokinetics (PBPK) model.

Although this work primarily focuses on pesticide exposure, the research is likely to be highly relevant to the inhalation of a variety of aerosols so that the implications may relate to environmental/occupational exposure, as well as pharmaceutical drug delivery.

Existing regulations and biological models of aerosol inhalation.

The research project focuses on understanding respiratory transepithelial bioavailability and comparative toxicology, in relation to occupational risk assessments required by the European Food Safety Authority (EFSA). Despite the relevance to EFSA regulation, due to the project's general focus

on the inhalation of aerosols, the research is highly relevant to various regulatory bodies such as the Environment Agency (which is affiliated with the Department for Environment, Food and Rural Affairs) and the Medicines and Healthcare products Regulatory Agency (MHRA), with an overall need for biological/computational models of respiratory transepithelial bioavailability and toxicology. There is significant overlap between occupational, environmental and pharmaceutical regulation, in terms of either respiratory exposure/drug delivery predictions. Subsequently, there is considerable need for the development of biological models which may be used to assess or predict aerosol deposition, disposition, metabolism, excretion (absorptive/non-absorptive clearance) and toxicity within various regions of the respiratory tract.

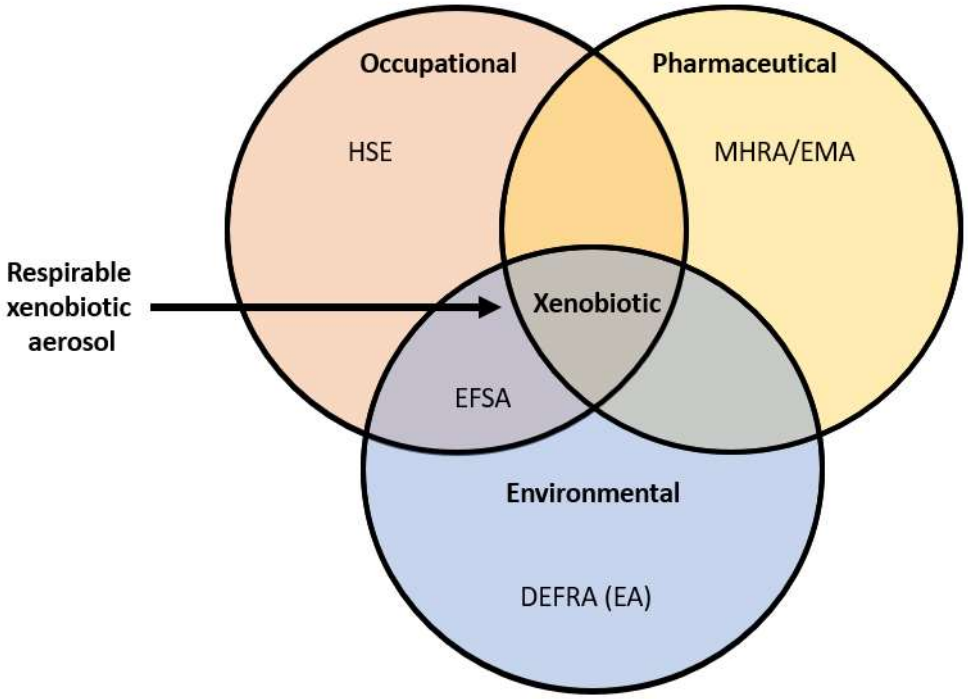


Figure 1. Overlap between different regulatory bodies interest in respiratory absorption and toxicity, following aerosol exposure/drug delivery.

Contrastingly, models for dermal and oral bioavailability are better characterised for occupational exposure/pharmaceutical drug delivery, e.g. *ex vivo* skin models, Caco-2 and PAMPA assays.

