

Application of biological models to inform research and development of inhalation products

Advancements in *in vitro* methodologies provide more options in drug development and could reduce the need for animal testing.

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Introduction

There has been significant progress and research activity within the field of inhalation therapy over the past decade but only a handful of new inhaled products have successfully progressed to market over the past five years [1, 2]. This is due to the complexity that is inherent in the development of inhaled formulations, where the respiratory system presents a unique challenge in terms of its complex anatomical structure, physiological and biological properties compared to other organs.

To establish relevant biological models for the development of inhalation products, it is essential to understand the unique and complex network of cells, structures and mechanisms that make up the respiratory system. The human respiratory system begins at the nasal cavity and follows through the pharynx and larynx to the series of sub-dividing airways with diminishing diameter from the trachea, through the bronchi and bronchioles and finally to the alveolar ducts and sacs, the sites of gas exchange with a rich bed of systemic vasculatures [3]. There are significant differences between the cells located at the different regions of the airways (trachea to the bronchioles), and the nasal cavity compared to the alveolar epithelium. The airways are composed of pseudostratified columnar epithelium containing goblet-secreting mucus cells and ciliated cells that constitute the mucociliary clearance mechanism of the lungs. In the alveoli, the columnar epithelial cells are replaced by pneumonocytes, the broad and thin type 1 and type 2 cells and alveolar macrophages. Therefore,

when selecting biological models for testing inhalation products, careful consideration must be made regarding the area of the respiratory tract that is the target site for a product. As an example, nasal cells and cell lines can be selected for products that aim to deliver locally to the nose and brain, whereas bronchial cells can be selected for testing products that aim for localized delivery to the airways, and for products to be delivered systemically, alveolar cell lines can be selected.

Secondly, the respiratory tract has several biological and physical barriers that can limit product uptake (transport, absorption) and subsequent efficacy (Figure 1) [4]. Following inhalation of an aerosol drug, the particles will first encounter a mucus barrier of varying thickness lining the conducting airways down to the alveolar regions [5]. This impaction and deposition process will subsequently create a concentration gradient across the mucus barrier that will help drive the drug across the epithelium. The epithelial cells that line the airways from the nasal region down to the alveolar region form tight junctions, which affect drug uptake and therefore therapeutic efficacy. These pulmonary processes are determined, or at least influenced, by one or more aspects of the inhaled drug, including physicochemical characteristics of the drug, the drug formulation and the inhalation device.

Figure 1

Epithelial cell transport mechanisms of inhaled products

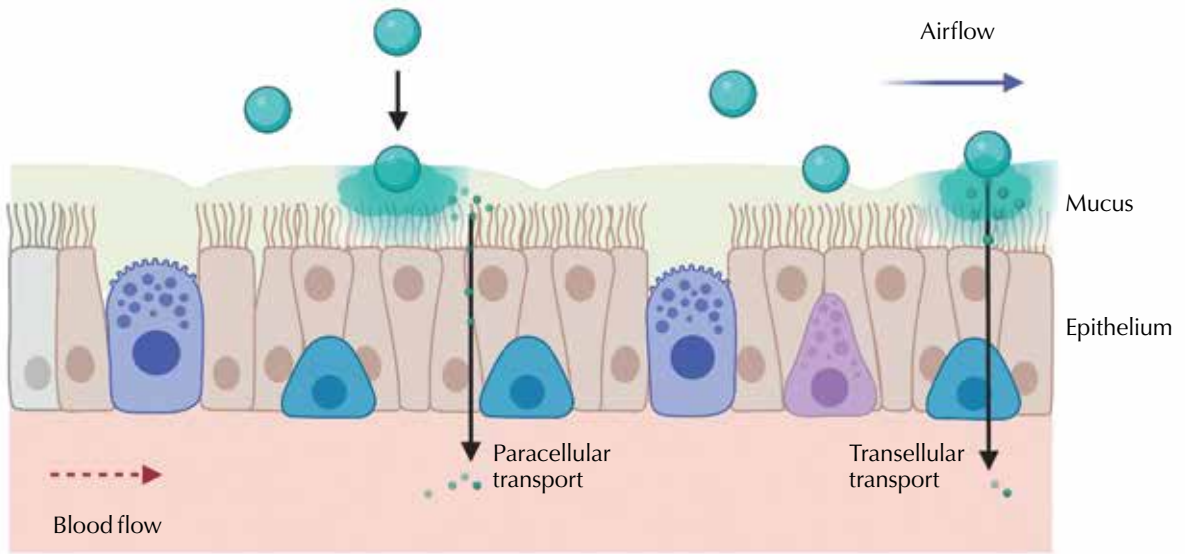
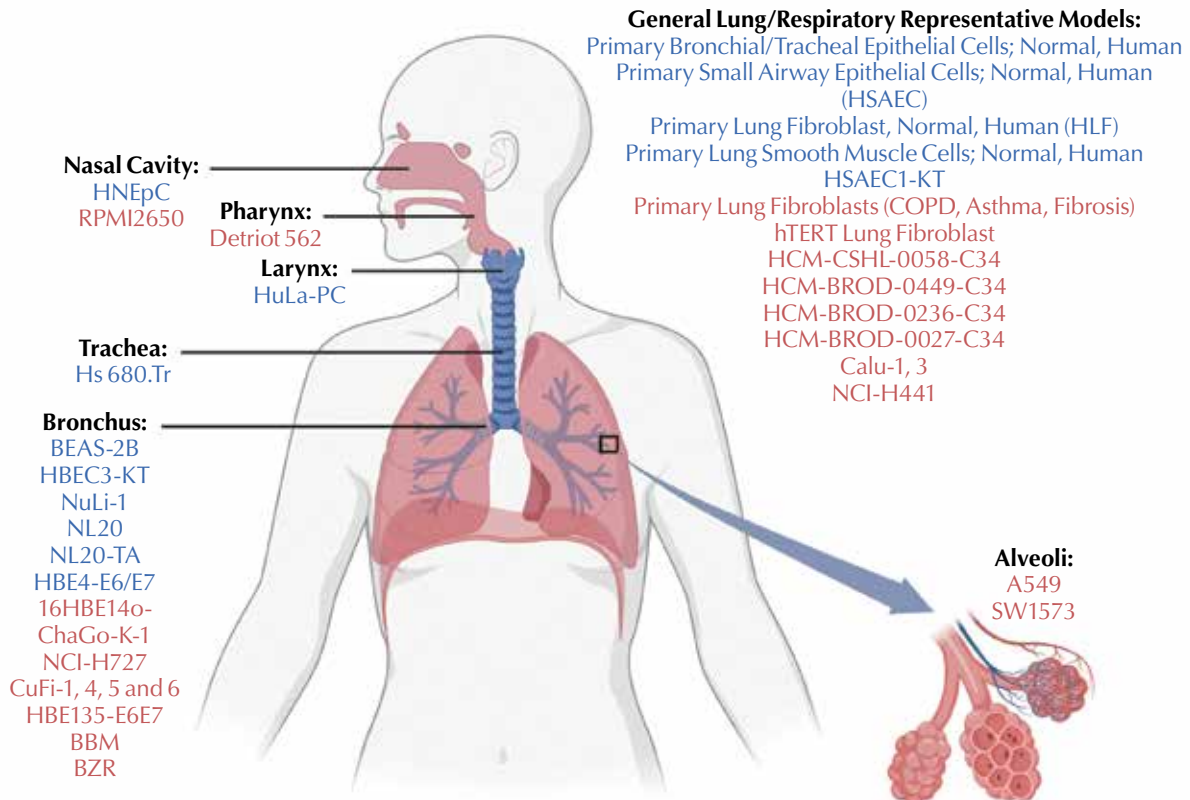


