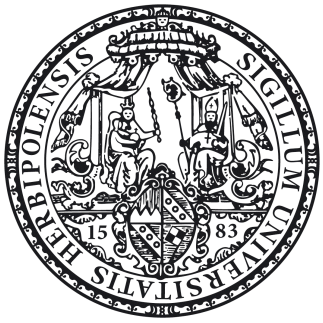
Pharmacokinetic and pharmacodynamic characterization of inhaled β_2 - agonists using the isolated human lung perfusion model



DISSERTATION

zur Erlangung
des naturwissenschaftlichen Doktorgrades
der Julius – Maximilians – Universität Würzburg

vorgelegt von
Mirjam Gnadt
aus Unsleben

Würzburg 2010

Eingereicht bei der Fakultät für Chemie und Pharmazie am

……

Gutachter der schriftlichen Arbeit

1. Gutachter: ……..
2. Gutachter: ……..

Prüfer des öffentlichen Promotionskolloquiums

1. Prüfer: ……
2. Prüfer: ……
3. Prüfer: ……

Datum des öffentlichen Promotionskolloquiums:

……

Doktorurkunde ausgehändigt am:

……

Die vorliegende Arbeit wurde auf Anregung und unter Anleitung von

Frau Prof. Dr. Petra Högger

am Lehrstuhl für Pharmazeutische Chemie

des Instituts für Pharmazie und Lebensmittelchemie

der Julius-Maximilians-Universität Würzburg angefertigt.

Meinen Eltern

Danksagung

An dieser Stelle möchte ich allen Personen herzlich danken, die mich direkt oder indirekt bei der Entwicklung und Durchführung dieser Arbeit unterstützt haben.

Allen voran gilt mein Dank Frau Prof. Dr. Petra Högger für die Aufnahme in Ihren Arbeitskreis und die Überlassung des interessanten und spannenden Themas. Das mir entgegengebrachte Vertrauen und die Bereitschaft zur Diskussion bei den unterschiedlichsten wissenschaftlichen Fragestellungen, haben wesentlich zum Fortschritt dieser Arbeit beigetragen. Desweiteren möchte ich mich für die Übertragung von verantwortungsvollen Aufgaben in der Lehre bedanken, die es mir ermöglichten, mich im Fach Klinische Pharmazie umfassend weiterzubilden.

Dr. Martin Bayliss und Chris Edwards von GlaxoSmithKline, Stevenage, UK danke ich für Ihren Aufwand regelmäßig nach Würzburg zu reisen, um den Verlauf und die Ergebnisse der Arbeit zu diskutieren und für die Bereitstellung der β_2-Agonisten und die finanzielle Unterstützung der Projekte.

Bei Dr. Andreas Kirschbaum, der momentan in der Thoraxchirurgie der Uniklinik Freiburg tätig ist, und bei meinem ehemaligen Arbeitskreiskollegen Dr. Matthias Freiwald möchte ich mich herzlich für die sehr gute Einarbeitung in der Handhabung des doch recht komplexen humanen Lungenperfusionsmodells bedanken, ohne die die spätere Durchführung eigener Projekte nicht möglich gewesen wäre. An dieser Stelle seien auch Herr Prof. Dr. Godehard Friedel und sein OP-Team genannt, die in der Anfangsphase dieser Arbeit das benötigte Lungengewebe und die Räumlichkeiten in der Klinik Schillerhöhe in Gerlingen bereitstellten.

Ganz besonders danken möchte ich Herrn Dr. Boris Kardziev, Chefarzt der Thoraxchirurgie an der Klinik Michelsberg in Münnerstadt, der es mir durch sein Engagement ermöglichte das Projekt dort fortzuführen. Die gute und angenehme Kommunikation und Kooperation vereinfachten so manche organisatorische Hürde und brachten die Arbeit in der entscheidenden Phase wesentlich voran.

Herrn Prof. Dr. med. Michael Schmidt, Oberarzt der Medizinischen Klinik und Poliklinik I und Leiter der Abteilung Pneumologie im Uniklinikum Würzburg, danke ich für die wertvolle Unterstützung im Zusammenhang mit der Antragstellung des Forschungsprojekts bei der Ethik-Kommission, welche die Durchführung überhaupt erst ermöglichte und für die Übernahme des Co-Referats.

Da die experimentellen Untersuchungen am Lungenperfusionsmodell stets vier Hände benötigten, war eine alleinige Durchführung nicht möglich. Daher bin ich meinen lieben Kollegen Beatrice Trammer und Daniel Baumann zu sehr großem Dank verpflichtet, die trotz des Zeit- und Kraftaufwands immer spontan und mit voller Einsatzbereitschaft assistierten. Diesen hohen Grad an Kollegialität wusste ich stets sehr zu schätzen.

Für die Planung und Anfertigungen der benötigten technischen Ausstattung für das Lungenperfusionsmodell möchte ich mich besonders bei den Mitarbeitern der institutseigenen

Werkstatt Matthias Völker, Karl Vollmuth, Georg Walter und den Auszubildenden sowie beim Glasbläser des Instituts für Organische Chemie Berthold Fertig für ihren Beitrag bedanken.

Bei Herrn Prof. Dr. Martin Müller möchte ich mich für die Möglichkeit bedanken, die LC-MS/MS Analytik in der Pharmazeuischen Biologie in Würzburg durchführen zu dürfen. Eine ganz große Hilfe war hierbei vor allem Dr. Markus Krischke, der mir bei den LC-MS/MS Messungen immer sehr bereitwillig und geduldig unter die Arme gegriffen hat.

Für die Unterstützung bei der notwendigen Datenverarbeitung danke ich besonders Magnus Matz aus dem Arbeitskreis von Herrn Prof. Dr. Knut Baumann, der mich von Braunschweig aus in die Geheimnisse des Programmierens eingeweiht hat.

Weiter danke ich recht herzlich Frau Prof. Dr. Cordula Stamme von der Abteilung für zelluläre Pneumologie im Leibniz-Zentrum für Medizin und Biowissenschaften in Borstel für die freundliche Überlassung von humaner Surfactant-Protein A Referenzsubstanz.

Bei den Mitarbeitern des Instituts für Klinische Transfusionsmedizin und Hämatologie des Universitätsklinikums Würzburg, insbesondere bei Herrn Dr. Andreas Opitz bedanke ich mich für die gute Kooperation bezüglich der Beschaffung von Blutprodukten, die ich regelmäßig für meine Forschungsarbeiten benötigte.

Auch wenn die Lehre im Fach Klinische Pharmazie manchmal zeitaufwendig war, hat mir diese Tätigkeit viel Spaß bereitet. Dies lag vor allem an der sehr guten und effektiven Zusammenarbeit innerhalb des KlinPharm Teams. Daher möchte ich mich bei Beatrice Trammer, Daniel Baumann sowie bei Armin Welker, der sich später nahtlos in das Team einfügte, für das tolle Arbeitsklima in diesem Zusammenhang bedanken.

Den ehemaligen Kollegen des Arbeitskreises Roswitha Skrabala, Dr. Samar Afify, Dr. Angelika Schäfer, Dr. Julia Thern und Dr. Matthias Freiwald danke ich für die freundschaftliche Aufnahme in die Gruppe, ihre Hilfsbereitschaft gerade in der Anfangszeit der Promotion und auch die lustigen Feierabende. Nicht vergessen möchte ich an dieser Stelle Dr. Anagnostis Valotis, der zwar zu diesem Zeitpunkt schon im Lehrstuhl für Lebensmittelchemie tätig war, mir jedoch stets mit wissenschaftlichem Rat zu Seite stand.

Natürlich danke ich auch allen aktuellen Mitgliedern des kleinen aber feinen Arbeitskreises Daniel Baumann, Max Kurlbaum, Roswitha Scheblein, Beatrice Trammer und Klaus Uhlenhut für die tolle Zusammenarbeit und das freundschaftliche Verhältnis. Die anregenden Diskussionsrunden beruflicher aber auch privater Natur sowie die lustigen Abende werden mir sehr fehlen.

Zu guter Letzt möchte ich mich ganz besonders bei meinen Eltern Elisabetha und Jürgen Machon und meinem liebsten Steff bedanken. Durch ihre Liebe und bedingungslose Unterstützung haben sie mir stets geholfen Zeiten der Frustration zu bewältigen und neue Motivation und Kraft zu schöpfen, um meine Ziele nicht aus den Augen zu verlieren.

The important thing is not to stop questioning

A. Einstein 1879 - 1955

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A. Introduction

1 The human lung as the site of drug application and pharmacological action

1.1 Anatomy of the human lungs

The structure of the human lungs ensures the essential physiological function of respiration defined by the oxygenation of the circulating blood and the elimination of carbon dioxide from the body. The organ responsible for the gas exchange includes the right and the left lung, the left lung consists of two and the right lung of three lung lobes, respectively. The human airways are divided into two functional regions: the conducting and the respiratory zone (see *Table 1*). The bronchial tree repeatedly branch dichotomously into two smaller daughter branches. The conducting airways from the trachea to the terminal bronchioles (airway generations 0-16) filter and transport inspired air to the respiratory region where the gas exchange takes place (airway generations 17-23) [1].

Table 1: Airway structure of the human lungs according to the model of Weibel [2].

	Generation		Diameter (cm)	Length (cm)	Number	Total cross sectional area (cm^2)
Conducting zone	trachea	0	1.08	12.0	1	2.54
	bronchi	1	1.22	4.8	2	2.33
		2	0.83	1.9	4	2.13
	↓	3	0.56	0.8	8	2.00
	bronchioles	4	0.45	1.3	16	2.48
		5	0.35	1.07	32	3.11
	↓	↓	↓	↓	↓	↓
	terminal bronchioles	16	0.06	0.17	$6*10^4$	180.0
Respiratory zone	respiratory bronchioles	17 ↓ 19	↓ 0.05	↓ 0.10	↓ $5*10^4$	↓ 10^3
	alveolar ducts	20 ↓ 22	↓	↓	↓	↓
	alveolar sucs	23	0.04	0.05	$8*10^4$	10^4

Two distinct circulatory systems supply the lungs with blood. The bronchial circulation is a part of the systemic circulation and comprises about 1 % of the cardiac output and provides the

conducting airways, pulmonary blood vessels and lymph nodes [3]. In contrast, the pulmonary circulation carries the entire right cardiac output. The blood vessels of the pulmonary circulation rise from the pulmonary arteries continue along the large airways to the respiratory bronchioles and alveoles where they finally split into a dense low-pressure vascular bed and therefore are the major supply for the alveolar walls and ensure efficient gas exchange.

Inflammatory pulmonary diseases such as chronic obstructive lung disease (COPD), asthma or long-term exposure to allergens, viruses or chemical toxins may result in chronic structural alterations in the lungs. The pathophysiological process of airway remodelling includes airway wall thickening, subepithelial fibrosis, hyperplasia of smooth muscles and goblet cells and hypersecretion of mucus [4, 5]. These changes may severely affect the mucociliary clearance, the characteristics of endo- and epithelial barriers and thus the permeability of inhaled drugs and the deposition pattern of aerosols in the airways [6-9].

1.2 Asthma diagnosis and lung function measurements

A clinical diagnosis of asthma is based on the occurrence of symptoms as episodic breathlessness, cough, wheezing and chest tightness. Measurements of lung function and the demonstration of reversibility of lung function after application of inhaled β_2 – agonists enhance the diagnostic confidence (see *Table 2)*. Furthermore, the assessment of lung function parameters allows the clinical monitoring of the effectiveness of asthma therapies.

Spirometry is the recommended method for the determination of lung function that offers the measurement of the most common lung function parameters in clinical practice, mainly the forced volume expired in one second (FEV1) and the forced vital capacity (FVC). FEV1 is one of the most commonly used lung function parameters for the classification of the asthma severity and control (evidence grade 1). The measurement of FEV1 is performed according to a standardized protocol. In general, the measurement of FEV1 is repeated three times to obtain at least two reproducible values. A successful diagnostic procedure is therefore strongly dependent on the cooperation of the patient, thus exact instructions for the forced expiratory manoeuvre by the medical staff is essential to minimize the efforts of the asthmatic patient. The measured FEV1 is then based on the reference value adjusted for age, sex and body height according to the general population and demonstrated as percent predicted FEV1 [10-12].

Table 2: Asthma severity classification in adults according to the GINA and German guidelines [13, 14]

Severity	*Symptoms*	*Lung function as percent predicted*
IV severe persistent	persistent symptoms with high intensity and variability in the daytime and at night strong impairment of physical activities frequent exacerbations	FEV1 ≤ 60 % or PEF ≤ 60 % PEF variability > 30 %
III moderate persistent	symptoms daily at night > 1/week exacerbations impair daily activities and sleep daily necessity of short-acting β_2 – agonists	60 % < FEV1 < 80 % PEF 60 – 80 % PEF variability > 30 %
II mild persistent	1/week < in the daytime < 1/day at night > 2/month exacerbations impair daily activities and sleep	FEV1 ≥ 80 % PEF ≥ 80 % PEF variability < 20 – 30 %
I mild intermittent	in the daytime < 1/week at night ≤ 2/month short exacerbations (hours to days)	FEV1 ≥ 80 % PEF ≥ 80 % PEF variability < 20 %

FEV1 – forced expiratory volume in one second; PEF – peak expiratory flow

The peak flow meter represents a further important tool for the diagnosis and the assessment of the effectiveness of asthma therapy. The device is cheap, easy to handle and appropriate for the daily domestic asthma monitoring because it has been shown that a decline in PEF (peak expiratory flow) signals an asthma exacerbation several days prior to the event [14-16]. To assess the lung function status of the patient, current PEF values are compared with the personal best value, measured during the last 2 – 3 weeks in a stable period of the respiratory disease [17]. But again precise instructions for the procedure and documentation of PEF measurement are very important for accurate and reproducible results.

Additional diagnostic methods of lung function are required if patients suffer from severe asthma, if patients are unable to perform forced expiratory manoeuvres, e.g. infants or very old persons or if the results of previous spirometric measurements are questionable. In these cases body plethysmography is the diagnostic method of choice which is less dependent on the precise cooperation of the patient. To determine the extent of pulmonary obstruction or the reversibility of obstruction after inhalation of a bronchodilator the airway resistance R is often determined via plethysmography [13, 14].

Up to the present day pulmonary administered drugs were been used in the treatment of various diseases. Especially the pharmacologic management of obstructive pulmonary diseases

like asthma and COPD are less well controlled without drugs that are locally administered via inhalation. Predominantly aerolized glucocorticoids and β_2 – agonists remain the mainstay in those indications according to recent guidelines [13, 14, 18].

1.3 The lung as a site of drug application and pharmacological action

The local application of medication within the airways has been proven as the optimal route of administration of first-line therapy of asthma and COPD. Inhalation of drugs has many advantages compared to the oral route. It offers the chance to achieve high drug concentrations directly at the target site, minimizes the risk of systemic side effects and bypasses potential poor gastrointestinal absorption and the first-pass metabolism in the liver. The rapid onset of the clinical effect after inhalation of salbutamol assures the decrease of acute asthma symptoms within a few minutes, whereas the maximum effect after oral intake occurs after about 2-3 hours [19]. Thereby, 100-200 µg of salbutamol administered as an aerosol is therapeutically equivalent to and causes fewer side effects than 2-4 mg given orally.

The efficient deposition and the distribution of an aerolized drug within the lung are the main challenges of inhalation therapies because different substance classes have different target structures that are not uniformly distributed throughout the airways. For example the receptors for β_2 – agonists are present at high density in the airway epithelium of the large bronchi and the terminal bronchioles. However, the effect of bronchodilation is induced by the activation of β_2 – receptors in the airway smooth muscles which have a higher receptor density in the bronchioles than in the larger bronchi [20, 21]. The location of these receptors suggests that inhaled salbutamol needs to be delivered preferentially to the peripheral regions of the lungs. In contrast to bronchodilators the anti-inflammatory effect of inhaled glucocorticoids is probably most effective when evenly distributed throughout the lungs, since inflammatory cells are present throughout the airways and the alveolar tissue in asthma [22, 23].

The deposition pattern of an aerosol largely depends on its physical and chemical properties and the physiological characteristics of the individual patient (breathing pattern, pulmonary disease) [24]. The particle size, standardized by the median mass aerodynamic diameter (MMAD), affects the lung deposition of the administered aerosol. The MMAD relates the particle to the diameter of a sphere of unit density that has the same settling velocity as the particle of interest regardless of its shape or density. On the basis of the MMAD, the deposition pattern of inhaled particles can be predicted using mathematical models [25, 26] as shown for solid aerosol particles in *Figure 1*. However, it should be noted that these theoretical considerations do not reflect *in vivo* situations precisely because only the physical properties of

the inhaled particles are accounted for and not the individual airway anatomy of the patient and the breathing pattern during inhalation.

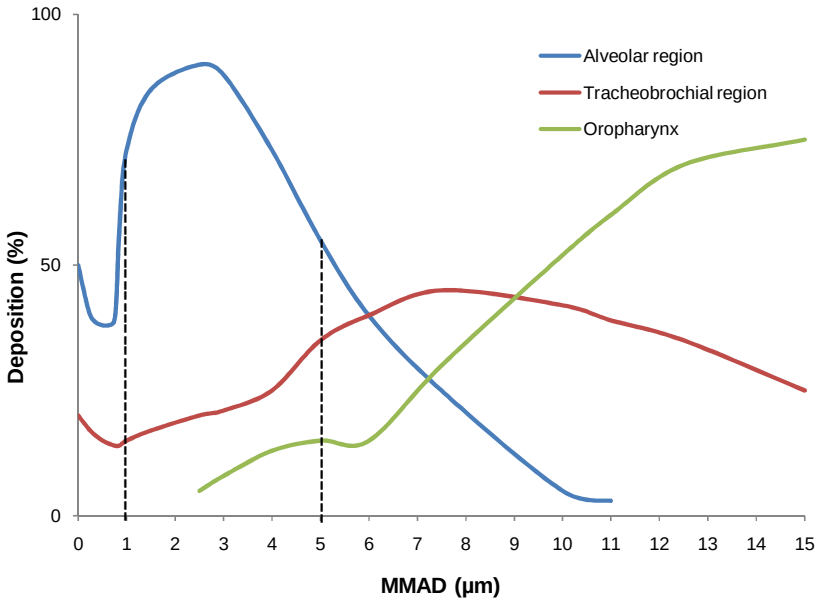

Figure 1: Deposition pattern of aerosolized solid particles with certain median mass aerodynamic diameters (MMAD) in different airway regions following a slow inhalation and a 5 s breath hold [25].

Basically, particles can be deposited by inertial impaction, gravitational sedimentation or diffusion depending on their size. Impaction takes place in the first 10 generations of the lung particularly at bends and airway bifurcations where the airflow is fast and turbulent [27]. Most of the large particles (MMAD > 10 µm) impact already in the oropharyngeal region (see *Figure 1,* green line), especially if the drug is inhaled with a device requiring a high inspiratory flow rate during administration (e.g. dry powder inhaler). Particles with a MMAD of 5-10 µm are mainly deposited in the conducting airways (see *Figure 1,* red line). Particles with a size between 1-5 µm (dashed area in *Figure 1)* favour a deposition in the smaller airways and the alveolar region (blue line), while particles < 3 µm have an 80 % chance to reach the lower airways [28]. In the bronchi and bronchioles, where the air velocity is low, particles are deposited by sedimentation and diffusion processes, whereas impaction is negligible. Particles down to 0.5 µm in diameter sediment, below 0.5 µm the mechanism of deposition is predominated mainly by diffusion [29]. Aerosol particles smaller than 1 µm not deposited during inhalation are exhaled again.

The therapeutic efficiency of the inhalation therapy with β_2 – agonists aerolized particles with a MMAD between 1-5 µm are preferred to achieve an optimal deposition of the drug in the peripheral parts within the airways [30]. Thus, the type of inhalation device, drug formulations

and the inhalation technique of the patient are the main determinants for the targeting to the appropriate lung region. For example the pulsatile administration of an aerosol with a slow and deep inhalation followed by a breath hold has been shown to reduce impaction and to enhance the retention time of the particles in the airways [26].

1.4 Substance class of interest: β_2 – agonists

1.4.1 The pharmacological effect of β_2 – agonists

The β_2-adrenoceptor is composed of approximately 413 amino acids and is a member of the seven- transmembrane family of receptors coupled to a G_s-protein unit [31]. They are widely distributed in the airway smooth muscle cells (30 000-40 000 per cell) but also occur in other cells of the lungs such as the epi- and endothelial cells, type II and mast cells [32, 33]. The β_2-receptor exists in an active form and in equilibrium in an inactive form. After the binding of the β_2-agonist to the receptor the adenyl cyclase is activated by the G_s protein, which increases the synthesis of 3`- 5`- cyclic adenosine monophosphate (see *Figure 2*). Then cyclic adenosine monophosphate (cAMP) catalyzes the activation of the proteinkinase A, which phosphorylates key regulatory proteins involved in the regulation of muscles tone. Furthermore, an increase of cAMP results in an inhibition of Ca^{2+} release from intracellular stores and influx into the cells, leading to relaxation of airway smooth muscle.

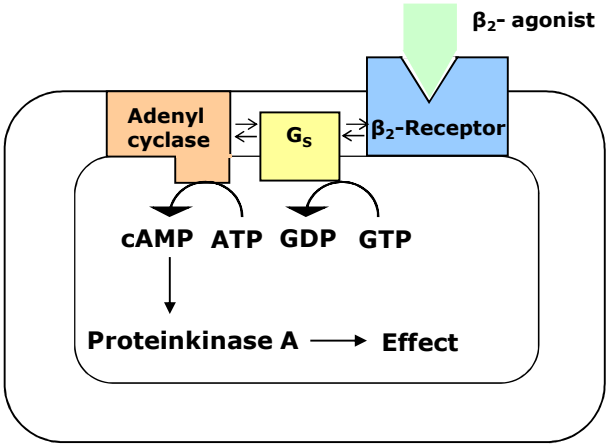

Figure 2: Mechanism of action of β_2 – agonists.

Beside the pulmonary effect of bronchodilation, the activation of extrapulmonary located β_2-receptors by β_2-agonists leads to dilation of blood vessels, contraction of the uterus, increase in heart rate and tremor.

The chemical structure of a β_2-agonist determines how it interacts with the receptor. A selection of important physicochemical properties of different short- and long-acting β_2-agonists is shown in the following *Table 3.*

Table 3: Selection of physicochemical properties of different β_2-agonists.

	Salbutamol*	Fenoterol*	Formoterol*	Salmeterol*	GW597901[#]
pK_a	9.22, 9.83	8.5, 10.0	7.9, 9.2	9.26, 9.99	9.23, 9.82, 10.08
log P	0.015	0.89	1.57	3.07	1.26
Water solubility (mg/mL)	14	0.2	0.04	0.16	0.3
PSA ($Å^2$)	72.7	93	90.8	82	142.11

pK_a - logarithmic acid dissociation constant, log P - logarithmic partition coefficient, PSA ($Å^2$) - polar surface area (Angstrom); * data were available at [34] and linked databases; [#] GSK, data on file.

A hypothesis being that while salbutamol, which is hydrophilic in nature, activates the receptor from the extracellular compartment, the lipophilic chain of the salmeterol molecule diffuses into the cell membrane resulting in a receptor binding that is slowly reversible and non-competitive [35]. Therefore, the onset of action induced by salmeterol is delayed but longer lasting (about 12 h) in comparison to the effect of salbutamol (4-6 h).

To prevent overstimulation due to the excessive use of β_2-agonists the β_2-receptor reacts with the autoregulatory process of desensitization occurring in response to binding of the agonist to the receptor leading to tachyphylaxis [32]. The underlying mechanisms are composed of three different processes that limit the β_2-receptor function: the receptor uncouples from the adenyl cyclase, the uncoupled receptor internalises and is phosphorylated by kinases. It has been shown that the underlying mechanism of desensitization occurring after short-term use of β_2-agonists is mainly phosphorylation which is transient and reversible within minutes after removal of the β_2-agonist. In contrast, long-term exposure results in internalisation of the receptor which takes hours to resolve. The extent of desensitization is dependent on the type of tissue. Whereas the autoregulatory process occurs rapidly in human lymphocytes and mast cells, the human smooth muscle is rather resistant towards desensitization due to β_2-agonist exposure. These differences may explain that the bronchodilating effect as a response of smooth muscle activation is maintained by regular β_2-agonist therapy while the bronchoprotective effect against provocative stimuli mainly mediated by mast cells and systemic side effects such as tachycardia and tremor decline [36]. Furthermore, the degree of desensitization is dependent on the agonist efficacy. Clinically it was shown that chronic exposure to the full agonist formoterol led to a lower bronchodilating effect over time than the treatment with the partial agonist salmeterol [36, 37]. Corticosteroids seemed to have a positive

influence on β_2-receptor downregulation as they increase β_2-adrenoceptor gene transcription and regulate the number and the coupling processes of the receptors [38].

Hypothesizing that defects in the genes for the β_2-receptor may contribute to the therapeutic control of asthma bronchiale, Reihsaus et al. identified nine point mutations in a study done with 51 asthmatic and 56 healthy subjects [39]. Three of the discovered polymorphisms appeared to alter the functional behaviour of the β_2-receptor and thus the response to endogenous catecholamines and exogenously administered β_2-agonists. Focused on the polymorphism at position 16 that can be either the amino acid arginine (Arg) or glycine (Gly) clinical studies have shown that the Gly 16 form may be associated with a higher extent of receptor downregulation after β_2-agonist exposure, impaired bronchodilator response, airway hyperreactivity and worsening of nocturnal asthmatic symptoms [39-42]. The second clinical relevant polymorphism was found to be at position 27, which can be either glutamine (Gln) or glutamate (Glu). The Glu 27 form seemed to protect against receptor desensitization in comparison to the Gly 16 form [39]. A clinical study investigating the association between Glu/Gln 27 polymorphism and airway hyperreactivity in asthmatic patients observed that Glu 27 homozygotes were four times less responding to the bronchial provocation than patients homozygote for Gln 27 [43]. The third polymorphism in the position 164 can be either threonine (Thr) or isoleucine (Ile). It's occurance is quite rare with an allelic frequency of < 1 %, but might be potentially relevant due to its location within the forth transmembrane domain of the receptor that has been predicted to interact with β_2-agonists [40]. Data assessing the clinical importance of the Thr-Ile polymorphism are not published yet.

Thus, beside the compliance of the patient and the pharmacological action the success of inhaled β_2-agonist therapy depends on many factors such as the intrinsic efficacy and potency of the β_2-agonist, the frequency of drug administration and the individual genotypes for the β_2-receptor.

1.4.2 Pharmacokinetic properties of β_2 – agonists

The airway efficacy of topically applied drugs is not only defined by their potency, but by their pharmacokinetic properties as well. Furthermore, safety issues are always of concern to ensure a balanced risk-benefit ratio and therefore to specify their therapeutic index [44]. For the entire pharmacokinetic evaluation of inhaled drugs, the systemic components oral bioavailability, clearance and plasma and tissue binding have to be considered in addition to the pulmonary parameters like the efficiency of pulmonary deposition, the pulmonary absorption rate and residence time [45-47].

The safety profile of β_2 – agonists depends largely upon their formulation, dosage and their classification into the group of short-acting (SABA) or long-acting (LABA) β_2 – agonists. Salbutamol, fenoterol and terbutaline as representatives of SABAs are commonly used as rescue medication in asthma exacerbations to relieve acute symptoms and can be given via inhalation, orally and parenterally whereas LABAs like salmeterol and formoterol are solely available for inhalation delivery, either as single substances or in combination with glucocorticoids. Patients' use of LABAs improves asthma symptoms and pulmonary function and is therefore effective in the long-term control of chronic asthma [48]. Character and extent of adverse events resulting from the use of β_2 – agonists are mainly dependent on the proportion of dose that reaches the systemic circulation. These undesired side effects range from headache and tremor that may resolve after one to two weeks of usage, to severe cardiac arrhythmias. The inhalation route is generally preferred, the β_2 – agonists reach the bronchial site directly and achieve a distinct pulmonary effect in the minimal effective dose and the systemic exposure is avoided to a very large degree in comparison to oral or parenteral application [49-51]. Thus, the inhalation therapy delivers high drug concentrations in the lung, whereas systemic concentrations are reduced. The final extent of systemic exposure is composed of the pulmonary and the orally absorbed fractions of the inhaled β_2 – agonists (see *Figure 3*). Especially in the development of new β_2 – agonists with improved pharmacodynamic profiles, it is desirable to create a drug that complies with optimal pharmacokinetic properties regarding pulmonary efficacy and safety. Thus, a high pulmonary residence time, a reduced or decelerated pulmonary absorption rate and a negligible oral bioavailability due to high first-pass inactivation are major goals to optimise pulmonary therapy. These aspects are preferentially affected by compound characteristics like the particle size of the aerolized drug and the physicochemical properties like the molecular polar surface area and lipophilicity [52, 53].

