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MINI-REVIEW

The isolated perfused lung: a bridge between *in vitro* and *in vivo* studies of inhaled medicinal products

Virginia Patterlini[1] · Fabio Sonvico[1,2] · Alessandro Fioni[1,3] · Francesca Buttini[1,2]

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Abstract

Understanding the complex relationship between drug deposition, pulmonary pharmacokinetics, and clinical efficacy is essential to developing effective inhaled therapies. Harnessing formulation physicochemical properties can improve aerodynamic properties and enhance lung retention, yet predictive *in vitro* and *in silico* models remain essential for advanced formulation development. A range of *in vitro* lung epithelial models has been explored to study transepithelial transport. However, these systems often lack physiological complexity. Advanced platforms such as organoids and lung-on-a-chip models attempt to replicate the structural and mechanical features of the lung, though they face limitations in airflow simulation, aerosol characterization, and operational complexity. Consequently, preclinical *in vivo* rodent studies continue to play a key role due to their ability to capture systemic interactions. However, several challenges remain, particularly in isolating lung-specific pharmacokinetics. The review focuses on the use of the isolated perfused rat lung (IPL), an *ex vivo* model that emerged as a valuable tool allowing for the direct measurement of pulmonary absorption while minimizing systemic confounding variables. This model supports accurate control of lung dosing and enables evaluation of local metabolism, clearance, and drug retention. The review outlines IPL setup, applications, and its role in supporting the 3Rs principles (Replace, Reduce, Refine) in animal testing. As an innovative tool, IPL enhances the understanding of pulmonary drug behaviour and holds promise for improving inhaled drug development through more ethical and precise preclinical research.

Keywords *ex vivo* lung model · inhalation toxicology · inhaled drug development · isolated perfused lung · pulmonary pharmacokinetics

Introduction

The study and understanding of the interdependence between pulmonary pharmacokinetics and both pulmonary drug deposition on one side as well as the ultimate clinical efficacy of the drug remains a critical cornerstone in the development of new inhaled products [1]. Recent research has contributed to advance the knowledge concerning actual lung drug concentrations and strategies for the maximization of local exposure in order to achieve the desired pharmacological response at a dose compatible with the creation of an inhaled product [2]. The primary objective of these studies has been to elucidate the inhaled product efficacy through the control and optimization of the delivered inhaled dose. Indeed, an accurate estimation of lung dose is critical for evaluating the inhaled drug pharmacokinetics and pharmacodynamics. In preclinical inhalation studies in rodents, dose estimation is typically performed by measuring the drug aerosol concentration within the inhalation chamber, as described by Cooper*et al.* [2]. On the other hand, by modifying the physicochemical properties of the active ingredient, such as its basicity or polarity, and by reducing its aqueous solubility, it is possible to enhance lung retention and modulate pulmonary absorption [3]. Nonetheless, the development of predictive models of lung pharmacokinetics

✉ Alessandro Fioni
a.fioni@chiesi.com

✉ Francesca Buttini
francesca.buttini@unipr.it

[1] Advanced Drug Delivery Research Laboratory, Food and Drug Department, University of Parma, Parco Area Delle Scienze, 27/A, 43124 Parma, Italy

[2] University Research Centre for the Innovation of Health Products, Biopharmanet-TEC, University of Parma, Parma, Italy

[3] Chiesi Farmaceutici S.p.A., Largo Belloli 11, 43122 Parma, Italy

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and more robust *in vitro* assays remains vital for optimizing inhaled drug formulations and achieving consistent therapeutic outcomes [3]. Indeed, as mentioned by Selo *et al*. various model systems have been developed to mimic biological barriers and replicate *in vivo* conditions, facilitating non-clinical safety testing and pre-clinical drug development [4]. When grown in monolayer conditions, primary epithelial cells isolated from lung tissue and some lung epithelial cell lines represent a relevant model to elucidate the intricate kinetics and processes of transepithelial drug transport [5, 6]. Both primary and immortalized epithelial cell lines are capable of forming monolayers, which allow for the quantification of transepithelial transport properties (*e.g.,* apparent permeability coefficient) and can serve as *in vitro* tools for estimating pulmonary drug absorption, as reported by Sakagami *et al*. [5]. However, despite their growing popularity in research practices, the extent to which these simplified *in vitro* systems accurately reflect the complex dynamics of drug absorption and distribution in native lung tissue remains a matter of ongoing discussion [5]. Despite their physiological relevance, primary cell cultures present significant limitations in terms of handling, reproducibility, and long-term maintenance. Several human lung epithelial cell lines—such as A549, Calu-3, 16HBE14o, and BEAS-2B—originating from cancerous tissues or virally transformed cells, have been evaluated for their utility and relevance [4, 6]. Due to their ability to proliferate continuously *in vitro*, these immortalized cell lines provide enhanced reproducibility and user-friendliness for experimental applications [4]. Advanced *in vitro* cell models have even incorporated key physiological components, such as the extracellular matrix, and mechanical aspects of breathing, to better replicate the airflow and deposition patterns of inhaled therapies [4]. Lung organoids and lung-on-a-chip have been designed to mimic structural features of the airways, supporting the growth and differentiation of various cell types and enabling studies on drug efficacy and toxicity [7]. Complex models, as reported by Zhu *et al*. such as the lung-on-a-chip, attempt to simulate crucial physiological and mechanical parameters of the respiratory system, such as breathing dynamics, airflow, cellular heterogeneity, and vascular perfusion [7]. Precision-cut lung slices represent an advanced primary cell culturing approach offering the advantage of containing all the lung resident cells and preserving the complex tissue architecture; however, similarly to primary cell cultures, they are characterized by challenging maintenance and handling requirements [8]. These models are particularly effective for early-phase screening of small molecules, assessing physicochemical-driven absorption behaviour, and comparing formulation performance under controlled conditions [4, 9]. For more complex formulations, such as biologics, nanoparticles, or inhaled formulations requiring assessment of mucociliary interaction, advanced *in vitro* models like lung organoids and lung-on-a-chip devices provide structural and functional relevance, enabling the study of tissue-level responses, local toxicity, and the interaction of aerosols with airway architecture [10]. Nevertheless, each of these *in vitro* approaches presents significant limitations. Commonly used cell lines, such as A549 and Calu-3, do not fully recapitulate the *in vivo* respiratory epithelium; A549 cells fail to form tight junctions, while Calu-3 cells lack functional ciliary activity [11]. Organoids, while structurally relevant, do not replicate airflow or pressure variations and lack the anatomical features necessary for studying the behaviour of inhaled therapeutics [11]. Even the most advanced systems, like lung-on-a-chip models, are not yet optimized for analysing aerosol characteristics and drug-particle interactions at the biological interface. Moreover, these systems are often complex to operate and expensive. Despite significant progresses have been made in the development of advanced *in vitro* and *ex vivo* models, no single experimental platform can fully reproduce the complexity of pulmonary absorption, distribution, and metabolism. Each model offers specific advantages while also presenting intrinsic limitations that reduce its predictive power when used in isolation. For these reasons, a reliable evaluation of the behaviour of inhaled drugs requires the integration of multiple complementary methodologies, selected according to the experimental objective or the clinical context of interest. The combined use of cell-based models, advanced systems such as organoids or lung-on-a-chip platforms and *ex vivo* approaches, provides a more comprehensive and robust understanding of the mechanisms governing pulmonary pharmacokinetics and pharmacodynamics, ultimately enhancing predictive capability in preclinical development.