Figure 2

Commercially available cellular models to target the respiratory system. The models listed in blue are extracted or derived from normal (healthy) subjects and the models listed in red are from diseased subjects.



Physiologically relevant *in vitro* conditions for biological characterization of inhaled products

Currently there are several cellular models that are available commercially (Figure 2), that can be used for *in vitro* testing of inhalation products targeting the respiratory system. These include immortalized cells lines that can proliferate indefinitely through passaging and primary cells that are freshly isolated directly from donor organs that have a finite life span. These cells and cell lines can be extracted and established from healthy human tissue or from specific disease states. Figure 2 highlights the models that can currently be purchased from the American Tissue Culture Collection (ATCC) and are representative of the specific regions of the respiratory tract epithelia cells or fibroblasts. They are color-coded based on the tissue origin, either normal (blue) or diseased (red) subjects. In addition, Table 1 summarizes some referenced examples of their use in inhalation studies. For instance, among the cell lines in red, the CuFi models are derived from a patient with cystic fibrosis and therefore can be used as a model for therapeutic inhalation products targeting cystic fibrosis.

Once the appropriate cellular models are selected, there are several cell culture techniques that can be utilized to mimic the physiologically relevant conditions for testing of inhalation products. Biological characterization of a product includes tests aimed to conduct a complete assessment of the product when exposed to the respiratory system. These include testing whether the product induces any adverse effects on the respiratory cells, such as cytotoxicity effects of the product (viability assays), inflammation and oxidative stress (addition of inflammatory mediators or oxidants and adding therapy to look at pro/anti-inflammatory and pro/antioxidant effects), and effect on tight junction formation (measurement of transepithelial electrical resistance) and permeability (sodium fluorescein assays) of the epithelial layer. Testing the efficacy of the product is another part of characterization. Functional assays can be used to test the specific mechanism of action of the drug on the relevant respiratory cells. These include assays that measure downstream effects of the treatment such as enzyme activity (e.g., cAMP assay) or variations in protein expressions. Lastly, cellular models are also particularly useful to determine the mechanisms by which the formulation interacts with the lungs and is absorbed into the systemic circulation by evaluating drug uptake (quantitate intracellular levels), dissolution in the epithelial lining fluid, drug metabolism and transport through the epithelium.

