How to Keep Breathing – The Future of Inhaled Medication Testing

The delivery of therapeutics by inhalation is a medical process dating back thousands of years. The first written evidence of this can be found ca. 1550 BC in Egypt, where vapours of the black henbane plant were used to treat patients with breathing difficulties.[1] We now know this vapour likely contained anticholinergic chemicals similar to those now used in modern inhalers to reduce inflammation and bronchoconstriction in the airways.

Despite major advances over the past three millennia in the treatment of pulmonary disease patients with inhaled medications, there is still no single therapy that controls the underlying lung disease, only temporary, symptom management-based approaches. This presents a significant opportunity for the development of novel medications which can be used to treat – and ultimately, cure – diseases that areresponsible for the deaths of approximately 8 million people per year.[2]

Delivering drugs through the pulmonary route has several benefits. It is non-invasive, with relatively little metabolic activity compared to other therapeutic routes. Our lungs also provide a large, thin surface area with direct access to the whole systemic bloodstream. This means that as well as being the optimal route for drugs directly targeting the lungs, it is also a rapid and simple way to transport drugs to other target organs.

However, the lung presents many hurdles that must be overcome for the successful delivery of a compound to the target cell or bloodstream. Understanding these challenges is key for the design of inhaled medications, not only to ensure drugs are delivered to the site of action, but also that they remain efficacious relative to their role. Current models for measuring the ADME (absorption, distribution, metabolism, excretion) properties of compounds through the lung are limited due to lack of predictivity, high cost and time. However, new human-relevant techniques are emerging which are changing the face of preclinical testing. Organ-on-a-chip (OOC) technology, also known as microphysiological systems (MPS), is a key innovation which can be used to reduce our reliance on traditionally used animal testing, by providing a physiologically-relevant and clinically translatable model that is both time and cost effective. Engaging with these new technologies will allow more analytical and rapid understanding of the multi-faceted journey of inhaled medications.

Breathe In

The first, and potentially most inhibitory challenge in a drug's ability to deliver results is the physical act of inhalation from the device into the patient's lungs. At this stage, a high proportion of the drug can already be lost due to poor technique, including lack of shaking the inhaler, or poor inhalation, which can lead to drug being swallowed rather than inhaled into the lungs. Recent innovations in inhaler technology, including spacers and even smart inhalers connected to smart phones, have enabled more effective medication delivery to tackle this initial inefficiency.[3]

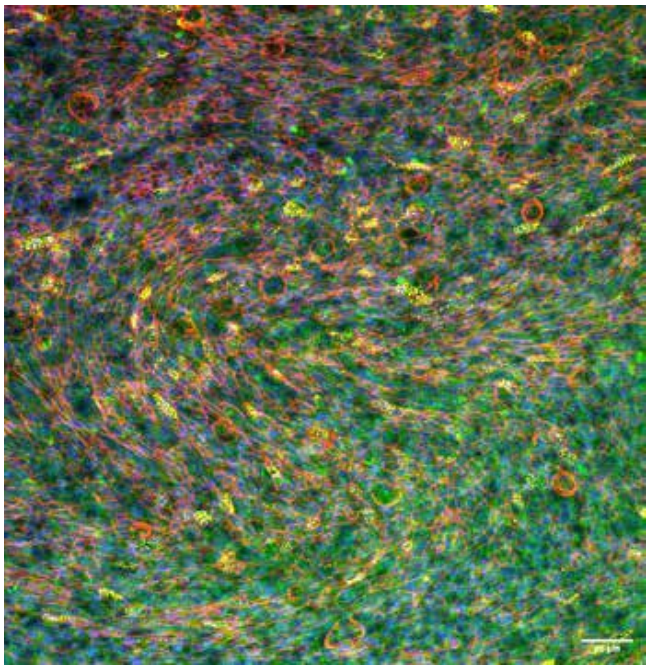

Figure 1. The bronchial epithelium of bronchial lung MPS cultured in the CN Bio PhysioMimix™ OOC. Cilia are shown in yellow, mucus in green, cytoskeleton in red and nuclei in blue. Image by Emily Richardson.