Despite the relevant results and biological processes understanding that can be obtained with the cited *in vitro* models, pre-clinical *in vivo* studies in small rodents remain the primary means of assessment of drug behaviour after pulmonary administration due to the pharmacokinetic data obtainable. These methods become even more relevant when emphasis is placed on accurate and reproducible dosing and control of lung regional deposition [5]. For pulmonary drug administration, the most commonly used method is orotracheal intubation, often performed using a variety of devices—many of which are custom-made—such as metered-dose inhalers, microsprayers, or insufflators. While this approach helps overcome technical challenges related to delivery, several limitations persist even in the *in vivo* models, particularly for the quantification of locally acting drugs. Notably, plasma concentrations primarily reflect the drug's fate after systemic absorption and clearance from the airways, rather than providing direct information about the drug present at the pulmonary site of action [12]. This disconnection complicates the accurate assessment of drug absorption, distribution, and local pharmacodynamics within

the lung [12]. Additional limitations include the need for animal acclimatization and training before aerosol administration, as well as the use of anesthetics, which can alter normal respiratory physiology and affect drug deposition and absorption [5, 13].

The isolated perfused lung (IPL) *ex vivo* model appears to address several of the shortcomings mentioned for *in vitro* and *in vivo* models. This unique *ex vivo* model, combined with advanced mathematical modelling, allows for the independent determination of the kinetic descriptors for lung absorption and non-absorptive clearances, such as mucociliary clearance, phagocytosis, and/or local metabolism, while controlling lung-regional distribution [5]. For example, the mathematical model proposed by Sakagami *et al*. considers that the passive absorption and non-absorptive loss are assumed to follow first-order kinetics. The non-absorptive loss includes processes such as mucociliary clearance, phagocytosis, and/or metabolism. In contrast, active absorption is described using Michaelis–Menten kinetics, defined by a maximum rate and an affinity constant. After experimentally confirming which lung deposition pathways are involved for each test compound, nonlinear least-squares regression was applied to the IPL data to estimate the kinetic parameters. The fraction of the dose that actually reached the lung lobes was experimentally determined and kept constant during the fitting process. This approach makes it possible to quantify the relative contribution of each lung deposition pathway, thereby clarifying the mechanisms underlying not only absorption but also the lung's clearance processes [5]. Despite its intrinsic drawbacks, this *ex vivo* model has been demonstrated to be kinetically predictive of *in vivo* animal distribution at a macroscopic level.

IPL is an *ex vivo* absorption model that allows a controlled lung-delivered dose, allowing the calculation of the pulmonary drug absorption while avoiding the confounding systemic PK effects, such as absorption after swallowing of the extra pulmonary dose fraction. Indeed, pulmonary drug absorption is measured directly from the pulmonary vein, and even if the bronchial region is not perfused in the IPL, since in vivo it is supplied by the systemic circulation, it is relevant for the drug local metabolism [14]. Although the IPL can not reproduce hepatic metabolism, it allows the investigation of the first-pass metabolic processes in the lung tissue such as bronchial epithelial metabolism, i.e. enzymatic degradation in the lung tissue before the compound reaches the perfusate [15]. Especially for inhaled compounds the bronchial compartment represents the first metabolic barrier, and the IPL allows this to be studied in isolation, without confounding hepatic clearance, thanks to the preservation of bronchial and alveolar enzymatic activity. In this respect, it has been demonstrated that the IPL model presents particular advantages for studying local drug metabolism as well as pulmonary pharmacology and toxicology [16]. This model offers different applications including the study of pharmacodynamic and pharmacokinetic parameters, as well as processes relevant for absorption, distribution, and metabolism; moreover the IPL model allows for the evaluation and comparison of inhaled formulations [1].

The present short review focuses on the description, use, and potential of the isolated perfused lung (IPL) model in pre-clinical studies of inhaled drug products. A detailed description of the experimental setup with the indication of the key parameters of the IPL system will be provided. The review then will report a selection of the most interesting applications in the preclinical development of inhalation medicinal products, along with a critical evaluation of advantages and limitations of the IPL *ex vivo* model. Finally, the potential of this technique for PK/PD correlations and for the agreement with the 3Rs principles in preclinical experiments will be briefly discussed.

Isolated Perfused Lung Experimental Set-up

Animal preparation

As reported by different articles mentioned below, male Wistar rats (250–300 g) are the most employed animals in this procedure, because they belong to a well-characterized and standardized line, which makes the results of the experiments more reproducible and comparable between different studies. The surgical part requires, as suggested by the procedures presented in different studies [17–21], a deep anesthesia of the rat. In preparation for the lung explant, the common procedure includes that the skin from the abdomen to the neck and the salivary glands are removed with scissors, without cutting the abdominal or thoracic space [17]. The trachea is transected halfway through its diameter between the fourth and fifth cartilage rings caudally to the thyroid gland, after an incision into the muscle tissue above the trachea. A stainless-steel tracheal cannula with internal and external diameters of 2 and 3 mm is inserted for 0.5 cm and secured with a tie to keep the lungs breathing through mechanical ventilation [20]. After the thorax is opened by median sternotomy, the diaphragm is resected from the thoracic wall. The thorax halves are spread with two hooks. The pulmonary artery is likewise catheterized with an arterial cannula, for the inlet of the perfusate, through an incision on the right ventricle; a tight ligation is wrapped around the vessel's major trunk [21]. Through the left ventricle and mitral valve, a second catheter for perfusate outflow is inserted into the left atrium and secured at the heart's apex using a ligature. An extra ligation is done above the atrioventricular junction to stop backflow into the ventricles [22]. After cannulation, the lung and the heart are removed from the thorax, cutting