Various cell culture techniques have been developed to carry out these standard biological tests of inhalation products. They can be sub-categorized into 2-dimensional (2-D) and 3-dimensional (3-D) cell culture (Figure 3). The 2-D category includes sub-

Table 1

Usage of cell models in inhalation studies

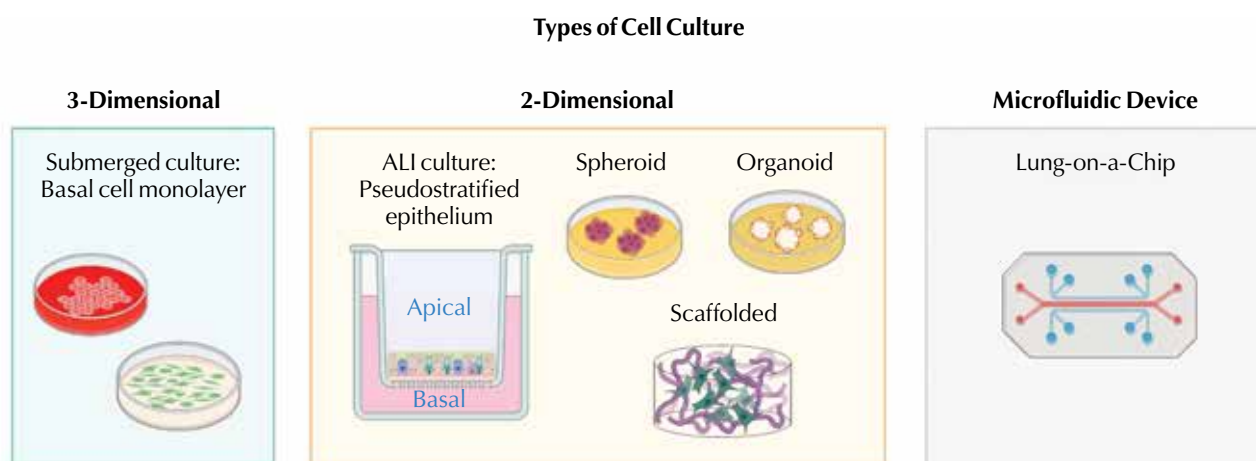
| Cell Line Name | Type | References |
|--------------------|---------------------------|--------------------|
| HNEpC | Nasal epithelial | 6-8 |
| RPMI2650 | Nasal epithelial | 9-11 |
| Detroit 562 | Nasopharyngeal epithelial | 12, 13 |
| HuLa-PC | Larynx epithelial-like | 14 |
| Hs 680.Tr | Trachea fibroblast | 15 |
| BEAS-2B | Bronchial epithelial | 16-20 |
| HBEC3-KT | Bronchus epithelial | 21 |
| NuLi-1 | Bronchus epithelial-like | 22-26 |
| NL20 and NL20-TA | Bronchus epithelial | 27-29 |
| HBE4-E6/E7 | Bronchus epithelial | 19 |
| 16HBE14o- | Bronchus epithelial | 26, 30-32 |
| ChaGo-K-1 | Bronchus epithelial | 33 |
| NCI-H727 | Bronchus epithelial | 34 |
| CuFi-1, 4, 5 and 6 | Bronchus epithelial | 22, 24-26, 35 |
| HBE135-E6E7 | Bronchus epithelial | 20, 36 |
| BZR | Bronchus epithelial | 32 |
| A549 | Alveolar epithelial | 16, 17, 21, 28, 37 |
| SW1573 | Alveolar epithelial | 38 |
| Calu-1 and 3 | Lung epithelial | 27, 31, 39-41 |
| NCI-H441 | Lung epithelial | 42, 43 |

merged culture. In a submerged culture, the cells are grown in a monolayer in media supplemented with growth nutrients. Both primary cells and immortalized cell line can be grown using this type of culture technique. It is ideal for initial cytotoxicity studies of products as it enables high-throughput data output and testing of broad concentration ranges. Additionally, it can be used to determine if the inhalation products have any pro-/anti-inflammatory and pro-/anti-oxidant properties on the specific cell models being tested. These tests are normally used as a starting point for “lead” formulation development and optimization in terms of dosing and determination of efficacy and toxicity prior to performance of more specialized and targeted assays using more physiologically relevant 3-D models.

Air-liquid interface (ALI) cultures

3-D cell culture models (Figure 3) were developed to recreate the spatial organization of tissue. They

Figure 3

***In vitro* cell culture techniques for testing inhaled products**

can be subdivided into scaffold-free, scaffolded and a hybrid of the two [44, 45]. The air-liquid interface (ALI) culture is predominantly used to mimic respiratory tract epithelia *in vitro*. To establish ALI culture, respiratory epithelial cells are seeded in compartmentalized culture systems on a porous membrane (Transwell® inserts (Sigma-Aldrich, St. Louis, MO, US) or Snapwell™ inserts (Sigma-Aldrich, St. Louis, MO, US)), which physically separates the cells from the underlying media. Cells are allowed to attach and proliferate to form of a confluent monolayer, then the culture medium on the apical chamber (Figure 3) is removed. This interface of the cells to the surrounding air forces them to differentiate and generate a pseudostratified epithelium with different cell populations, while from the basal chamber, the cells have access to the culture medium nutrients and additives via diffusion through the porous membrane [46]. When human nasal or bronchial or alveolar epithelial cells are grown on an ALI, ciliated and mucus-producing cells differentiate to take on the proper apical-basal morphology, including functional apical cilia and/or mucous secretion from goblet cells, mimicking the *in vivo* physiology [43, 47-50].