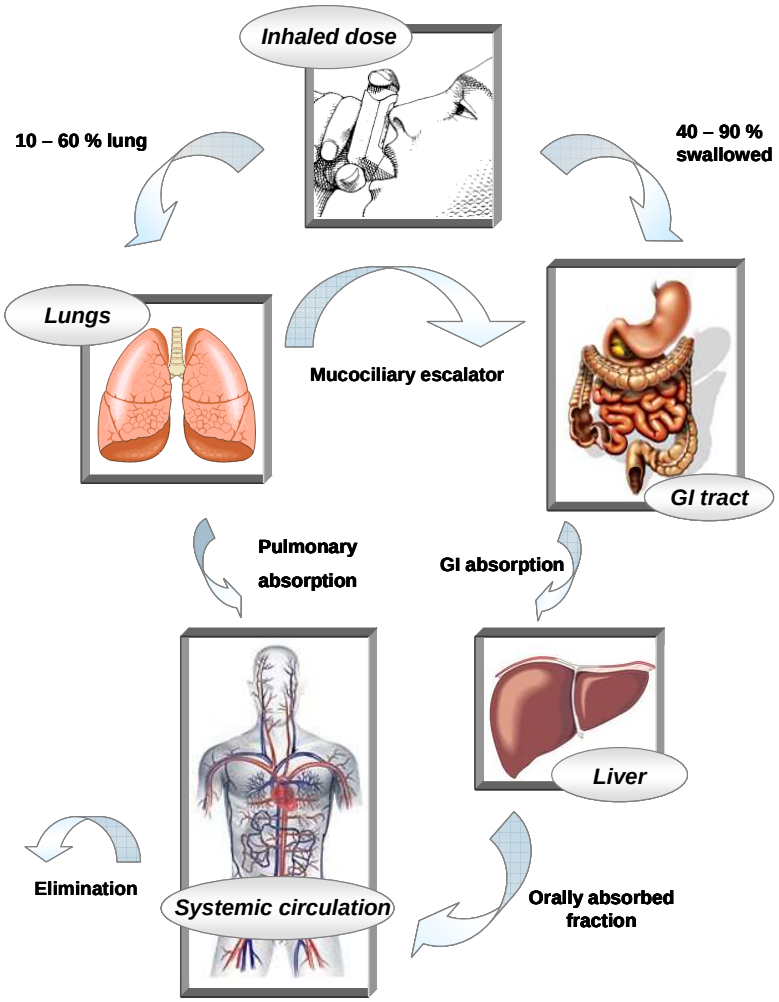

Figure 3: Pharmacokinetic model to describe pulmonary targeting.
After inhalation of an aerolized drug by a metered-dose inhaler (MDI) 10-60 % of the nominal dose effectively reach the lungs, the main proportion of aerosol particles deposit in the deeper bronchial airways and up to 10 % may be breathed out again [29]. Larger particles (≥ 5 µm mass median aerodynamic diameter, MMAD) impact on the oropharynx during inhalation and are subsequently swallowed resulting in a gastrointestinal absorption. Dependent on the oral bioavailability and rate of pulmonary absorption, the drug reaches at the systemic circulation and may cause systemic effects before it is eliminated [47, 54] (Figure modified according to [47]).

Methods for pharmacokinetic characterization of pulmonary applied drugs include many *in vitro*, *in vivo* and *ex vivo* techniques (see *Figure 4*). At the simplest level, *in vitro* methods start with the analysis of physicochemical properties and the determination of the octanol/water partitioning coefficient in lipid solvents and the partition in immobilized artificial or biological membranes. Epithelial cell models to predict absorption processes in the intestine and the lung form part of preclinical studies. In fact, *in vitro* techniques may just provide an idea of the rate and extent of redistribution of an inhaled drug from the lung into the systemic circulation, but they are not able to mirror the complexity and the interaction of different human organ structures. Thus, *in vitro* data needs to be combined with those of *ex* and *in vivo* experiments or used as input into a physiological based pharmacokinetic model [55, 56].

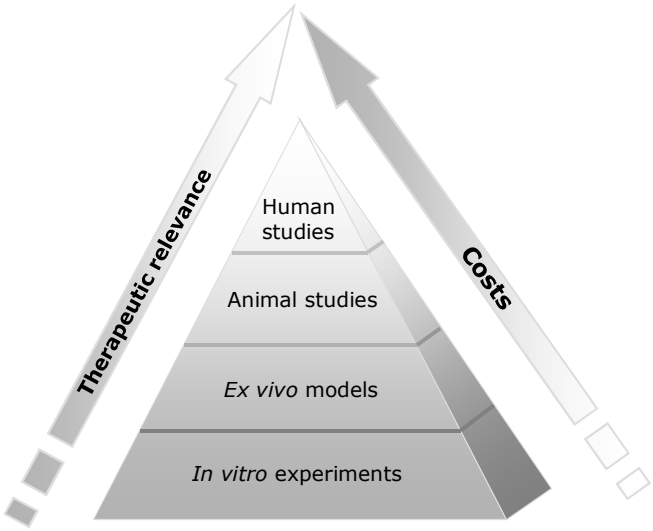

Figure 4: Schematic illustration of the hierarchy of systems used for preclinical PK data collection of inhaled drugs (image modified according to [57]).

Investigation of pulmonary targeting in animals advanced in the 1970s and 1980s [58-64]. Introducing special dosing techniques for intratracheal instillation and aerosol inhalation in mice, rats, guinea pigs and dogs were the first approaches to establish substantial data sets on drug kinetics in the lung within its physiological context. The limitations of animal models are obvious and the derived pharmacokinetic data have to be regarded with caution. Commonly used laboratory animals such as rodents or dogs show significant differences to humans in pulmonary anatomy on molecular and physiological basis, biochemistry and cell biology that may affect the deposition pattern of inhaled particles and pulmonary drug absorption and clearance processes [65, 66]. Furthermore, some studies demonstrated that anaesthesia of experimental animals in absorption studies of pulmonary applied drugs alter physiological functions such as mucociliary clearance and alveolar epithelial permeability [67, 68]. In addition, pulmonary dosing techniques in preclinical laboratory settings usually include intratracheal instillation or pulmonary administration with special self-constructed inhalation devices. However, conventional pressurised metered-dose inhalers or dry powder aerosol formulations are rarely applied in animal studies [69], although Ewing et al. used dry powder formulations in isolated perfused rat lungs with a recently developed DustGun aerosol technology [70]. Furthermore, Penn-Century Inc. introduced a new MicroSprayer® device allowing intratracheal delivery of liquid sprays that has been used in several animal studies [71, 72]. Nevertheless, the translation of pharmacokinetic data from animal to human models is restricted. Human studies may gain insight into traditional pharmacokinetic parameters such as oral bioavailability and clearance. If the drug is labelled, the site of pulmonary deposition can be visualized. But discrete processes like drug distribution from the lung into the systemic circulation and the

systemic bioavailability after inhalation are not accessible in these experimental settings, first of all for ethical reasons and secondly due to very low circulating levels being below the limits of detection [73, 74].

1.5 The isolated perfused human lung model

The isolated perfused lung (IPL) model resolves some of these difficulties which cannot be addressed by *in vitro* and *in vivo* models mentioned above. The first attempts to establish the IPL were made in the early 1970s, exclusively using resected organs of animal origin. In these studies the characterization of pharmacodynamic or metabolic processes in the lung was the primary interest [75-77], the model was extended to pharmacokinetic questions in the following decades [78-81].

By reperfusion of the lung lobe the pulmonary circulation is re-established, although the bronchial and pulmonary lymph circulations are not considered. Since both blood circulations are connected additionally via the bronchial vein and right atrium and the pulmonary vein and left atrium, respectively, the contribution of bronchial circulation to pulmonary absorption of inhaled drugs is mainly unknown [82-84]. Although Wagner and colleagues could demonstrate in a study with sheep that pulmonary absorption of particles is influenced strongly by bronchial circulation if deposited in the afferent airways [85]. Nevertheless, it is assumed that due to the great alveolar surface area and the particle size distribution of common aerosols, the initial pulmonary absorption mainly relies on the pulmonary circulation [86].

The power of the IPL model is limited mainly because of tissue edema and cell destruction. Thus, reperfusion procedures *ex vivo* for longer than five hours with entire animal lungs were rarely achieved when using physiological flow rates. The choice of the flow rate is dependant on the perfusion pressure generated in the capillaries, because the edema formation and impairment of lung tissue is forced by excessive pressures. In the human isolated lung perfusion model those unfavourable conditions are already achieved at much lower perfusion flow rates [87]. Beside the flow rate the composition of the perfusion fluid plays an important role. Generally, either different blood products (whole blood, erythrocyte concentrates, buffy coats) or buffer solutions are possible options. If buffer solutions are chosen the addition of glucose for cell metabolism and macromolecular substances for the adjustment of a physiological oncotic pressure are essential. Ventilation parameters have to be monitored over the whole duration of the experiment. “Overventilation” due to too high ventilation pressures and volumes may cause irreversible tissue damage and may influence the quality of perfusion of discrete tissue regions.

Nevertheless, the IPL model allows a realistic simulation of pulmonary physiology by reperfusion and ventilation of resected lung lobes. Whereas models using animal lung tissue can use the whole organ, the reperfusion of human material is confined to a lung or single lung lobes. Cellular integrity, permeability barriers, biochemical activity and the structure of the organ with its anatomical constitution remains well-preserved as far as possible. Pharmacokinetic parameters in the lung can be determined under nearly physiological conditions, without any systemic influences such as redistribution into other tissues, hepatic metabolism or elimination processes.

2 Aim of the thesis

The objective of the thesis was to determine the absorption kinetics of pulmonary applied β_2-agonists from the lung into the systemic circulation *ex vivo* in relation to their physicochemical properties. For that purpose the isolated perfused lung model developed by Linder et al. [87] was to be modified. The short-acting agent salbutamol and the new long-acting β_2-agonist GW597901 were chosen to be applied to *ex vivo* perfused lungs via the pulmonary route. GW597901 as potential representative for a new generation of ultra long-acting β_2 – agonists is a clinical research compound which proved to be encouraging in early phase II studies [88]. For the first time, the pharmacokinetic data derived from the IPL model were to be supplemented by the analysis of the pharmacodynamic properties of the bronchodilators GW597901 and salbutamol. Furthermore, potential biochemical markers of edema in perfusion fluids were sought to be identified.

B. Results and Discussion

1 The pharmacokinetic enabling study: Comparison of the pharmacokinetic properties of GW597901 and salbutamol employing the human lung lobe perfusion model

1.1 Objective

So far human studies describing pharmacokinetic (PK) behaviour of inhaled β_2 – agonists were mainly concerned with the determination of systemic bioavailability of drugs and testing for bioequivalence of different drug delivery devices or drug combinations. For this purpose Lipworth et al. initiated extensive research into this field since the early 90s. In most of the studies the pharmacokinetic parameters C_{max}, t_{max} and AUC were determined and compared by analysis of blood samples after administration of β_2 – agonists via different inhalation techniques. Sampling started at the earliest time point 5 min, post-dose by drawing blood from the basilic vein. Besides, the recording of common side effects such as finger tremor, alterations in heart rate and serum potassium of test subjects allowed conclusions about extrapulmonary responses to β_2 – agonists and thus their safety profiles [89-93]. Others examined the amounts of β_2 – agonists in collected urine samples allowing assessments of the renal excretion of parent compounds and metabolites [94].

In general, human studies are aimed at the accurate description of pharmacokinetics after the inhalation route. But this methodology reported only plasma concentrations in venous samples, in which recovered drug concentrations were altered by dilution in the systemic circulation that comprehends about 4 – 6 L of blood and also by metabolism processes in the liver [52, 95]. Consequently, the very early time of release of an inhaled drug from the target site into the systemic circulation was not defined, therefore it would be necessary to obtain samples directly from the pulmonary venous output during dosing procedures. Thus, early data points were not available in pharmacokinetic studies *in vivo*. Furthermore, the information about the actual deposited dose of the inhaled drug in the lung and the swallowed proportion was not known in human studies. Therefore, the percentage of applied dose reaching the systemic circulation generated exclusively by distribution processes from the lung into the blood could not be determined. One method to prevent drug absorption from the gastrointestinal tract is the peroral intake of an aqueous slurry of charcoal prior to and after inhalation (charcoal –block method). This technique for determination of exclusive pulmonary availability of inhaled drugs has been described in several studies [96-98]. However, the efficiency of the charcoal block may vary among individuals and it remains unclear, which amount of ingested charcoal inhibits absorption of a certain drug by 100 % [99, 100]. Thus that depends to a large extent on the type and physicochemical properties of the drug and the dose swallowed. Furthermore, absorption

processes from the buccal cavity due to oral deposition of inhaled drugs leading to systemic drug levels without the first past effect should also be considered. Some studies performed mouth rinsing to remove the drug from the buccal cavity subsequently after inhalation [101, 102]. These essential PK processes have to be analyzed in advance to giving any statements about absorption profiles of drugs solely from the pulmonary site after inhalation. To date, the Food and Drug Administration (FDA) does not support the charcoal-blocking method in bioequivalence studies for regulatory approval of inhalation drugs, whereas the European Medicines Agency (EMA) recently included this methodology for PK determination *in vivo* [103, 104]. For the evaluation of tissue binding and deposition characteristics of inhaled drugs sampling of lung tissues *in vivo* would be necessary but is obviously not possible, mainly due to ethical reasons. Hence, the data base concerning distribution kinetics and drug disposition *in vivo* is not complete yet.

For these reasons, the *ex vivo* human lung lobe perfusion model would appear to be a model that might supplement detailed PK information. Recent results of external lung perfusion experiments substantially contributed to scientific knowledge gain during the last years. Freiwald et al. showed that the redistribution kinetics of inhaled glucocorticoids seemed to be strongly dependent on the particle size and characteristics defined by the used application form. It was elucidated that the dissolution of drug crystals, upon recrystallisation in human bronchial fluid, specified the rate – determining step for systemic release of glucocorticoids [105]. Other settings focussing on PK of cytostatics contributed to the understanding of the poor clinical outcomes in the treatment of non-small bronchial cell carcinoma. Local concentrations of cyclophosphamide and adriamycin tended to be ten times higher in healthy than in tumour lung tissue, concluding that further approaches have to be made to enhance drug concentrations in relevant lung regions to improve prognosis [106, 107].

In the present study, for the first time the pharmacokinetic redistribution of endobronchially delivered compounds from the β_2 – sympathomimetics class was to be demonstrated in relation to the drug lipophilicity and other characteristics. The goal of the present pharmacokinetic enabling study was to determine the initial pulmonary absorption phase of the long-acting β_2 – agonist GW597901 on the basis of the isolated perfused lung (IPL) settings using the human lung lobe model implemented by Linder et al. [87]. The short-acting β_2 – agonist salbutamol was administered concomitantly in the same experimental settings (see *Figure 5*), denoted as “cassette dosing procedure”.

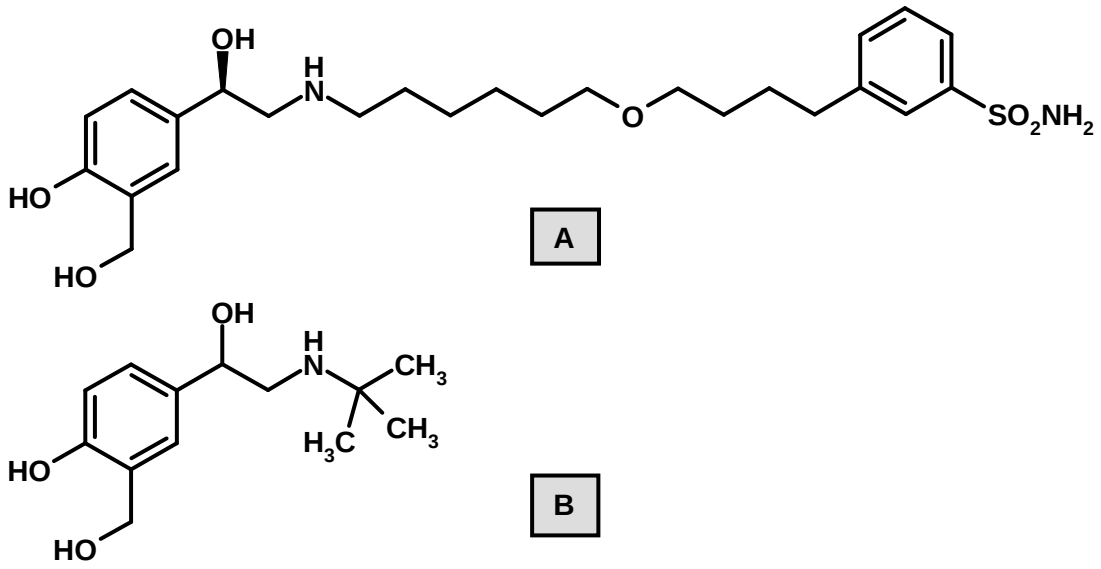

Figure 5: Chemical structures of GW597901 (A) and salbutamol (B).
M_r (A) = 494.65 g/mol; molecular formular (A) = $C_{25}H_{38}N_2O_6S$
M_r (B) = 239.3 g/mol; molecular formular (B) = $C_{13}H_{21}NO_3$

The clinical PK profile of the LABA salmeterol has been examined in combination with a glucocorticoid due to its therapeutic use [108, 109]. Very low systemic plasma concentrations of LABAs after inhalation of therapeutic doses lead to further difficulties in the determination of their PK parameters. Inhaled formulations of combined SABAs and LABAs do not have any therapeutic relevance and therefore no human studies exist addressing the pharmacokinetic evaluation of these agents, when applied concomitantly. Thus, the IPL provided an opportunity to examine β_2 – agonists given in combination and to apply doses to assure sufficient concentrations in the perfusion fluid while maintaining ethical aspects. Salbutamol was chosen to be directly compared with GW597901 due to its wide therapeutically use and wealth of pharmacokinetic and – dynamic data available in the literature [13, 14, 18, 32, 45, 51]. As the most prominent short – acting β_2 – agonist with its hydrophilic chemical properties, it was expected to observe differences between salbutamol and the lipophilic comparator GW597901 with respect to the pharmacokinetic behaviour in relation to compound attributes. Furthermore, by analysis of drug disposition within perfused lung tissues complementary pharmacokinetic data were pursued. The pharmacokinetic enabling study was to define and ensure the applicability of the lung lobe perfusion model in describing the pharmacokinetic properties and differences between salbutamol and GW597901 in six isolated human lung lobe perfusion experiments.

1.2 Experimental procedure of the lung lobe perfusion

For the pharmacokinetic enabling study the human lung perfusion model described by Linder et al. [87] was modified [105]. Human lung lobes were obtained from lung cancer patients, who had undergone either a lobectomy, bilobectomy or pneumectomy. For the patients no further burden resulted during the surgery due to the pharmacokinetic enabling study. The experiments were performed immediately after resection of the lung lobes in a room adjacent to the operating theatre. After termination of the isolated lung lobe perfusion, the human lung tissue was promptly transferred to pathology for further routine examination. Permission for the study protocol was obtained from the Ethics Committee of the Medizinische Fakultät, Eberhard – Karls – Universität Tübingen, Germany. A signed informed consent form was a mandatory requirement for inclusion of tissue from a patient in the pharmacokinetic enabling study. Furthermore, patients were excluded if they were treated neoadjuvantly due to bronchial cell carcinoma or if they had infectious diseases. To prevent corruption of obtained pharmacokinetic data, inhaled or oral $ß_2$ – agonist was to be withdrawn four weeks prior to surgery to guarantee a sufficient wash-out phase.

Subsequent to resection of lung tissue, the bronchus was anastomised and the pulmonary arteries were catheterised. The lung lobe preparation was connected to the perfusion circuit and the respirator. Ventilation procedure was pressure controlled and performed with a commercial available respirator. Reperfusion was carried out through a half open system. Perfusion fluid exiting the opened vein was collected in a fluid reservoir and returned to the fluid circulation leading in the cannulated pulmonary arteries (see *1.3.1*, C – Experimental Setup). To deliver the drug aerosol into the respiratory flow, a self-made application device was placed between respirator and lung lobe, with the distance between bronchus and “spacer” being as short as possible to minimize drug adsorption on to plastic or silicone material (see *Figure 29* in *1.2.2*, C – Experimental Setup). The transparent spacer allowed visual control of the “inhalation procedure” of the nebulized drug plume. After administration of the $β_2$ – agonists the spacer remained in the experimental setup for the duration of the experiment. In modification of the original composition of perfusion buffer from Mürdter and colleagues [110], the macromolecular fraction was replaced with bovine serum albumine (BSA) (see *1.1.2.1,* C – Experimental Setup).

It has been shown that some $β_2$ – agonists are significantly distributed into red blood cells. The blood/plasma ratio of salbutamol was reported to be approximately 1 (0.96 ± 0.13) [111, 112]. *In vitro* assays showed a concentration dependent distribution ratio of GW597901 into red blood cells with extent of binding being 45 – 94 % (100 – 0.01 µg/ mL) after a 30 min incubation. Thus, to simulate physiological conditions and to approximate the absorption and distribution kinetics of salbutamol and GW597901 to the *in vivo* situation, a mixture of perfusion

buffer and pooled buffy coat products was used in six IPL experiments as perfusion fluid (see *2.1.2.1,* C – Experimental Setup). The fraction of red blood cells in the perfusion fluid was set at 20 % (= HCT %).

GW597901 and salbutamol were applied to the lung lobe with a commercial available PARI LL® nebulizer (see *2.2.2,* C – Experimental Setup) in nominal doses of 45/150 µg (n= 3) and 180/600 µg (n= 3) (GW597901/salbutamol), respectively. The interrupter technique was used for dosing procedure while nebulization was activated during inspiration phase and stopped throughout expiration. During the dosing procedure the ventilation pressure was enhanced by 5 mbar to warrant optimal deposition of the β_2 – agonist in the deeper airways and returned to normal level afterwards. The administration of targeted nominal doses of aerolized β_2 – agonists took about 12 minutes. Already during the application of the aerosol, samples of perfusion fluid were obtained from the venous output to observe the early absorption phase of the β_2 – agonists. Subsequently 6 mL samples of perfusion fluid were drawn from the open vein and reservoir in equal measure at particular times for the remaining duration of the experiment. Removed liquid was immediately replaced with fresh pre-warmed perfusion fluid. Perfusion fluid samples of each time point were aliquoted either as whole perfusion fluid (WPF) or as cell – free perfusion fluid plasma (PFP) obtained by centrifugation (see *2.3.3.1,* C – Experimental Setup). Besides, quantitative measurement of GW597901 and salbutamol in PFP samples, WPF samples were also analyzed as well to allow additional conclusions about the distribution of GW597901 or salbutamol into red blood cells in this experimental setting. The study protocol aimed at collecting fluid samples from the venous output and reservoir for up to 60 minutes subsequent to completion of dosing procedure. After termination of the perfusion experiment lung tissue specimens of at least 4 different sites of the lung lobe were attained and a bronchoalveolar lavage (BAL) was carried out (see *2.3.3.3,* C – Experimental Setup).

To ensure physiological conditions over the whole duration of the perfusion experiment the concentration of hydrogen carbonate, pH value and temperature of perfusion fluid were continuously monitored. If necessary, the perfusion fluid was supplemented with gaseous CO_2 by a secondary circulation system (see *2.3,* C – Experimental Setup). For determination of the effectively administered dose of GW597901 and salbutamol all parts of the dosing device including the PARI LL® nebulizer, application device and diverse connection tubes were rinsed with MeOH after termination of the experiment and the concentration of the β_2 – agonists was quantified. By subtraction of the totally used amount of β_2 – agonists from the residual quantity, the actually applied dose was determined (see *2.4,* C – Experimental Setup).

To evaluate the pulmonary absorption process of GW597901 and salbutamol, complete sample sets of six different human lung lobe perfusion experiments, including perfusion fluid,

lung tissue as well as broncho-alveolar (BAL) fluid samples, were quantitatively analyzed for GW597901 and salbutamol.

1.3 Results

In total six human lung lobes were included in the pharmacokinetic enabling study while a predefined composition of perfusion buffer and pooled buffy coats served as perfusion fluid in every experiment. Detailed characteristics of perfused lung lobes are shown in *Table 4*, IPL experiments are numbered chronically in ascending order. Four patients provided lung lobes deriving from lobectomies, patient 4 had to undergo a pneumectomy and patient 6 was bilobectomized, respectively. The actual mean haematocrit of perfusion fluids was 20.5 ± 1.3 % (see *1.1*, D – Appendix) determined by centrifugation.

Table 4: Characteristics of donor lungs and perfusion experiments of the enabling study

Perfusion experiment	Donor		Resected part of the lungs	Number of catheterised pulmonary arteries	Duration of lung lobe perfusion (min)	
	Gender	Age (y)			After dosing	Total
1	m	65	UL (r)	1	60	85
2	f	58	UL (r)	1	90	116
3	m	76	UL (l)	2	60	88
4	m	72	Lung (r)	3	60	83
5	m	68	LL (r)	2	60	82
6	m	44	ML + LL (r)	2	60	86

m – male; f – female; UL – upper lobe; LL – lower lobe; ML – middle lobe; (r) – right part of the lungs; (l) – left part of the lungs

Target doses of GW597901/salbutamol in experimental settings 1 - 3 were 45/150 µg. After quantitative analysis of these three complete sample sets, the decision was made to increase the nominal doses fourfold for both compounds due to low but reproducibly detected concentrations of GW597901 and salbutamol in perfusion fluid samples over the duration of the experiments. Therefore, total doses of 150 µg GW597901 and 600 µg salbutamol were applied via cassette dosing to additional three human lung lobes. Other experimental settings of the pharmacokinetic enabling study like ventilation and perfusion pressure values are shown in *Table 11, 2.3.2*, C – Experimental Setup.

Based on total amounts of aerolized drugs, the mean loss due to adsorption on to the dosing device were similar for both β_2 – agonists: 17.6 % (SD 5.0 %) of administered salbutamol

and 17.4 % of GW597901 (SD 6.9 %) were recovered at the inner sides of the glass spacer and connection tubings to the lung lobes. It was calculated that the mean actual deposited doses of GW597901/salbutamol were 43 ± 8/139 ± 35 µg in the first three experiments and 117 ± 3 µg and 400 ± 110 µg in the following three lung lobe perfusions, respectively (see *1.1*, D – Appendix). By applying the dose only during the inspiration phase, drug losses were kept low. However, the method of dose determination (see *2.4,* C – Experimental Setup) did not account for the exhaled fraction of drugs so that the calculated deposited doses might be slightly overestimated [21, 47].

After quantitative analysis of fluid sample sets of six human lung lobe perfusion experiments differences emerged in the pharmacokinetic profiles of GW597901 and salbutamol (see *Figure 6*).

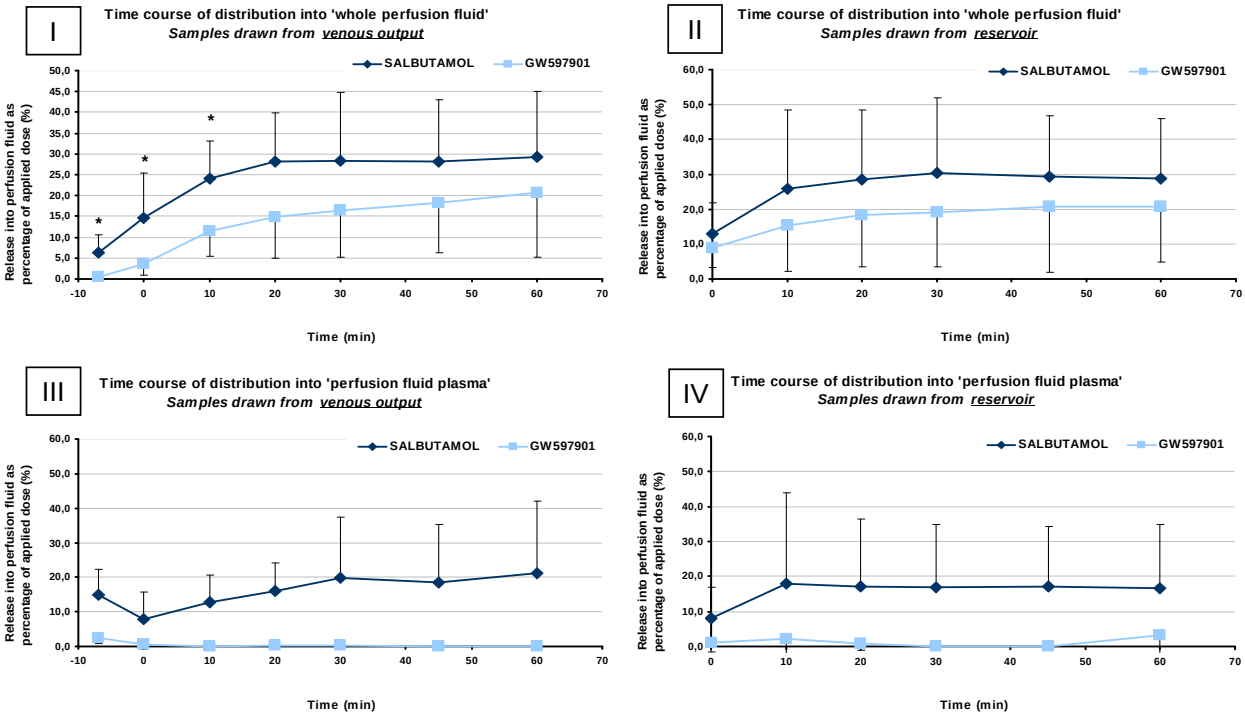

Figure 6: Pharmacokinetic time courses of salbutamol (dark blue line) and GW597901 (light blue line) in the IPL experiments (n= 6). Values are mean concentrations ± SD expressed as percentage of applied doses into whole perfusion samples drawn from venous output **(I)**, whole perfusion samples drawn from the reservoir **(II)**, perfusion fluid plasma samples of the venous output **(III)** and perfusion fluid plasma samples of the reservoir **(IV)**.