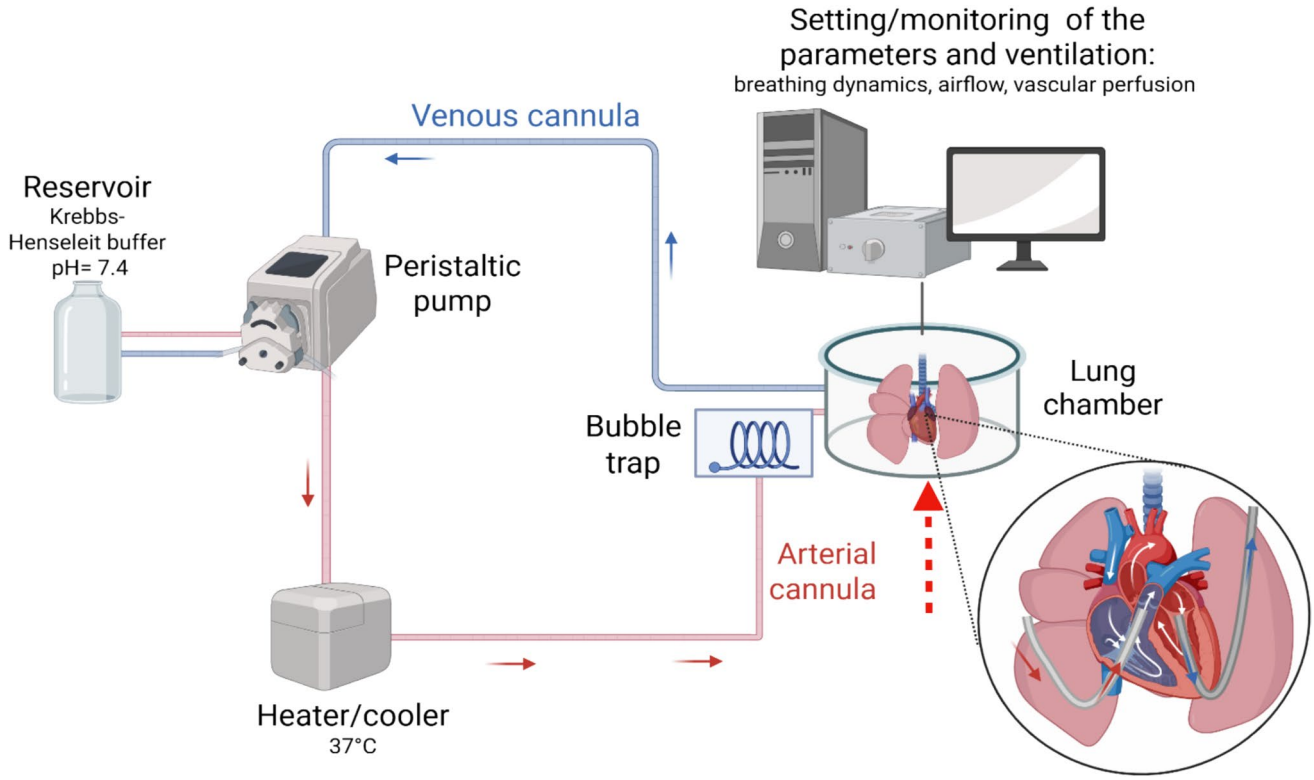


Fig. 1 Schematic representation of the isolated perfused lung system developed by Harvard Apparatus. The red arrow indicates the lung chamber in which the lung is placed after the explant (created with BioRender)

the connective tissue junctions of the caudal mediastinal tissue with the diaphragm and the anatomical connections (vena cava, thoracic aorta, esophagus) [23]. The isolated lung, with the cannula inserted, is carefully placed into the lung chamber, simulating the artificial thorax, and the perfusion starts. Figure 1 represents the schematic and simplified configuration of a system designed to host the isolated perfused lung (Harvard Apparatus, Holliston, MA, USA). The lung chamber (shown by the red arrow in Fig. 1) is a sealed glass container designed to hold the excised rat lung and maintain negative pressure to keep it expanded. The isolated perfused lung system illustrated in Fig. 1 comprises several key components. The lung chamber is equipped with a water jacket that allows precise temperature control. The perfusion circuit includes a peristaltic pump to circulate the perfusate, a heat exchanger to maintain the perfusate temperature, a bubble trap to eliminate air bubbles, and pressure transducers for monitoring pulmonary artery and left atrial pressures. System parameters are monitored using a pneumotachometer for respiratory airflow measurements, alongside software for real-time data acquisition and analysis. The ventilation system is formed by a ventilation control module to adjust the respiration rate, inspiratory and expiratory pressures through a tube linked to the tracheal cannula [1].

Instrument settings

Once the lungs are explanted, they are transferred to a humidified thermostated chamber and perfused via the pulmonary circulation [24] through a peristaltic pump with constant flow rate or constant pulmonary pressure [15]. At the constant flow rate, the perfusion pump maintains a fixed flow rate (*e.g.,* µl/min), and perfusion pressure will vary depending on vascular resistance. In the constant pressure, the pulmonary artery pressure is held constant, and flow varies depending on vascular resistance [25]. The choice of the perfusion type is based on the experimental objective and physiology being studied. The constant flow perfusion is applied when the goal is to study vascular resistance or vasoreactivity, while the constant flow rate is employed mainly for the permeability and fluid balance studies [25]. The system is kept at 37°C – to replicate the physiological environment- with a thermostatic bath which heats the perfusate and the lung chamber thanks to a water-jacket [18].

Various parameters, such as perfusion flow rate, ventilation, and temperature, can be adjusted in the system depending on the type of experiment being conducted, whether it is absorption, toxicology, or metabolism studies [19]. The perfusate circulates throughout the entire *ex vivo* system: entering the heart and exploiting the pulmonary circulation

to pass through the lungs. In the original experiments, blood was considered the best perfusate because it closely replicated physiological conditions [26] and the metabolic effects. Later, Roth *et al.* introduced perfusion using artificial media containing salts and enriched with serum albumin to prevent pulmonary edema, despite acknowledging the deviation from physiological conditions [19]. More recently, the most commonly used perfusate has been a modified Krebs–Henseleit buffer at pH around 7.4 containing $CaCl_2$, KCl, KH_2PO_4, $MgSO_4$, NaCl, and $NaHCO_3$. It can be enriched with HEPES, glucose, and bovine serum albumin [27]. Regarding the enrichment, adding albumin increases osmotic pressure and reduces edema, but albumin may alter cytokine signaling and drug binding. Krebs–Henseleit buffer is very easy to reproduce, thanks to its well-defined composition, but it does not mimic blood's oxygen-carrying capacity or protein content [28]. The absence of immunoglobulins and complement proteins has important implications for data interpretation, as these components play key roles in opsonization, immune complex formation, and complement activation [29]. Their absence is particularly relevant for proteins, antibodies, and nanoparticle-based biopharmaceuticals. *In vivo*, many biologics and nanoparticles rapidly adsorb immunoglobulins and complement proteins, forming a "protein corona" that can substantially affect immunogenicity, biodistribution, and cell-binding properties [29]. Thus, data obtained using the IPL model may not fully reflect the complexity of *in vivo* immune activation, where multiple circulating proteins critically affect the fate and behavior of biopharmaceuticals. In particular, the absence of physiological blood proteins in the IPL system precludes complex protein-binding interactions and enzymatic metabolism, which may otherwise reduce the free drug fraction *in vivo* and complicate the correlation between IPL-derived results and *in vivo* pharmacokinetic data [19].