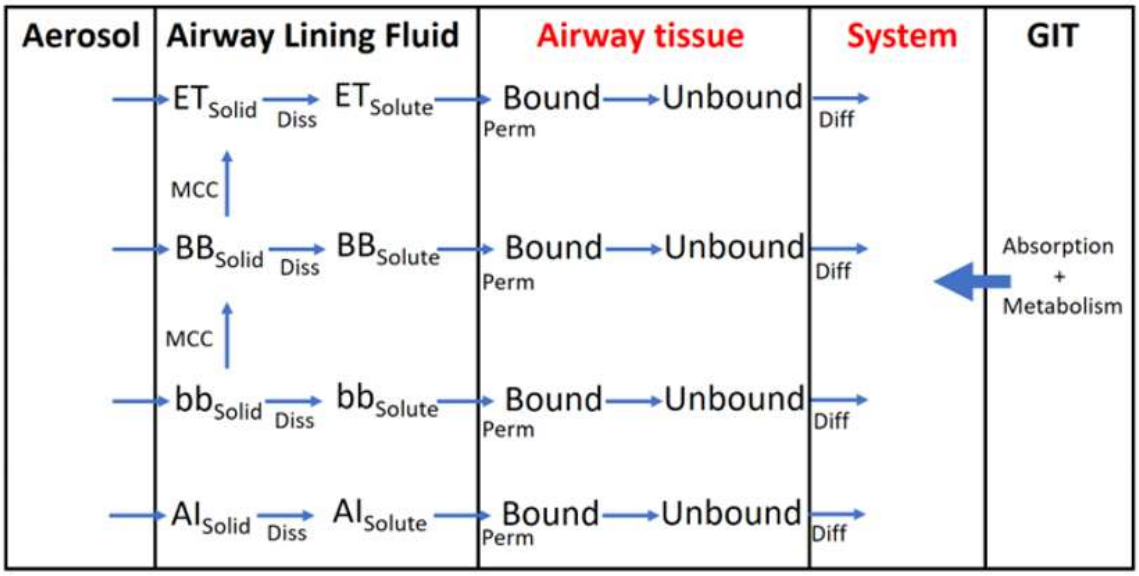
However, despite models such as MucilAir™, isolated perfused lung (IPL) and multiple respiratory cell lines, there is a lack of standardised model for transepithelial bioavailability. The issue is confounded by the highly variable morphology of the different regions of the respiratory tract, including the variations in function.

Another limitation is the lack of focus on nasal inhalation, despite this being a well-known site of absorption particularly due to the high density of capillaries in the nasal region (Alagusundaram, 2016). Additionally, nasal inhalation may represent a more likely route of passive exposure in comparison to oral inhalation/"mouth-breathing". Whilst this is useful for *in vivo* toxicology studies

using rodents, as rats and mice are obligate nose breathers, with regards to *in vitro* studies local bioavailability and toxicity specifically in the nasal cavity is rarely investigated for respiratory occupational exposure assessments. Although the use of *in vivo* toxicology studies for this purpose, is both highly useful and well established, there are a number of differences between the rodent and human respiratory system (Matute-Bello et al., 2008, Vanoirbeek et al., 2010). Additionally, as fields in biomedical research try to replace, refine and reduce the number of animals used, developing physiologically relevant *in vitro* models of respiratory permeability and toxicology becomes increasingly important. Furthermore, the use of this data to develop a predictive *in silico* model, also advances the future effort to aid efficiency and reduce time and resource consumption in the traditional 'wet-lab' setting.

Finally, one of the greatest limitations is aerosol deposition, with regards to aerosolised pesticides the size of the liquid droplets is generally quite large e.g. 50-200µm, and therefore very unlikely to reach the deep lung/lower respiratory tract to be 100% absorbed systemically (Ross et al., 2001). Subsequently, the bioavailability and toxicity in other regions of the respiratory tract become a more important focus, as dose and aerosol deposition in these regions will determine the extent of the pesticide that is absorbed systemically (Bäckman et al., 2017). Additionally, whilst the geometric diameter of particles is useful, the aerodynamic diameter may be more important and other considerations include; molecular weight, vapour pressure and water solubility (Whiteaker and Prather, 2003). There are many factors that may influence the properties and overall fate of the inhaled aerosol, however there is evidence that even the low molecular weight and highly water soluble pesticides rarely exceed 70% inhalation uptake (Ross et al., 2001). Furthermore, the volatility of commercially used pesticides varies considerably and this is also likely to effect the extent to which aerosols may be inhaled and absorbed (Coscollà et al., 2013, Sauret et al., 2008). Despite these limitations, generally there are well established parameters for measuring aerosol deposition after inhalation, such as measuring the emitted or delivered dose, total lung dose or lung deposition pattern. Deposition modelling may be used to link the aerosol deposition to the biological models through dose, this includes considerations about the aerosol formulation, volatility and composition. Experimental modelling options also include the introduction of aerosols directly to organotypic or tissue models.