The ALI culture with respiratory epithelial cells provides a powerful and a physiologically relevant tool to test and develop inhalable products. This is due to ALI culturing, which provides a platform to simulate dissolution in the lung because it can mimic important aspects of the biological milieu in the lung such as pH, clearance mechanisms, mucus and residence time. The lungs are covered with a thin mucus layer, which is a gel lining with varying thickness along the conducting airways down to the alveolar regions. In chronic respiratory diseases, this mucus lining may be dehydrated and thickened, further impairing drug dissolution and absorption. Furthermore, surfactant

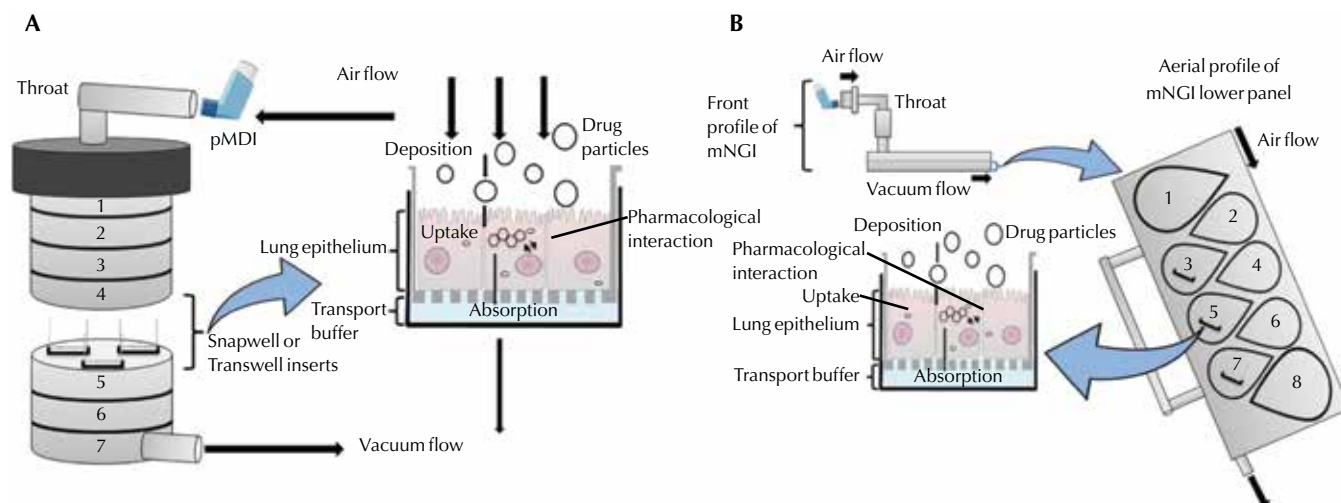
composition within the mucus layer may have a dual role of either facilitating dissolution of inhaled drug such as corticosteroids but more often than not acting as a permeability barrier [51, 52]. The critical fate of inhaled drugs once deposited in the various respiratory regions is therefore often overlooked, resulting in failures of many developmental products in clinical trials reaching the market. Studies such as effects of formulation on epithelial permeability, tight junctions and transport of products through the epithelial layer can be determined using these ALI grown epithelial layers [40, 53]. These studies can elucidate how the product behaves once deposited on the epithelia, by quantitating the levels of the product remaining on top of the epithelia, determining how much product is systematically cleared through the epithelia via paracellular or transcellular mechanisms (Figure 1) and how much permeates inside the epithelial cells.

Scaffold-free models

The scaffold-free system produces spheroids and organoids, which involves cellular aggregation via the process of organogenesis. The spheroids can be generated by using respiratory cellular models (Figure 1) or extracting cells from lung or transbronchial tissue and then applying diverse techniques, such as non-adhesive surfaces, gravity, centrifugation, constant stirring, electric fields, magnetic force or ultrasound, to produce lung spheroid cells (LSCs). Organoids, on the other hand, are derived from human stem cells and patient-derived, induced, pluripotent stem cells (PSCs) that mimic some specific organ functions. A recent example of this are the lung bud organoids that were generated from human PSCs, which developed into branching airway and early alveolar structures after *in vivo* transplantation [54, 55]. The advantage of using scaffold-free models is determin-

Figure 4

Integrated models that assess aerodynamic distribution in the lungs, as well as responses of epithelial layers. (A) Andersen cascade impactor (ACI) modified model (B) Next Generation Impactor (NGI) modified model. Adapted from Cidem, et al. 2020 [4].



ing how well a drug product can permeate through a mass, such as those prevalent in disease states like fibrosis and cancer.

Scaffolded models

The scaffolded models of 3-D cell culture involve using biomaterials to generate structures that can support cell growth and provide a physical structure for cell organization and differentiation. In the context of the respiratory system, the biomaterials are very often components of the extracellular matrix (ECM), hydrogels or commercially available bio-inks such as GelMA (gelatin methacrylate) and PEGDA (poly (ethylene glycol) diacrylate). Recent developments in 3-D bioprinting technologies have enabled the ability to generate biologically accurate structures of the airways onto which cells can be seeded and grown. Scaffolds can simulate cell/ECM and cell/cell interactions. They can also help elucidate ECM and tight cell junctions that influence the cytotoxic effect of inhalable products [56].

Unlike 2-D cell culture, these 3-D cell culture techniques can reproduce some of the hallmarks of *in vivo* models, which can be very beneficial in drug discovery and development studies. However, it is important to note the disadvantages of these models, which include a complex and labor-intensive process, a high degree of variability that makes reproducibility difficult, high costs associated with producing these models and the inability to test aerosolized products.