The next challenge is understanding where, in the lungs, a particle will be deposited. Ultimately, this depends on its size and chemical properties. Smaller particles will be deposited into the distal lung (smaller bronchioles and alveoli) whereas larger, heavier particles will deposit in the proximal lung (trachea and bronchi). Therefore, if the drug is designed to target bronchial function – such as a bronchodilator – then a larger particle (>5 µm diameter) will be advantageous, whereas if the drug is designated to act elsewhere in the body – such as insulin – the particle size should be smaller (1–5 µm) to allow it to reach the alveoli and then bloodstream. Any particle less than 1 µm would likely not be dense enough to settle and would instead be suspended in the air and exhaled.

Dissolution – The Race to the Epithelium

Depending on where the drug lands along the pulmonary tree, the next influencing step is the dissolution of the drug into lung fluid. In the airways, a 10 µm layer of viscous liquid covers the epithelium, the top layer of which is a gel-like layer of mucus. Any inhaled molecule therefore needs to dissolve and diffuse through these layers before being absorbed by cells. At this rate-limiting step, the drug is at risk of being eliminated from the lung by mucociliary clearance. Cells in the epithelium contain cilia on their cell surface which rhythmically beat, moving the mucus layer up and out of the airways. This is a good system for clearing unwanted invaders from the lung,

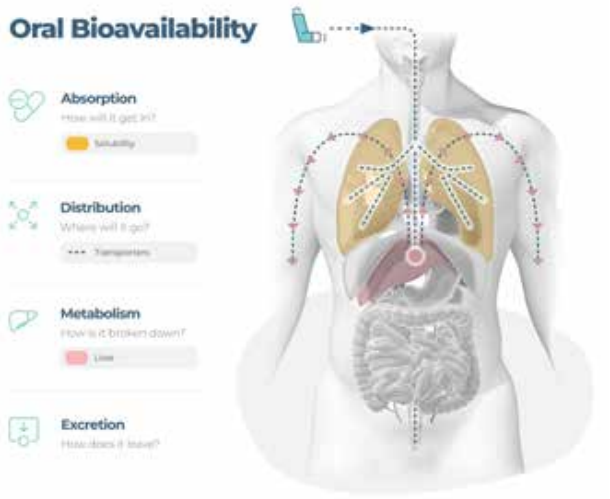

Figure 2. ADME routes of inhaled medications through the body. Drug is inhaled into the lungs where it absorbs into the bloodstream and distributes around the body. It is then metabolised by the liver and excreted.

such as environmental particles or pathogens, however not so useful for drug delivery.

In contrast to other regions of the lungs, if a drug reaches the alveoli, there is no mucus or ciliary bodies to inhibit its journey to the epithelium. The alveolus is an extremely thin (0.1–0.3 µm) cell barrier with a small layer of non-sticky, immobile liquid. Therefore, in comparison to the thick bronchial airways, the alveolar passage is (on paper) an easier way of gaining access to the bloodstream. However, alveoli do contain alveolar macrophages, the resident immune cell within the lung, that patrol around the 500 million alveoli, engulfing any foreign material they come across. Alveolar macrophages will engulf and try to digest drug compounds, however, any that are indigestible can remain sequestered within lung macrophages for years.

Permeability and Absorption – It's All About the Chemistry

Taken together, the quicker an inhaled medication can dissolve into the lung fluid the more likely it is to reach its target before being eliminated or sequestered. So which drug type is best placed? One would assume a highly water-soluble (hydrophilic) molecule would quickly dissolve into fluid and reach the epithelium. However, a hydrophilic molecule, once it reaches the cell surface, will not absorb as rapidly into cells. In a rat model study comparing compounds with a range of chemical properties, it was broadly found that hydrophilic molecules have an absorption half-life into the lung of around 60 minutes.[4] Lipophilic compounds, which do not dissolve well in aqueous solutions but will interact with lipids – like those in the cell membrane – were found to have a lower half-life of around 60 seconds.