Depending on the application of the system, two perfusion methods can be chosen: recirculation of a set volume of perfusion fluid and one-through perfusion. Perfusate recirculation appears crucial for evaluating substances that present slower metabolization rates [19]. A few possible drawbacks of the recirculation method are: the formation of harmful metabolites for the lung from either endogenous or exogenous sources; the accumulation of xenobiotic metabolites that limit the rate of perfusion; the depletion of substrates and other critical components during perfusion [19]. On the other hand, the single-pass perfusion requires a larger volume of buffer medium, but it is useful for determining the drug transport parameters, the absorption and distribution profile [27]. The perfusate can be oxygenated through an oxygenator to control perfusate gases and temperature and to protect the lung from hypoxia during periods of compromised ventilation, or to enable control of perfusate pO_2/CO_2 independently of lung function. If no de-oxygenator is used, hypoxic perfusate can alter pulmonary vascular tone and metabolism, which may change drug uptake/metabolism and edema formation [30].

About the mechanical ventilation, there are two methods: positive pressure or negative pressure inflation. For positive pressure ventilation, a respirator must be connected directly to the lung through the trachea cannula in which the air is directly insufflated [31]. In negative pressure ventilation, sub-atmospheric pressure values are cycled in the chamber where the lung is suspended, using a reverse-connected respirator [31]. Since positive pressure ventilation may cause overinflation, leading to lung architectural deterioration, edema, and progressive atelectasis, sub-atmospheric ventilation is generally recommended [19]. Moreover, in order to keep the lungs compliant, every 5 min a deep inspiration is initiated to maintain the lung vital [32]. Mechanistic physiological lung data, such as tidal volume, dynamic compliance, air flow, pulmonary artery pressure, and resistance, are monitored continuously to check the viability of the tissue [17]. For instance, a significant drop in tidal volume is thought to be a sign of pulmonary edema [27]. Different administration routes can be exploited in the IPL, producing different lung deposition patterns, regional concentrations, and kinetics of transfer into the perfusate. Aerosolized delivery produces particle size–dependent, more uniform distal airway and alveolar deposition when particle aerodynamic diameter is in the respirable range (typically 1–5 μm) [33]. Different types of devices can be employed, such as jet, ultrasonic, and vibrating mesh nebulizers, which affect the deposition, like the flow path (through endotracheal tube or tracheal cannula), and ventilatory settings (tidal volume, inspiratory time, flow) [34]. Delivery of the formulation in form of finely dispersed aerosol represents an effective approach to mimic exposure by, however, thorough characterization of the particle size distribution and of the delivered dose are necessary. Intratracheal instillation of a liquid bolus, instead, is a simple and reproducible way for delivering a known nominal dose to a defined airway segment but typically results in highly focal deposition (central airways or dependent lobes) and can cause transient airway flooding and uneven spreading; it does not reproduce aerosol deposition patterns [35]. Several classical isolated-lung studies show large differences in deposition and pharmacokinetics between instillation and aerosol routes [36]. PennCentury microsprayer can create a fine, controlled spray from a catheter inserted into the trachea; intermediate between instillation and nebulized aerosol in terms of distribution—better dispersion than bolus instillation but still dependent on placement and ventilation [20]. Then, in the IPL, it is also possible to administer a drug directly in the pulmonary artery, mimicking an intravascular administration, bypassing airway deposition entirely. It delivers the drug directly to the pulmonary endothelium and interstitium via the perfusate;

it is useful to study uptake from the blood side or metabolism, but not inhalation deposition [37]. Classic isolated lung pharmacokinetic studies demonstrate substantially different metabolism/clearance depending on vascular *vs* airway routes [37].

Current Applications

The isolated perfused rat lung is a valuable experimental model with diverse applications in pulmonary research [1]. It allows for precise control of the lung environment, enabling investigations into various physiological and pathological processes [31].

Toxicological assessments

The IPL model has been widely utilized to study pulmonary vascular function, inflammation, and the effects of various drugs and toxins on lung mechanics. The IPL model has been instrumental in elucidating the mechanisms of acute lung injury and evaluating the efficacy of potential therapeutic interventions. An example is provided by P. Ewing *et al*. who administered budesonide through the IPL and assessed a decrease in the perfusate flow rate caused by a significant pulmonary vasoconstriction, induced by the drug within 40 min after inhalation [38]. By splitting the perfusion post-exposure period into three distinct time periods—from 0 to 10 min, from 10 to 40 min, and from 40 to 100 min—the decrease in perfusate flow rate after treatment was examined. The average relative pulmonary perfusion flow rate for each control and treatment was determined for the three distinct perfusion periods as well as for the entire post-exposure perfusion period. The effect of corticosteroid exposure on perfusion flow rates, as observed in the presented IPL model following the administration of sufficiently high doses of inhaled budesonide, is most likely mediated by the retention of norepinephrine at the neuromuscular junction, which induces vasoconstriction in pulmonary arteries, causing the rapid decrease in perfusion flow rate [38]. The isolated perfused rat can serve as a model to investigate corticosteroid-sensitive vasoconstriction in the human bronchi, given that rats and primates exhibit similar noradrenergic innervation of the pulmonary veins and bronchial arteries. Therefore, the model can be a useful alternative or supplement to more time-consuming clinical models for examining quick, corticosteroid-dependent effects on the pulmonary/bronchial circulation, which could have significant effects on how these drugs are disposed of and how well they work in the lung, either by themselves or in conjunction with other inhaled medications [38]. IPL also allows for the direct assessment of the pulmonary toxicity of various substances, including environmental pollutants, drugs, and industrial chemicals. By exposing the isolated lung to these agents, researchers can investigate their effects on key physiological parameters such as pulmonary vascular resistance, lung compliance, and gas exchange.

The IPL model enables the study of inflammatory responses induced by toxicants, including the release of inflammatory mediators and the recruitment of immune cells. As demonstrated by Chian *et al.* an intratracheal administration of lipopolysaccharide (LPS) in an isolated and perfused rat lung model resulted in lung damage. Fifteen minutes before administering LPS, apocynin, a natural compound with potent selective inhibitory effects on NADPH oxidase, was added to the perfusate to reduce the generation of reactive oxygen species (ROS) [39]. Assessments included hemodynamic parameters, evaluated by continuous online monitoring of pulmonary arterial and venous pressures and by calculation of pulmonary vascular resistances, as well as inflammatory responses, indices of lung injury, and activation of apoptotic pathways. Increased pulmonary vascular permeability, inflammatory cell infiltration, and pulmonary edema were all consequences of LPS-induced lung damage [39]. This study demonstrates that LPS can affect the isolated lung permeability, and the apocynin therapy effectively reduced the formation of ROS and enhanced antioxidant capability while reducing the inflammatory and apoptotic responses.