After estimating aerosol deposition, other considerations include xenobiotic release and solubility, non-absorptive clearance (e.g. mucociliary clearance, alveolar macrophages and metabolism), absorptive clearance, tissue retention and local/lung concentration. As shown in **Figure 2**, these processes can be modelled in different regions of the lung, including the extrathoracic region (e.g. nasal cavity, pharynx and larynx), the large airways such as the trachea and bronchus, smaller airways such as the bronchioles, and the alveolar interstitial tissue region that is often termed the 'deep lung'. Data derived from these studies may then be used in mechanistic *in silico* models for simulating both respiratory and systemic exposure after inhalation, current *in silico* models include Gastroplus ADRM™, PK-SIM™ and SimCyp Simulator™ (Backman et al., 2017).



Al= alveolar interstitium, BB= large conducting airways, bb= small conducting airways, ET= Extrathoracic compartment, Diff= diffusion, Depo= deposition, Diss= dissolution, MCC = mucociliary transport, Perm= permeation

Figure 2. Outline of the respiratory absorption model. Adapted from Backman et al. (2017).

Proposed research methods

A total of 7 pesticides have been chosen as illustrative examples, these compounds have varying physicochemical characteristics e.g. molecular weight, LogP, aqueous solubility and volatility. Additionally, the range of pesticides chosen include those with both high and low dermal bioavailability, established *in vivo* metabolism pathways and various degrees of rodent *in vivo* respiratory toxicity (including two known respiratory irritants).

As recently highlighted by Backman et al. (2017), the respiratory tract may be divided into 4 key regions; extra-thoracic, thoracic, bronchiolar and alveolar interstitial. Subsequently, it is proposed to identify and perform the studies using cell lines relevant to these regions, starting with Calu-3 as a model of the bronchiolar region. Calu-3 has been suggested due to being a well-established and commercially available physiologically relevant cell line, that forms tight junctions and has key transporter proteins required for permeability assays (Bäckman et al., 2017).

Although studies will primarily focus on permeability and toxicity assays, the study will also assess mucociliary transport, dissolution in mucus and lung fluid, permeation, tissue binding and metabolism.

Permeability assays will include the testing of various pesticide concentrations across the apical layer to the basolateral layer of Calu-3 cells in a Transwell® plate. Additionally, transepithelial electrical resistance studies will also be performed.

Toxicity assays, will initially focus on generating dose-response curves using the MTT assay as a measure of cytotoxicity, and depending on these results later investigation of pesticide exposure in relation to oxidative stress, inflammation and irritation.

Following initial toxicity and permeability assays using Calu-3, additional cell lines will be identified and used to model toxicity and permeability in other relevant regions of the respiratory tract. The permeability assays may be supplemented with western blot protein analysis of potential 'drug' transporters, that may be present and or induced in the chosen cell lines.

Metabolite identification studies will be performed to see if any of the relevant respiratory cell lines used, produce any previously characterised or unknown metabolites, with this contributing to non-absorptive clearance of the pesticides. Whilst the use of Calu-3 is well established for the previously described assays, BEAS-2B is a commonly used bronchial cell line for metabolism studies and therefore is also likely to be included (Ehrhardt et al., 2008).

In addition to the metabolism studies, chemical stability assays will also be performed in biologically relevant fluids, such as simulated lung fluid, so as to identify any likely degradation of the pesticides once inhaled but prior permeation across the airway tissue.