Most drugs require absorption into or across the epithelium to reach their site of action. The permeability of the barrier is influenced by several factors such as the thickness of the epithelium, cellular phenotype, the number of intercellular junctions and types of transporters expressed on the cell surface.[5] Molecules can move, or be moved, across the lung epithelium in several manners. Very small hydrophilic compounds can travel passively through the junctions between cells – this is called the paracellular route. Small lipophilic molecules can passively diffuse through cells via their interaction with the cell lipid membrane – the transcellular route. Accordingly, these small molecules are absorbed quickly, as described previously.

In some cells, particularly alveolar type I cells, drugs can be transported across and into the endothelium via vesicle-mediated mechanisms. The drug is encapsulated by an extension of the cell membrane and is brought into the cell interior as a vesicle, which is then transported across the cell and deposited in the opposite manner from the cell membrane on the endothelial side. Larger or more complex compounds can be actively transported across cells using specific protein transporters (e.g., organic cation transporters OCT-1). Transporters are expressed at different levels on specific cell types within the lung, which affects the rate a drug may be transported in particular sections of the lung.[6]

Whilst transiting the epithelium, certain drugs can become "trapped" within cells in vesicles, mitochondria or lysosomes due to the lower pH inside cells. Non-ionised, basic drugs (usually lipophilic) which enter the cell easily are protonated by the more acidic environment, and thus unable to diffuse back across the cell membrane. Highly lipophilic drugs may also become sequestered in the cell membrane, resulting in longer retention within the lung.

Overall, despite the lung being a relatively simple and rapid way to administer drugs, careful consideration must be given to the chemistry of the drug with respect to the requirements of time of onset, longevity and organ-targeting. The 'correct' chemistry of the drug can be predicted, however only until it is tested in a biological system can the full implications of the chemistry be understood.

How to Predict the Complex

The challenges described for designing a drug for pulmonary administration result from the complex biology and physiology of the human lung. Currently, drugs are required to be tested on animals for preclinical testing, particularly for toxicity testing. This includes mice, rats, rabbits, dogs and non-human primates, which are exposed either in whole-body tubes (small rodents) or are restrained and exposed through head or mouth pieces (larger mammals). However, there are many differences between human and animals respiratory tracts, including lung anatomy (architecture and cell type distribution), physiology (manner and rate of breathing), immunology (cell types and distribution) and biology (expression of transporters and enzymes).[7] This diversity has resulted in an extremely

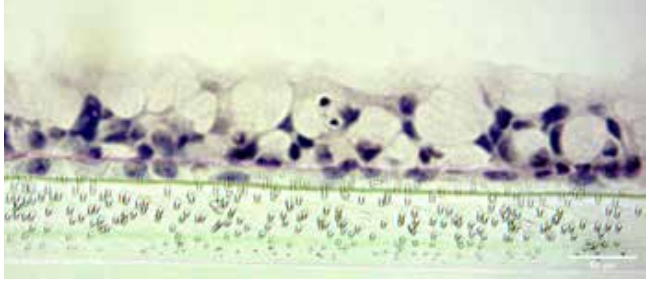

Figure 3. The alveolar epithelium of the alveolar lung MPS cultured in the CN Bio PhysioMimix™ OOC. Tissues were sliced and stained with H&E. Image by Emily Richardson.