Microfluidic platforms

A lung- or organ-on-a-chip (Figure 3) is a microfluidic device in which cells are seeded and perfused in a chip-like array. The aim of these devices is to recapitulate the multicellular architectures, tissue/tissue

interfaces, physicochemical microenvironments and vascular perfusion of the lungs, which is not possible with conventional 2-D or 3-D culture systems [57]. They also enable high-resolution, real-time imaging for *in vitro* analysis of biochemical, genetic and metabolic activities of living cells of the lung, making them an ideal tool for pharmacokinetics studies and toxicity tests [58]. To recreate the physiological conditions of the respiratory system, microfluidic platforms with a dual-chamber system were developed to enable the manipulation of air-liquid interfaces and flow conditions [59-61]. Further, these microfluidic devices can be integrated with a computer-controlled vacuum to produce cyclic stretching of the tissue/tissue interface to mimic physiological breathing movements [62]. Generally, the design and the fabrication of the microfluid platforms comprise microchannels made of polydimethylsiloxane (PDMS), sandwiching a semi-permeable membrane, and bonded with two plexiglass covers. In addition, integration with a microsensors system within the devices can enable high throughput screening of novel inhalation therapies. However, challenges with these models include the level of complexity in set up and operation, the requirement for highly trained operators and the inability to test aerosolized formulations.

Integrated models for deposition to study interactions of aerosols with biological barriers in the respiratory tract

Conventional, *in vitro*, cell model testing to evaluate toxicity and efficacy is still performed using a solution or suspensions of test compounds. This may be representative of the environment in organs such as the gastrointestinal tract, brain, kidney and liver. How-

ever, such testing is significantly different than processes that occur after inhalation of “real” airborne aerosols or involve the complex geometry and respiratory mechanics of the lungs. An alternative model with which to study aerosolized inhalation products is the integration of ALI culture models with the pharmacopeia-approved impaction models used to assess lung delivery and nasal expansion chambers to assess intranasal delivery.

For lung delivery, inertial impaction studies are considered the gold standard for *in vitro* assessment of the aerodynamic deposition of inhaled formulations. Such tests are regulatory requirements to estimate the amount of formulation that is delivered to the lungs [63]. Impactors can classify particles according to the aerodynamic diameter of the entire delivered dose [64] and quantitate the delivered amount of the formulation at the various stage of the human respiratory system. The type of impactor to use for analysis depends on the inhalation product tested and can be used on dry powder inhalers (DPIs), nebulizers, pressurized metered dose inhalers (pMDIs) and nasal sprays [65]. The impactors alone, however, cannot determine efficacy, drug uptake, dissolution and transport through the epithelium. To address these parameters, hybrid approaches and modifications have been developed that involve combining the biological representation of the lung epithelium (ALI models) with the impaction instrumentation.

ALI cultured epithelial models generated from cell/cell lines representing the various regions of the lung can be inserted into the respective stages of an impactor to generate a model that not only can assess the regional lung deposition of an aerosolized formulation but also its biological impact. Figure 4 illustrates such an integrated model using the Andersen cascade impactor (ACI) in Figure 4A and the Next Generation Impactor (NGI) in Figure 4B. In Figure 4A, an

ACI stage plate is 3-D printed to enable housing of Snapwell inserts. Similarly in Figure 4B, a printed, modified NGI stage plate to house Snapwell inserts is used to replace the NGI stage. These modified plates can be replaced with an ACI or NGI plate at any of the impactor stages that represent the trachea through the alveoli, depending on the target for the inhalation product, and can be used to evaluate the differential interactions of aerosols with the various regions of the respiratory tract. After the aerosolized dose has been delivered, the Snapwell inserts containing the epithelia can be removed and tests for biological characterization can be conducted [4, 9, 66-68]. Similarly, the pharmacopeia-approved nasal expansion chamber used to assess delivery of nasal spray formulations can also be 3-D printed to include housing for inserts [9].

Co-cultures

Co-culture systems are used to study the fundamental interactions and behaviors between different cell populations. Co-cultures are cell cultivation setups in which two or more different populations of cells are grown with some degree of contact between them. This enables the study of natural interactions between populations and generates a microenvironment (involving cytokines, growth factors and transcriptional regulators) that reflects *in vivo*-like human tissue and can be much more relevant than animal models when performing drug studies [69]. The most popular types of co-culture in respiratory research are bi-cultures and triple-cultures (Table 2). Co-cultures of airway epithelial cells, macrophages, dendritic cells and fibroblasts are instrumental in disease modeling, drug discovery and development of novel therapeutic targets [70].

Table 2

Co-culture types used for inhalation studies

| Type | Description | Examples |
|----------------|--|--|
| Bi-Culture | <ul style="list-style-type: none"> • Uses two cell types • Can be used with submerged culture, ALI, spheroids, organoids and scaffolded culture models • Can be mixed or separated by membrane | <ul style="list-style-type: none"> • A549 cells and fibroblasts spheroids to test anti-cancer nanoparticle delivery [71] • Human bronchial epithelial cells and airway smooth muscle cells to test inflammatory modulation of salbutamol and budesonide [72] • Calu-3 cells grown with bacterial biofilm (<i>P. aeruginosa</i>) to test the efficacy of antibiotic treatment with ciprofloxacin [73] |
| Triple-Culture | <ul style="list-style-type: none"> • Uses three cell types • Generally used with chambered culture with ALI, and spheroid and organoids • Usually, two cell types mixed or seeded with one in a different chamber | <ul style="list-style-type: none"> • A549 or 16HBE14o- epithelial cells co-cultured with macrophages and dendritic cells to study interaction with xenobiotics [74] • Human pulmonary microvascular endothelial cells co-cultured with NCI-H441 cells and differentiated blood monocytes (THP-1) to study inflammation and interaction of nanocarriers [75] • A549 cells co-cultured with macrophages and dendritic cells to test the effects of allergens [76] |

Further considerations

This article discusses options and advancements in *in vitro* methodologies that can be used to mimic *in vivo* conditions during research and development of inhalation products. The development of novel models that are physiologically relevant to the human lung can improve the predictability of pharmacokinetics and pharmacodynamics. They can also accelerate the translation of novel therapies to clinical trials and help provide better health outcomes for patients with chronic respiratory conditions.