To combine all data, distribution rates are presented as release of GW597901 and salbutamol into perfusion fluid samples, expressed as a percentage of actual deposited doses. The time scale of the experiments is given on the x – axis and displayed data points are related to the termination of dosing procedure (= 0 min). Since sampling from the venous output started during the administration phase (about 12 minutes, see *2.3*, C – Experimental Setup), the first

values in diagrams I and III were available at time -6 min to cover the onset of initial distribution into fluid circulation.

Salbutamol showed measurable concentrations in the whole perfusion fluid drawn from the pulmonary vein during the dosing procedure with a mean extent of 6.4 % (SD ± 4.2 %). The degree of initial redistribution of GW597901 at this time was much less pronounced (0.4 ± 0.3 %) as expected due to its lipophilic nature. In comparison both β_2 – agonists in samples drawn from the venous output at the times -6, 0 and 10 min, the statistical evaluation showed significant differences in the extent of redistribution of salbutamol and GW597901 (two-tailed unpaired student t – test, $p < 0.05$). In the further course of the lung lobe perfusions the extents of absorption of salbutamol and GW597901 were constantly increasing, reaching stable values at time 20 min after termination of dosing. The mean concentrations of GW597901 were always below the concentrations of salbutamol at any time point, confirming a delayed and lower "systemic" availability after inhalation, which is the hypothesis for long – acting β_2 – agonists to reduce the systemic component and therefore the side effects. The recovered amounts of GW597901 in perfusate at the end of the experiment were approximately 20 % of applied dose (see *Figure 6*, *diagram I*), whereas salbutamol achieved a plateau mean extent of absorption of 30 % after 20 min. Diagram II, representing the levels of β_2 – agonists in the WPF samples obtained from the reservoir, showed similar extents for both compounds versus WPF samples drawn from the venous output. In contrast to the pharmacokinetic profiles of the β_2 – agonists in perfusion fluid plasma samples (= cell - free fraction) in the diagrams III and IV, it is noticeable that GW597901 was detected just in very low plasma concentrations underneath the LoQ, suggesting the high degree of binding affinity and drug distribution into blood cells. The fraction of GW597901 bound to blood cells was therefore calculated back to 97.1 ± 7.1 % (mean ± SD). In contrast, salbutamol displayed variable but low binding to blood cells (55.5 ± 20.4 %), eventually caused by the varying levels of haemolysis of the blood material used for the perfusion fluid.

To complete the description of the pharmacokinetic behaviour of GW597901 and salbutamol, small lung tissue specimens from perfused lung lobes and BAL fluid samples were obtained subsequently to the termination of experiments and the absolute amounts of β_2 – agonists were determined (see *2.3.3.3,* C – Experimental Setup). The tissue samples were cut from four different anatomical sites of the lung lobe to draw conclusions about the distribution pattern within different areas of the reperfused lung lobe (see *Figure 31* in *2.3.3.3*, C - Experimental Setup). Tissue samples were then analyzed for concentration of GW597901 and salbutamol in ng/g tissue.

To determine the ratio of the amount of drug in the perfusion fluid circulation, serving as a model for the "systemic" availability, and the disposition pattern within the targeted organ

depending on the anatomical region, the concentration of β_2 – agonists in WPF samples drawn from the venous output at time 60 min was related to the amount of drugs recovered in 1 g lung tissue (see *Figure 7*).

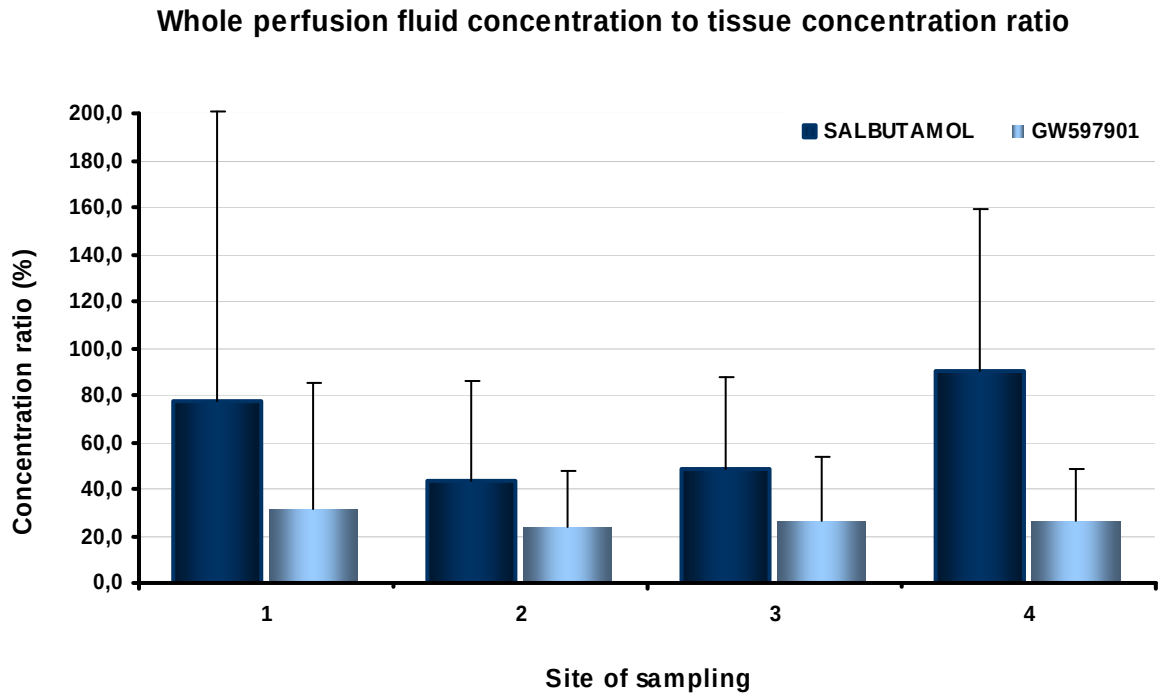

Figure 7: Whole perfusion fluid to tissue concentration ratios of salbutamol (dark blue bars) and GW5979091 (light blue bars) calculated for 4 different anatomical sites of the perfused lung lobe (mean + SD, n= 6).

Site 1 was localized near the ventilated central airway, site 4 represents the most peripheral region and samples from site 2 and 3 were removed from the area in between. Both β_2 – agonists were recovered in the tissue samples at each site of sampling, due to the cassette dosing technique. As expected, GW597901 and salbutamol demonstrated similar deposition patterns. However, the assumption that the extent of deposition decreased from the central site 1 to the peripheral site 4 for the β_2 – agonists was not confirmed by analysis of tissue samples. GW597901 showed constant concentration ratios in a range of 24 – 31 % independent on the site of sampling. As expected for a more hydrophilic drug, salbutamol seemed to have a more distinct tendency to diffuse into the fluid circulation, as seen by the higher concentration ratios (44 – 90 %). Nevertheless, the differences in concentration ratios of salbutamol and GW597901 at each site of tissue sampling were not statistically significant (two-sided unpaired student t – test, $p > 0.05$). The total fractions of inhaled β_2 – agonists recovered in lung tissue samples, calculated as the mean percentage of actual deposited dose found in site 1 – 4, ranged from 18.5 – 86.3 % for salbutamol and from 15.1 – 98.5 % for GW597901 (see *1.3.4*, D – Appendix) and were not significantly different. These results demonstrated that the tissue binding

characteristics of GW597901 and salbutamol within the lung after inhalation were highly variable throughout the individuals.

The amounts of both β_2 – agonists recovered in the BAL fluid samples were low, up to 4 % and 3 % of applied doses for salbutamol and GW597901 were detected, respectively. But these percentage values might be underestimated due to very low recovery rates (4 – 26 %) of inserted volumes of lavage fluid (see *1.3.5,* D – Appendix). In general, a BAL procedure as a diagnostic tool for various lung diseases in clinical practice was successful, if the rate of regained instilled volume was at least 40 – 50 % [113, 114]. Others recommended a minimum recovery of BAL fluid of 25 – 30 % to be reliable for the determination of contained inflammation markers [115, 116]. Thus, in the enabling study the determined amounts of salbutamol and GW597901 in BAL fluid were not reliable due to insufficient recovery. It was concluded that the instilled volumes of isotonic NaCl – solution (5 – 10 mL) was not adequate as the majority of the liquid drained away into the deeper dilated airways and therefore was not accessible for BAL sample recovery.

Summing up all percentaged fractions of applied doses of β_2 – agonists analyzed in obtained specimen (perfusion fluid, lung tissue and BAL) the relative recoveries were 66.9 ± 27 % for salbutamol and 68.4 ± 26.5 % (mean ± SD) and therefore comparable. However, there was a tendency for salbutamol to display a shorter tissue retention and a faster distribution into perfusion fluid.

1.4 Discussion

1.4.1 Validity of the human IPL model for the description of pharmacokinetics of short- and long-acting β_2 - agonists

Although isolated perfused animal lungs have been applied since the early 80s, reflecting the growing interest in the non-respiratory functions of the lungs e.g. drug delivery, the first work by Linder et al. describing the refined methodology to perfuse and ventilate resected human lungs under physiological conditions was in 1996 and opened up new opportunities to address pharmacokinetic analysis that were closer to the *in vivo* situation [87]. In these studies the redistribution of anticancer drugs from the perfusate into the lung tissue was examined, Freiwald et al. were engaged with the pharmacokinetic behaviour of pulmonary delivered glucocorticoids, two different commercially available HFA-propelled aerosols of BDP were applied to isolated human lung lobes and the pulmonary absorption profiles over time were compared. It was demonstrated that absorption rates varied between the BDP preparations used, attributed to differences in delivered drug particle size and the rate of dissolution processes of the solid

particles within the human bronchial fluid. Furthermore, the results were consistent with the conclusions of a clinical human study completed by Woodcock et al. in 2002, confirming the suitability of the human IPL to demonstrate the absorption kinetics of pulmonary applied drugs [105, 117].

Here for the first time, the human IPL model has been extended to the field of β_2 - agonists, to improve the knowledge about their pharmacokinetic characteristics. Since the compounds were in solution before administration via nebulizer, dissolution processes of salbutamol and GW597901 within the airways did not preceed absorption. The time profiles of pulmonary absorption of salbutamol and GW597901 were therefore only dependent on their ability to pass from the alveolar region into the capillaries to be further distributed into the perfusion circulation and the binding affinity to certain components of the lung tissue. Thus, the current observations exclusively isolate the absorption kinetics as the rate-determining step.

Based on an hypothesis with respect to physicochemical properties of both molecules it may be expected that the time courses of distribution into the perfusion fluid of salbutamol and GW597901 differed (see *Figure 6*, *1.3*) regarding the rate and extent of absorption. The more lipophilic agent GW597901 showed a more pronounced tendency to remain in the lung tissue, whereas the mean recovery of salbutamol in the perfusion fluid was always above that of GW597901. The observed differences were statistically significant until 10 min after the termination of dosing (see *diagram I*). This was further supported by a higher tissue affinity of GW597901 leading to a mean whole perfusion to tissue concentration ratio of 26.1 - 31.4 % (see *Figure 7*, sampling site 1 - 4), whereas salbutamol showed a perfusate:tissue concentration ratio of 43.7 - 90.1 %. Thus, GW597901 demonstrated a higher tendency to remain in the lung tissue *ex vivo* resulting in a lower “systemic” availability and, if transferred to the *in vivo* situation, it could be postulated that the lower systemic exposure results in a lower probability of systemically mediated side - effects. Both compounds were recovered at every sampling site from the lung tissue illustrating a similar deposition pattern. This observation was not surprising regarding the concomitant application of GW597901 and salbutamol. However, the amounts of the nebulized drugs analyzed in tissue samples did unexpectedly not differ across the sites of sampling. It was expected that the recovered amounts would be higher in the central lung tissue near the main bronchus than in the peripheral areas as it has been seen with inhaled radiolabelled β – adrenoceptor ligands or pulmonary applied saline droplets [118, 119]. However, the present results demonstrate that the lungs were optimally ventilated and the nebulizer device used for application of the aerosols produced droplet sizes with a median mass diameter ideal for reaching all ventilated parts of the pulmonary airways to the same extent including the transitional and respiratory zones with inner airway diameters of ≤ 0.5 mm [2]. By using a spacer, the lung deposition is additionally improved towards the peripheral lung regions

[120]. Furthermore, the observations might be explained by sufficient perfusion of the lung lobes reaching an equilibration between fluid and tissues. Since elimination processes are generally not present in the IPL model the establishment of equilibrium between the two compartments might have been completed by the end of the perfusion experiment. By complete perfusion, GW597901 and salbutamol were equally distributed across the tissue regions with a higher tendency of the lipophilic LABA to be retained in the lung than the SABA.

However, high standard deviations of PK data produced non - significant differences between salbutamol and GW597901 perfusate profile in the further experimental time course, reflecting the interindividual variabilities between the IPL experiments. Experimental settings and procedures were comparable and could be ruled out as reasons for this observation. Applied perfusion fluids and other conditions were always kept constant throughout the lung lobe perfusions. Adsorbed fractions to the dosing device (see *Figure 29* in *1.2.2,* C - Experimental setup) not accessible for absorption were similar. Mean amounts of salbutamol and GW597901 adsorbed at components of the dosing device were about 17.5 % of applied dose for both with a SD of ±5.0 % for salbutamol and ±6.8 % for GW597901. Therefore variances of up to 15 % of applied dose recovered in perfusate samples of the venous output at time 60 min (see *1.3*, D - Appendix) could not be explained by differences in adsorption to the device.

As mentioned in *Table 4* (see *1.3*)*,* donor lung lobes used in the experiments differed. Three single upper and one lower lung lobe were perfused, but one right lung and two coherent lung lobes were available for perfusions. Thus, the pulmonary surface exposed to inhaled drugs and the number of catheterised pulmonary arteries varied, being an important factor for the extent of fluid circulation within the lung tissue. Assuming that the release of pulmonary deposited β_2 - agonists was dependent on the quality of perfusion, the amounts of salbutamol and GW597901 recovered in the perfusion fluid samples were higher the better the perfusion fluid circulated within the lung tissue. For example in experiment 4, the right lung, consisting of three lung lobes, was perfused via all available pulmonary arteries, implementing a complete perfusion performance. The extents of release of $ß_2$ – agonists were 44.6 % of applied dose of salbutamol and 47.1 % of applied dose of GW597901 in samples drawn from the venous output at time 60 min and thus significantly higher than the rates of redistribution shown in other experiments, explaining the large SD of averaged data. However, PK data analysis normalized for the weight of the lung lobes as indicator for the quality of perfusion did not show a correlation between the weight of the perfused lung tissue and the extent of release into the perfusate.

In general, the quality of perfusion was determined by the visual appearance of the lung lobe based on the method of Friedel et al. [121]. Well perfused lung areas appeared light – coloured after flushing with perfusion buffer prior to the experiment because erythrocytes

maintained in the lung tissue after resection were washed out. Other methods for the assessment of the quality of fluid circulation were not undertaken. Furthermore, interindividual variablities between each single lung lobe preparation regarding the initial state of the inner airways had to be an additional cause for divergent results. Patients' characteristics such as age, smoking history and especially cancer stage and associated lung diseases, COPD, may impact the condition of lung tissues and therefore the ability to absorb and redistribute inhaled drugs like a healthy subject. Due to suspected low availabilities of resected lung lobes, the inclusion criteria of the pharmacokinetic enabling study did not include restrictions in such patient details in advance. Even in healthy volunteers, large intersubject variabilties in pharmacokinetics of inhaled drugs are known due to different inhalation techniques, individual differences in metabolism processes and blood flow within organs responsible for absorption and elimination [97].

Thus, the results of the pharmacokinetic enabling study demonstrated the validity of the human IPL model for investigating distribution and tissue binding characteristics of intrapulmonary applied short – and long – acting ß$_2$ – agonists.

1.4.2 Comparison of the results with clinical trials

1.4.2.1 Salbutamol a short-acting β_2 – agonist

Before discussing the time course and extent of β_2 – agonists release into the perfusion fluid, several factors have to be taken into consideration if comparing the current data with PK results of human *in vivo* studies. C_{max} and t_{max} values largely depend on the drug formulation, e.g. whether an oral or inhaled drug is administered, and the targeted dose of the drug. Therefore, the knowledge of nominal doses and type of drug administration/formulation is essential for further accurate comparisons with our data and for deducting conclusions. Comparisons for t_{max} values can only correctly be made with human trials involving the same dosing device and formulation, in this case the application of aqueous solutions via nebulizer as used in the IPL experiments. Comparisons of absolute C_{max} levels between clinical trials and our data must be made with caution since targeted doses of inhaled drugs are not equal in most of the cases, only plasma concentrations presented as percent of applied dose would allow such discussions. Unfortunately, the actual intrapulmonary applied dose is rarely determined in human trials.

Newnham and Lipworth [122] investigated the PK profiles of salbutamol after nebulization to eight asthmatic subjects. Application of salbutamol via two different nebulizer devices lead to relatively low plasma concentrations regarding the large nominal inhaled doses. To compare Ventstream and Hudson nebulizer systems 1.25, 2.5 and 5.0 mg were

administered to patients and the plasma concentrations of salbutamol were observed over 40 min and 240 min (see *Figure 8*).

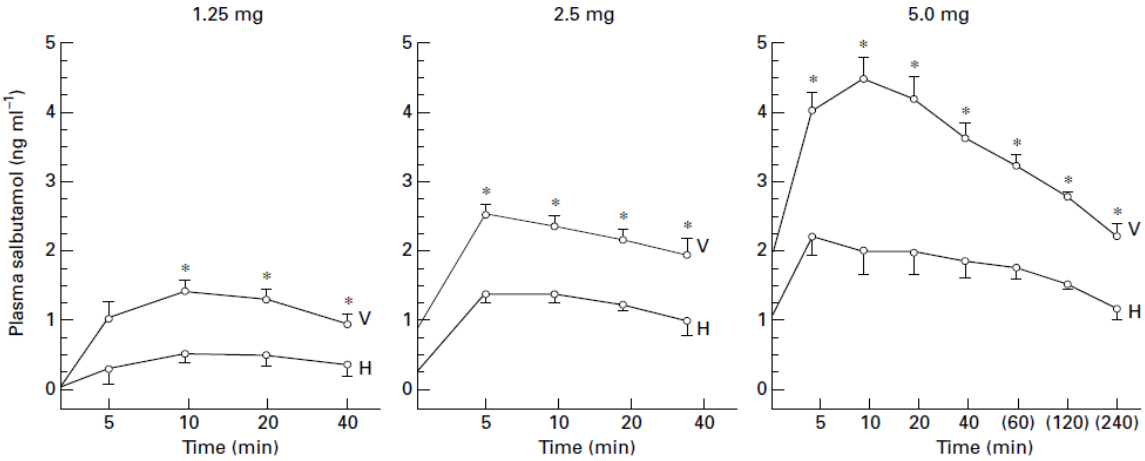

Figure 8: Pharmacokinetic time profiles for plasma salbutamol after inhalation of 1.25, 2.5 and 5 mg administered via a Ventstream (V) or Hudson Updraft II (H) nebulizer (n= 8). Values are means ± SEM, asterisk denotes significant differences between the two nebulizer brands [Images were taken from the literature [122] with permission of the BMJ publishing group].

After application of salbutamol via Ventstream nebulizer plasma concentrations were twofold higher compared to the Hudson device. Administered salbutamol at a nominal dose of 1.25 mg produced a mean C_{max} of 1.53 ng/mL (Ventstream) and 0.62 ng/mL (Hudson) at a median t_{max} of 10 min (see *Table 5*) showing a large influence of the type of nebulizer device used on pulmonary deposition of nebulized dose and the resulting extent of absorption.

Despite different doses of salbutamol, the observed PK time profiles originated after nebulization in this trial are very similar to those obtained in our experiments. Already at time 0 min (here: termination of dosing procedure) plasma levels of salbutamol were detectable. In the further time course t_{max} was reached at 5 – 10 min, whereas in the IPL perfusion experiments the maximum of the plasma concentration was around 20 min. However, the duration of the dosing procedure via nebulizer in the clinical trial of Newnham is not specified. The administration of 600 µg of salbutamol by nebulizer in the IPL setting took around 12 – 13 min, the administration of double or higher doses must have lasted even longer leading to "earlier" t_{max} values corresponding to the termination of dosing procedure 0 min. Thus, the rate of absorption profile of salbutamol observed by the IPL experiments is qualitatively comparable to the human *in vivo* study conducted by Newnham and Lipworth.

Many human clinical trials investigating the PK profile of salbutamol have been published, differing mainly in applied nominal doses and routes of administration of salbutamol. A selection of clinical trials with the main results is listed below in *Table 5.*

Table 5: Selection of clinical trials analysing PK characteristics of salbutamol in healthy volunteers.

Reference	Dose	Application	C_{max} (ng/mL)	t_{max} (min)
Schmekel at al. [123]	4 mg	oral tablet	7.2*	206*
	200 µg	pulmonary instillation	1.6*	68*
	800 µg	dry powder inhaler	2.0*	38*
Clark et al.[89]	1200 µg	pMDI	2.93 – 3.33	10 (5 – 10)
Fowler et al. [124]	400 µg	pMDI	4.0	10 - 20
Goldstein et al. [125]	1.5 mg	i.v. infusion	20-30	5
	4.0 mg	liquid/tablet/capsule	12.7-13.0	104-127
Reggio et al [126]	200 µg	pMDI	7.59	13
	1.5 mg	nebulizer	9.64	17
Newnham et al.[122]	1.25 mg	nebulizer	1.53	10
	2.5 mg		2.61	5
	5.0 mg		4.59	10

* PK data refer to the S – enantiomer of salbutamol
pMDI – pressurized metered-dose inhaler

The C_{max} and t_{max} values of salbutamol listed in *Table 5* emphasize that PK data are highly variable throughout the clinical studies. It is not surprising that t_{max} values increase from the i.v., inhaled to the oral route of administration. As expected, the best correlation of the t_{max} value of salbutamol measured in our IPL experiments can be drawn to *in vivo* studies with nebulization as route of administration.

In the present experiments, on average, 29.4 ± 15.6 % of the applied dose of salbutamol were recovered in whole blood perfusion fluid samples drawn from the venous output at 60 min, corresponding to mean absolute concentrations of 27.6 ng/mL for salbutamol in the first three experiments (nominal dose 150 µg) and 69.8 ng/mL in the last three perfusion experiments (nominal dose 600 µg). Thus, analyzed concentrations of salbutamol in perfusion fluid samples of the human lung lobe perfusion experiments were extensively higher when being compared to the *in vivo* studies mentioned above. Several factors contribute to this observation. The blood circulation of a human adult comprises a total volume of 4 – 6 L, while in the IPL experiments 1500 mL of perfusion fluid were used. Therefore the concentration of the absorbed β_2 – agonist was up to four times higher in perfusion fluids than in the *in vivo* situation due to a lower apparent volume of distribution. Furthermore, nominal doses used in the perfusion experiments

were set at 150 and 600 µg, respectively, whereas the amounts given to the patients/volunteers via inhalation varied from 400 – 5000 µg. Elimination processes by hepatic metabolism and renal clearance of salbutamol, which decrease the concentration in perfusion fluids over time in humans, were not present in our setting. Generally, in clinical PK studies blood samples were drawn from the basilic vein, thus the inhaled β_2 – agonist had been massively diluted after absorption from the inner airways and systemic circulation. In the IPL setting only minimal dilution occurred, when the first perfusion fluid samples were drawn directly from the output of the pulmonary vein and higher concentrations of the redistributed agent were comprised.

But the major determining factor accounting for the difference in analyzed concentrations of an inhaled β_2 – agonist between blood and perfusion buffer is the actual fraction that reaches the lungs after inhalation, which determines the pulmonary bioavailability. Numerous studies were carried out to examine the pulmonary deposition pattern regarding different agents, dosing devices and inhalation techniques. The degree of lung deposition can be determined for example with gamma scintigraphy. Scintigraphic studies of aerosol inhalers are commonly performed with drugs labelled with the radionuclide ^{99m}Tc. After inhalation of the radiolabelled agent, a gamma camera detects the gamma - ray emission and quantifies the disposition in the lung, oropharynx and the fraction retained in the dosing device [127-129]. Other approaches for the determination of the amount of pulmonary deposited drugs are pharmacokinetic methods. By blocking the gastro - intestinal absorption of a swallowed drug after inhalation by orally given charcoal suspension (= “charcoal – block method”) the pulmonary bioavailability can be calculated, if the systematically available amount of drug is compared with an analogue intravenous dose [96, 98]. Lung depositions of salbutamol were reported to be around 10 % and 20 % of nominal doses after applications by dry powder and pressurized metered - dose inhalers, respectively [120, 130]. Studies using gamma scintigraphy have found the mean percentage of the dose deposited in the airways from a jet nebulizer to be between 2 % and 12 % in humans [131, 132]. In comparison the mean percentage of actual applied dose of nebulized salbutamol was 79.8 % in this present pharmacokinetic enabling study (see *1.1,* D-Appendix), thus about eight times higher than in the *in vivo* situations mentioned above. By impaction of aerosol particles in the oropharyngeal region and consecutive swallowing after inhalation about 60 – 80 % of the applied doses reach the gastro - intestinal tract [54]. Obviously, this process does not occur in the IPL setting. Including the experimental volume of distribution, targeted nominal dose and percentage of pulmonary applied dose, the analyzed concentrations of salbutamol in perfusion fluid samples would probably be in the same lower ng/mL range as demonstrated in human trials after inhalation.

Thus, the extent and variabilities of redistribution into perfusion fluids recovered after pulmonary deposited salbutamol *ex vivo* are in agreement with the findings of human clinical trials, in consideration of different doses, used devices and patient groups.

1.4.2.2 GW597901 a long-acting β_2 – agonist

Human clinical trials describing the pharmacokinetic characteristics of GW597901 are not published yet. Thus, LABAs already used in the treatment of obstructive lung disorders with sufficient PK data available have to be consulted for comparison with the PK of GW597901. However, pharmacokinetic characteristics of LABAs such as formoterol or salmeterol deduced from human clinical trials are limited due to the challenge of developing analytical methods sensitive enough to determine very low plasma concentrations resulting after pulmonary application of therapeutic doses. For example 50 µg salmeterol given to 22 asthmatic patients produced a mean C_{max} of 0.34 ng/mL and 24 µg formoterol fumarate administered to 29 volunteers resulted in maximum serum levels of 40 pg/mL. Maximum plasma concentrations of salmeterol and formoterol were measured at 5 min (= t_{max}) after dosing [108, 109, 133]. Clinical trials examining PK of LABAs after administration via nebulizer systems are not available since this plays no role in clinical practice. Furthermore, investigations concerning the determination of pharmacokinetic properties of newly developed and commercially available ultra – long acting β_2 – agonists like indacaterol are not published yet.

Of the applied dose of GW597901 20.7 ± 15.6 % were recovered in whole blood perfusion fluid samples drawn from the venous output at time 60 min, corresponding to mean absolute concentrations of 5.6 ng/mL for GW597901 in the first three experiments (nominal dose 45 µg) and 17.3 ng/mL in the last three perfusion experiments (nominal dose 180 µg). In consideration of the experimental volume of perfusion fluid (1500 mL) in comparison to the volume of human blood circulation (4 – 6 L) and the high mean actual pulmonary deposited amount of 79.8 % of nominal dose in our experiments (see *1.1*), analyzed concentrations of GW597901 in perfusion fluid samples would be in the low ng/mL upper pg/mL range, if transferred to the *in vivo* situation. Interestingly, the time of analyzed C_{max} was about 20 – 30 min after termination of dosing in our experiments and thus later than t_{max} values for salmeterol and formoterol reported in the literature, suggesting a slower rate of redistribution of GW597901 into the “systemic” circulation. This differing behaviour might be based on its higher lipophilicity and thus more pronounced affinity to lung tissues. Another factor for the rate of redistribution into perfusion fluid could be a lower perfusate flow within the lung lobe. In our experiments the flow rate was set around 0.5 – 0.75 mL/min per g tissue, whereas human lungs are perfused with about 14 mL/min *in vivo* [134]. However, since the PK data of salbutamol obtained by the IPL experiments are consistent with the results achieved in *in vivo* situations, a good *in vivo*

correlation might be assumed for the PK characteristics of GW597901 as well. Due to the application of aqueous solutions of GW597901, dissolution processes played no role for later t_{max} values.

Furthermore, the results of the IPL experiments have shown that GW597901 was almost completely redistributed into red blood cells and binds to proteins to a high extent (see *Figure 6, 1.3*) confirming preclinical *in vitro* data of GW597901 (GSK, data on file). Concentrations of GW597901 in perfusion fluid plasma samples (= samples without blood components) were under the LoQ at almost every time point of sampling. To our knowledge, data of LABAs concerning their distribution behaviour into blood cells are not published yet.

1.4.3 Conclusions

It was shown that the enhanced human IPL setting is a valuable model in the laboratory for the PK analysis of the inhaled β_2 – agonists GW597901 and salbutamol. The pharmacokinetic time course of the agents after concomitant pulmonary application revealed the suitability of the model.