A recent study by Nosratabadi *et al.* employed the isolated perfused lung (IPL) model to investigate cytokine release induced by wear particles generated at the road–tire interface and from vehicle brakes [40]. In their experimental setup, lungs were exposed to an aerosol of these particles for 25 min, followed by monitoring functional parameters, such as tidal volume, for an additional 50 min. To evaluate the impact of inhaled particles on inflammatory cytokine release, perfusate samples were collected before exposure (baseline), immediately after exposure, and at the end of the experiment. Potential inflammatory markers were first screened in the perfusate and subsequently quantified using ELISA assays. The pulmonary effects of different types of wear particles were assessed through changes in tidal volume and cytokine concentrations such as NF, CXCL1 and CCL3 [40]. The IPL model provided a unique opportunity to investigate the impact of environmental agents on respiratory physiology and the pulmonary immune system, while preserving the native architecture and immunocompetent cell populations of the lung. Several particle types were evaluated, revealing marked particle-specific effects on lung mechanics. Quartzite particles produced the most pronounced functional response, inducing a rapid and significant reduction in tidal volume that became evident within 5 min of exposure, at a dose corresponding to approximately 20% of the total administered particle load. This decrease in tidal volume occurred earlier and at lower doses compared to

Table 1 Pharmacokinetic parameters calculated by Ewing et al. [42] on the perfusate after the isolated perfused lung experiment of Budesonide, Formoterol, and Terbutaline

Drug	Estimated deposition (µg)	T_{max} (min)	IPL C_{max} (nM)	$T_{½}\beta$	IPL C_{max}/Clin C_{max}
Budesonide					
Low dose	3.3 ± 0.1	0.9 ± 0.2	20 ± 1.0	N/A	1–2
High dose	27 ± 5.5	2.2 ± 0.2	110 ± 44	40 ± 12	
Formoterol					
Low dose	5.2 ± 0.9	3.1 ± 0.2	37 ± 7.5	N/A	100–300
High dose	37 ± 2.3	6.7 ± 4.0	180 ± 20	32 ± 11	
Terbutaline					
Low dose	9.7 ± 2.4	1.3, 1.6	125, 140	N/A	10
High dose	73 ± 14	2.2 ± 1.1	1030 ± 260	14 ± 4.5	

T_{max} is the time to maximum concentration (min); IPL C_{max} is the maximum concentration in perfusate (nM); $t_{1/2}\beta$ the second half-life of the drug in the perfusate (min); IPL C_{max}/Clin C_{max} is the ratio of perfusate C_{max} to clinical concentration of the drug in plasma

all other tested particles, indicating a higher airway-reactive potential of quartzite material.

In parallel, inflammatory biomarkers were quantified in the perfusate, including pro-inflammatory cytokines (e.g., TNF-α, IL-6, and IL-1β). Although exposure to particles resulted in measurable cytokine release, changes in cytokine levels were not consistently proportional to the magnitude of tidal volume reduction. This finding suggests a partial dissociation between functional airway constriction and inflammatory mediators release.

Compared with whole-animal studies, the IPL preserves pulmonary structure and functional integrity without the systemic influence of circulating immune cells and cytokines from other organs [40]. Overall, this work demonstrates that the toxic effects of airborne particles on the lung can be effectively studied by combining functional readouts, such as tidal volume, with molecular endpoints, such as cytokine release.

However, the IPL model for toxicology evaluations presents some limitations related to the reduction of the potential hemodynamic effects on lung injury and to the lack of interactions with other organ systems. For example, metabolites generated in the liver that contribute to pulmonary toxicity such as reactive intermediates of cyclophosphamide, as reported by Patel, are not produced in the isolated lung preparation, limiting the ability to assess metabolite driven injury [41].

Pharmacokinetic evaluation

IPL also has significant applications in pharmacokinetics, particularly in assessing pulmonary drug absorption and deposition. This model allows for the direct measurement of drug uptake from the lung space into the pulmonary circulation, providing valuable insights into key pharmacokinetic parameters such as absorption rate, permeability, and tissue distribution. Ewing *et al.* brought an example of the absorption and deposition profile of three different widely used inhaled drugs, *i.e.* formoterol fumarate, budesonide, and terbutaline, administered by the DustGun aerosol generator (Inhalation Sciences Sweden AB, Stockholm, Sweden) [42]. Two different dose levels were tested for each compound. The estimated theoretical pulmonary deposition of the aerosol for budesonide, formoterol, and terbutaline was 27 µg, 37 µg, and 77 µg, respectively, at the high dose, and 3.3 µg, 5.2 µg, and 9.7 µg, respectively, at the low dose (Table 1). This example has been chosen because it reports the comparison of different dry powders administered by DustGun aerosol technology, which is a method for dry powder administration frequently used in *in vivo* studies for its reproducibility. The analysis was performed in a one-through perfusion mode, and the perfusate samples at different timepoints were analyzed by HPLC–MS, and the tissue drug concentration was determined in homogenized lungs. For all the drugs, the pharmacokinetic parameters were calculated (T_{max}; C_{max}; $t_{½}$) and the profiles were compared (Table 1). The inhaled drugs rapidly appeared in the perfusate. Compared to formoterol, the concentrations of budesonide and terbutaline reached their peak at a significantly lower T_{max} for both low and high dose exposures. By scaling each perfusate sample volume to the total volume, the overall clearance into the perfusate was determined, and the fraction of drug retained in the lung tissue could be calculated and was 0.19 ± 0.05, 0.19 ± 0.06, and 0.04 ± 0.01 in terms of fraction of deposited dose for budesonide, formoterol, and terbutaline, respectively [42]. The higher tissue retention shown for budesonide and formoterol was in good agreement with previous pre-clinical and clinical results and attributed to the higher lipophilicity of those substances. The more water soluble β2-agonist terbutaline, on the contrary, presented a more rapid absorption and higher perfusate concentrations. The difference in T_{max} observed for the same compound administered at two different doses is most likely attributable to the dissolution process, which occurs more rapidly at lower doses compared with higher ones [42]. Dissolution also accounts for the greater

absorption observed with the more hydrophilic terbutaline compared with formoterol and budesonide [43]. For the latter two compounds, which are more lipophilic, dissolution may acts as a rate-limiting step for absorption [43]. Since the IPL perfusate reflects only the fraction of the drug that becomes available, the measured fraction is often low for poorly soluble compounds.

The calculated data on absorption rates in the IPL model of budesonide, formoterol, and terbutaline and tissue retention rates in the rat IPL are in reasonable agreement with reported clinical results (Table 1), with the larger discrepancy evidenced for formoterol fumarate [42]. The observed divergence between *ex vivo* and *in vivo* absorption profiles of formoterol is likely explained by its pronounced first-pass hepatic metabolism, which is not highlighted by the IPL model, resulting in rapid clearance via phase II glucuronidation and phase I O-demethylation pathways [44]. Conversely, the greater lipophilicity of budesonide promotes pulmonary tissue retention, thereby slowing its systemic availability and metabolic clearance [45]. In contrast, terbutaline's high hydrophilicity limits tissue retention and metabolism, leading to partial renal excretion of the unchanged parent compound [46].