In vivo models continue to be the mainstay assessment for inhalable products by virtue of their ability to administer an aerosolized formulation, using the ideal dose, on individual organisms. However, differences in lung anatomy and physiology between humans and animal models, have limited the translation of experimental results from animal studies. Animal models can also be expensive, time consuming to use and have ethical restrictions for use in research studies. Consequently, researchers should be aware of these fundamental principles underlying the use of animals and consider “the 3R’s” of replacement, reduction and refinement. At the same time, the concerns listed above reinforce the unmet need for relevant data collected using *in vitro* respiratory model systems that can be realistically translated *in vivo*.

References

- Petersson, G., Pipeline trends and challenges in pulmonary delivery. *ONdrugDelivery Magazine*, 2018. (92): p. 4-8.
- Sedo, K., et al., Global drug delivery & formulation. *JIP*, 2020. 20: p. 18-23.
- Weibel, E.R., et al., Morphometry of the human lung. Vol. 1. 1963: Springer.
- Cidem, A., et al., Modifying and integrating *in vitro* and *ex vivo* respiratory models for inhalation drug screening. *Front Bioeng Biotechnol*, 2020. 8: p. 581995.
- Haghi, M., et al., Across the pulmonary epithelial barrier: Integration of physicochemical properties and human cell models to study pulmonary drug formulations. *Pharmacol Ther*, 2014. 144(3): p. 235-252.
- Jabba, S.V., et al., Chemical adducts of reactive flavor aldehydes formed in e-cigarette liquids are cytotoxic and inhibit mitochondrial function in respiratory epithelial cells. *Nicotine Tob Res*, 2020. 22(Suppl 1): p. S25-S34.
- Jia, J., et al., Investigation of the impact of PM(2.5) on the ciliary motion of human nasal epithelial cells. *Chemosphere*, 2019. 233: p. 309-318.
- Huang, J., et al., Toxicity of micro(nano)plastics with different size and surface charge on human nasal epithelial cells and rats via intranasal exposure. *Chemosphere*, 2022. 307(Pt 4): p. 136093.
- Pozzoli, M., et al., Application of RPMI 2650 nasal cell model to a 3D printed apparatus for the testing of drug deposition and permeation of nasal products. *Eur J Pharm Biopharm*, 2016. 107: p. 223-233.
- Fallacara, A., et al., *In vitro* characterization of physico-chemical properties, cytotoxicity, bioactivity of urea-crosslinked hyaluronic acid and sodium ascorbyl phosphate nasal powder formulation. *Int J Pharm*, 2019. 558: p. 341-350.
- Gholizadeh, H., et al., Smart thermosensitive chitosan hydrogel for nasal delivery of ibuprofen to treat neurological disorders. *Expert Opin Drug Deliv*, 2019. 16(4): p. 453-466.
- Hotomi, M., et al., Nontypeable *Haemophilus influenzae* isolated from intractable acute otitis media internalized into cultured human epithelial cells. *Auris Nasus Larynx*, 2010. 37(2): p. 137-144.
- Kallio, A., et al., Role of Pht proteins in attachment of *Streptococcus pneumoniae* to respiratory epithelial cells. *Infect Immun*, 2014. 82(4): p. 1683-1691.
- Wang, K., et al., UBR5 regulates proliferation and radiosensitivity in human laryngeal carcinoma via the p38/MAPK signaling pathway. *Oncol Rep*, 2020. 44(2): p. 685-697.
- Soni, S., et al., MAPKAPK2 plays a crucial role in the progression of head and neck squamous cell carcinoma by regulating transcript stability. *J Exp Clin Cancer Res*, 2019. 38(1): p. 175.
- Mostafa, M.M., et al., Glucocorticoid-driven transcriptomes in human airway epithelial cells: Commonalities, differences and functional insight from cell lines and primary cells. *BMC Med Genom*, 2019. 12(1): p. 29.
- Hillyer, P., et al., Differential responses by human respiratory epithelial cell lines to respiratory syncytial virus reflect distinct patterns of infection control. *J Virol*, 2018. 92(15).
- Chen, C.H., et al., Tiotropium/Olodaterol treatment reduces cigarette smoke extract-induced cell death in BEAS-2B bronchial epithelial cells. *BMC Pharmacol Toxicol*, 2020. 21(1): p. 74.
- Little, F.F., et al., Il-9 stimulates release of chemotactic factors from human bronchial epithelial cells. *Am J Respir Cell Mol Biol*, 2001. 25(3): p. 347-352.
- Wei, W., et al., HDAC4 induces the development of asthma by increasing slug-upregulated CXCL12 expression through KLF5 deacetylation. *J Transl Med*, 2021. 19(1): p. 258.
- Murray, J.R., et al., Role of human aldo-keto reductases in the metabolic activation of the carcinogenic air pollutant 3-nitrobenzanthrone. *Chem Res Toxicol*, 2018. 31(11): p. 1277-1288.