By considering different conditions of the *ex vivo* IPL model and the *in vivo* situation as the differing volume of distribution, employed drug formulation and particularly the actual deposited dose in our setting and in clinical practice, the relations of achieved plasma concentrations of β_2 – agonist are both in the lower ng/mL range. T_{max} values reported in the literature of about 10 – 30 min after nebulization of salbutamol were reproduced in our experimental setting [122, 126]. The time absorption profile of GW597901 showed a delayed t_{max} of 20 - 30 min in comparison with other LABAs emphasizing its lipophilic properties with improved PK characteristics and the potential for lower systemic exposure which may result in a lower side effect profile.

2 Determination of pharmacodynamic and pharmacokinetic characteristics of β_2 – agonists

2.1 Objective

2.1.1 Pharmacodynamic measurements of β_2 – agonists

The results of the experimental setting of the lung lobe perfusion model used in the pharmacokinetic enabling study (see *2.3*) had shown that the model was suitable to simulate the pulmonary absorption and distribution processes of inhaled β_2 – agonists *ex vivo* when administered via cassette dosing (see *1*, B – Results and Discussion). Thus, the proof of concept was confirmed. The question remained would the isolated perfused lung model (IPL) be able to reflect the pharmacodynamic activities of β_2 – agonists nebulized to an *ex vivo* ventilated lung lobe. The limitation in this setting was the relatively short duration of the experiment of upto two hours. However, the basic requirement of a fast onset of measurable pharmacodynamic effects was fulfilled by this substance class, because their main therapeutic effect of bronchodilation occurs quickly within a few minutes after administration via the airways.

A variety of parameters are available to monitor the pharmacodynamics of β_2 – agonists regarding, either pulmonary or systemic effects. In human studies direct bronchodilation can be easily measured with standard pulmonary tests using a spirometer. Standardized parameters are for example the forced expiratory volume in one second (FEV1) or the peak expiratory flow measured before and after the application of inhaled drugs to determine the efficacy or to prove the therapeutic equivalence [135-140]. The airway resistance presents an alternative parameter to monitor the airway function under normal breathing patterns [141, 142]. These parameters are traditionally used for asthma monitoring in clinical practice [143]. For assessing systemic effects of β_2 – agonists, with respect to adverse events after inhaled administration, the finger tremor response, hypokalemic effects and heart rate are usually monitored [139, 144-146]. Literature described methods of pharmacodynamic characterization exhibit some disadvantages. Sensible effect measurements as bronchodilation are not feasible in healthy subjects due to absent pathohysiological conditions of the airways. Thus, pharmacodynamic recordings in healthy individuals are limited to systemic side effects and are therefore not the focus of PK/PD studies mainly due to ethical reasons. Furthermore, to capture a fast onset of action of β_2 – agonists or effects occurring already during nebulization, the methods for the determination of pharmacodynamics can not be utilized rapidly enough. In contrast, the lung lobe perfusion model offers the possibility to detect simultaneously any influences on several ventilation parameters initiated by inhaled GW597901 or salbutamol already during the dosing

procedure without interruption of the experiment. Online recording allows continuous documentation.

2.1.2 Bronchial provocation

For PD measurements *ex vivo* there is a requirement to induce a bronchoconstriction prior to the application of the β_2 – agonists. This concept has been adopted from clinical practice and numerous human clinical trials with agents such as methacholine (MCh) and histamine [147-151].

Asthmatic patients suffer from shortness of breath due to a bronchospasm triggered by a variety of causes and an underlying inflammation of the airways. So called hyperreactive airways respond pathologically to sensitisers e.g. to inhaled allergens, pollutants or cold air. To test whether bronchial hyperreactivity is present in an asthmatic patient, challenge tests serve as a diagnostic instrument. For this purpose, bronchoconstrictive agents like the direct parasympathomimetic agent MCh (see *Figure 9*), histamine and cAMP are administered in aerolized formulations to the patients' lungs and to provoke narrowing of the airways [152].

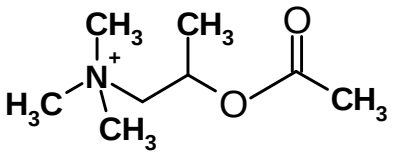

Figure 9: Chemical structure of methacholine, $C_8H_{18}NO_2^+$, M_r = 160.23 g/mol [153]

People with pre-existing airway hyperreactivity react to low doses of the challenge agents. Spirometric measurements then quantify the degree of airway tightening. The PC_{20} value, the provocative concentration of the bronchoconstrictive agent causing a 20 % fall in FEV1, serves as an index for the degree of hyperreactivity or as an index to classify the efficiency of an anti asthmatic treatment [154, 155].

MCh was chosen as bronchoconstrictive agent due to its regular clinical use in the diagnostic field of bronchial hyperreactivity, its long lasting duration of pharmacological action and the higher reported responder rates in comparison to other agents [151, 154, 156]. Bronchial challenge with MCh is frequently described in the context of the effectiveness of β_2 – agonists in human *in vivo* studies [157-161]. It is a parasympathomimetic synthetic analogue of acetylcholine and thus highly active at all subtypes of the muscarinic receptors. The molecule has a permanently charged quaternary amine structure and is physiologically not able to pass through lipid cell membranes and the blood-brain barrier and is also poorly absorbed from the gastrointestinal tract. Stimulation of muscarinic receptors in the lung after inhalation leads to

bronchial smooth muscle constriction. It is metabolized at a relatively slow rate within the body, due to its resistance to acetylcholinesterases. Cartier [162] determined the time courses of bronchoconstriction measured by the change in the pulmonary conductance (= reciprocal of airway resistance) induced by inhaled MCh versus inhaled histamine in asthmatic patients. The onset and extent of bronchoconstriction was comparable. But pulmonary applied histamine showed a relative short duration of action of 16.8 ± 9.8 min, whereas the bronchoconstriction induced by MCh lasted significantly longer over 74.6 ± 53.7 min.

Several national and international guidelines exist which standardize challenge tests in clinical practice using the direct-acting MCh as stimulus. Protocols differ in applying MCh concentrations and dosing schedules [154, 163, 164]. The American Thoracic Society guideline recommends either the two – minute tidal breathing method or five – breath dosimeter protocol with doubling and quadrupling dosing steps. MCh at a concentration of 0.0625 or 0.03 mg/mL is administered to the patient after performing baseline spirometry. Then the FEV1 is determined again. If the FEV1 falls less than 20 %, the next highest (double or quadruple) concentration of MCh is nebulized, otherwise the challenge test is complete. The highest acceptable concentration of MCh according to the American guideline is 16 mg/mL. The degree of bronchial responsiveness is categorized according to the concentration of MCh, which leads to a decline of 20 % in FEV1 defined as PC_{20} value (see *Table 6*).

Table 6: Categorization of bronchial responsiveness [154]

PC_{20} (mg/mL)	Interpretation
>16	Normal bronchial responsiveness
4.0-16	Borderline bronchial hyperresponsiveness
1.0-4.0	Mild bronchial hyperresponsiveness
<1.0	Moderate to severe bronchial hyperresponsiveness

PC_{20} - provocative concentration of MCh causing a 20 % decline in FEV1

To eliminate factors that decrease bronchial hyperreactivity, the patient has to avoid medication and food (e.g. caffeinated beverages) before the test that may interfere. According to the guideline, therapies with short- and long-acting bronchodilators have to be stopped 8 h and 48 h prior to the challenge test, respectively. The treatment with inhaled tiotropium should even be paused for one week. Therefore, the discontinuation of bronchodilator therapy for an appropriate period prior to our IPL experiments was very important and crucial for the success of induced bronchoconstriction. Patient inclusion criteria took this wash out period into account (see *1.2.1*, C – Experimental Setup).

The PK/PD study addressed the central question, whether the IPL was an appropriate *ex vivo* model to describe the pharmacodynamic characteristics of inhaled bronchodilators on the basis of the long-acting β_2 – agonist GW597901 and the short-acting β_2 – agonist salbutamol, and, if any differences in the pharmacodynamics between the β_2 – agonists would be observed. By defining of the pharmacodynamic behaviour of the β_2 – agonists, the onset of action and the extent of bronchodilation were to be monitored in six perfusion experiments for GW597901 and salbutamol, respectively. The extent of the duration of bronchodilation was not achieved in the IPL setting due to the short experimental period of 2 – 3 h. Finally, combined pharmacodynamic and corresponding pharmacokinetic data sets were analyzed in terms of existing PK/PD correlations of the compounds. In this context bronchial provocation of an *ex vivo* ventilated human lung lobe should be established and valid ventilation parameters for the measurement of pharmacodynamic characteristics of β_2 – agonists be determined.

2.2 Experimental modifications

In contrast to the pharmacokinetic enabling study, drug solutions of GW597901 and salbutamol were administered in separate lung lobe perfusion experiments to differentiate between their pharmacodynamic effects. The combined PK/PD study consisted of six IPL experiments for GW597901 and salbutamol, respectively. The experimental design and setting of the lung lobe perfusion model allowed sampling of the perfusion fluid, lung tissue and BAL samples for determination of the PK of β_2 – agonist and remained the same as in the pharmacokinetic enabling study (see *2.3*, C – Experimental Setup). For the purposes of the pharmacodynamic characterization of the β_2 – agonists VentView® PC - software was employed. The ventilation machine EVITA 4 allowed online recording of all ventilation data during the lung lobe perfusion experiments via an external notebook (see *Figure 10).*

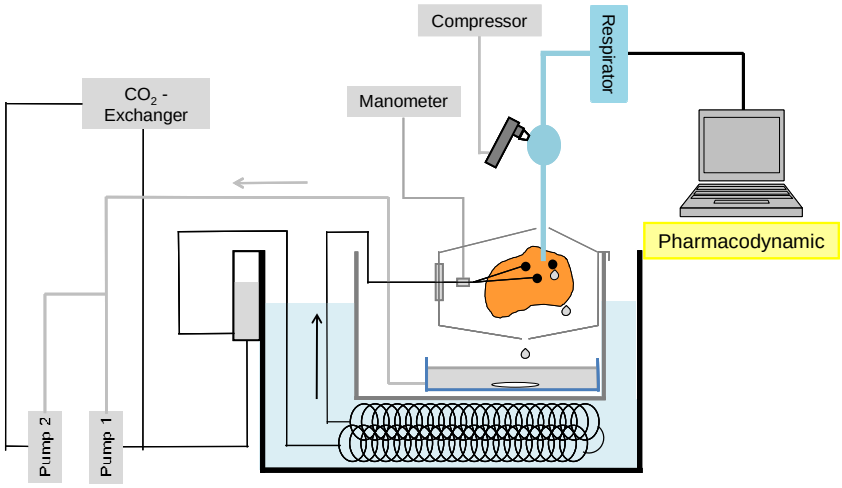

Figure 10: Online documentation of ventilation parameters during lung lobe perfusion. For detailed explanations for single elements in the setup see *1.2.2,* C – Experimental Setup.

In clinical practice, VentView® PC - software is routinely used in intensive care units to monitor and interpret the ventilation status of critically ill patients. The recorded data allows for later analysis and support for further therapeutic decisions [165-168]. Types of ventilation parameters describe different respiratory volumes and mechanical factors of the lung. The most important ventilation variables for later pharmacodynamic analysis of GW597901 and salbutamol are explained in more detail below.

The expiratory tidal volume V_{Te} is defined as the absolute amount of air exhaled in one respiratory cycle. In mechanical ventilation procedures, typical recommended values range around 7 mL/kg bodyweight [169, 170]. The minute volume (MV) represents the volume of air inhaled in one minute and is therefore calculated by the product of the tidal volume and breathing rate [171]. The **p**ositive **e**nd-**e**xpiratory **p**ressure (= PEEP) refers to the pressure in the airways at the end of an expiration that exceeds atmospheric pressure and is set by the ventilator. In humans it has been shown that applying PEEP prevents alveolar collapse, usually improves oxygenation and lung compliance, stabilizes lung units and minimizes ventilator-induced lung injury [172-174]. Because the BIPAP mode (**Bi**phasic **P**ositive **A**irway **P**ressure) was used for the *ex vivo* ventilation of the human lung lobe, the PEEP and P_{Peak} were kept constant at 1-2 mbar and 20 mbar, respectively, throughout all perfusion experiments. The pulmonary compliance C (mL/mbar) specifies the amount of respiratory volume reaching the lungs that enhances the pressure in the alveolar region by one unit and is therefore a measurement of the lung tissue elasticity. Respiration activity has to work against frictional resistances composed of tissue deformation of the lungs during breathing movement and the flow resistance. This flow resistance occurring in the lungs is also described as airway resistance R (mbar/L/s). As examples of respiratory mechanical parameters, the airway resistance and the pulmonary compliance are important variables for the diagnosis of

obstructive lung diseases. It was expected that the application of GW597901 and salbutamol by inhalation would improve ventilation of the lung lobe leading to changes in the ventilation volume and mechanical parameters mentioned above. Due to drug induced bonchodilation the V_{Te} and MV should increase despite constant ventilation pressure, the elasticity (= C) of the lung tissue should be improved and the pulmonary resistance (= R) reduced.

For the present IPL experiments a narrowing of the airways, bronchoconstriction, leading to deterioration in the ventilation parameters of 40 % of the reference values was targeted. Successful preliminary provocation tests confirmed the efficiency of MCh to significantly constrict the airways of human lungs ventilated *ex vivo* (see *3.3.2*, C – Experimental Setup). Furthermore, the pharmacodynamic action of MCh was shown to last over 45 min. Thus, it was assumed that the bronchoconstriction of the airways was still present at the time of subsequent administration of GW597901 or salbutamol and had to be reversed by the pharmacodynamic actions β_2 – agonist.

In the PK/PD study, applied aqueous MCh solutions contained between 10 – 50 mg/mL. Target doses were 180 µg GW597901 and 600 µg salbutamol, respectively. For administration procedures PARI LC® SPRINT nebulizer with blue nozzle insert was used for MCh application (MMD 2.9 µm, 75 % < 5 µm, output rate 590 mg/min) and a red nozzle insert was used for nebulization of the β_2 – agonist (MMD 2.2 µm, 89 % < 5 µm, output rate 450 mg/min). Thus, the aerosol particle size produced was in the respirable range of 2 – 5 µm MMD with an expected deposition within the peripheral and central parts of the airways [175].

Perfusion fluid containing blood cells was used with GW597901 studies and plain perfusion buffer in salbutamol perfusion experiments (see *3.1.2.1*, C – Experimental Setup). In contrast to the pharmacokinetic enabling study, the analysis of GW597901 in fluid samples was done in whole perfusion fluid without separating the blood cells from the plasma. The LC-MS/MS method for quantification of GW597901 and salbutamol in corresponding matrices was developed and validated (see *4.3* and *5*, C – Experimental Setup).

2.3 Results

2.3.1 Conditions of the IPL experiments

In contrast to the pharmacokinetic enabling study, GW597901 and salbutamol were administered independently to resected human lung lobes in separate IPL experiments to determine their pharmacodynamic and pharmacokinetic characteristics. Overall six human lung lobes were included in the PK/PD study for GW597901 and salbutamol, respectively. The experiments were performed at the Klinik Schillerhöhe, Gerlingen and in the Thoraxzentrum

Unterfranken, Münnerstadt, Germany. Details of donors and experimental conditions of the perfused lung lobes are shown in *Table 7*, IPL experiments are numbered chronologically in ascending order for each compound. Ten lung tissue samples originated from lobectomies, in the experiments SB 5 and SB 6 total left lungs were provided for perfusion and ventilation *ex vivo*.

Table 7: Characteristics of donor lungs and perfusion experiments of the PK/PD study

Perfusion experiment	Donor		Resected part of the lungs	Number of catheterised pulmonary arteries	Duration of lung lobe perfusion (min)	
	Gender	Age (y)			After dosing	Total
GW 1	m	69	UL (l)	1	60	124
GW 2	m	64	UL (l)	2	60	167
GW 3	f	62	UL (r)	2	60	133
GW 4	m	70	LL (l)	2	60	73
GW 5	m	77	LL (r)	1	60	165
GW 6	m	81	LL (l)	2	80	133
SB 1	m	73	UL (r)	1	60	122
SB 2	f	76	LL (r)	2	30	138
SB 3	-	-	UL (r)	1	60	130
SB 4	m	75	UL (r)	2	20	71
SB 5	m	63	Lung (l)	3	80	115
SB 6	m	65	Lung (l)	2	60	93

GW -GW597901 was applied in the PK/PD experiment; SB -Salbutamol was applied in the PK/PD experiment;
m – male; f – female; UL – upper lobe; LL – lower lobe; (r) – right part of the lungs; (l) – left part of the lungs
- Data not recorded

The mean total duration of the lung lobe perfusions was 122 ± 30 min. In two experiments the lung perfusion did not achieve 60 min post-dose due to the lung lobe in experiment SB 2 showing significant pulmonary edema formation and in SB 4 the fluid output of the pulmonary vein stopped at 20 min after dosing and therefore fluid sampling was not possible.

The results of the pharmacokinetic enabling study have confirmed the complete distribution of GW597901 into blood cells of the perfusion fluid (see *1.3* and *1.4*). Expecting that blood cells within the perfusion fluid have a certain impact on the redistribution of GW597901, perfusion fluid containing red blood cells had been used in the perfusion experiments GW 1 – GW 6. The actual mean haematocrit of perfusion fluids was 20.2 ± 1.5 % (see *2.1.1*, D – Appendix) as determined by centrifugation. Further IPL experimental details for the PK/PD

studies are summarized in *Table 16, 3.4.2*, C – Experimental settings. Ventilation and perfusion parameters were kept constant throughout the IPL experiments with the perfusion pressure always in the range of 20 – 40 mmHg to prevent pulmonary edema formation. The mean pH of the perfusion fluid was 7.6 (range 7.4 – 7.8) and was measured at least once during a perfusion experiment. If the pH increased it was adjusted to 7.4 with gaseous CO_2.

2.3.2 Pharmacokinetic parameters

Dosing procedures and target doses of GW597901 and salbutamol were the same as in the previous three enabling experiments with the exception that GW597901 and salbutamol were applied to lung lobes separately. Nominal doses were 600 µg salbutamol and 180 µg GW597901, respectively. To define the early redistribution processes of the compounds through the lungs and into the perfusion fluids, samples from the venous output were taken at three sequential time points already during dosing (see *3.4.5*, C – Experimental Setup).

The quantitative analysis of GW597901 and salbutamol adsorbed to the inner surfaces of the glass spacer and tubes connecting the lung lobes after nebulization showed that 16.9 % (SD 4.8 %) of GW597901 and 16.7 % (SD 8.6 %) of salbutamol bound non-specifically to the dosing apparatus. Therefore, the mean loss of aerolized drugs due to adsorption was comparable to the adsorbed amounts in the previous enabling study (see *1.3*) showing consistent delivery and reproducible experimental procedure. On average 130 ± 17 µg GW597901 (≙ 72 % of targeted dose) and 380 ± 129 µg salbutamol (≙ 63 % of targeted dose) were deposited within the airways (see *2.1.1* and *2.2.1*, D – Appendix). Exhaled fractions of nebulized dose were not captured by the method of dose determination. Thus, the calculated percentages of actual applied doses might be slightly overestimated.

Concentrations of GW597901 and salbutamol in perfusion fluid samples of six IPL experiments were determined by a validated LC – MS/MS method (see *4.3* and *5*, C – Experimental Setup). Amounts (in ng/mL) of salbutamol and GW597901 in samples taken at different times were converted to the percentage of actual deposited dose in the corresponding experiments. PK data were then pooled and the mean value and standard deviation (SD) determined. A summary of results are shown in *Figure 11*. For detailed PK data analysis for each experiment see *2.1* and *2.2*, D – Appendix.

Diagram I (see *Figure 11)* depicts the release of compounds into the perfusion media when samples were taken from the venous output. Time 0 min signifies the termination of dose administration. Samples were also taken at predose times of -10, -7 and -2 min to define the onset of the initial distribution processes following dosing. The time course of recovered

amounts of β_2 – agonists in samples obtained from the reservoir starts at time 0 min and is shown in diagram II.

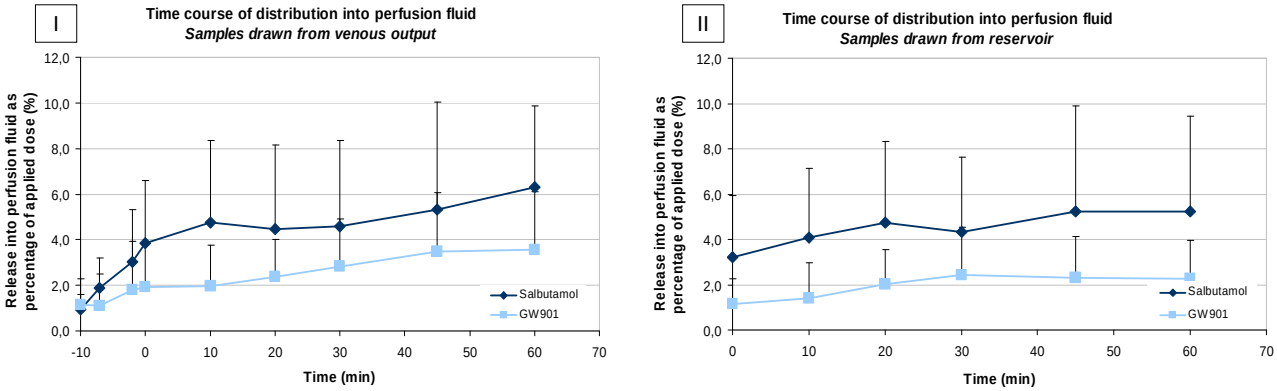

Figure 11: Pharmacokinetic time courses of salbutamol (dark blue line) and GW597901 (light blue line) in the PK/PD study (n= 6). Values are mean concentrations + SD expressed as percentage of applied doses recovered in perfusion samples drawn from venous output (I) and perfusion samples drawn from the reservoir (II).

Concentrations of β_2 – agonists in fluid samples were always higher than the limit of quantification. Comparing the distribution of the compounds, the mean release of salbutamol into perfusion fluid was greater than the absorption time course for GW597901 for the duration of the lung lobe perfusion experiments. Both compounds were already detected 2 min after initiating the dosing procedure to a similar extent and comprised about 1 % of initial deposited dose. Subsequently salbutamol showed a higher rate of distribution than GW597901. At the termination of dosing at time 0 min, 3.9 ± 2.8 % of the actual applied dose of salbutamol was recovered in the perfusate sampled from the pulmonary vein, whereas the mean release of GW597901 was 1.9 ± 1.9 % for a similar time interval. After completion of dosing the absorption profiles of both compounds continued approximately in parallel. At 60 min post-dose 6.3 ± 3.6 % of the deposited dose of salbutamol has been released into the perfusion fluid via the venous output. The distribution of GW597901 averaged at 3.5 ± 2.6 % at this time point. However, large standard deviations and small differences between GW597901 and salbutamol of mean PK data did not allow statistically significant differentiation (two-tailed unpaired students t – test, $p > 0.05$).

BAL sampling was not feasible in three of the lung perfusion experiments following administration of salbutamol after bronchial provocation as no fluid was recovered after instillation. In other experiments the mean volume of lavage fluid delivered was 12.7 mL (10 – 20 mL) with a mean fluid recovery of 5.3 mL (1 – 15 mL) corresponding to a mean recovery rate of 38 % (10 – 86 %). In comparison to the previous pharmacokinetic enabling study, the fluid recovery rates were much better (see *1.3*). After appropriate dilution and analysis of BAL samples the mean recovered amounts of GW597901 and salbutamol were 3.1 % (1.8 – 5.2 %;

n= 6) and 5.1 % (3.6 – 6.5 %; n= 3), respectively, expressed as percentage of the delivered doses. Concentrations of compounds in BAL were in the same range as in the pharmacokinetic enabling study (see *1.3*). However, low fluid recovery rates due to penetration of lavage fluid into the deeper dilated airways confound the interpretation of the results. To perform the lavage process to obtain BAL is a difficult technique requiring practical experience. The practicalities of performing the lavage process resulted in variable recovering of BAL fluid.

2.3.3 Pharmacodynamic parameters

2.3.3.1 Bronchial challenge of *ex vivo* ventilated lung lobes

Prior to the application of GW597901 and salbutamol to lung lobes, a well defined bronchoconstriction was induced via a challenge agent. Preliminary tests confirmed that the IPL model is functional and a successful bronchial provocation *ex vivo* by application of aerolized MCh solution can be achieved (see *3.3.2*, C – Experimental Setup). In these experiments the total applied doses of MCh ranged from 12.5 – 63.9 mg and were therefore highly variable. Cumulative nebulized doses of MCh leading to a measurable bronchoconstriction were estimated by using the *Formula 1* detailed below. This formula is based on the different parameters that define the dosing procedure.

Formula 1:

$$\text{Total dose}_{MCh} = \sum_{i=1}^{n} [(t_i \times f) \times T_{insp}] \times 0.59\ \text{mL/min} \times c_i$$

n = concentration step
t_i = total time of c_i application intervals (min)
f = breathing rate (1/min)
T_{insp} = time of one inspiration cycle (min)
c_i = concentration of MCh solution administered in t_i (mg/mL)

In each experimental setting different concentrations of MCh solution were available (= c_i). If the application of the first nebulized MCh solution led to no bronchoconstriction, an increased concentration was administered for at least 3 min (= concentration step n). The breathing rate and the duration of one inspiratory cycle was kept constant at 15 /min (= f) and 1.7 s (= T_{insp}). Assuming a relative density of 1 g/mL for the aqueous MCh solutions, the output rate of the PARI nebulizer with blue nozzle insert was set at 0.59 mL/min (≙ 0.59 g/min) according to the data sheet of the device [176].

The concentrations and approximate cumulative doses of nebulized MCh in twelve IPL experiments for the PK/PD study are detailed in *Table 8.* Rows highlighted in grey (GW 1, 4, 6 and SB 1, 5, 6) mark perfusion experiments with successful bronchial provocation. Total amounts applied to the lung lobes were calculated according to *Formula 1.* On average, MCh

solution with a mean concentration of 26.5 mg/mL (10 – 50 mg/mL) was nebulized to the lung lobes for 8.4 min (1 – 25 min) resulting in a mean total dose of 85.2 mg (2.5 – 180.5 mg) MCh.

Table 8: Concentrations and calculated cumulative doses of applied MCh in perfusion experiments and total time for each provocation step to achieve bronchoconstriction (n= 12).

Perfusion experiment	MCh concentration step n						
	1		**2**		**3**		Total dose (mg)
	c_1 (mg/mL)	t_1 (min)	c_2 (mg/mL)	t_2 (min)	c_3 (mg/mL)	t_3 (min)	
GW 1	40	12	-	-	-	-	120.4
GW 2	40	18	-	-	-	-	180.5
GW 3	40	15	-	-	-	-	150.5
GW 4	50	3	-	-	-	-	37.6
GW 5	10	7	25	8	50	8	168.0
GW 6	10	9	-	-	-	-	22.6
SB 1	10	5	-	-	-	-	12.5
SB 2	10	10	25	15	-	-	119.1
SB 3	10	9	50	9	-	-	135.4
SB 4	10	8	50	4	-	-	70.2
SB 5	10	1	-	-	-	-	2.5
SB 6	10	1	-	-	-	-	2.5

Grey highlighted rows - successful bronchial provocation with measurable bronchoconstriction

By assessing the ventilation parameters during provocation procedures, a significant narrowing of the airways after administration of nebulized MCh was observed in six perfusion experiments. The estimated actual applied MCh doses did not correlate with a successful bronchoconstriction. In IPL experiments with a pharmacodynamic effect of MCh the mean dose was 33.0 mg (range 2.5 – 120.4 mg) and was lower than in lung lobe perfusions without any effect (mean estimated dose 137.3 mg, range 70.2 – 180.5 mg). Calculated aerolized amounts of MCh cannot be equated with the actual pulmonary deposited doses and therefore specified total doses in *Table 8* might be an overestimate. It was expected that interindividual variability to the sensitivity of the bronchial challenge would be present. Nevertheless, it was presumed that every lung lobe would react with bronchoconstriction at a high enough dose of MCh. However, this was not the case. Thus, additionally yet unknown factors obviously influenced successful bronchoconstriction.

2.3.3.2 Analysis of ventilation data

In experiments with successful bronchial challenge, ventilation parameters such as the minute volume (MV), pulmonary compliance (C) and expiratory tidal volume (V_{Te}) were reduced to 60 % and the airway resistance (R) increased to 140 % of the baseline reference value (= 100%) that was recorded at the beginning of the ventilation procedure after the equilibration phase. Online recordings of ventilation parameters were analyzed graphically as demonstrated in *Figure 12.* Example time courses of the MV of the successful lung lobe perfusions GW597901-1 and salbutamol-5 are displayed in diagram Ia and IIa, averaged data for the time sections of the experiments (reference, bronchoconstriction, bronchodilation) are shown in Ib and IIb, respectively. Pharmacodynamic data and graphics of GW 1-6 and SB 1-6 are detailed in *2* and summarized in *2.3.3*, D – Appendix.