Eriksson *et al.* explored drug absorption via the IPL model, measuring cumulative absorption percentages of different drugs and formulations [47]. Budesonide, fluticasone furoate, and propionate were studied as solutions, suspensions, and dry powders. The system successfully highlighted and discriminated between the distinct characteristics of the tested compounds. For budesonide, it was evidenced that in this experimental set-up dissolution was not the rate-limiting step, as both the suspension and the solution exhibited comparable absorption profiles. In contrast, for fluticasone, the three formulations displayed markedly different profiles: solutions were absorbed most rapidly, suspensions showed intermediate absorption, and dry powders were the slowest. The latter can be attributed to the need for powder particles to undergo wetting in the lung fluid before dissolution and subsequent absorption. This substantial variance underscores the impact of formulation type (solution, suspension, or dry powder) on pulmonary absorption efficacy and especially the capacity of the system to investigate the dissolution of poorly soluble inhaled drugs [47].

In all the examples provided, the isolated perfused lung has demonstrated its validity as a model for preliminary preclinical evaluation of inhaled drugs and different formulations.

IPL recent applications: from novel formulations to the elucidation of absorption mechanisms

The isolated perfused rat lung (IPL) model is a valuable tool in biomedical research, still offering several promising future perspectives and applications. By controlling the experimental conditions, the influence of various factors on drug absorption, including particle size, solubility, and the presence of surfactants, can be studied [48]. This information is crucial for optimizing drug delivery strategies for inhaled medications, such as those used in the treatment of asthma, chronic obstructive pulmonary disease (COPD), and other respiratory disorders. This could be even more interesting for the administration of systemically active drugs for a broader range of pathologies and therapeutics. Henceforth, the IPL model is increasingly used to study the pulmonary absorption of inhaled drugs, as it provides a realistic environment that mimics lung function and maintains physiological tissue barriers, making it a reliable predictor of drug pharmacokinetics [48]. For this reason, it could be used to investigate the fate of different formulations (liquids, powders, liposomes, nanosuspensions) and compare their behavior in terms of absorption and drug release, helping the optimization of drug formulation for better therapeutic outcomes. For example, in the research of Gupta et al. two different liposome formulations of Fasudil and superoxide dismutase (SOD) used in pulmonary arterial hypertension were compared in terms of lung retention using IPL studies: plain liposomes and liposomes conjugated with a pulmonary-specific targeting peptide, *i.e.,* CARSKNKDC (CAR). The results, obtained from the perfusate, highlighted that the conjugated liposomes in both cases were better retained in the lungs, increasing the uptake by pulmonary endothelial cells after intratracheal aerosolization, as demonstrated in Fig. 2 [49]. Lung retention can be inferred from the perfusate concentration profile, which was lower and reached a plateau for the liposomal formulations. At the end of the experiments, lung tissue homogenates revealed that the amounts of Fasudil and SOD retained in the lung were 2.5- and 5.3-fold higher, respectively, compared with the plain drug [49].

A very recent preclinical study demonstrated that the IPL can also be employed for evaluating gene-therapy approaches. Gao et al. used the isolated perfused rat lung model to investigate adenovirus-associated virus 9 (AAV9)-mediated gene delivery [50]. In this study, lungs were perfused *ex vivo* for one hour, during which the viral vector encoding a luciferase reporter was administered via the airways, pulmonary artery, or directly into the perfusion circuit. Afterwards, the left lung was transplanted and the graft and other organs examined after 2 weeks by bioluminescence imaging. The authors reported that airway delivery resulted to provide the highest transgene expression within the graft compared to the other *ex vivo* administration modalities. Furthermore, off-target expression in other organs was minimal in all animals [50]. This work illustrates the feasibility of applying the *ex vivo* perfused lung model for gene therapy studies, highlighting

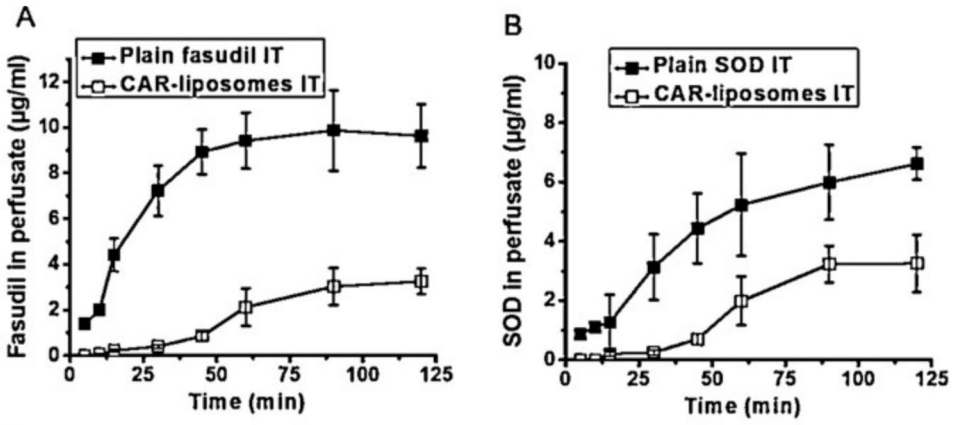


Fig. 2 Isolated perfused rat lung studies showing the concentration of plain and CAR-liposomes of Fasudil (**A**) and SOD (**B**) in pulmonary perfusate over time (reproduced with permission from [49])

its potential to evaluate vector delivery, tissue targeting, and early transduction efficiency in a physiologically intact organ while minimizing systemic exposure [50]. Although limited to preclinical proof-of-concept, these findings indicate that the IPL approach can be a valuable tool for testing complex biologics and gene-therapy strategies before *in vivo* pre-clinical or clinical application.

In another work, Dalla-Bona et al. reported on the absorption dynamics of sildenafil when delivered using an in situ forming controlled release formulation via the IPL model. The findings indicated rapid absorption across the air-perfusate barrier, especially for the free drug solution, emphasizing the role of formulation strategies in enhancing drug transport within pulmonary contexts [51].

Dong *et al.* exploited IPL to assess the distribution and local tolerability of aerosolized chitosan-coated poly(lactide-co-glycolide) based nanoplexes containing antisense 20-O-Methyl RNA (OMR) for lung cancer targeted therapy [52]. Therefore, an aerosol of OMR-nanoplexes or OMR alone was administered intratracheally, and the physiological parameters (pulmonary compliance, resistance, tidal volume) were assessed to predict toxicity post-administration. The distribution and intracellular uptake were evaluated using immunohistochemistry. After perfusion, the lung was fixed, stained, and sectioned for microscopic visualization. Different cell types were highlighted, and the drug distribution was assessed (Fig. 3) [52]. This approach allowed for early-stage differentiation between the behavior of formulated and non-formulated OMR, demonstrating the formulation's ability to enhance the uptake of oligonucleotides by the respiratory epithelium targeting lung tissue [52].