As well as investigating metabolism within the respiratory tract as a route of non-absorptive clearance, mucociliary clearance may also be investigated in cell lines representing the small and large conducting airways.

Finally, in order to validate any *in vitro* models developed, *in vivo* or *ex vivo* studies may be performed. This may include the isolated perfused lung model, in order to measure absorptive clearance. The industry standard rodent nasal inhalation model may also be used, with this allowing for the assessment of deposition and pharmacokinetics studies, with the latter providing absorption, disposition, metabolism and excretion data, as well as the possibility of histopathology.

Research outcomes

Alongside aerosol deposition predictions derived from the complementary *in silico* modelling approaches, key regions of the respiratory tract will be selected and physiologically relevant models of transepithelial bioavailability and comparative toxicology will be developed. These models, may be useful in risk assessments for exposure to future pesticides developed for widespread use within the agricultural industry or may find use as models for drug delivery within the pharmaceutical industry. The outcomes of this research will hopefully be of some use for assessing potential respiratory effects following exposure to aerosols, with regards to an environmental, occupational or pharmaceutical viewpoint. Furthermore, by focusing on a range of key regions and processes within the respiratory tract, the immediate implications of this research may aid improved regulatory guidelines by EFSA for pesticide exposure assessments. Currently as EFSA estimate that 100% of inhaled pesticide aerosols are systemically absorbed, which does not consider many of the non-absorptive clearance mechanisms, this research is likely to provide a more accurate estimation of respiratory bioavailability, ideally providing more logic-based estimations similar to those currently available for oral and dermal bioavailability predictions.

Conclusion

In conclusion, currently research into the effects of respiratory inhalation of pesticide aerosols during occupational exposure is limited. More specifically, *in vitro* bioavailability and toxicity models of the respiratory tract, could be further developed and used both for occupational, environmental and pharmaceutical purposes, so as to assess the biological effects of a range of inhaled aerosols. Whilst the research is highly relevant to both governmental regulatory bodies and also within various private industrial settings, the immediate implications of this research are likely to be applicable to risk assessments required by EFSA for occupational exposure to pesticides.

Overall, there are many factors that must be taken into consideration when studying the biological effects of respirable aerosols such as; introducing the appropriate aerosol fractions to the models, establishing doses of relevant concentration and durations of exposure. Additionally, the use of different deposition models also highlights the challenges in linking predicted dose to lung toxicology and inhaled xenobiotic bioavailability. Nevertheless, by estimating absorption, local toxicity and various clearance mechanisms, valuable information on the biological fate of respirable aerosols may be gained. Furthermore, the experimental data generated from this could be used to inform and develop *in silico* deposition and PBPK models.

Ultimately, this research will aid the identification of *in vitro* and *in silico* models that evaluate the likely fate of aerosols once inhaled, an area of research that is highly relevant to a number of scientific disciplines with far-reaching implications.

About the applicant

Before starting my PhD research, I completed my Biochemistry BSc at King's College London, with the final year research project researching genotoxicity in two different alveolar epithelial cell lines. Having been generously awarded a student bursary by the UK Environmental Mutagen Society, I continued this research, which has recently been published, listing me as a co-author.

It was this experience that gave me the understanding to be able to appreciate the broad implications of aerosol science, and the determination to research the toxic effects of respiratory exposure to aerosols.

I subsequently completed a Toxicology MRes degree at the University of Birmingham, with the research project being completed within industry, focusing on *in vivo* DMPK (Drug Pharmacokinetics and Metabolism) studies, in addition to a range of *in vitro* cytotoxicity assays. Having completed this project, I was fortunate enough to be able to present this work as a poster at the Drug Metabolism Discussion Group 2017 open meeting.

As well as these experiences, extracurricular activities such as part-time work and volunteering have also helped me hone a wider range of interpersonal skills that will be essential as part of my career in research, including organisation, teamwork and creative problem solving.

Based on my previous experience, I am highly motivated to complete this research as part of my PhD and am confident that I have the skills required to strive towards making this ambitious and exciting research project a success.

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