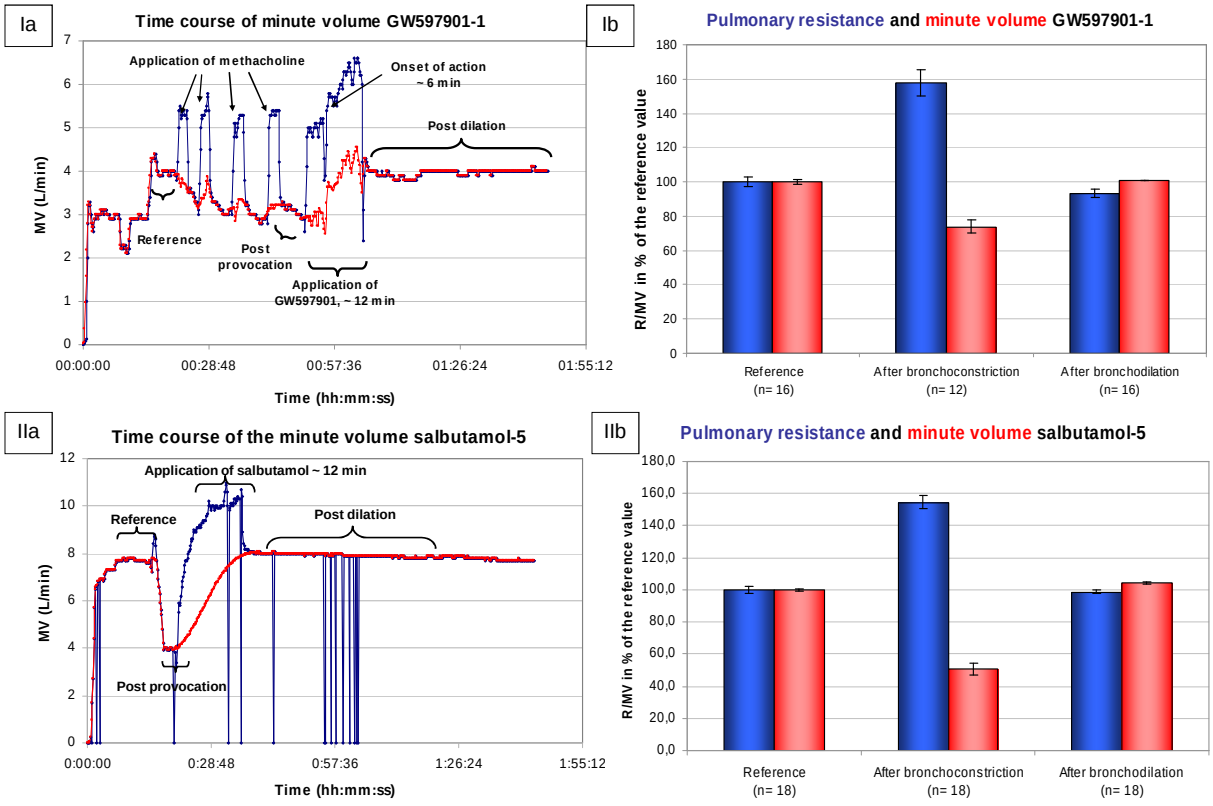

Figure 12: Time courses of the minute volume of the experiment GW597901-1 (**Ia**) and salbutamol-5 (**IIa**): dark blue data points are original raw data, mathematically smoothed data points (Matlab software) are depicted in red. Means (± SD) of the MV (red) and pulmonary resistance (blue) in of the same experiments are shown in **Ib** and **IIb**. Data points of MV and R measured during the reference interval, after bronchoconstriction and after bronchodilation were averaged.

The zero time on the x – axis represents the start of data recording and the beginning of the IPL experiment. During the equilibration phase, the maximum ventilation pressure was adjusted to 18 mbar and constant ventilation parameters were achieved for at least five consecutive minutes to obtain a consistent 100 % reference value. In perfusion experiment GW597901-1 the

reference value was recorded between 15 and 20 min after experiment initiation (= 0 min). Then the application of nebulized MCh was initiated to achieve bronchoconstriction. During application procedures, ventilation parameters were tampered by attaching the nebulizer to the air conducting system as seen in diagram Ia, overlaying the actual signal.

To smooth data curves, occurring during dosing procedures, a validated algorithm of Matlab software (see *3.3.1*, C - Experimental Setup) was used to edit available data sets of the lung lobe perfusion experiments. Curve smoothing was done similar to Phillips GR and Eilers P with modifications to detect outliers that consisted of more than one data point [177, 178]. First, data pairs were entered as vectors with n elements into the Matlab program and then the resulting curve was smoothed. Next, irregularities within the data set were identified with a quadratic Savitzky – Golay filter, standardized residuals between the original and the smoothed values were formed and the standard deviations calculated. All observations greater than a given threshold (= 3.5 by default) were considered to be outliers derived from an overlying signal (= influence of the nebulizer). The outliers were then adjusted by a certain degree of smoothing resulting in the data shown as red curve in the diagrams Ia and IIa, *Figure 12.* A width of 5 data points was found to work satisfactorily as a given limit of smoothing.

In the perfusion experiment GW597901-1, MCh was administered four times for 3 minute intervals. After removal of the dosing device, ventilation parameters such as the MV returned either to initial values or decreased if the airways reacted to the challenge by constricting. If either the bronchial provocation was successfully achieved or no effect was seen after several dosing intervals with higher MCh concentrations, the experiment was completed with the application of either GW597901 or salbutamol. In the lung lobe perfusions GW 1, 4 and 6 and SB 1, 5 and 6 the bronchial challenge was successful.

To analyze and quantify the extent of bronchoconstriction and bronchodilation, consecutive values of ventilation parameters recorded every ten seconds were averaged in the time interval of the reference value, after bronchoconstriction and after bronchodilation. Means and standard deviations were calculated for data of at least two minutes intervals (at least n= 12). Averaged values of the reference interval were compared with 100 % values and means of the sections after pharmacodynamic interventions were expressed as a percentage of the reference value. If the bronchial provocation was successful, the difference between the value after bronchoconstriction and after bronchodilation was calculated, demonstrating the extent of the pharmacodynamic effect of the applied $ß_2$ - agonist. If the provocation with MCh failed and no bronchoconstriction was observed, the difference between the value after bronchodilation and 100 % reference value was calculated. The mean data for the successful bronchoconstriction of tissue samples in the IPL experiments are shown in *Table 9.*

Table 9: Extent of achieved bronchoconstriction prior to drug administration of GW597901 or salbutamol demonstrated in ventilation parameters as percentage of reference value.

Parameter	Percent of reference value after bronchoconstriction (%)					
	Overall (n= 6)		GW597901 exp.(n= 3)		Salbutamol exp.(n= 3)	
	Mean	SD	Mean	MDM	Mean	MDM
MV	50.9	17.1	60.5	10.3	41.2	12.5
V_{Te}	51.1	20.1	60.6	11.9	41.5	17.0
R	134.0	26.0	147.7	6.5	120.3	22.7
C	64.3	15.5	75.1	9.8	53.5	7.3

SD – Standard deviation; MDM – Mean deviation of the mean

The targeted values of bronchoconstriction (decrease/increase of reference values by 40 %) was achieved as shown by mean overall data of the successful bronchial challenge procedures (n= 6; GW597901 and salbutamol perfusions). The broad range of these data shows the difficulties in achieving a defined bronchoconstriction and thus a uniform initial position of narrowed airways prior to bronchodilation. However, the degrees of obtained bronchoconstriction were not significantly different between GW597901 and salbutamol perfusion experiments except the pulmonary compliance.

Administration of GW597901 and salbutamol after a successful provocation always led to a significant improvement of ventilation parameters and thus dilation of the airways, whereas in IPL`s without bronchoconstriction prior to the application of the $ß_2$ – agonist the values remained almost the same as the reference values except for lung lobe perfusion GW -2. In the pharmacodynamic data summary (see *2.3.3*, D – Appendix and *Figure 13*) the extent of bronchodilation is presented as the difference between the ventilation parameters measured after bronchoconstriction and after the application of the β_2 – agonist, expressed as percentage of reference value. In experiments without bronchoconstriction the percental change is related to the 100 % reference value.

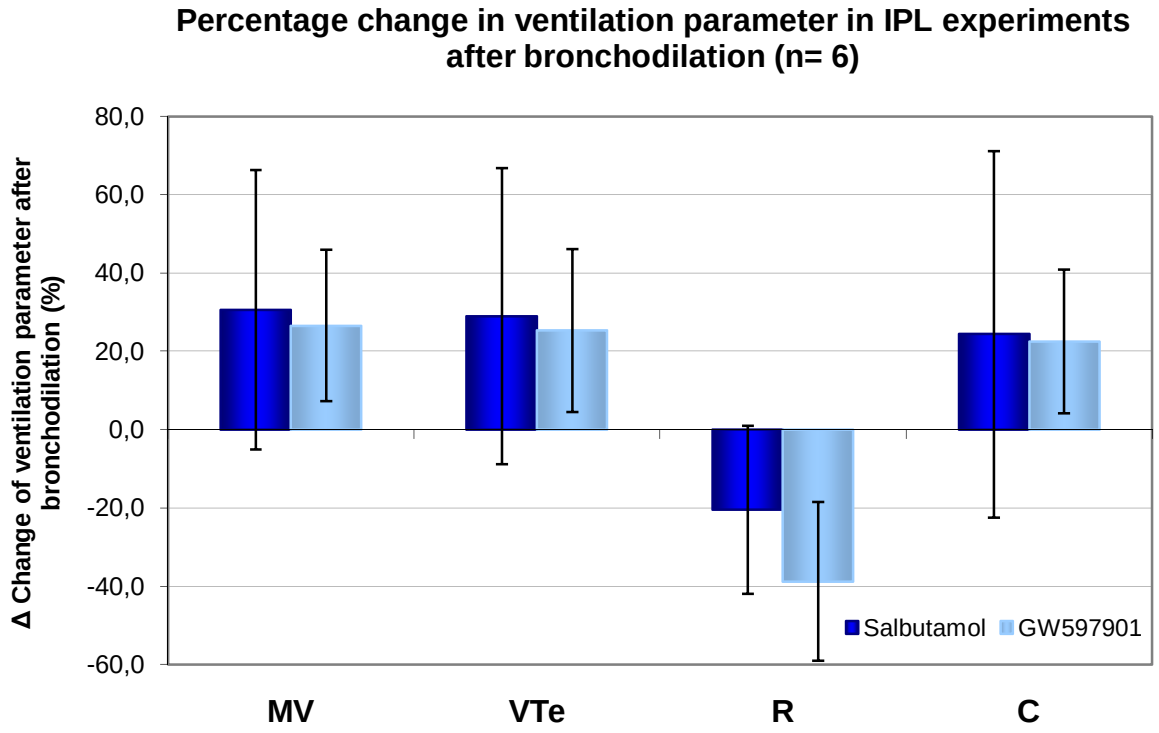

Figure 13: Percental change in ventilation parameters MV, V_{Te}, R and C after bronchodilation by application of 180 µg GW597901 (light blue bars) and 600 µg salbutamol (dark blue bars) in the PK/PD - study. Mean data (± SD) are summarized for six perfusion experiments regardless of successful bronchial challenge.

Beside the analysis of the effects of applied β_2 – agonist on the airways in the current experiments, the data allows further conclusions about the onset of action. During dosing procedures of GW597901 or salbutamol the inspiration pressure was enhanced by 5 mbar to improve deposition of the aerosols within the peripheral airways. As mentioned above, ventilation parameters react to the attachment of the nebulizer to the spacer with a prompt increase only due the enhancement of the air conducting system. The underlying data curve without that influence was extracted by statistical analysis (see *3.5*, C – Experimental Setup) as shown in *Figure 12* (diagram Ia and IIa, red curves). Interestingly, the onset of action of salbutamol in experiments SB 1, 4 and 6 after bronchial provocation occurred almost immediately. The values of ventilation volumes MV and VT_e escalated very fast up to the post dilation values. In contrast, the onset of action in experiments GW 1, 4 and 6 during dosing of GW597901 was observed with a time delay of about 6 min (5.5 – 6.5 min).

2.4 Discussion

2.4.1 Validity of the IPL model to determine the pharmacodynamic parameters of inhaled β_2 – agonists

Based on current information the clinically relevant effect of bronchodilation of inhaled β_2 – agonist was elucidated in an *ex vivo* ventilated human lung lobe model for the first time. Thus, the direct onset of the desired pharmacological and resulting pharmacodynamic effect was recorded in real time and visualized. The PK/PD study included six perfusion experiments evaluating the pharmacodynamic characteristics of GW597901 and salbutamol, respectively. Prior to the administration of β_2 – agonists to the airways the human lung lobes were challenged by pulmonary delivered MCh leading to a bronchoconstriction of the airways. In this context, the validity and the clinical relevance of the brochodilation effects observed in the experimental ventilation data have to be discussed.

The IPL model provided the opportunity to study the pharmacodynamic characteristics *ex vivo* of GW597901 and salbutamol under standard experimental conditions. The experimental interventions performed with the human lung lobes including bronchoconstriction followed by bronchodilation were similar to the procedures used in clinical pharmacodynamic trials. Unfortunately, forced expiratory manoeuvres to record FEV1 or FVC values were not possible in the human IPL setting due to experimental design.

To maintain the proximity to the clinical practice, most of the *in vivo* trials use FEV1 and PEF as outcome measurements for the response to bronchodilator therapies [179-183]. The measurement of the airway resistance or the pulmonary compliance as parameters for lung mechanics are less well described in this context, likely due to a higher variability than FEV1 in terms of reproducibility [184, 185]. However, others hypothesized that the variation of lung mechanic parameters may be characteristic for asthmatics. Therefore, the airway resistance and pulmonary compliance were suggested as useful variables for monitoring effective bronchodilation [186-190]. Additionally, the determination of the lung function or treatment response by body plethysmography might be more appropriate and comfortable for infants than repeated spirometry measurements.

In the present study, ventilation volume parameters such as the minute volume MV and the expiratory tidal volume V_{Te} were used as measurements to assess bronchodilation. It was expected that the *ex vivo* pharmacodynamic effect of bronchodilation would result in an enlargement of airway diameter and thus in an enhancement of recorded ventilation volumes while using a pressure – controlled ventilation mode. The additional online observations of lung mechanics including airway resistance, pulmonary compliance and airway flow should also complement the determination of the pharmacodynamic influence of inhaled β_2 – agonists on

constricted airways *ex vivo*. Thus, pharmacodynamic data recordings are of informative value similar to the FEV1 or PEF because they are parameters that support FEV1.

In the present PK/PD study, the targeted extent of induced bronchoconstriction was set at 40 % alterations of recorded baseline ventilation parameters. Including the preliminary tests (see *3.3.2*, C - Experimental Setup) and the PK/PD study, nine out of sixteen human lung preparations showed a significant bronchoconstriction due to the bronchial challenge with MCh. In the PK/PD study the minute volume MV, the expiratory tidal volume V_{Te} and the pulmonary compliance C were reduced to 50.9, 51.1 and 64.3 % of reference values, respectively. The airway resistance R increased to 134.0 % compared to the basic levels recorded at the beginning of the experiments (see *Table 9* in *2.3.3.2*). Thus, the method chosen for the bronchial challenge was suitable to achieve the tightening of the airways in the human lung lobes. The estimated cumulative doses of MCh leading to a measurable response ranged from 2.5 - 120.4 mg and seemed to be independent of the initial weight and type of the lung lobes. Changes in ventilation parameters in the PK/PD study after successful bronchoconstriction procedures are shown in *Figure 14.*

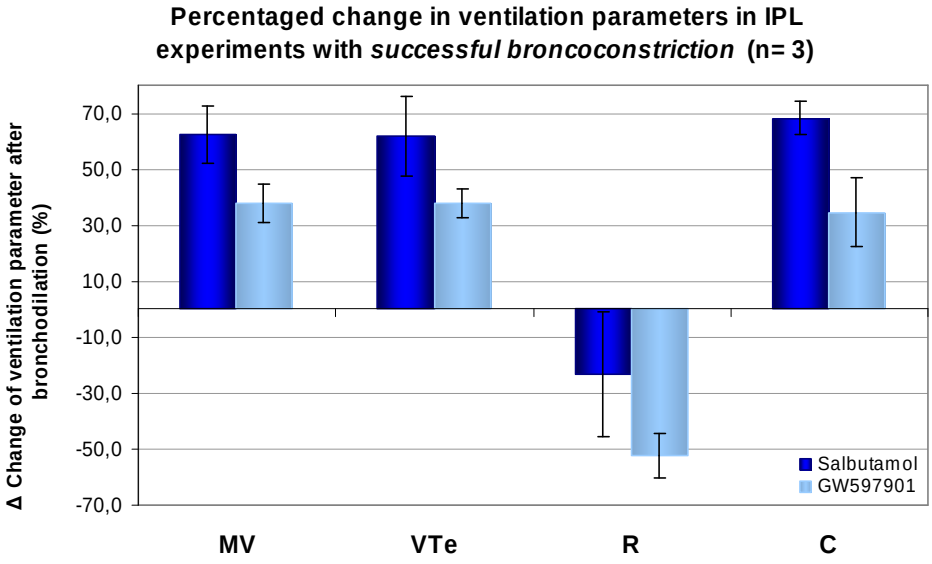

Figure 14: Percent change in ventilation parameters MV, V_{Te}, R and C after bronchodilation by application of 180 µg GW597901 (light blue bars) and 600 µg salbutamol (dark blue bars) in the PK/PD - study. Mean data (± MDM) were summarized for three perfusion experiments with successful bronchoconstriction.

Improvements of lung parameters after application of salbutamol seemed to be more pronounced in this experimental setting, whereas the mean reduction of the pulmonary resistance was greater in the GW597901 perfusion experiments. Nevertheless, observed differences did not reach statistical significance. The positive impact of salbutamol on

pulmonary compliance was more distinctive in comparison to GW597901. But it should be noted that the extent of bronchodilation shown in *Figure 14* was not dose - normalised. The targeted dose of 600 µg salbutamol was not equivalent to 180 µg GW597901 with regard to expected effect size. Thus, these data suggest that GW597901 had a higher intrinsic activity and was more potent than salbutamol. Furthermore, the greater observed change in C in the three salbutamol perfusion experiments could be due to a larger reduction of this parameter by the bronchial challenge agent prior to the application of the short-acting β_2 – agonist. It is even more interesting that the reduction in R was greater in the GW597901 perfusion experiments and thus the airflow limitation of the obstructed airways was improved to a higher extent than in the salbutamol perfusion experiments. Beside the different nominal doses and intrinsic activities of the administered β_2 – agonists, observed differences in the effectiveness of GW597901 and salbutamol were strongly dependent on the interindividual variabilities of each human lung lobe.

Unfortunately, the IPL model is not able to show a difference in the duration of action of inhaled β_2 – agonists reflecting the main pharmacodynamic difference between LABAs and SABAs. This is due to the short time frame of the experimental observations of only 2 – 3 hours. As expected, the onset of action of salbutamol was observed almost immediately after starting the nebulization, whereas the bronchodilation effect of GW597901 started after about 5 – 6 min. No clinical data of inhaled GW597901 are available yet, but the similar chemical structure in comparison to salmeterol implies related physicochemical properties. The onset of action of salmeterol was reported to start after 10 min or even later regarding the effect of bronchodilation [191]. Since salmeterol is usually administered as solid particles to the lung, the compound has to dissolve within the bronchial lining fluid before interacting with the receptors. Since GW597901 was already in solution, this process did not take place explaining the earlier onset of action compared to salmeterol.

Thus, the data from the PK/PD study has shown that the human IPL model is appropriate for the determination and comparison of the pharmacodynamic properties of inhaled β_2 – agonists, namely the effect of bronchodilation and the onset of action.

2.4.2 Response of *ex vivo* ventilated human lung lobes to the bronchial provocation with methacholine

Despite the successful approach showing pharmacodynamic responses of human resected lung lobes to MCh and β_2 - agonists the obtained results of the PK/PD study pose several questions. In total, sixteen human lung lobes were exposed to nebulized MCh including the preliminary tests (n= 4) and the lung lobe perfusion experiments from the PK/PD study (n= 12). Estimated cumulative doses of MCh applied to the lung lobes varied with a broad range from 2.5 - 180.5 mg (see *2.3.3.1,* B - Results and Discussion and *3.3.2*, C - Experimental Setup), which might be

slightly overestimated because the exhaled and adsorbed fractions of MCh were not accounted for in the calculation of the pulmonary deposited doses. Nevertheless, the responses of the human lung lobes to the MCh challenge were very divergent. Interindividual variabilities in the responsiveness towards the bronchial provocation were expected. But only nine out of sixteen lung lobes reacted with a measurable bronchoconstriction, corresponding to a responder rate of 56.3 %. It was expected that almost every lung lobe would respond to the challenge if the MCh dose was high enough, but this assumption was not confirmed. On the contrary, no positive correlation between the dose of applied MCh and a successful bronchoconstriction was observed. The mean MCh dose leading to a significant narrowing of the airways was 54.7 mg (n= 9). However, an average dose of 118.9 mg MCh was administered to seven lung lobes which did not show any response. Studies determining the effect of such high doses of MCh applied to either healthy subjects or patients with respiratory disorders have not been published. In general, for clinical studies MCh solutions with concentrations not higher than 16 - 32 mg/mL are used for bronchial challenge procedures. Therefore, the amounts of MCh nebulized to human lung lobes *ex vivo* in the present study exceeded the doses applied to humans *in vivo* many times. Several reasons might explain the high nonresponder rate towards the bronchial provocation in the perfusion experiments despite high cumulative doses of MCh.

First of all, the donor characteristics might have contributed to a successful bronchial challenge especially in terms of the pathological condition of the resected lung lobes. Several clinical trials investigated patient responsiveness to MCh in relation to certain clinical attributes. The European Community Respiratory Health Survey studied the variation of bronchial responsiveness in 16 countries using randomly selected men and women [192]. The authors observed significant varying results in Europe. However, adjusted for age and sex the percentage of individuals with a high level of responsiveness, defined as 20 % decline in FEV1 with a provocative dose of less of 1 mg MCh, were found in Australia, New Zealand, Britain, USA, Germany and France. The responder prevalences found included two centres in Germany and were 12.0 % in Erfurt and 17.5 % in Hamburg. The median prevalence of bronchial responsiveness in all countries was 13 % (range 1.6 - 33.4 %). In comparison to these results the responder rate towards MCh challenge of 56.3 % found in the PK/PD study seemed to be high. However, considering the different doses of MCh leading to a positive challenge (< 1 mg vs. 2.5 - 180.5 mg in the PK/PD study), the low responder rate in the present study can not be completely explained.

Schwartz et al. aimed to quantify the importance of different predictors for MCh responsiveness in the general population such as age, sex, smoking, atopic status and airway calibre [193]. In total, 7126 individuals in the age range 18 to 60 years were challenged with nebulized MCh according to the European Respiratory Health Survey protocol. The overall

prevalence of increased responsiveness in terms of PD_{20} was found to be 11.7 % in men and 21.8 % in women. According to this study smoking, atopy and female sex were strong predictors for bronchial hyperreactivity towards inhaled MCh. However, the author stated that the sex difference among former or non-smokers in responsiveness disappears when the results were adjusted for FEV1 or lung function, respectively, explained by physiologic differences between men and women. Furthermore, the probability of induced bronchoconstriction by inhaled MCh declined with increased age of the subjects. Other publications found prevalences of bronchial hyperresponsiveness in the general population ranged from 6 - 55 % [194, 195]. In the present PK/PD study, the majority of the patients were men and the age ranged from 62 - 81 years. Thus, the low responder rate of the *ex vivo* perfused lungs despite high MCh doses are consistent with the patients demographics and the findings of Schwartz.

Besides the patients characteristics mentioned above, the main factor influencing the degree of bronchial hyperreactivity is clearly the pathological condition of the airways. It has to be assumed that the patients suffering from bronchial cell carcinoma included in the present study also had COPD or at least a previous history of long term airflow limitation, probably caused by former or current tobacco use. Detailed patient information´s about these characteristics were not available. However, it is evident that smoking is the most prominent risk factor for the development of COPD and lung cancer. About 85 - 90 % of COPD patients are or were chronic smokers and around 50 % of lung cancer patients have COPD [196-199].

Thus, it can be hypothesized that at least 50 % of the patients in the current study had COPD and therefore pathologically altered parenchyma. As already mentioned above, bronchial challenge testing is normally a measure for the diagnosis of asthma than for COPD. Therefore, less is known about the prevalences of bronchial hyperreactivity to MCh or other irritants in COPD patients. The Lung Health Study, a multicentre trial designed to evaluate early interventions in COPD, found bronchial hyperresponsiveness to MCh to be 63 % in men and 87 % in women, defined as a 20 % decline of FEV1 induced by inhalation of MCh solutions with a concentration ≤ 25 mg/mL. By adjusting the data for baseline FEV1, the higher prevalence in females was finally associated to smaller airway calibres in women compared to men [200, 201]. In the present PK/PD study, the mean concentration of MCh solution used for bronchial challenge was 26.5 mg/mL (10 - 50 mg/mL) achieving a responder rate of 56.3 % and these findings are very similar to the findings of the Lung Health Study.

In addition to patient condition´s, the dosing technique or inhalation procedure of MCh might have an influence on bronchoconstriction. Several authors have examined these considerations. In a study of Sunblad et al. [202] two different dosing protocols compared the PC_{20} value of MCh. The agent was applied in doubling (long protocol) and fourfold (short protocol) concentrations between dose steps to 30 healthy non-smoking volunteers. Maximum

concentrations used were 32 mg/mL and 64 mg/mL, respectively. Application of the long dosing protocol led to a higher proportion of subjects with successful provocation than the short dosing protocol used in the same cohort. Furthermore, the doubling protocol resulted in an estimated cumulative dose of MCh that was less than half the value of the fourfold protocol. The authors could not fully explain these observations.

Others have discussed the contribution of MCh dose, deep inspirations during dosing (e.g. dosimeter vs. tidal breathing technique) and the effects of lung volumes in the context of airway responsiveness. They concluded that mainly deep inspirations during inhalation of aerolized MCh prevented bronchoconstriction in healthy subjects. In the absence of deep inspirations the responsiveness to MCh in healthy volunteers was similar to that of atopic patients. This observation was explained by the ability of deep inspirations to relax the airways and thus to work against the bronchoconstriction on a physical basis [203-207].

Applying these conclusions to the bronchial provocation in the *ex vivo* ventilated lung lobes, starting with a low MCh concentration for nebulization, not more than doubling the concentration to the next dosing step and the reduction in inspiration pressure during provocation should result in a higher responder rate. These conditions were not applied in the experimental setup during the preliminary test (n= 4) and the first six perfusion experiments of the PK/PD study. On the contrary, the inspiration pressure was enhanced by 5 mbar during dosing (= deep inspirations) to enhance the deposition of MCh in the peripheral regions of the airways. After four consecutive experiments without successful bronchial provocations and extensive research for possible reasons for this observation, the ventilation parameters during dosing of MCh were changed in the following six experiments according to the literature. Four out of these six challenged lung lobes showed significant bronchoconstriction. However, further studies are needed to confirm whether these changes in provocation procedures contribute to the higher responder rate.

Methacholine exhibits its mode of action by passing the airway epithelium to the airway smooth muscle cell followed by stimulation of G_q - coupled receptors, assuming that the concentration at the site of action is appropriate. Thus, it can be hypothesized that the transport of MCh to the smooth muscle cell is impaired or the pharmacological effect itself is antagonized or impeded, the reason for unsuccessful bronchoconstriction might also have a cellular basis. The MDR1 P-glycoprotein, a membrane bound protein that acts as an ATPdependent efflux pump, is one of the most studied epithelial drug transporters in the airways of the lung [208-210]. Even though the function of the P-glycoprotein for drug absorption and efflux processes in the lung has been extensively investigated in different cell culture as well as in isolated rat lung models, the relevance *in vivo* remains unclear [211-214]. To conclude if the crucial cause for a 50 % nonresponder rate despite high MCh doses was based on an impaired passage to the

smooth muscle cell, the route of administration of MCh would have to be changed from the inhaled to systemic application to bypass the air - blood barrier.

Lung tumors show elevated secretion of prostaglandin - E 2 (PGE - 2), interleukin - 6, interleukin - 10 and vascular endothelial growth factor *in vivo* providing immune suppressive functions [215]. Also, PGE - 2 serves as a mediator involved in different biological processes including the stimulation of human lung cancer growth, invasion and apoptosis resistance [216-218]. PGE - 2 is an interesting endogenous agent due to its potent relaxant effects on airway smooth muscle by activation of EP2 and EP4 receptors. However, inhaled PGE - 2 demonstrated paradox effects in human and animal studies, both bronchodilation and - constriction. Furthermore, aerolized PGE - 2 has been shown to reduce the responsiveness to methacholine stimulated bronchoconstriction in mice [219, 220]. Assuming that the human lung lobes perfused and challenged in the present study also had elevated PGE - 2 levels in the lung lobe containing tumor tissues, then the failed bronchial challenge with nebulized MCh could have been partly due to the preventive effect of PGE - 2 against bronchoconstriction. To determine whether PGE - 2 levels were indeed different in successfully challenged and unsuccessful challenged lung lobes, the PGE - 2 concentrations would have to be determined in the perfusion samples drawn 5 min after the start of lung lobe perfusion experiments.