To evaluate the deposition of different formulations and their ability to penetrate lung cells, lung tissue collected after IPL can be analyzed by fluorescence-activated cell sorting (FACS). This technique enables quantification of intracellular drug uptake and, using cell–type–specific fluorescent antibodies, allows identification of the specific lung cell populations that internalize the formulation or compound, thereby confirming whether the intended target is reached [53].

Through the online monitoring of the tidal volume, compliance, and resistance, the IPL is useful to assess the acute toxicity of the new formulations and their components, helping understand the mechanisms leading to lung injury, which is crucial for developing protective strategies and treatments [36].

Moreover, the IPL model could be useful for assessing pulmonary clearance and drug metabolism. By recirculating the perfusate and administering the drug repetitively, it is possible to evaluate metabolite formation in the lung, thereby studying the organ-specific aspects relative to ADME (Absorption, Distribution, Metabolism, and Excretion) of various compounds. IPL is particularly useful for assessing the efficacy and safety of inhaled therapies. A very interesting application mentioned by Ehrhardt and colleagues using IPL is the role of drug active transport mechanisms on the lung permeability [54]. Indeed, through this system, it is possible to administer transporter substrates or inhibitors at concentrations not admissible *in vivo* [54]. Related to this topic, Tronde *et al.* reported that losartan, a known P-glycoprotein substrate, exhibited high absorption in the isolated perfused rat lung. This finding suggests that P-glycoprotein does not exert a significant absorption-retarding effect in this model, thereby providing limited evidence for the involvement of active transport pathways in pulmonary drug uptake [14]. The same observation was made with digoxin, another P-glycoprotein substrate, which did not exhibit any significant change in pulmonary absorption, even when co-administered with a P-glycoprotein inhibitor [55]. On the other hand, a different behavior for idarubicin was observed, where an increase in drug absorption was observed when the drug was administered to the IPL via the perfusate with P-glycoprotein inhibitors [56]. Indeed, other IPL studies with inhibitors or knock-out animals have shown the substrate-specific impact of P-glycoprotein on

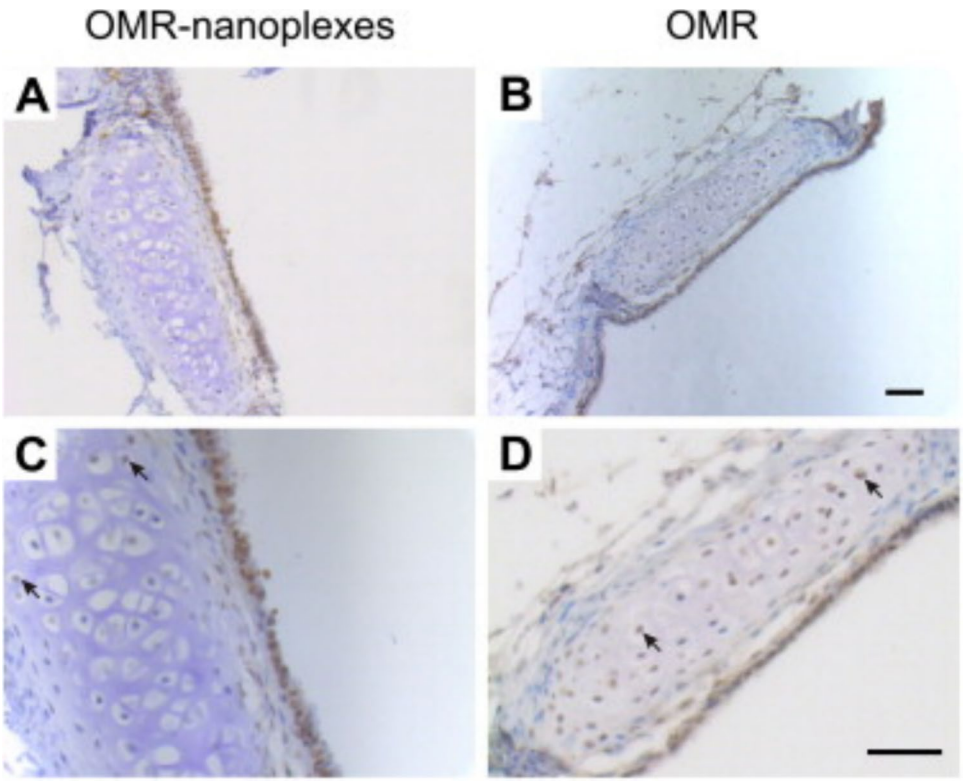


Fig. 3 Distribution of 2'-O-Methyl RNA (OMR) in rat lung tissue following aerosol administration in the isolated perfused rat lung system of OMR (**B**, **D**) or OMR-nanoplexes (**A**, **C**), visualized by immunohistochemistry (reproduced with permission from [52])

the lung permeability [54]. Unfortunately, the application of P-glycoprotein substrates in the isolated perfused lung model remains controversial. The discrepancies observed in the studies cited above may be influenced by factors such as lung viability and physiological conditions during the experiments, which are not consistently reported. Establishing standardized practices for carrying out such studies, regarding the type of inhibitors or genetic knockouts, would be helpful to fully utilize the potential of isolated perfused lungs to investigate the impact of drug transporters [54]. In particular, the use of P-glycoprotein knockout animals would certainly provide a more robust approach to delineate the contribution of this transporter to pulmonary uptake of specific substrates. Additionally, fundamental studies are required to clarify transporter activity—particularly P-glycoprotein—in the *ex vivo* lung model and to better define its role when evaluating compounds that are known transporter substrates.

Finally, the impact of tight-junction modulation on pulmonary drug absorption has also been explored using the IPL model. Selg et al. developed a respirable horseradish peroxidase (HRP) powder to evaluate whether opening alveolar tight junctions with poly-L-arginine—an agent known to alter the localization of tight-junction components and facilitate paracellular transport of macromolecules—could enhance the absorption of inhaled HRP in the rat IPL [57]. Aerosols of the HRP powder were generated using the DustGun technology (Inhalation Sciences Sweden AB, Stockholm, Sweden) and delivered directly to the ventilated and perfused rat lung. Administration of poly-L-arginine at concentrations above 0.2% resulted in a ninefold increase in HRP absorption, demonstrating that biochemical modulation of tight junctions can substantially enhance paracellular uptake. Increasing the concentration to 2% did not produce further enhancement, indicating a steep dose–response relationship for this absorption promoter [57]. Overall, the study showed that pulmonary absorption of HRP is inherently slow and that tight-junction–targeting enhancers exert a measurable but ultimately limited effect. This inhalation approach, combined with the IPL model, provides a valuable platform for assessing the pulmonary absorption of biopharmaceuticals and for characterizing how tight-junction modulation influences macromolecule transport across the alveolar barrier.