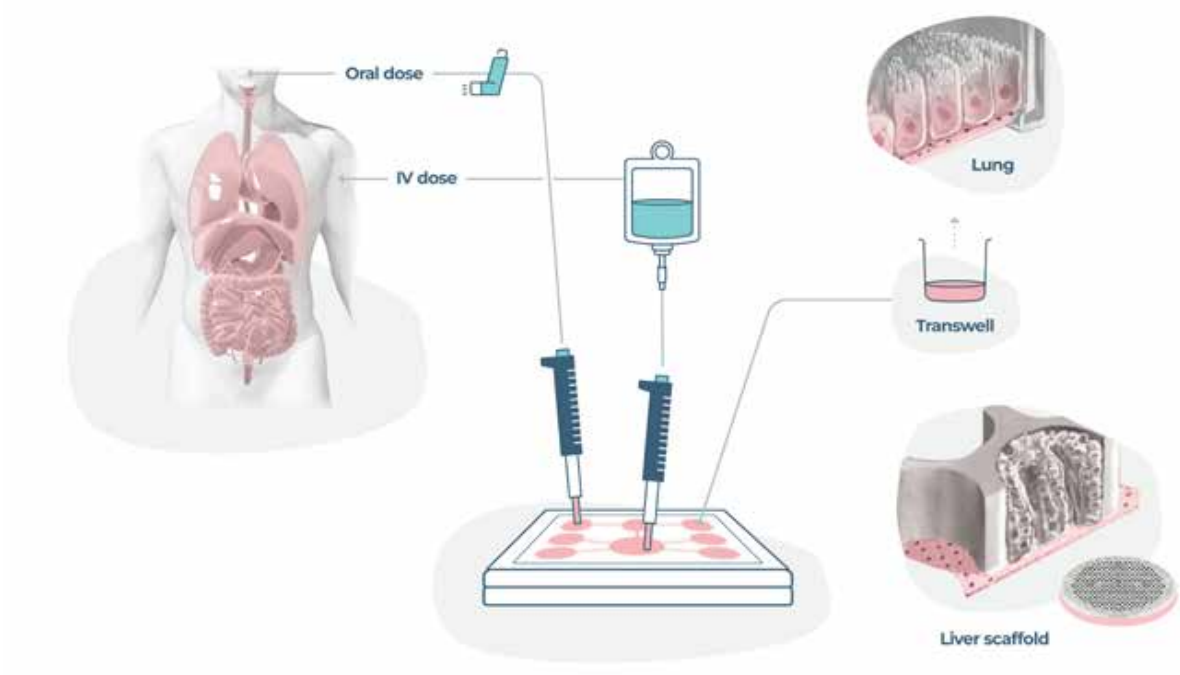

Figure 4. Fluidically linked multi-organ MPS allows modelling of the biodistribution of inhaled medications and organ-organ crosstalk.

high attrition rate of pulmonary medications, with only 3% of drugs reaching the market compared to 6–14% for other disease therapeutics.[8] As testing *in vivo* can take many years and millions of dollars, the current investment in drug development is very risky.[9,10]

However, more than ever before there is a clear desire by regulatory agencies to reduce the requirement for animal testing by using new alternative methods (NAMs) such as advanced *in vitro* models, systems biology and mathematical modelling. This year, the US House of Representatives passed the FDA Modernisation Act which aims to reform the drug approval process by driving the use of NAMs.[11]

Traditional *in vitro* models of cultured lung cell lines are not best placed for this replacement. Cell lines such as Calu-3, 16HBE14o- (bronchial) and A549 and NCI-H441 (alveolar) have been used for a variety of applications. However, despite them each being able to mimic an aspect of lung biology, they are not able to replicate the full depth and complexity of the complete human organ. As an example, in air-liquid-interphase cultures Calu-3 can form a cell layer and secrete mucus, so can be useful for the study of dissolution of drugs. However, there are disparate reports of cilia production ability (or lack thereof) and responses to stimuli.[12,13] Without the full spectrum of bronchial cell phenotypes, translating the results of a drug study from cell line cultures to patient outcomes can become challenging.