Thus, there are several reasons to consider when explaining the differing responsiveness of the human lung lobes towards the bronchial provocation *ex vivo.* In comparison to the responder rates found in healthy subjects and patients with respiratory obstructive disorders, the responsiveness to the MCh challenge of 56.3 % found in the PK/PD study is in accordance to *in vivo* data. Nevertheless, further studies are needed to clarify whether impaired MCh transport or impeded pharmacological efficacy also contributed to the observations.

2.4.3 Pharmacokinetic findings of the PK/PD study in comparison to the results of the pharmacokinetic enabling study

After confirming the suitability of the lung lobe perfusion model for the evaluation of the pharmacokinetics of inhaled β_2 - agonists in the pharmacokinetic enabling study (see *1.4.1*) the experimental design was maintained in the PK/PD study with respect to the targeted doses of pulmonary applied GW597901 and salbutamol in the last three perfusion experiments. It was expected that the mean release into perfusion fluids of administered β_2 - agonists would be altered if the airways were successfully constricted by the bronchial challenge with MCh. But the application of MCh without notable bronchoconstriction of the lung lobe was not anticipated to influence the pharmacokinetics of salbutamol and GW597901 and that the PK data would be comparable to the results found in the enabling study. This assumption was not supported for

the redistribution of either GW597901 or salbutamol. Whereas the mean percentages of applied doses recovered in perfusion fluid samples drawn from the venous output at time 60 min were 29.4 ±15.6 % for salbutamol and 20.7 ±15.6 % for GW597901 in the pharmacokinetic enabling study (see *1.3*) the mean release into the perfusion fluid in the PK/PD study were only 6.3 ±3.6 % for salbutamol and 3.5 ±2.6 % for GW597901 (see *2.3.2*). By differentiation of the pharmacokinetic time courses between IPL experiments of the PK/PD study with and without successful bronchoconstriction the discrepancy remained as shown for GW597901 (diagram I) and salbutamol (diagram III) in *Figure 15*.

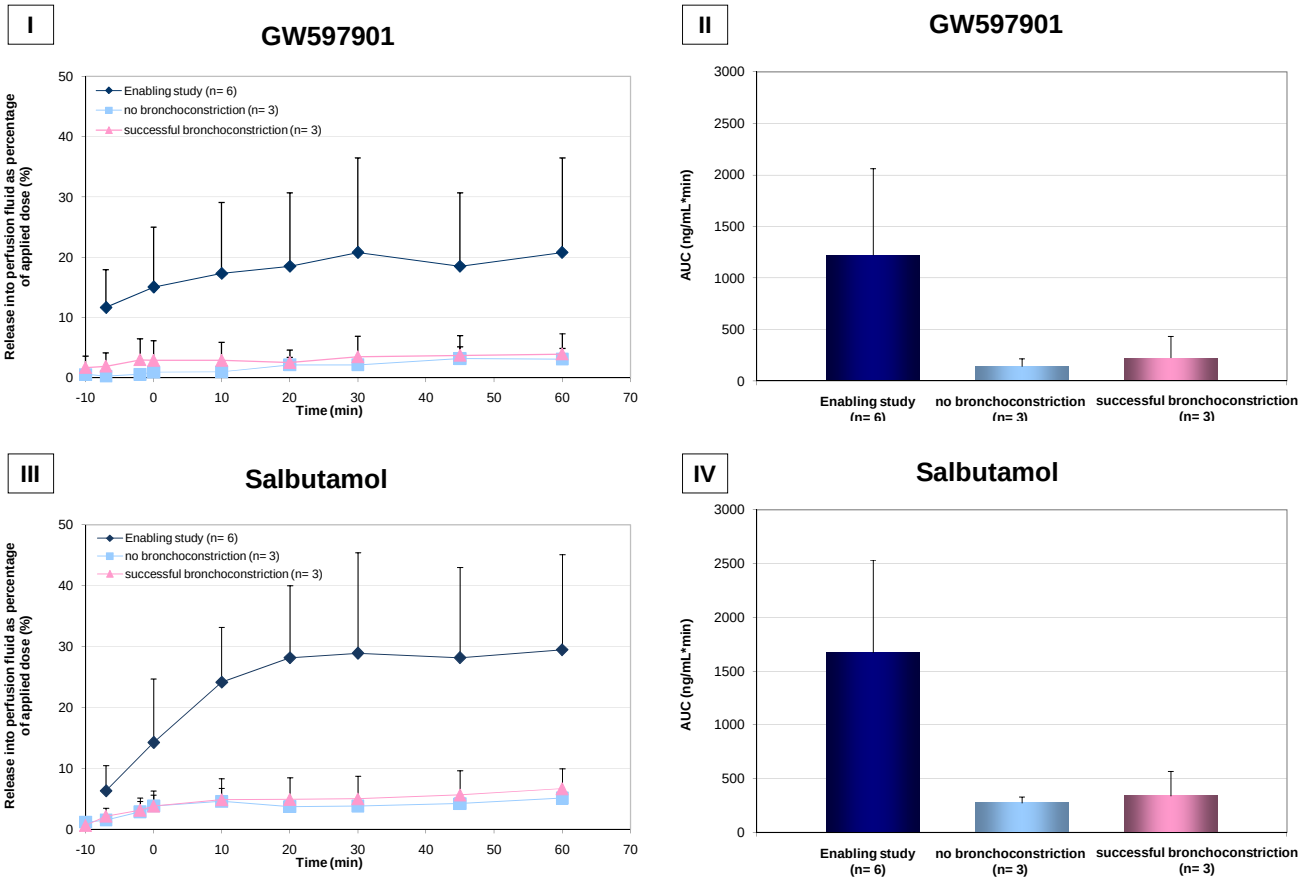

Figure 15: Comparison of the release of GW597901 and salbutamol into perfusion fluid samples drawn from the venous output (I and III) and the corresponding areas under the curves (II and IV) found in the pharmacokinetic enabling study (n= 6, dark blue), in perfusion experiments of the PK/PD study with unsuccessful bronchoconstriction (n= 3, light blue) and with successful bronchoconstriction (n= 3, light pink). Data were represented as means + SD

Beside the time course of redistribution, the areas under the curves (AUC) were calculated by using the trapezoidal method (diagrams II and IV). They also reflect that the extent of redistribution into the perfusion fluid over 60 min was strongly reduced in the PK/PD study. This was even more pronounced in perfusions without successful bronchoconstriction than in experiments with successful bronchoconstriction as seen in the relationship of calculated AUCs 1 : 0.12 : 0.18 for GW597901 perfusions (diagram II) and 1 : 0.16 : 0.20 for salbutamol

perfusions (diagram IV) , respectively ($AUC_{enabling\ study}$: $AUC_{PK/PD\ study}$ without bronchoconstriction : $AUC_{PK/PD\ study}$ with bronchoconstriction). To explain this divergent pharmacokinetic behaviour of applied β_2 - agonists the differing experimental conditions of the enabling study and the PK/PD study have to be examined carefully.

As mentioned above, the actual amounts of salbutamol and GW597901 delivered to the airways were comparable between studies and were not significantly different. On average, 79.8 ±18.5 % of nebulized salbutamol and 79.8 ±23.0 % of GW597901 were actually deposited within the ventilated airways in the enabling study versus 63.3 ±21.5 % and 72.0 ±9.6 % of administered salbutamol and GW597901 in the PK/PD study, respectively (see *1.1*, *2.1.1* and *2.2.1* in D - Appendix). Thus, any difference in the doses of β_2 - agonists accessible for absorption and redistribution in the airways can be ruled out as a reason for the low recovery rates found in the PK/PD study.

Low distribution of inhaled drugs into plasma was observed in an *in vivo* study with twenty asthmatics investigating the influence of induced bronchoconstriction by MCh challenge on the plasma concentrations of inhaled fluticasone proprionate and budesonide. AUC and C_{max} values were significantly diminished after a 33 % reduction in FEV1, while t_{max} and the shapes of the pharmacokinetic profiles remained similar. This result was explained by a decreased rather than delayed drug absorption. Thus, the influence of bronchoconstriction induced by MCh administration on the pharmacokinetic time course was more pronounced for the compound fluticasone proprionate than for budesonide [137]. Earlier the effect of bronchoconstriction on the plasma concentrations of fluticasone proprionate was already observed in several human studies in patients with obstructive airway diseases as asthma and COPD leading to a reduced systemic exposure of the drugs and thus fewer side effects [221-223]. It was suggested that these findings were mainly due to differences in absorption rates and/or pulmonary deposition patterns of inhaled doses of glucocorticoids between subjects with and without airway obstruction. Bronchoconstriction was proposed to cause a deposition of inhaled drugs more centrally within the airways, and then being removed by mucociliary mechanisms and therefore unavailable for pulmonary absorption. The mucociliary clearance is not likely to be a reason for the reduced redistribution of salbutamol or GW597901 in the IPL setting, since it is questionable whether the clearance mechanism is effectively functionable in *ex vivo* ventilated lung lobes. Also salbutamol and GW597901 delivered as solutions are unlikely to be present in the airways as solid particles and will be therefore less prone to mucociliary clearance. Furthermore, the duration of the perfusion experiment after the application of β_2 - agonists of one hour is unlikely to be long enough for a complete clearance as the rate of bronchial mucociliary clearance is 30 - 42 cm/h and the absence of the mucociliary clearance in the distal airways of the lung lobe preparation [224].

Nevertheless, reduced plasma concentrations due to airway obstruction did not explain the diminished redistribution behaviour despite unsuccessful bronchial challenge observed in the PK/PD study. Most likely, the major experimental modification may play a substantial role in this context, the application of nebulized MCh prior to the administration of the β_2 - agonists. It cannot be excluded that MCh itself contributed to the observed effect. Yet it is not clear whether pharmacodynamic effects, certain concurrence mechanisms on molecular basis or both altered the redistribution behaviour of nebulized β_2 - agonists as seen in the PK/PD study. Though some evidence exists in the literature that may explain our findings.

Recently, several polyspecific uptake transporters have been identified that facilitate the passage of structurally diverse cationic molecules through different membrane barriers. The organic cation transporters (OCTs) are members of the solute carrier transporter family (SLC22A) and comprise two distinct classes that work with different mechanisms. OCT1, OCT2 and OCT3 are driven by the transmembrane potential difference. The pH - dependent organic cation/carnitine transporters OCTN1, OCTN2 and OCTN3 act electroneutral [225-227]. They all have the capacity to translocate endogenous and exogenous substances across the plasma membrane in both directions and are distributed in various human tissues such as the intestine, kidneys, brain, liver and the lungs and their activity seem to affect drug absorption and disposition in these organs [228-231].

It has been shown that the OCTNs are highly expressed at the apical side of airway and tracheal epithelial cells that transport organic cations in a pH - dependent manner (see *Figure 16*).

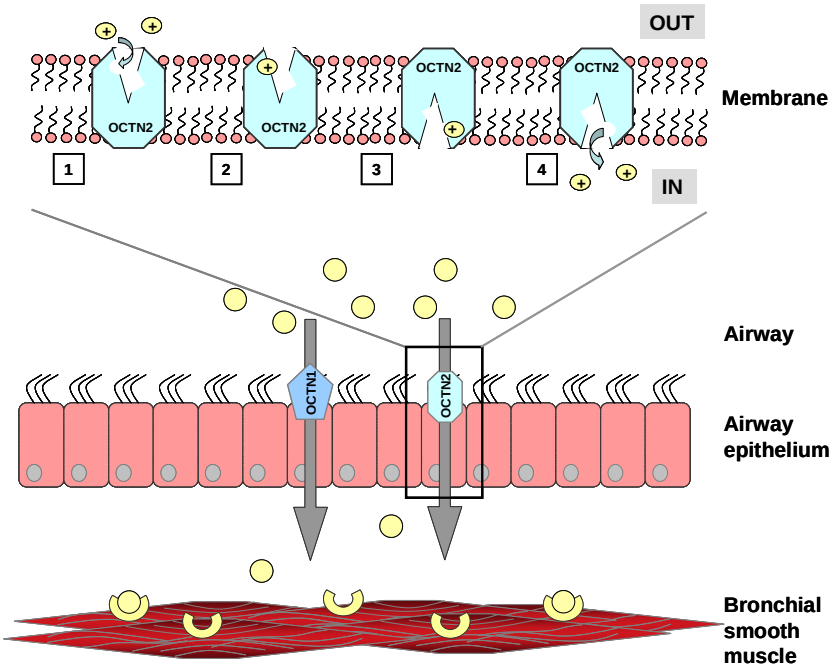

Figure 16: Schematic illustration of the proposed model of the transport mechanism of organic cations by OCTN2 and apical localization on airway epithelium (Figure modified according to [232, 233]). The steps 1-4 depict the binding of cations to the outer surface of the OCTN2 followed by the release into the epithelial cells.

For reaching and activating the pharmacological relevant receptors in the airway smooth muscle cells, inhaled bronchodilators as β_2 - agonists and anticholinergic drugs have to pass the airway epithelium as the major physicochemical barrier. However, the molecular mechanisms for the translocation of inhaled drugs from the airways towards the smooth muscle are not completely understood. Since the majority of inhaled bronchodilators are transient or permanently positively charged at physiologic pH, among those β_2 - agonists as well, several *in vitro* investigations focussed on the role of the OCTNs in the pulmonary absorption mechanism of these drugs.

Horvath et al. investigated the carrier-mediated mechanism via OCTN1 and OCTN2 in the absorption of salbutamol, formoterol and salmeterol [234]. The ability of different concentrations of β_2 - agonists to inhibit the uptake of the fluorophore ASP^+ in *in vitro* cultures of human epithelial cells was measured. The study has shown that salbutamol and formoterol, but not salmeterol reduced the active uptake of ASP^+ by competition for the same transport mechanism, indicating that salbutamol and formoterol are substrates for OCTN2. Indeed previously in 2005, Ehrhardt et al. showed a bidirectional active transport of salbutamol in monolayer cell cultures from apical to basolateral and from basolateral to apical sites. The addition of tetraeethylammonium (TEA^+) to the apical donor fluid significantly inhibited the transport of salbutamol from apical to basolateral [235] therefore indicating that absorption of salbutamol and formoterol in the lung could be associated with OCTN2. Other findings report further inhibitory potencies of various endogenous compounds and xenobiotics towards the uptake of carnitine via OCTN2, among those acetylcholine and methacholine [233]. Published data suggests that the active uptake of inhaled β_2 - agonists beyond the airway epithelium might play an essential role in the distribution process of the drugs to the target site and the systemic circulation. Salbutamol is a known substrate for OCTN2, but data are not available about the active transport mechanisms via OCTNs of GW597901, even though it might be feasible since GW597901 is also positively charged at a physiologic pH of 7.4 (GSK, internal data on file).

In the recent PK/PD study, the release of salbutamol and GW597901 into the perfusion fluid was extensively reduced in comparison to the enabling study. These observations might be partly explained by the previous administration of relatively high doses of nebulized MCh prior to the pulmonary application of the β_2 - agonists probably leading to a significant competition for the carrier-mediated transport via OCTN2. As a consequence, the pharmacokinetic time courses of the compounds were strongly altered in this setting as shown by an 80 % reduction in the AUC (60 min) for GW597901 (see *Figure 15*). At this point it should be noted that the AUCs were slightly more decreased in perfusion experiments with no successful bronchial provocation. As mentioned in *2.3.3.1* the mean estimated dose of MCh was four times higher in failed than in successful provocation procedures (137.3 mg vs. 33.0 mg). Thus, larger amounts of pulmonary deposited MCh resulted in a greater reduction in the extent of redistribution of

salbutamol and GW597901 supporting the hypothesis of a concurrence mechanism in the transmembrane passage.

The altered pharmacokinetic profiles of inhaled β_2 - agonists after induced bronchoconstriction observed in the present study are similar to the observations of several *in vivo* studies investigating plasma concentrations of inhaled glucocorticoids in patients with obstructed airways [137, 221-223], but the underlying reasons might be different. The explanations for differing pharmacokinetic profiles seem to be multifactorial and complex and potential involvement of altered transport mechanisms and pharmacodynamic effects of MCh may have an additive impact on the pharmacokinetic behaviour of the β_2 - agonists. Thus, a definite conclusion about the underlying processes resulting in different pharmacokinetics of inhaled salbutamol and GW597901 found in the PK/PD study in comparison to the enabling study cannot be drawn yet and needs further investigations.

2.4.4 Conclusions

The PK/PD study was the first approach to simulate the pharmacodynamic actions and pharmacokinetic time courses of inhaled β_2 - agonists using the human *ex vivo* isolated lung lobe perfusion model. An experimental design was established with the ability to demonstrate the clinically relevant effect of bronchodilation by online recording of ventilation volume and mechanics. Coherent results were obtained regarding the onset of action and extent of the pharmacological effect of nebulized salbutamol and GW597901. As expected, the administration of salbutamol led to an immediate onset of action. The induced bronchodilation following the application of the lipophilic compound GW597901 occurred after a delay of about 6 min. In the IPL setting both β_2 - agonists considerably improved monitored lung function parameters but were not significantly different. Thus, GW597901 seemed to have a higher intrinsic activity and bronchodilating potency than salbutamol regarding the differing applied doses. However, the determination of the duration of action is not possible in this IPL setting due to the short duration of the perfusion experiments.

Furthermore, bronchial challenges with pulmonary administered MCh were performed successfully for the first time in isolated ventilated human lung lobes, even though the results raised several questions. The low responder rate of 56.3 % towards the bronchial provocation was not expected. However, comparison with published clinical data of responder rates in healthy and pulmonary disordered patients our findings were consistent with observations of clinical studies, though it is not clear why lung lobes did not respond to excessive MCh doses that cannot be used in clinical studies due to ethical reasons. Other possible reasons as

endogenous mediators that inhibit a bronchoconstriction or molecular mechanisms that prevent the passage of MCh to the airway smooth muscle cells are of interest for further research.

The pharmacokinetic time courses in perfusion fluids determined for salbutamol and GW597901 in the PK/PD study were significantly different to those analyzed in the pharmacokinetic enabling study. While t_{max} and the shape of the fluid time profile were similar, C_{max} values and calculated AUCs were considerably lower in perfusion experiments with previous MCh application. The most reasonable cause for this observation might be competition processes for the transmembrane passage to the systemic circulation between MCh and the β_2 – agonists. To investigate this theory further experiments have to be performed. For example instead of the pulmonary application, MCh could be given into the perfusion fluid to analyze if an inhibition of translocation of nebulized substances takes place from the apical (air) or the basolateral (blood) site. Other molecules like carnitine or tetraethylammonium, substrates with a high affinity to the OCTN2, could be administered prior to the bronchodilator to clarify the contribution of OCTN2 for the transport to the smooth muscle cells. Bronchial provocations with cold air could be another approach to observe the redistribution of salbutamol and GW597901 without any influence of another substance deposited within the airways in parallel.

Thus, many different approaches are possible to investigate the molecular mechanisms determining the pharmacokinetic characteristics of β_2 – agonists that remain incompletely understood by now. To expand the knowledge about PK/PD relationships of inhaled drugs the isolated lung lobe perfusion model offers various experimental options with the advantage of being more representative of the *in vivo* situation compared to *in vitro* assays.

3 Evaluation of valid biomarkers for edema formation in the human isolated lung lobe

3.1 Objective

The development of pulmonary edema was a major experimental problem in the lung lobe perfusion because it often led to an increased perfusion pressure and therefore lung tissue damage. As specified in the experimental protocol, the lung lobe perfusions were immediately terminated if significant lung edema occurred. There is not much known about the influence of pulmonary edema on the PK behaviour of inhaled drugs in the IPL model. But it was assumed that this complication most probably impaired the passage of pulmonary administered drugs across the air-blood barrier into the perfusion fluid and thus made the interpretation of obtained PK data difficult.

The term pulmonary edema describes the diffuse accumulation of fluid in the lung tissues and air spaces due to either increased hydrostatic pressure or enhanced epithelial capillary permeability (see *Figure 17*) [236, 237].

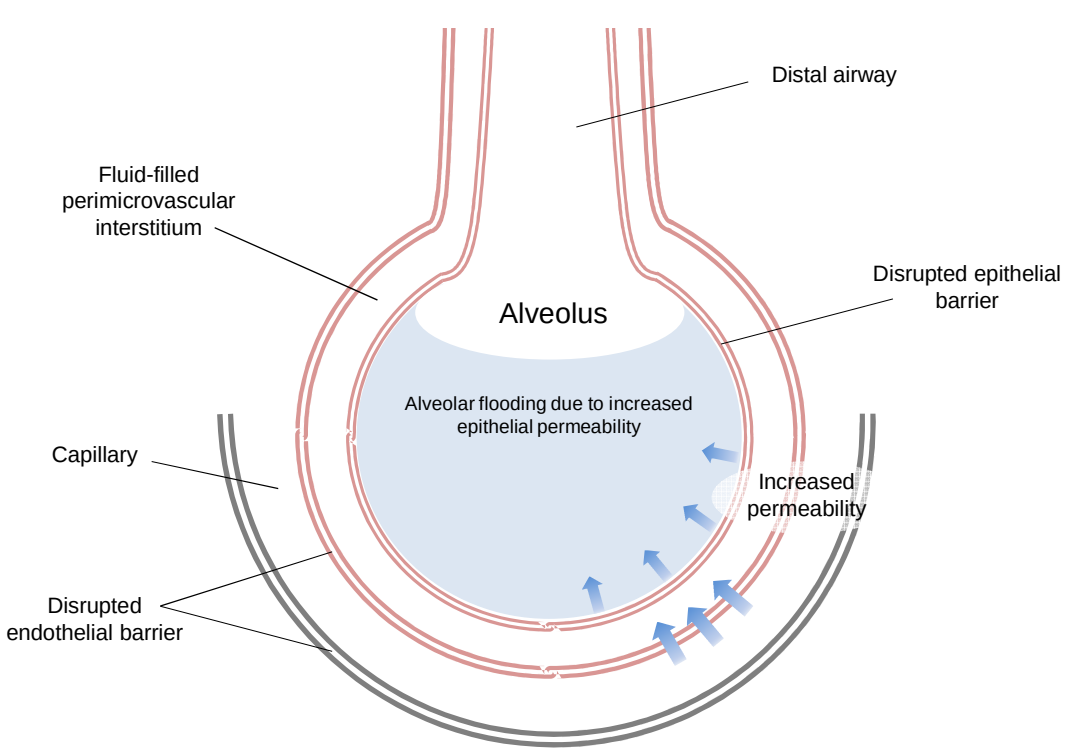

Figure 17: Schematic illustration of a pulmonary edema formation. The permeability of vascular membranes increases due to direct or indirect lung injury leading to interstitial edema and flooding of the alveolus (image was modified according to [238]).

There are various potential reasons for the development of pulmonary edema in the *ex vivo* ventilated human lung lobe model. High perfusion pressures during the experiment due to inadequate perfusate flow rates and the composition of perfusion fluid were reported to be the major influencing factors [239-243]. Furthermore, the maintenance of the physiological pH 7.4 of the perfusate during the experiments, the settings of ventilation parameters or the prevention of damage of the lung parenchyma by cautious preparation of the lung lobe were essential for the viability of the lung lobe without edema.

The determination of optimal experimental conditions to maintain the normal physiological and biochemical status of the lung lobes throughout the *ex vivo* lung perfusions was the challenging task in the development of the human IPL model as described by Linder et al. [87]. Factors such as the composition, buffering capacity and osmolarity of the perfusate was tested for the viability of the perfused lung, but the problem of edema formation had not been completely solved. The weight gain of the perfused lung lobe and the wet-to-dry weight ratio of lung tissue samples were used as criteria for edema formation. For this purpose, the lung lobe was weighed before, during and after perfusion and tissue samples were taken before and after the experiment. In that study, out of 52 perfused lung lobes 11.5 % showed considerable pulmonary edema defined as a weight increase of more than 75 %, 15.4 % lung preparations were classified as critical (weight gain between 50 - 75 %). In terms of edema formation, the use of whole blood as a perfusion fluid was reported not to be superior to the buffered solution with 5 % albumin (pH 7.4).

Weighing of the lung lobe preparation before and after the IPL experiment had been established as an accurate method for the determination of edema development. However, the net weight gain of the perfused lung provided the information to which extent a pulmonary edema developed, but did not specify the time of its initiation during the experiment. For the present study, the knowledge of the onset of edema formation was expected to help interpreting especially irregular PK data of inhaled drugs. For this purpose, suitable biomarkers analyzed in perfusion fluid samples drawn at distinct times throughout the lung lobe perfusion needed to be identified. Thus, four endogenous substances that were either already proven or discussed to be potential markers for lung cell damage and loss of tissue integrity were chosen to be determined in the perfusate.

The cytoplasmic enzyme lactate dehydrogenase (LDH) is almost ubiquitous in the human organism. Therefore, the determination of LDH activity in body fluids and cell culture supernatants has been frequently used in clinical practice and *in vitro* studies, respectively, since an increase in enzyme activity indicates cytolysis or cytotoxicity of xenobiotics [244-250]. Besides, the LDH activity is easy to measure via commercial available non-radioactive colorimetric assays.

Urea is synthesized as a degradation product either from the oxidation of ammonia or amino acids as part of the urea cycle in the human organism. The urea production occurs mainly in the cytosol of liver cells when the amino acid L-arginine is metabolized to L-ornithine catalyzed by the enzyme arginase I. Dissolved in the blood (reference range 12 - 50 mg/dL), urea undergoes renal elimination. In addition, arginase I and the mitochondrial isoform arginase II are constitutively expressed in the bronchial epi - and endothelial cells, responsible for the degradation of L-arginine to either nitric oxide and L-citrulline or L-ornithine and urea [251-253]. Thus, bronchial cell damage due to pulmonary edema formation should release urea into the perfusion fluid that can be quantified in fluid samples by a fast enzymatic assay.

Additionally, lung-typically proteins were analyzed since their increase in perfusion fluids should specifically indicate a loss of lung tissue integrity. The peptidase angiotensin converting enzyme (ACE) is a luminal ectoenzyme that is distributed at high concentrations throughout the surface of the pulmonary endothelium [254]. Extensive research has been undertaken to investigate the suitability of ACE as a biomarker for lung injury. While some studies have shown a positive correlation between elevations of ACE in serum or BAL fluids of patients suffering from acute respiratory distress syndrome (ARDS, including alveolar edema) or sarcoidosis [255-258], others doubt the prognostic value of ACE levels [259, 260]. Thus, the clinical relevance of the determination of ACE in different body fluids concerning lung diseases has not been clarified yet.

Pulmonary surfactant is formed exclusively in the lung, lining the surface of the pulmonary epithelium to prevent the collapse of the alveolus by reducing the surface tension. Surfactant is composed of phospholipids and surfactant proteins (SP), which are classified as the isoforms SP-A, -B, -C and -D also known as collectins [261-263]. These proteins differ in their molecular weight, hydrophobicity and main functions [264]. Evidence exists that increased plasma levels of SP-A are correlated with the clinical outcome in patients with ARDS and pulmonary fibrosis [265-267]. An ELISA method was developed for the quantification of SP-A in perfusion fluid samples obtained in the IPL experiments.

Thus, the aim of the present investigations was to analyze the LDH activity, urea, ACE and SP-A concentrations in perfusion fluid samples over time of each experiment to uncover possible correlations with pulmonary edema formation in addition to the weight gain of the lung lobes.

3.2 Methods

3.2.1 Detection of edema formation and sampling of perfusion fluids

For measurement of weight gain of the perfused lung, the prepared lung lobe was weighed before and immediately after the perfusion experiment. During the lung perfusion, a formation of edema was estimated by monitoring of the perfusion fluid level in the reservoir (see *Figure 30* in *2.3.3.2*, C - Experimental Setup).

Perfusion fluid samples included into the analysis for potential biomarkers for pulmonary edema formation derived entirely from human IPL perfusion experiments performed for different pharmacokinetic projects by our research group during the last five years. Sampling procedure of fluids within the pharmacokinetic enabling and the PK/PD study was done as described in *2.3.3.2*, C - Experimental Setup. Matrices used as perfusion fluid were either artificial perfusion buffer or a mixture of blood components (buffy coats, red blood cells) and perfusion buffer (see *2.1.2.1* and *3.1.2.1*, D - Experimental setup). For long-term storage, blood components were separated by centrifugation, supernatants were aliquoted, shock frozen in liquid nitrogen and stored at -40 °C until further analysis. Sample sets from earlier perfusion experiments were stored in the same manner.

3.2.2 Test principles for LDH, urea and ACE

Methods used for the determination of LDH activity, the quantification of urea and ACE were based on colorimetric enzymatic assays and enzyme-linked immunosorbent assay technique (ELISA) with UV-Vis detection.

The measurement of LDH activity was performed with a commercial available validated cytotoxicity detection kit (see *6.2*, C - Experimental Setup). In a first step NAD^+ was reduced to $NADH/H^+$ by the LDH catalyzed conversion of lactate to pyruvate. In the second step H/H^+ was transferred from $NADH/H^+$ to the pale yellow tetrazolium salt which was reduced to the red formazan salt (see *Figure 18)*.