Advantages and Limitations of Isolated Perfused Lung

The isolated perfused rat lung (IPL) model provides a valuable platform for investigating various aspects of pulmonary physiology and pathophysiology. Its advantages include precise control over experimental conditions, such as ventilation, perfusion, and the administration of drugs or toxins. This level of control allows researchers to study specific mechanisms underlying various pulmonary

processes in a more isolated and controlled manner compared to *in vivo* models [58]. For instance, it is possible to regulate blood flow and perfusion pressure, which are likely to change in an intact animal during an experiment, especially in response to the administered test chemical. Isolated perfused lung studies allow for the precise control of endogenous or exogenous stimulatory drug concentrations and other variables. Additionally, perfused lung preparations facilitate multiple and frequent blood or perfusate sample collections. This capability enables the management of perfusate volume, allowing for comprehensive qualitative and quantitative analysis of the test compound's major and minor biotransformation products. Unlike *in vitro* homogenate preparations, intact perfused lungs maintain the organ's structural and functional integrity [58]. The preservation of appropriate membrane barriers between individual cells and between the vascular and parenchymal sides is a significant advantage [4, 58].

The IPL foresees the lung withdrawal, which ensures that animals do not experience post-operative pain or stress, as they are not revived after anesthesia. Despite the sacrifice of the animal, the procedure does not cause further suffering. This approach avoids additional interventions or manipulations, thereby enhancing overall animal welfare [59]. Furthermore, this method often aligns better with ethical guidelines and regulations that promote humane treatment of laboratory animals. Research institutions and ethics committees generally favor procedures that minimize pain and distress [60]. Indeed, as demonstrated in the applications mentioned above, the IPL model is fully consistent with the 3Rs principles (Replacement, Reduction, and Refinement) [23]. It can replace experiments in live, conscious animals, particularly for the assessment of pulmonary vascular responses, edema formation, and permeability. Moreover, a single lung preparation allows multiple experimental conditions to be tested, yielding extensive data and thereby reducing the number of animals required. Finally, as the animal does not experience the experimental manipulations, the model avoids pain and distress, thus contributing to refinement [61].

The isolated perfused rat lung (IPL) model is a valuable tool for studying pulmonary drug absorption and other lung-related phenomena. However, it has several limitations, such as a limited duration because the viability of the isolated lung is limited (3–4 h), and this restricts the duration of the experiments [14]. The complexity of the setup requires specialized equipment and expertise to set up and maintain the IPL model; moreover, there can be variability in the results due to the differences in lung preparation and perfusion techniques. Indeed, false positives are particularly relevant in toxicity studies, as suboptimal setups may lead to premature edema. Both the surgical procedure and the setup of the IPL are critical factors that can influence outcomes in terms of toxicity and tissue viability. In addition, potential interactions may arise from interference of the test compound with the experimental system itself (*e.g.,* tubing or perfusion buffer). This issue can be addressed by flushing the compound through a lung-free circuit and quantifying its concentration in the perfusate at the end of the experiment, thereby allowing evaluation of system-related interactions independent of lung tissue or metabolism [14]. Moreover, the IPL model does not fully replicate the immune responses that occur in a living organism, and the results from rat lung studies may not always be directly applicable to human physiology due to species differences. Indeed, substantial interspecies differences in lung architecture, cellular composition, and metabolic capacity have to be taken into account when looking for predictability in human outcomes. Variations in airway branching patterns between rat and humans influence regional deposition, absorption, and clearance of inhaled compounds, while differences in alveolar–capillary barrier thickness and cellular organization further constrain direct extrapolation to humans [62]. To date, no studies have demonstrated clinical validation of Isolated Perfused Rat Lung data in humans, and the structural disparities outlined above constitute a major barrier, particularly regarding particle deposition and distribution patterns. Conversely, as shown by Eriksson *et al*. the technique is a well-validated and predictive method for assessing pulmonary absorption and transport [14]. As all complex *ex vivo* models also IPL presets some critical limitations regarding the statistical robustness and reproducibility of experimental outcomes. IPL preparations are technically demanding, time-consuming, and require high operator expertise, which often limits throughput and consequently reduces the achievable statistical power. Moreover, substantial inter-laboratory variability has been reported, largely due to differences in perfusion systems, ventilation settings, buffer composition, warming and oxygenation methods, and criteria used to assess lung viability during the experiment. The lack of universally accepted standards for physiological monitoring—such as perfusion pressure, airway pressure, compliance, and gas exchange—further complicates comparisons between studies. To improve reproducibility and facilitate data integration, future work would benefit from harmonized protocols, standardized reporting of physiological parameters, and clearly defined quality-control criteria for inclusion of IPL preparations. Despite these limitations, the IPL model remains a useful *ex vivo* tool for gaining insights into lung physiology and inhaled drug pharmacokinetics and pharmacology. Certainly, this approach is not intended to replace traditional *in vivo* methods completely, but it can serve as a valuable and cost-effective option for preliminary compound screening. Catheterized rats represent

a well-established *in vivo* technique that enables multiple blood withdrawals from the same animal, thereby allowing detailed pharmacokinetic assessments while reducing the total number of animals required [63]. However, the surgical preparation and maintenance of catheterized animals make this approach relatively expensive. In this context, the IPL model, despite its inherent limitations—most notably the restricted experimental duration—offers a practical alternative for early screening and prioritization of candidate compounds before advancing to more resource-intensive *in vivo* studies.

Conclusion

This review has provided a comprehensive overview of the isolated perfused rat lung (IPL) model, detailing experimental procedures, current applications, and emerging future uses. The IPL represents an innovative *ex vivo* approach that actively supports the 3Rs (Replacement, Reduction, Refinement) in animal research by offering a reliable alternative to traditional *in vivo* studies, particularly for the screening of novel inhaled compounds and formulation strategies. Beyond reducing the number of animals required, the model enables repeated and precise sampling, thereby enhancing data quality and reproducibility. Importantly, the IPL preserves the architecture and integrated physiology of the intact lung, allowing simultaneous evaluation of ventilation, perfusion, drug deposition, and tissue responses in a controlled environment. Looking ahead, the IPL model holds strong potential for advancing our understanding of pulmonary physiology and pathology, optimizing drug delivery platforms, and accelerating the development of innovative therapeutic modalities such as mRNA, siRNA, and oligonucleotide-based treatments. Its continued refinement—through integration with advanced techniques such as imaging, precision cut lung slices, and computational modeling—will not only expand its translational value but also strengthen its role as an ethical and scientifically robust tool in pulmonary research.

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Data Availability All the data are present in the cited reference.

Declarations

Conflict of Interest Alessandro Fioni is employee of Chiesi Farmaceutici S.p.A. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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