Recently, more sophisticated models of *in vitro* cell culture have been employed. The increased availability of primary and stem cells has allowed production of more human relevant cultures. Use of OOC/MPS technologies further enhance the physiological relevance of primary or stem cell cultures by replicating the human lung microenvironment through media perfusion to mimic blood flow, stretch conditions and the inclusion of multiple lung cell types.[14] Multiple MPS models are now available that recapitulate the airways or alveoli. They demonstrate more relevant lung anatomy through the inclusion of relevant cell types, functionality (such as production of mucus or surfactant) and physiologically relevant permeability, thus allowing more informed decision making about the safety and efficacy of a therapeutic.

The Future of Pulmonary Drug Testing

So how can these novel approaches replicate the intricacies of pulmonary drug delivery to predict outcomes in preclinical testing? A collective, multi-disciplinary approach will be crucial. By combining lung MPS cultures and anatomically accurate drug impactor technologies the biological relevance of *in vitro* exposures can be maximised.[15,16,17] These novel engineering and bioengineering technologies can be combined or used independently to understand a variety of factors, including deposition, rate of absorption and permeabilisation of drugs through the lung epithelium, toxicity and pharmacology.

A selection of MPS providers offer the ability to link different organ MPS together into multi-organ systems that allow organ-crosstalk studies, for example to understand the metabolism of drugs by the liver, or interactivity of immune cells between organs.[18,19,20] Technologies that offer recirculating fluidic flow, designed to mimic the bloodstream, also enable components of the immune system to be incorporated into lung MPS models. This enables the study of immune interactivity between organs or specific immune responses to drugs. As many new drugs are based on immune-based therapies, these areas are becoming increasingly important to understand and reliably test in the preclinical stages.

A good example of where *in vitro* MPS offer clear advantages over *in vivo* testing is the understanding of inhaled drug deposition and dissolution. As previously mentioned, animals, such as mouse, have a significantly different lung

physiology and biology to humans[21] As well as these obvious disparities, practically measuring deposition and dissolution in animal models is challenging, and often requires an animal to be sacrificed at regular timepoints to obtain tissues which are subsequently analysed for drug abundance. As well as a distinct waste of animal life, it is extremely costly and time-consuming.

Furthermore, understanding the exact localisation of a drug is problematic in animal models. Upon isolation of tissue, often the whole lung tissue is homogenised for analysis, meaning drug concentration differences between lobes, areas and cell layers are undistinguishable.[22] *In vitro* studies using MPS and aerosolisation technologies not only allow human relevant drug exposures, but are also able to detect precise drug localisation, receptor specificity, absorption in cells and permeability through the barrier over time. Drug accumulation and release can be monitored over longer periods of time by repeated sampling in the same culture, making for more cost-effective testing which can be replicated both technically and biologically. Different cell donors can also be used in MPS to generate a "clinical trial-like" study to understand reactions of people of different genders, ethnicities, ages and disease states.

Over the past decade, development of MPS technologies has accelerated to the point where many different options are available for drug developers and regulators to evaluate. Their focus should now be firmly set on how these *in vitro* systems can best replace, or at the least reduce, animal testing in terms of i) predictivity ii) biological relevance iii) ability to test different parameters iv) ease of use v) cost and vi) turnaround for fast-track decision making earlier in the developmental pipeline. Regulators such as the FDA and the European Medicines Agency (EMA) will be key players in the adoption of NAMs for use in ADME and toxicology studies, and many have already begun to evaluate these systems for use in predictive preclinical studies.

In summary, the pulmonary route of drug delivery is a route vastly unappreciated and under-utilised. There are a plethora of applications for inhaled medications which are not limited

to pulmonary diseases. However, with the rise of respiratory diseases from environmental pollution and novel pathogens (such as SARS-CoV-2), rapid development of pulmonary medications will inevitably be required. Current preclinical animal models are clearly not sufficiently predictive of human responses, as evidenced by high drug attrition rates, therefore novel methods, such as MPS, must be brought to the forefront. The adoption of these state-of-the-art techniques will not only save countless animal lives, researcher time and money, but also revolutionise and accelerate drug development, to better the lives of millions of patients.

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