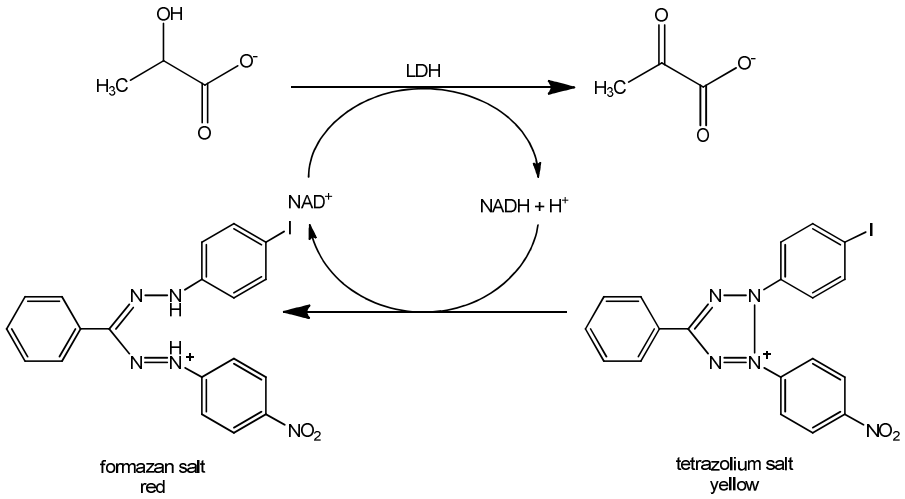

Figure 18: Test principle of the LDH detection kit.

An increase of LDH activity due to an enhanced release from damaged cells directly correlated to the amount of formazan formed. Therefore, the intensity of colour built in the assay was proportional to the enzyme activity and the degree of cell damage [268, 269].

The method for the determination of urea in perfusion fluid samples was established by Talke and Schubert [270] for the quantification in human blood and serum. The enzyme urease specifically catalyzed the hydrolytic decomposition of urea to ammonia and carbon dioxide. Next, α – ketoglutarate was reduced by the glutamate dehydrogenase (GDH) to glutamate, while the substrate NH_4^+ was completely implemented. $NADH/H^+$ was oxidized to NAD^+ leading to a decrease in absorption intensity at 340 nm of the sample to the degree as urea was present (see *Figure 19)*.

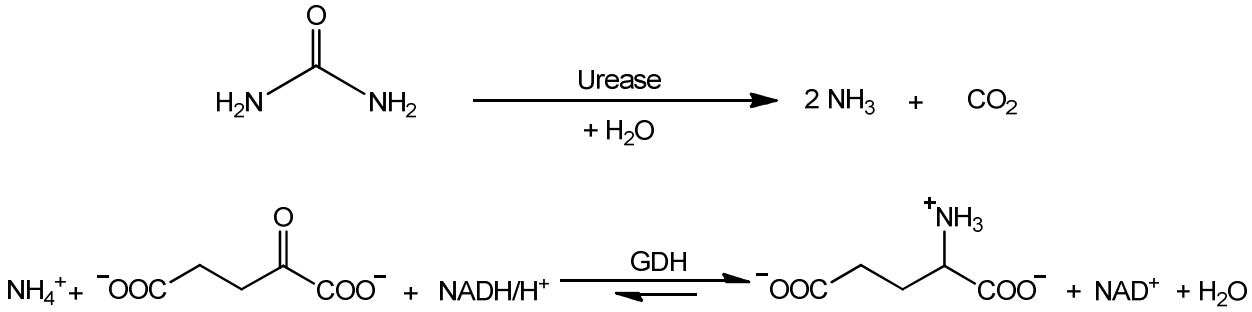

Figure 19: Test principle of the quantification of urea in perfusion fluid samples.

The reduction of absorption intensity compared to the absorption of urea-free samples was therefore directly proportional to the urea content (see *6.3*, C - Experimental Setup).

For the quantification of ACE a commercial available ELISA kit was used (see *6.1*, C - Experimental Setup). This assay employed the direct sandwich ELISA technique. The capture

antibodies, passively attached to the solid phase, specifically bound the antigen ACE if present in the fluid samples. Unbound ACE was then washed away and the polyclonal antibodies against ACE were added that were conjugated with the enzyme horseradish peroxidase. Following a wash to remove any unbound antibody-enzyme reagent, the substrate solution consisting of tetramethylbenzidine and H_2O_2 was added (see *Figure 20)*. The colour developed proportionally to the amount of ACE bound in the initial step. The reaction was then stopped by addition of sulphuric acid and the absorption intensity was measured at 450 nm with a correction wavelength of 560 nm.

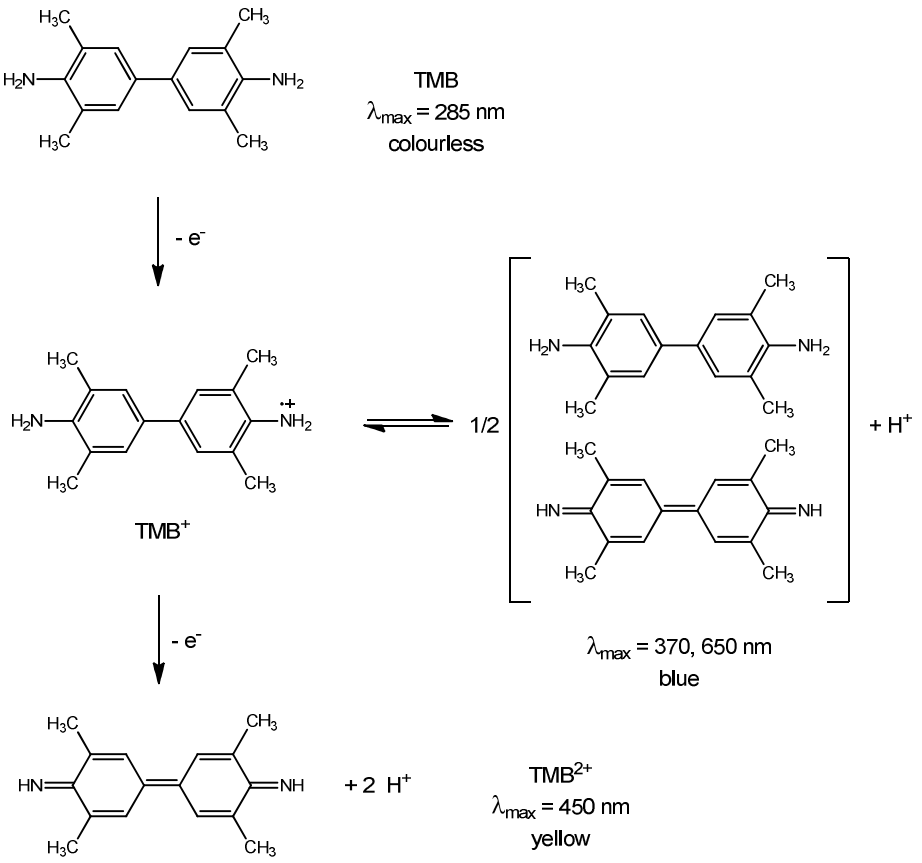

Figure 20: Colour reaction of tetramethylbenzidine (TMB) in the presence of horseradish peroxidase and H_2O_2 [271].

To correct for the background signal, blank matrix samples of every perfusion sample set were always analyzed in parallel to the unknown samples from the IPL experiments. Calibration standards were measured in each assay. If the absorption intensities of the unknown samples were above those of the calibration line the samples were diluted with the corresponding matrix and analyzed again.

3.2.3 Development of an indirect sandwich ELISA method

Since an assay for the quantification of human SP-A was not commercially available, a specific ELISA method had to be developed for the analysis of SP-A in biological fluids. An indirect sandwich ELISA technique was chosen (see *Figure 21*) because no enzyme-conjugated antibody directed against the antigen SP-A was available. After selection of antibodies specific for the detection of human SP-A the working concentrations of the antibodies leading to an optimal and sensitive performance of the ELISA method were determined according to Crowther [272]. For detailed information about used reagents, antibodies and detailed experimental procedures see *6.4* in C - Experimental Setup.

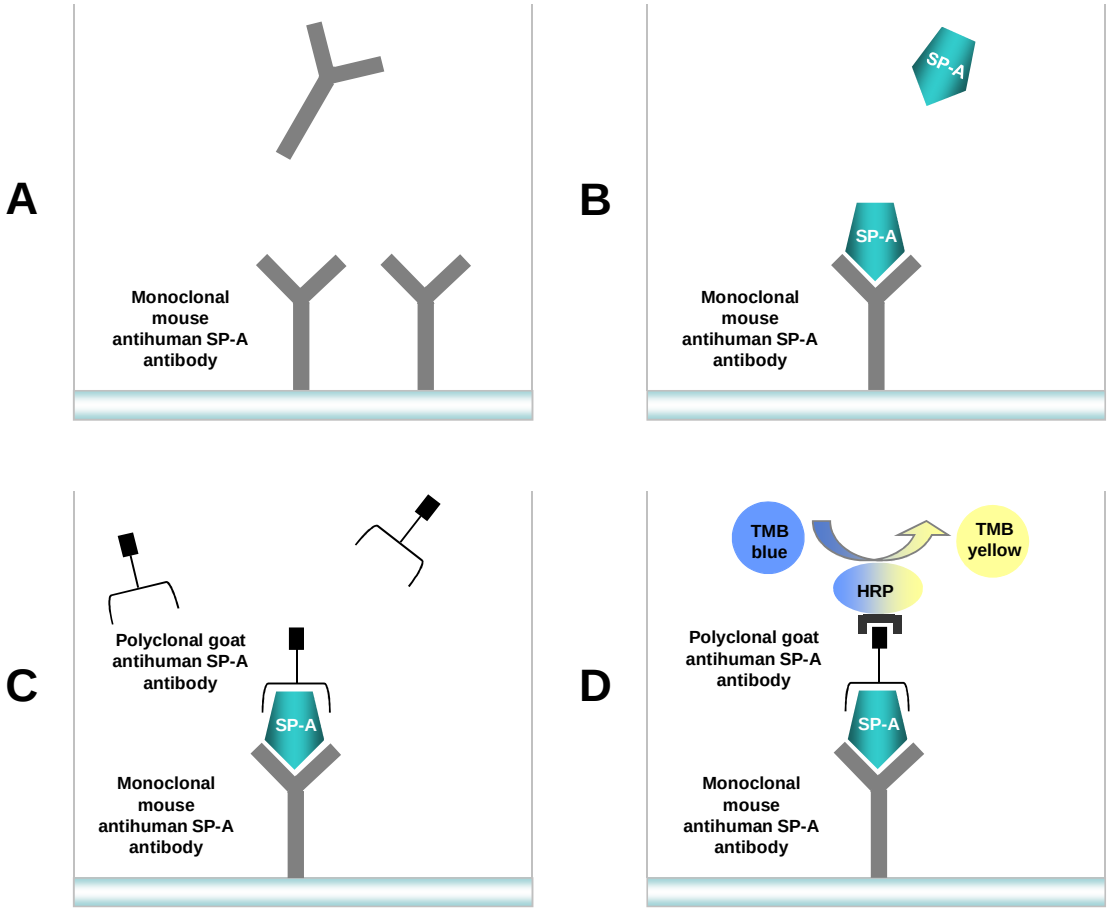

Figure 21: Reaction scheme of the indirect sandwich ELISA method. A: Each well was coated with the *capture antibody*; B: The *antigen* was added; C: The *detecting antibody* attached to the antigen; D: The *HRP – conjugated secondary antibody* bound to the detecting antibody and catalyzed the colour reaction from the blue to the yellow form of tetramethylbenzidine (TMB).

For the assessment of the optimal working concentrations of the antibodies for the final assay the chessboard titration (CBT) was used. This technique involved the dilution of two reagents against each other in the same assay to examine the activities at all resulting combinations.

First, the concentration of SP-A was titrated against the detecting antibody (see *Figure 22*), whereas the mouse anti-human SP-A monoclonal antibody (= capture antibody) and the dilution of the horseradish peroxidase (HRP) – conjugated secondary antibody were kept constant. After coating the wells with capture antibody (see **A** in *Figure 21*) except those in column 12, non-specific binding of SP-A was avoided by addition of blocking buffer to the wells for 30 min. Then SP-A solution was added to the first column (see **B** in *Figure 21*) and subsequently serially diluted across the *columns* producing a 1:2 dilution of the antigen in the second column, a 1:4 dilution in the third column ending in a 1:1026 dilution in column 11. The last column contained no SP-A for the later control of the background binding signal of the detecting antibody.

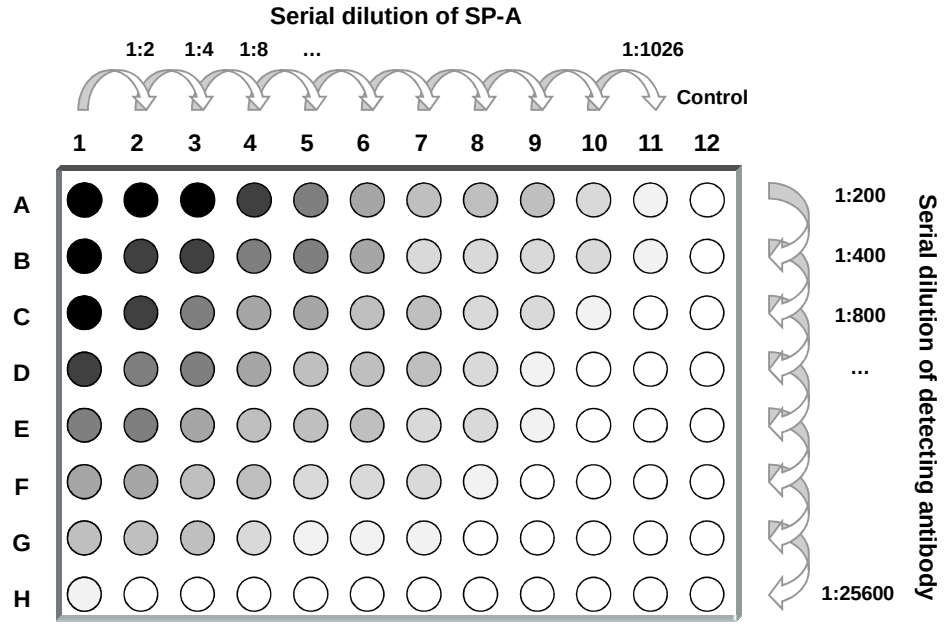

Figure 22: Chessboard titration scheme for the determination of the optimal working concentrations of human SP-A in the standards solutions and the detecting antibody.

Accordingly, the detecting antibody was serially double diluted across the *rows* A-H. Up to this step, the column 12 only contained a dilution range of the detecting antibody. Afterwards, the detecting antibody was incubated with the HRP-labelled anti-antibody followed by the colour reaction (see **D** in *Figure 21*) resulting in decreasing absorption intensities from A1 to H12 as shown in *Figure 22* due to the serial dilution procedures of SP-A from columns 1-11 and of the detecting antibody from the rows A-H, respectively. To determine the optimal concentration of the detecting antibody the measured absorption intensities of each row were plotted against the columns 1-12 (see *Figure 23*).

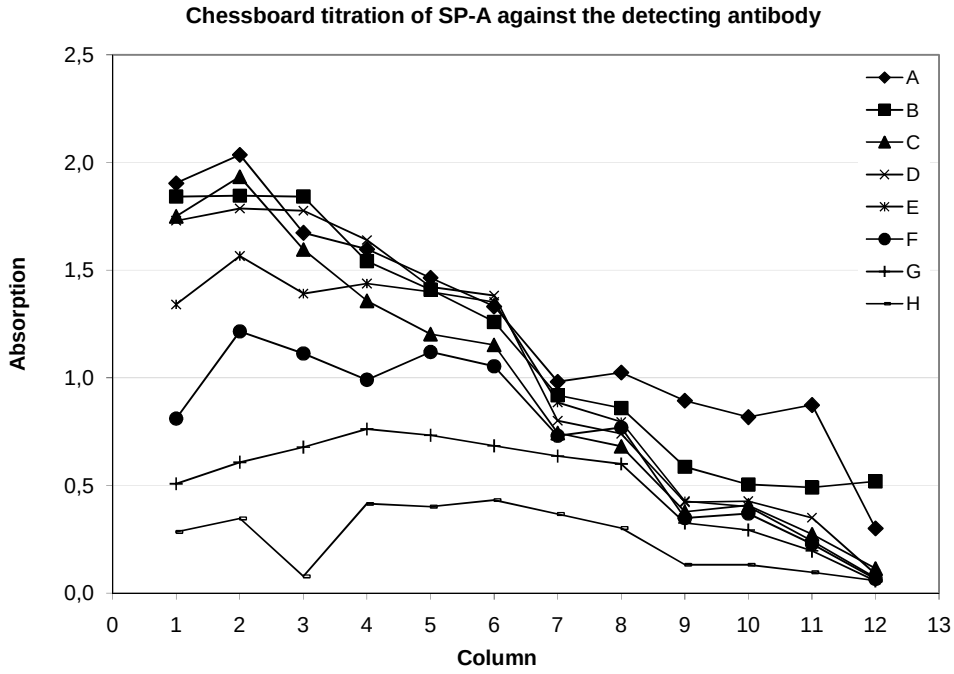

Figure 23: Absorption intensities plotted against the columns 1-12 measured in the rows A-H resulting from the chessboard titration of SP-A against the detecting antibody.

Since column 12 contained no antigen the colour here gave a measure of the binding of the detection system to the plate. Absorptions around 0.09 appeared to be the plate background in the presence of the same dilution of the HRP-conjugate. The rows A and B showed higher absorptions than other rows. Thus, the endpoint detection of SP-A was affected here. Examination of the plateau heights indicated that the trapping system was saturated in the columns 1-3, since similar absorption intensities were measured. The data showed that the optimal dilution of the detecting antibody was achieved in row C. Here, the maximum concentration of the antigen was bound in column 1, the reduction of the absorptions across the columns was sensitive towards the dilutions of SP-A and the background signal in column 12 was the plate background. Thus, for the further experimentals a 1:800 dilution of the detecting antibody was used.

Next, the amount of the capture antibody for coating the wells was optimized. For this purpose, the monoclonal mouse antihuman SP-A antibody was double diluted across the rows in the columns 1-4. The concentrations of the antigen, the detecting and conjugated antibody were kept constant. The highest absorption intensities were detected in row A indicating that the concentration of the capture antibody here was required to bind all available antigen.

To reassess the conjugate dilution SP-A and the detecting antibody were used in its optimal working concentrations according to prior experiments and the capture antibody was titrated against the HRP – conjugate. An optical density of around 1.2 was regarded as an

optimal result. In the last preliminary experiment the ELISA was tested for linearity. All components, except human SP-A, were used in the dilutions as determined before. Linearity in signal intensity was observed from 125 – 1.25 ng/mL with a correlation coefficient of 0.996. For the final ELISA method used for quantification of human SP-A in perfusion fluid samples with unknown concentrations of SP-A see *6.4.4* in C-Experimental Setup.

3.3 Results

In total, complete sets of perfusion fluid samples of 39 IPL experiments were available for the analysis of potential edema markers. Experimental protocols documenting the times of sampling, the type of fluid matrices and the weight of the lung lobe before and after the perfusion were available.

For further analysis of the results, the levels of edema formation were arbitrarily classified into three groups. Lung lobes with a weight gain of less than 30 % were rated as edema class I perfusions, lung lobes exhibiting weight increases between 30-59 % and above 60 % were placed in edema class II and III, respectively (see *Table 10* and *3.1*, D - Appendix). Furthermore, the IPL experiments were separated according to the used perfusion fluids because the plain artificial perfusion buffer did not contain any of the measured substances, whereas in the mixture of buffer and blood components the analyzed edema markers were already present in the blank samples.

Table 10: Number of lung lobes classified into categorized in to the edema classes I-III.

Edema class	*Buffer* perfusions		*Blood* perfusions	
	Number	Mean weight gain in % (± SD)	Number	Mean weight gain in % (± SD)
I (weight gain ≤ 29 %)	11	10.5 (± 11.4)	13	7.5 (± 9.0)
II (weight gain 30 - 59 %)	6	36.3 (± 5.5)	3	39.3 (± 4.6)
III (weight gain ≥ 60 %)	6	83.0 (± 27.3)	-	-

SD - standard deviation

About 60 % of all perfused lung lobes have shown a slight weight increase (edema class I) indicating no or a low degree of edema formation. Standard deviations of the mean weight gains were higher than the mean values in this class, since some of the lung lobes were lighter after the experiment than before and the “weight gain” became negative. In contrast to other findings

[87], the weight gain was significantly higher in lung lobes perfused with perfusion buffer than perfused with a mixture of blood and buffer (unpaired students t-test, $p< 0.01$) suggesting a preventive influence of blood components on the development of pulmonary edema. The monitored fluid level in the reservoir decreased in average by 9 mm (range 6-13 mm, n= 3) in edema class III perfusions, by 3 mm (range 3-4 mm, n= 8) in edema class II perfusions and by 2 mm (range 0-4 mm, n=18) in edema class I perfusions, respectively.

The LDH activity and the urea concentration were measured in samples drawn from 24 and 17 IPL experiments, respectively. SP-A and ACE concentrations were analyzed in fluid sample sets of 33 and 39 perfusion experiments, respectively. After the quantification of SP-A, ACE, urea and LDH activity, obtained data were plotted against the time course of the corresponding experiment (see *3.2*, D - Appendix). The time courses of the LDH activity and urea concentrations in analyzed perfusion fluids were very inconsistent in relation to the weight increases of the perfused lung lobes due to edema formation and thus did not allow conclusions about the onset of lung tissue damage. The release of SP - A and ACE into the perfusate over time is shown in *Figure 24.* Presented data were obtained from the analysis of samples of three representative buffer perfusion experiments rated as edema classes I, II and III according to the observed weight gains of the lung lobes, respectively. Time 0 min marks the start of the perfusion experiment and data points the time of sampling of perfusion fluid out of the reservoir.

Time courses of SP-A and ACE in buffer perfusion experiments

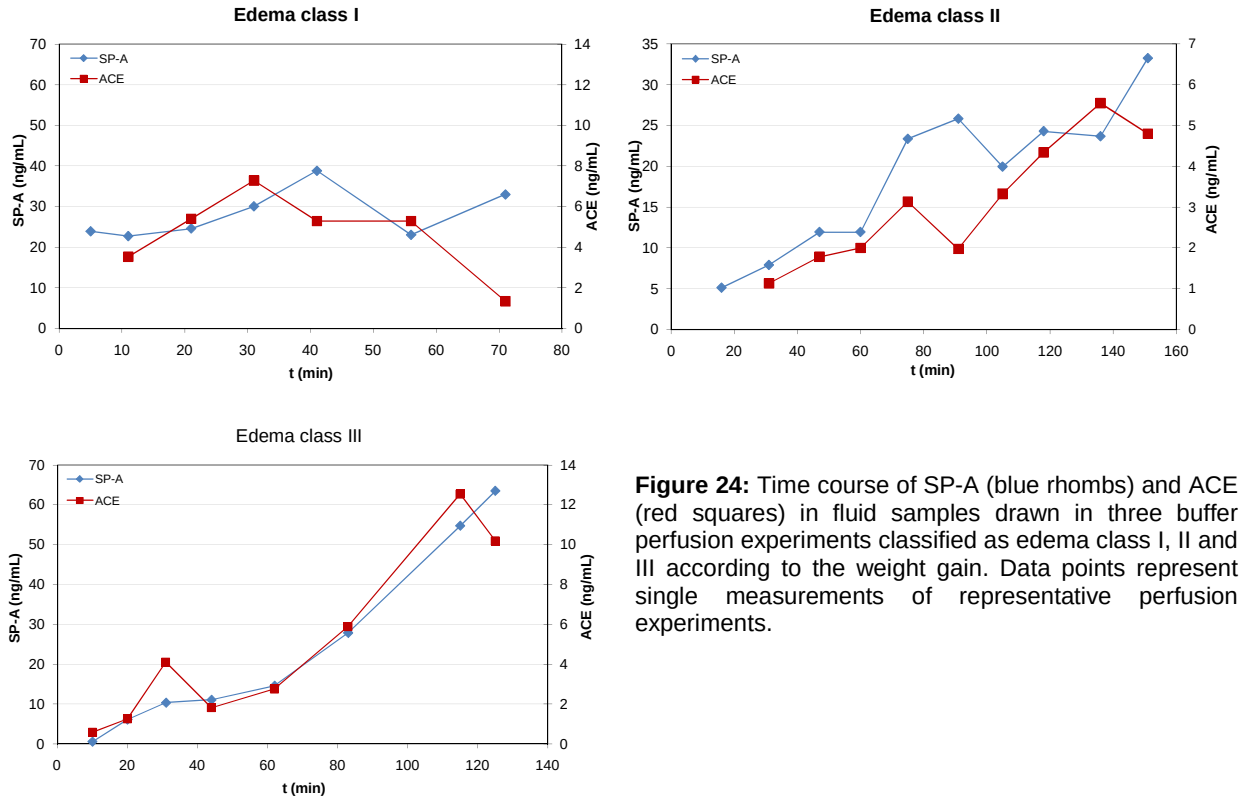

Figure 24: Time course of SP-A (blue rhombs) and ACE (red squares) in fluid samples drawn in three buffer perfusion experiments classified as edema class I, II and III according to the weight gain. Data points represent single measurements of representative perfusion experiments.

The data demonstrate that the release profiles of SP-A and ACE into the buffer perfusate were unexpectedly highly similar. In the lung lobe perfusion classified as edema class I (weight gain < 30 %) the concentrations of both markers remained nearly on the level of the initial values, whereas significant increases in the concentrations at time 60 min were observed in edema class II and III perfusion experiments marking the time of excessive lung tissue damage. Furthermore, the concentrations of SP-A and ACE exceeded 50 ng/mL and 10 ng/mL in the last analyzed fluid samples obtained from the edema III perfusion, respectively, indicating a high extent of damage of lung epithelia, while the concentrations of SP-A and ACE of the other two experiments were below these levels. However, the concentrations of the potential edema markers varied in between the perfusion experiments already in the first drawn samples, even in samples obtained from buffer perfused lungs so that additionally the maximum change of the concentrations over time was analyzed which allowed pooling of the data (see *3.2*, D - Appendix). Unfortunately, the time courses of each edema class could not be summarized because of the different sampling times in the experiments.

Overall, the concentrations of SP-A in analyzed samples from buffer perfusion experiments ranged from 2.0 - 135.8 ng/mL (mean 25.9 ng/mL, n= 23) and in blood perfused lungs from 2.3 - 126.0 ng/mL (mean 37.9 ng/mL, n= 16), respectively. The mean ACE content in samples obtained from experiments performed with perfusion buffer was 4.6 ng/mL (range 0.8 - 14.9 ng/mL, n= 19) and in 54.0 ng/mL (range 4.6 - 143.1 ng/mL, n= 14) in fluid samples containing blood components.

On average, 19.0 µg/mL (range 2.7 - 70.1 µg/mL, n= 17) urea and 131.4 µg/mL (range 57 - 186.8 µg/mL, n= 7) were recovered in plain buffer and buffer/blood samples drawn in the IPL experiments, respectively. The LDH activity was between 27.3 and 341.9 U/L (mean 115.8 U/L, n= 13) in samples obtained from experiments performed with perfusion buffer and fluctuated from 131.8 - 535.0 U/L (mean 348.7 U/L, n= 4) in fluid samples obtained from *ex vivo* lung lobes perfused with a mixture of blood and buffer. It should be noted that the overall mean concentrations or activity of analyzed compounds were significantly higher in blood/buffer than in buffer perfused lungs, except the contents of SP-A, concluding that these substances were already present in the blood components and did not originate from the perfused lung lobes.

Therefore, the differences between the first measured and highest value of the compounds in the perfusion fluid samples in each experiment were calculated and then displayed as either absolute changes (ng or µg /mL and U/L) or percent changes of the first measured values for further data analysis. Thereby, data were grouped into the edema classes I-III of buffer and blood perfusions, respectively. Then obtained data were summarized as means and standard deviations or mean deviations of the means depending on sample size (see *Figure 25* and *Figure 26*).