22. Sheikh, Z., et al., An *in vitro* model for assessing drug transport in cystic fibrosis treatment: Characterisation of the CuFi-1 cell line. *Eur J Pharm Biopharm*, 2020. 156: p. 121-130.
23. Klar, S., et al., Application of impedance measurement to investigate *in vitro* inhalation toxicity of bacteria. *J Occup Med Toxicol*, 2021. 16(1): p. 32.
24. Sheikh, Z., et al., Tobramycin and colistin display anti-inflammatory properties in CuFi-1 cystic fibrosis cell line. *Eur J Pharmacol*, 2021. 902: p. 174098.
25. Sheikh, Z., et al., Development and *in vitro* characterization of a novel pMDI diclofenac formulation as an inhalable anti-inflammatory therapy for cystic fibrosis. *Int J Pharm*, 2021. 596: p. 120319.
26. Useckaite, Z., et al., Increased extracellular vesicles mediate inflammatory signalling in cystic fibrosis. *Thorax*, 2020. 75(6): p. 449-458.
27. Min, K.A., et al., Functional and cytometric examination of different human lung epithelial cell types as drug transport barriers. *Arch Pharm Res*, 2016. 39(3): p. 359-369.
28. Ruan, T., et al., H5N1 infection impairs the alveolar epithelial barrier through intercellular junction proteins via itch-mediated proteasomal degradation. *Commun Biol*, 2022. 5(1): p. 186.
29. Jamal, M.H., et al., Rapamycin treatment correlates changes in primary cilia expression with cell cycle regulation in epithelial cells. *Biochem Pharmacol*, 2020. 178: p. 114056.
30. Manford, F., et al., Drug permeability in 16HBE14o- airway cell layers correlates with absorption from the isolated perfused rat lung. *Eur J Pharm Sci*, 2005. 26(5): p. 414-420.
31. Forbes, B., et al., Human respiratory epithelial cell culture for drug delivery applications. *Eur J Pharm Biopharm*, 2005. 60(2): p. 193-205.
32. Martinella-Catusse, C., et al., Down-regulation of MT1-MMP expression by the alpha3 chain of type IV collagen inhibits bronchial tumor cell line invasion. *Lab Invest*, 2001. 81(2): p. 167-175.
33. Varsano, S., et al., Cytokines modulate expression of cell-membrane complement inhibitory proteins in human lung cancer cell lines. *Am J Respir Cell Mol Biol*, 1998. 19(3): p. 522-529.
34. Sacewicz, M., et al., Inhibition of proliferation, VEGF secretion of human neuroendocrine tumor cell line NCI-H727 by an antagonist of growth hormone-releasing hormone (GH-RH) *in vitro*. *Cancer Lett*, 2008. 268(1): p. 120-128.
35. Zabner, J., et al., Development of cystic fibrosis and noncystic fibrosis airway cell lines. *Am J Physiol Lung Cell Mol Physiol*, 2003. 284(5): p. L844-L854.
36. Tsai, M.J., et al., Aryl hydrocarbon receptor (AhR) agonists increase airway epithelial matrix metalloproteinase activity. *J Mol Med (Berlin)*, 2014. 92(6): p. 615-628.
37. Shiratori, B., et al., Quinolone-induced upregulation of osteopontin gene promoter activity in human lung epithelial cell line A549. *Antimicrob Agents Chemother*, 2012. 56(6): p. 2868-2872.
38. Puerta, A., et al., Early pharmacological profiling of antiproliferative compounds by live cell imaging. *Molecules*, 2022. 27(16) p. 5261.
39. Harikrishnan, K., et al., Cell derived matrix fibulin-1 associates with epidermal growth factor receptor to inhibit its activation, localization and function in lung cancer Calu-1 cells. *Front Cell Dev Biol*, 2020. 8: p. 522.
40. Almazi, J.G., et al., Protective abilities of an inhaled DPI formulation based on sodium hyaluronate against environmental hazards targeting the upper respiratory tract. *Pharmaceutics*, 2022. 14(7): p. 1323.
41. Ong, H.X., et al., Pharmaceutical applications of the Calu-3 lung epithelia cell line. *Expert Opin Drug Deliv*, 2013. 10(9): p. 1287-1302.
42. Sakagami, M., *In vitro*, *ex vivo* and *in vivo* methods of lung absorption for inhaled drugs. *Adv Drug Deliv Rev*, 2020. 161-162: p. 63-74.
43. Salomon, J.J., et al., The cell line NCI-H441 is a useful *in vitro* model for transport studies of human distal lung epithelial barrier. *Mol Pharm*, 2014. 11(3): p. 995-1006.
44. Jensen, C., et al., Is it time to start transitioning from 2D to 3D cell culture? *Front Mol Biosci*, 2020. 7: p. 33.
45. Langhans, S.A., Three-dimensional *in vitro* cell culture models in drug discovery and drug repositioning. *Front Pharmacol*, 2018. 9: p. 6.
46. Miller, A.J., et al., *In vitro* models to study human lung development, disease and homeostasis. *Physiology (Bethesda)*, 2017. 32(3): p. 246-260.
47. Grainger, C.I., et al., Culture of Calu-3 cells at the air interface provides a representative model of the airway epithelial barrier. *Pharm Res*, 2006. 23(7): p. 1482-1490.
48. Haghi, M., et al., Time- and passage-dependent characteristics of a Calu-3 respiratory epithelial cell model. *Drug Dev Ind Pharm*, 2010. 36(10): p. 1207-1214.
49. Ren, H., et al., An optimised human cell culture model for alveolar epithelial transport. *PloS One*, 2016. 11(10): p. e0165225.
50. Bai, S., et al., Evaluation of human nasal RPMI 2650 cells grown at an air-liquid interface as a model for nasal drug transport studies. *J Pharm Sci*, 2008. 97(3): p. 1165-1178.

51. Sécher, T., et al., Correlation and clinical relevance of animal models for inhaled pharmaceuticals and biopharmaceuticals. *Adv Drug Deliv Rev*, 2020. 167: p. 148-169.
52. Guillon, A., et al., Insights on animal models to investigate inhalation therapy: Relevance for biotherapeutics. *Int J Pharm*, 2018. 536(1): p. 116-126.
53. Ong, H.X., et al., The effects of mannitol on the transport of ciprofloxacin across respiratory epithelia. *Mol Pharm*, 2013. 10(8): p. 2915-2924.
54. Lancaster, M.A., et al., Organogenesis in a dish: Modeling development and disease using organoid technologies. *Science*, 2014. 345(6194): p. 1247125.
55. Ramani, S., et al., Human organoid cultures: Transformative new tools for human virus studies. *Curr Opin Virol*, 2018. 29: p. 79-86.
56. Rijal, G., et al., A versatile 3D tissue matrix scaffold system for tumor modeling and drug screening. *Sci Adv*, 2017. 3(9): p. e1700764.
57. Bhatia, S.N., et al., Microfluidic organs-on-chips. *Nat Biotechnol*, 2014. 32(8): p. 760-772.
58. Lee, J., et al., *In vitro* toxicity testing of nanoparticles in 3D cell culture. *Small*, 2009. 5(10): p. 1213-1221.
59. Gholizadeh, H., et al., Development of a microfluidic nose-on-a-chip for real-time nasal drug assessment. *Respiratory Drug Delivery 2020*, 2020. Virginia Commonwealth University.
60. Ye, Z., et al., An adaptable microreactor to investigate the influence of interfaces on *Pseudomonas aeruginosa* biofilm growth. *Appl Microbiol Biotechnol*, 2022. 106(3): p. 1067-1077.
61. Zhang, Y., et al., Understanding the effects of aerodynamic and hydrodynamic shear forces on *Pseudomonas aeruginosa* biofilm growth. *Biotechnol Bioeng*, 2022.
62. Huh, D., et al., Reconstituting organ-level lung functions on a chip. *Science*, 2010. 328(5986): p. 1662-1668.
63. Rangaraj, N., et al., Insight into pulmonary drug delivery: Mechanism of drug deposition to device characterization and regulatory requirements. *Pulm Pharmacol Ther*, 2019. 54: p. 1-21.
64. Taki, M., et al., Aerodynamic deposition of combination dry powder inhaler formulations *in vitro*: A comparison of three impactors. *Int J Pharm*, 2010. 388(1-2): p. 40-51.
65. Zhang, Y., et al., Nanoparticle delivery platforms for RNAi therapeutics targeting COVID-19 disease in the respiratory tract. *Int J Mol Sci*, 2022. 23(5) p. 2408.
66. Haggi, M., et al., *In vitro* cell integrated impactor deposition methodology for the study of aerodynamically relevant size fractions from commercial pressurised metered dose inhalers. *Pharm Res*, 2014. 31(7): p. 1779-1787.
67. Wong, C.Y.J., et al., Validation of a cell integrated next-generation impactor to assess *in vitro* drug transport of physiologically relevant aerosolised particles. *Int J Pharm*, 2022. 624: p. 122024.
68. Ong, H.X., et al., Is the cellular uptake of respiratory aerosols delivered from different devices equivalent? *Eur J Pharm Biopharm*, 2015. 93: p. 320-327.
69. Goers, L., et al., Co-culture systems and technologies: Taking synthetic biology to the next level. *J R Soc Interface*, 2014. 11(96): p. 20140065.
70. Castellani, S., et al., Human cellular models for the investigation of lung inflammation and mucus production in cystic fibrosis. *Anal Cell Pathol*, 2018. 2018: p. 3839803.
71. Pant, T., et al., Establishment and characterization of lung co-culture spheroids for paclitaxel loaded Eudragit® RL 100 nanoparticle evaluation. *Biotechnol Prog*, 2021. 37(6): p. e3203.
72. Haggi, M., et al., Mono- and cocultures of bronchial and alveolar epithelial cells respond differently to proinflammatory stimuli and their modulation by salbutamol and budesonide. *Mol Pharm*, 2015. 12(8): p. 2625-2632.
73. Ong, H.X., et al., Establishment of a lung infection co-culture *in vitro* model for chronic obstructive pulmonary disease. *Respiratory Drug Delivery 2016*, 2016. RDD Online.
74. Lehmann, A.D., et al., An *in vitro* triple cell co-culture model with primary cells mimicking the human alveolar epithelial barrier. *Eur J Pharm Biopharm*, 2011. 77(3): p. 398-406.
75. Costa, A., et al., Triple co-culture of human alveolar epithelium, endothelium and macrophages for studying the interaction of nanocarriers with the air-blood barrier. *Acta Biomater*, 2019. 91: p. 235-247.
76. Schleh, C., et al., Surfactant protein D modulates allergen particle uptake and inflammatory response in a human epithelial airway model. *Respir Res*, 2012. 13(1): p. 1-10.

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