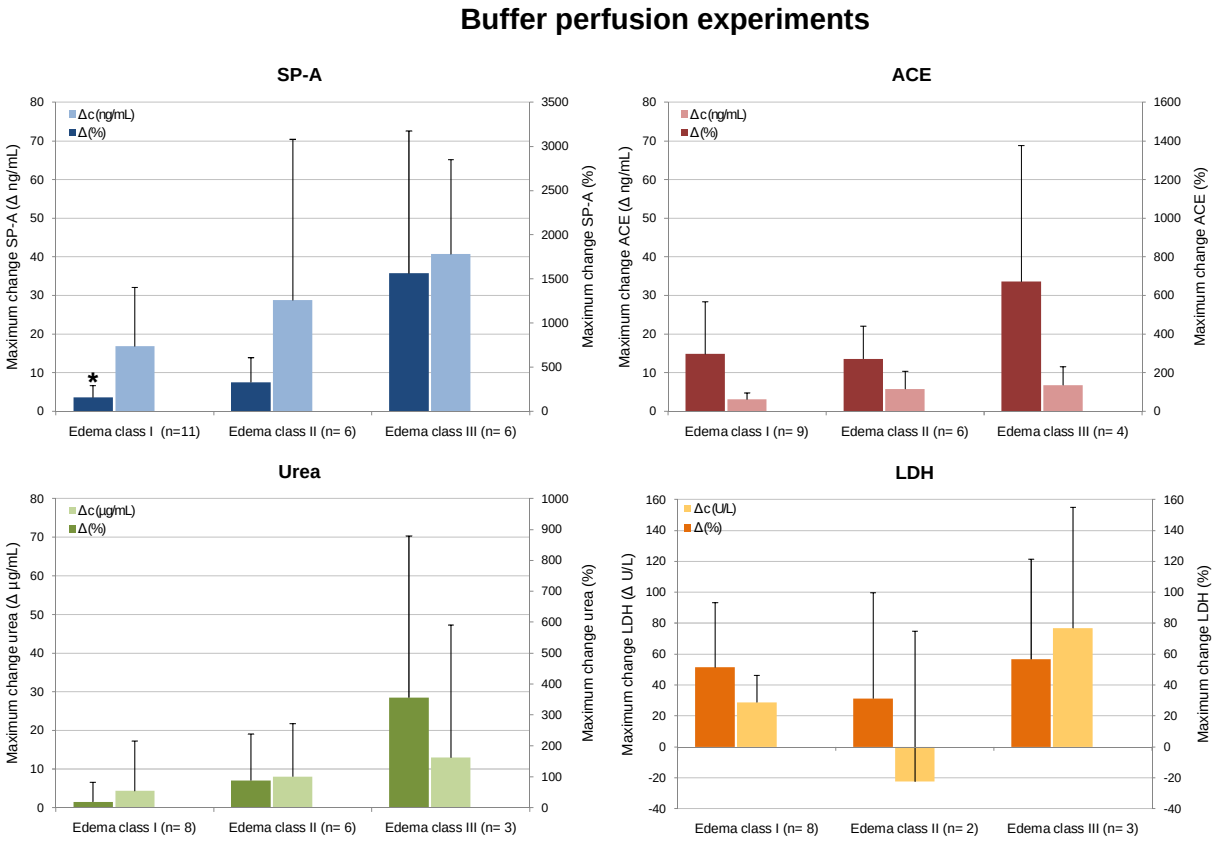

Figure 25: Absolute (left y-axis) and percent (right y-axis) change of SP - A (light and dark blue), ACE (light and dark red) and urea (light and dark green) content and LDH activity (light and dark orange) in perfusion experiments with buffer as perfusion fluid according to edema classes I-III. Data represent means and standard deviations (if n> 3) or mean deviations of the mean (if n≤ 3). * - significant difference between the edema class I and III ($p < 0.05$).

Detailed data of the maximum change in edema markers in IPL experiments are listed in *3.2*, D - Appendix. The one-way analysis of variance revealed a significant difference between the maximum percent changes (dark blue bars) of SP-A in buffer perfusion experiments regarding the edema class I and III, whereas the maximum changes in the absolute concentrations (light blue bars) were not (see diagram top left in *Figure 25*). A tendency was observed that the maximum changes in SP-A, ACE and urea concentrations increase according to the extent of weight gain, but the results for each edema class did not reach statistical significance (one-way ANOVA) due to the large deviations of the means. The change in activity of LDH in perfusion fluid samples did not show any correlation to the grade of edema formation in these experiments.

SP-A and ACE were analyzed in samples obtained from 16 and 14 perfusion experiments performed with blood/buffer perfusion fluid, respectively. The maximum changes (in ng/mL and percent) in the edema class I and II are shown in *Figure 26.* A weight gain of more than 59 % (= edema class III) did not occur in lung lobes perfused with a mixture of buffer and blood (see *Table 10*). Since only a few sample sets obtained from blood perfused lungs

were analyzed for urea concentration and LDH activity, the results were not presented here but are listed in *3.2,* D - Appendix.

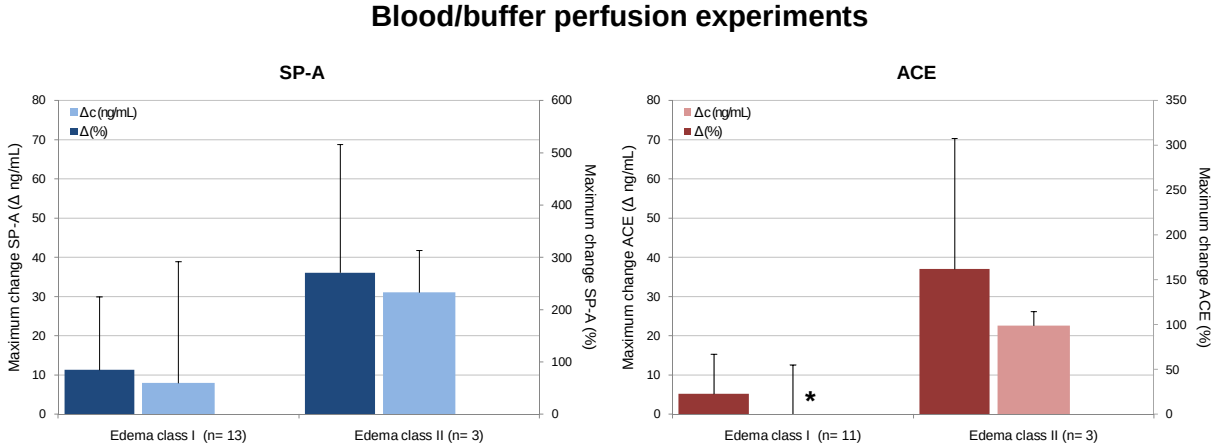

Figure 26: Absolute (left y-axis) and percent (right y-axis) change of SP - A (light and dark blue), ACE (light and dark red) in perfusion experiments with a buffer/blood mixture as perfusion fluid according to edema classes I and II. Data are means + standard deviations. * - significant difference between the edema class I and II ($p < 0.05$).

The mean changes of SP-A and ACE were higher in IPL experiments resulting in a weight gain of perfused lung lobes of 39.3 % (= edema class II) in comparison to lung lobes classified in edema class I indicating a correlation between the weight increase and the extent of lung tissue damage. However, statistical significance was only shown for the comparison of the change in the concentration of ACE between edema class I and II (Mann- Whitney test, $p < 0.05$).

3.4 Discussion

The development of pulmonary edema can be described by two distinct phases. Fluid first accumulates in the alveolar and bronchial perivascular spaces. When the intestinal fluid volume increases by approximately 50 % it disrupts the epithelial barriers and floods the alveoli and airways [273]. Within the IPL experimental model.edema formation frequently occurred, resulting in a weight gain of the lung lobe. Besides the detection of edema formation the knowledge about its onset was important for the assessment of the quality of the IPL experiment since it was intended to run perfusions as close to the physiological *in vivo* conditions as possible. It was hypothesized that a damage of pulmonary epithelium and a subsequent pulmonary edema led to an enhanced release of intracellular or membrane-bound compounds into the perfusion fluid which were usually not present or present in low amounts in the plain perfusion buffer. The evaluation of potential biomarkers for the early development of

pulmonary edema in the IPL experiment revealed promising tendencies, although the results did not reach statistical significance due to high inter-experimental variability.

To determine if the extent of weight gain of the perfused lung lobe was associated with a higher concentration of the analyzed compounds, the maximum changes in the recovered amounts in perfusion fluid samples were examined. It was observed that the change of the SP-A content over time was significantly higher in fluid samples obtained from buffer perfused lung lobes with edema formation > 60 % in comparison to lung lobes with a weight gain less than 30 %. Furthermore, the mean percent change of ACE recovered in blood/buffer perfusion samples was significantly more pronounced in lung lobe perfusions classified as edema class II (weight gain ≥ 30 %) than in IPL experiments with no edema formation. A similar tendency was seen for urea in buffer perfusion experiments (see *Figure 25* in *3.3*), however statistical comparisons did not reveal significant differences since pooled data had high standard deviations. This was primarily due to the fact that of sample sets of a few perfusion experiments revealed inconsistent results. Thus, a correlation between an increase in the concentrations and a significant weight gain due to pulmonary edema formation was confirmed for SP-A and ACE but not for urea and LDH activity.

To evaluate the onset of edema formation the time courses of the potential biomarkers were explored for each single perfusion experiment. It was assumed that a steep increase in the concentration between two sampling times characterized the time of massive cellular damage and loss of tissue integrity. Interestingly, the time courses of SP-A and ACE were closely correlated in most of the analyzed sample sets. For example in experiments with large weight increases of the lung lobes the concentrations of SP-A and ACE suddenly rose almost in parallel (see *Figure 24* in *3.3*) indicating a damage of the blood-air barrier due to the onset of alveolar flooding. The compounds SP-A and ACE were bound on the surfaces of alveolar epithelium and its disruption led to a release of these compounds into the perfusion fluid, whereas in experiments with no edema formation no increase was observed. In addition, a development of a massive pulmonary edema was sometimes accompanied by a deterioration of ventilation parameters, e.g. a reduction of the minute volume despite constant inspiration pressure, as represented in *Figure 27.*

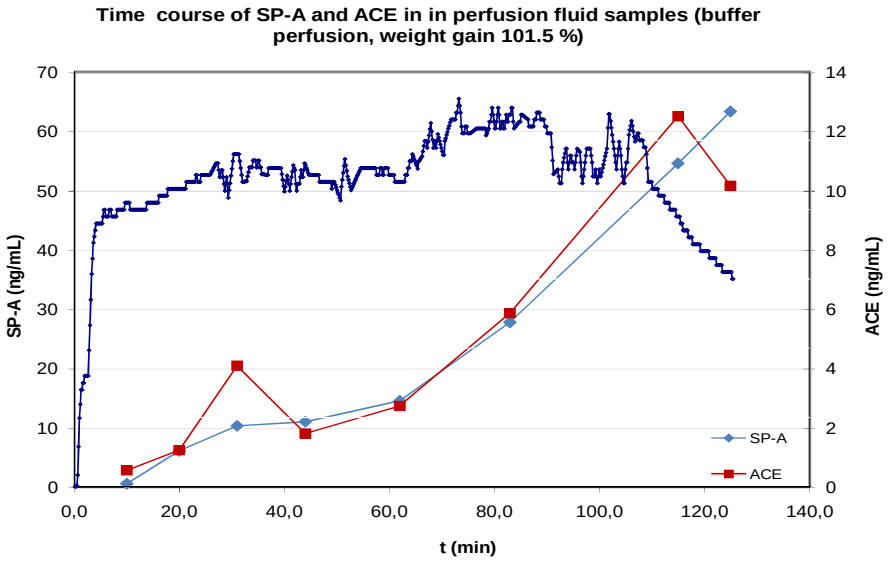

Figure 27: Time course of the minute volume (dark blue) in parallel to the analyzed concentrations of SP-A (left y-axis, light blue) and ACE (right y-axis, red) in a perfusion experiment with a massive edema formation (weight gain 101.5 % = edema class III).

The time 0 min marks the start of the perfusion and recording of ventilation data, at time 10 min the first fluid samples for analysis of edema marker were drawn resulting in the first data for SP-A and ACE concentrations. An apparently massive damage of the airways led to a reduction of the minute volume at time 110 min, in parallel SP-A and ACE reached their maximum in fluid samples drawn at that time. In this experiment, the weight of the perfused lung lobe increased by 101.5 %. Thus, these observations confirmed the correlation between edema formation and the extent of SP-A and ACE in the perfusion fluid.

However, time courses of SP-A and ACE in perfusion fluid samples obtained from other perfusion experiments were not as consistent in all perfusions. In two experiments classified as edema class II and in one experiments categorized as edema class III, respectively, SP-A and ACE levels remained stable or even decreased over time although the weight of the lung lobes markedly increased, (see *3.3.3*, D - Appendix). On the contrary, the concentrations of both increased in fluid samples especially in buffer perfused lungs, despite a constant weight of the perfused lung, clearly indicating no edema formation. These findings might be due to several reasons.

The analytical methods for quantification of the compounds were reliable. The assays for the determination of the LDH activity and ACE and urea contents were valid for the analysis in biological matrices. The indirect sandwich ELISA method for the quantification of SP-A was not fully validated, but standard and blank samples were measured always in parallel to the unknown samples, assuring an accurate analysis of each sample set. Obtained data were only used if the correlation coefficients of the calibration lines were higher than 0.97 and the

absorption intensities of unknown samples were within the calibration line. Since some sample sets derived from perfusion experiments dating back to 2004, the storage might have led to a degradation of the compounds. The stability of the analyzed proteins and enzymes had not been tested for long-term storage over several years. Furthermore, it could not be entirely excluded that all obtained samples were shock frozen in liquid nitrogen or repeating freeze and thaw cycles were avoided that would be very important for the maintenance of initial enzyme activity and protein composition. Decreased contents of SP-A and ACE and LDH activity due to suboptimal sample handling, respectively, would lead to false negative results concerning edema formation. However, by the measurement of blank samples of each sample set, drawn after the equilibration phase of the perfusion experiments, and by evaluating the change in concentrations or percentage of initial concentration over time for data analysis this concern was eliminated. Thus, an increase in concentrations over time despite constant mass of the perfused lung lobes (edema class I) could not be explained by degradation of the compounds due to inappropriate sample handling.

The detection of the onset of significant weight gain due to edema formation was not accessible in our experimental IPL setting. The lung lobe was only weighed before the start and after the termination but not during the *ex vivo* perfusion. As mentioned above, a massive increase in mass followed the destruction of epithelial barriers and enhancement of extravascular fluids. Therefore, cellular components might have been present in the perfusion fluid before the initiation of weight gain. If the perfusion experiment was terminated prior to a weight increase, the lung lobe would be classified e.g. in edema class I while a cellular damage already occurred and the biomarker for edema formation could be detected in perfusion fluids leading to false positive results.

As mentioned above these observations were mainly made in buffer perfused lungs rated as edema class I. Since the artificial perfusion buffer contained none of the analyzed substances, an increase in the concentrations reflected any cellular damage very sensitively, whereas the markers (with the exception of SP-A) were already present at higher concentrations in the blank samples drawn in blood/buffer perfused lungs (see *3.3*). Thus, a significant change in the concentrations/activities of released compounds into the blood/buffer perfusion fluid could be detected only when massive tissue damage occurred.

Since the resected lung lobes derived from patients suffering from bronchial cell carcinoma, it could not be excluded that the amounts of cellular markers detected within the perfusate resulted from other pathopysiological processes explaining the analyzed concentrations already in the blank sample drawn shortly after the beginning of the experiment. At that sampling time the perfusate already passed the lung lobe and it´s unlikely that a pulmonary edema was already present at that time. Furthermore, by arbitrary classification of

the obtained results into three groups according to the extent of weight gain, some of the edema class I lung lobes (weight gain < 30 %) might be misclassified. Nine out of 24 perfused lungs grouped in edema class I had a weight increase of more than 15 %. The edema categories were based on the experiences of Linder who set a weight gain of more than 50 % as a clear sign of pulmonary edema formation [87]. Except that other *ex vivo* studies investigating the correlation between weight gain and the time profile of biochemical markers in the perfusate are not published yet. Thus, observations for adequate comparison with our findings are not available.

Degradation of the compounds in the perfusion fluid samples and the termination of the experiment before a significant weight gain of the lung lobe occurred might be accountable for the heterogeneous results even though a correlation was observed between the weight increase and the release of SP-A and ACE into the perfusion fluid.

Strictly standardized sample handling and storage as well as long-term stability analysis of the compounds would clarify these influences on the recovery of ACE and SP-A in fluid samples. Focussing on massive edema formation (weight gain > 60 %) of *ex vivo* perfused lung lobes with weight monitoring during the experiment would extend the knowledge about the time course and correlation between weight increase and release of potential edema markers into the perfusion fluid. The decrease of the fluid level in the reservoir over the time of the experiment did correlate with the extent of the weight gain. However, monitoring of the fluid level alone at distinct time points was not sensitive enough to assess the onset of edema formation and could only give additional informations.

In the literature, valid specific biomarkers for pulmonary edema formation are still up for debate and an object of *in vitro* and clinical investigations. SP-A and ACE as lung-typically proteins have been extensively studied in the context of different lung diseases accompanied with loss of cellular integrity, but the findings were controverse. However, SP-A might be one of the most promising markers. It has been shown that a significant increase of SP-A in serum was associated with the onset of acute lung injury and patients prognosis [265, 266, 274], whereas others did not observe a correlation [275, 276]. However, these clinical studies monitored serum SP-A levels in acute situations for a longer period of time (about one day) than we did in our IPL setting (about two hours). Thus, the transferability of our findings to the *in vivo* observations might be difficult. Current data about the suitability of ACE as a biomarker for lung damage are inconsistent as well. Experimentally induced lung injury resulted in a fast elevation of ACE in serum and lavage fluid [255, 277, 278], while the ACE levels in a clinical situation did not reflect the intensity of pulmonary disease [259, 260].

3.5 Conclusions

SP-A and ACE seemed to be valuable biochemical markers for the development for pulmonary edema formation in the *ex vivo* lung lobe perfusion model. Significant differences between the changes in SP-A and ACE levels recovered in perfusion fluid samples over time in relation to the weight gain of the lung lobes was observed as a sign for edema formation. Furthermore, the observed time courses of both compounds in the perfusion fluid were very similar. A correlation between the time course of urea content and LDH activity and pulmonary edema was not seen.

Additionally to the monitoring of the weight of the lung lobe, the ventilation parameters, the change of the fluid level in the reservoir and the visual appearance of the lung lobe the combined analysis of SP-A and ACE in fluid samples over time could give further indications of about the onset of edema formation in the *ex vivo* IPL setting.

C. Experimental Setup

1 Experimental design of the *ex vivo* human lung lobe perfusion model

1.1 Reagents

1.1.1 Substances

GW597901 (cinnamic acid salt) and salbutamol base were generously provided by GlaxoSmithKline R & D (Stevenage, England). Bovine serum albumin (BSA, Type H1) was obtained from GERBU (Gaiberg, Germany) and glucose monohydrate from Gruessing (Filsum, Germany). A stock solution containing penicillin G (10 000 IU/mL) and streptomycin (10 000 µg/mL) in isotonic saline solution was purchased from Biochrom (Berlin, Germany). Heparin – natrium – 5000 - ratiopharm® (Ratiopharm, Ulm, Germany) was obtained from a local pharmacy. Water derived from an in – house demineralisation system (Milli - Q® - reagent grade water system) from Millipore (Schwalbach, Germany). Nitrogen in liquid form as well as in compression gas cylinders (quality 5.0) and carbon dioxide for medical purpose (DAB, Ph.Eur) was purchased from Linde (Munich, Germany). Other chemicals (p.a. quality) were obtained from E. Merck (Darmstadt, Germany).

1.1.2 Solutions

1.1.2.1 Perfusion buffer pH 7.4

The composition of the perfusion buffer was derived from Mürdter and colleagues [110]. Additionally, antibiotics and heparin were added to obtain the following concentrations:

BSA	5 % (m/V)
Calcium chloride dihydrate	2.5 mM
Glucose monohydrate	5.5 mM
Potassium chloride	3.5 mM
Potassium dihydrogen phosphate	2.5 mM
Magnesium sulphate hexahydrate	1.18 mM
Sodium chloride	85 mM
Sodium hydrogen carbonate	20 mM
Pen/Strep	10 000 IU/10 000 µg/L
Heparin – Na	5000 U/L

For 1000 mL buffer 50 g of BSA were dissolved in 900 mL Millipore® - water and the buffer electrolyte components except sodium hydrogen carbonate were added. The buffer was stirred until yielding a clear solution. Then 1000 µL of the antibiotic stock solution and 200 µL of the heparin – natrium – 5000 – ratiopharm® (see *1.1.1*) were added. Subsequently sodium hydrogen carbonate solution (10 % m/V) was added to adjust the pH value to 7.4 and the volume of 1000 mL was completed with Millipore® - water. The perfusion buffer was always freshly prepared and then stored at 4 – 8 °C one day prior to use in perfusion experiments.

1.1.2.2 Perfusion fluid

In lung lobe perfusion experiments either a defined mixture of different blood products (see *1.2.1*) and perfusion buffer (see *1.1.2.1*) or blank perfusion buffer served as perfusion fluids according to specifications of the perfusion experiments. For detailed composition of the perfusion fluids used in the studies see *2.1.2.1* and *3.1.2.1*.

1.1.2.3 Drug preparations for administration via nebulizer

The β_2 – agonists GW597901 and salbutamol were available as either single substances or combinations in lyophilised formulations prepared by GlaxoSmithKline. Substances for nebulization were reconstituted in Milli - Q® - reagent grade water to yield clear solutions and diluted if necessary to achieve defined drug concentrations. For detailed information about composition and concentrations of β_2 – agonists in the drug solutions used in the pharmacokinetic and pharmacodynamic studies see *2.1.2.2* and *3.1.2.3.*

1.2 Materials and equipment

1.2.1 Human tissue and blood products

Patients who had to undergo a lobectomy, bilobectomy or pneumonectomy due to primary bronchial cell carcinoma were included in the studies. Patients were excluded if they previously received neoadjuvant treatment due to tumour stage, if the tumour was located centrally within the lung lobe or if therapeutic interventions with inhaled or oral β_2 – agonists were applied during the last four weeks. Systemic and pulmonary infections including pneumonia had to be absent in the patient at the time of surgery. Lung lobe resections took place in the Klinik Schillerhöhe, Gerlingen and in the Thoraxzentrum Unterfranken, Münnerstadt, Germany. Every patient was thoroughly informed about the experiment prior to surgery according to the

Declaration of Helsinki [279] and was included if signed informed consent was obtained. After termination of the experiment the human lung tissue was delivered to the pathology unit for routine examination. Permission of study protocol was obtained from Ethics Committee of the Medizinische Fakultät, Eberhard – Karls – Universität Tübingen and Universität Würzburg, Germany. Blood material utilized for perfusion fluid (see *1.1.2.2*) was obtained from local blood donation services (Rotes Kreuz, Frankfurt; Transfusionsmedizin, Würzburg, Germany). Blood donors gave informed consent. Only blood products tested according to the guidelines of the Paul-Ehrlich institute were used [280].

1.2.2 Setup of the human lung perfusion model

To determine and compare the course of pulmonary absorption and distribution of different β_2 – agonists *ex vivo,* the human lung perfusion model described by Linder et al. was modified [87, 105]. It had been shown that this model is able to simulate lung lobe perfusion and ventilation under proximate physiological conditions. Each lung perfusion experiment took place next to the surgery room and after completion of the lung perfusion experiment, lung preparations were immediately delivered to pathology.

Reperfusion was carried out through a half open circulation system. Perfusion fluid was pumped from a reservoir through a heat exchanger, a CO_2 – exchanger and a bubble trap and was delivered with physiological pressure of 20 – 40 mmHg and flow rates of about 60 – 100 mL/min through a valve into cannulated segmental arteries. After leaving the opened vein, perfusate dripped into the reservoir, which was kept at 37 °C. To avoid desiccating of the lung lobe, the unit was enclosed with a transparent plastic cover designed by an in – house workshop (see *Figure 28)*.

Ventilation was performed with a commercial respirator (Evita 4, Draeger Medical, Lübeck, Germany) through an anatomised bronchus connected to a silicone bronchial tubing (I.D. 8 mm, wall thickness 2 mm; WITEG Labortechnik GmbH, Wertheim, Germany). To monitor pH, pCO_2 and pO_2 about 500 µL of perfusion fluid were withdrawn from venous output two to three times during the experiment and analyzed immediately with a blood gas analyzer ABL® 510 (Radiometer, Willich – Schiefbahn, Germany).

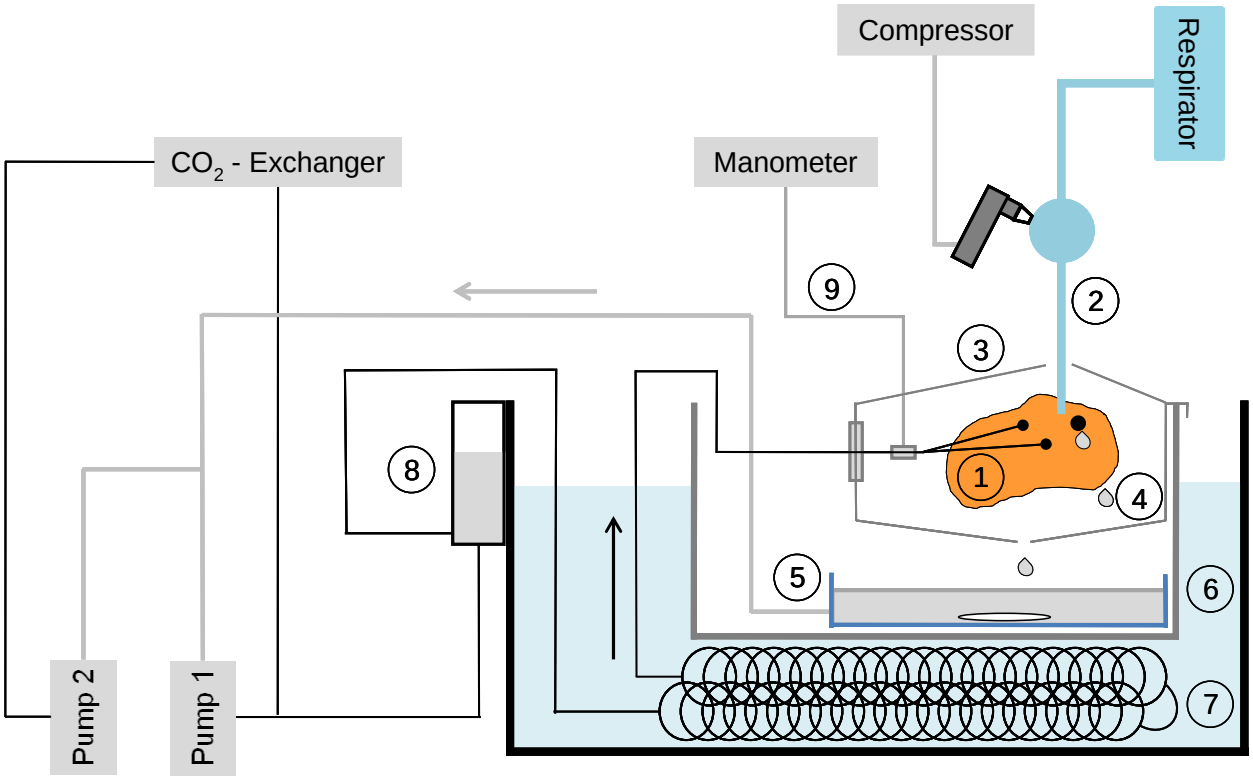

Figure 28: Schematic illustration of human lung perfusion model according to Linder et al. [87] with modifications [105]. Arrows mark the direction of perfusion fluid flow. 1) lung lobe preparation with catheterised arteries, opened vein and anastomised bronchus; 2) connection from bronchus to respirator with dosing device; 3) transparent plastic cover; 4) collection container for perfusion fluid; 5) perfusion fluid reservoir with magnetic stirring bar; 6) water bath tempered at 37 °C; 7) heat exchanger; 8) bubble trap; 9) measurement of arterial perfusion pressure.

For administering solutions of active compounds commercial available PARI Boy® compressors, PARI nebulizers and PARI Interruptors (PARI, Weilheim, Germany) were used (see *2.2.2* and *3.2.2*). The nebulizer was connected via adapter to a glass spacer with a net volume of 100 mL (see *1.2.3*). This air tight dosing device was then installed between respirator and bronchial tube. For this application a round bottom flask with center neck (WITEG Labortechnik GmbH, Wertheim, Germany) was modified by an in – house workshop. Two additional screw threads attached at the flask in an angle of 135° to each other provided an access to the respirator and the bronchus of the lung lobe, respectively (see *Figure 29*).

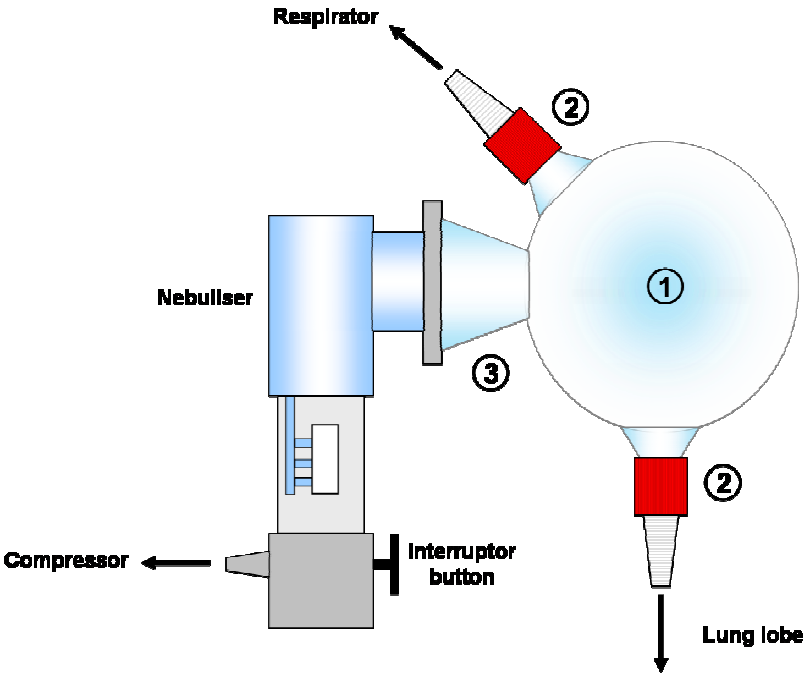

Figure 29: Schematic construction of the application device: 1) hollow round glass component with one standard ground joint and two screw threads (volume: 100 mL, outer diameter: 64 mm); 2) adaptor to screw threads for connection to respirator and bronchus; 3) standard ground joint ST 29/32 for attachment of the nebulizer.

1.2.3 Other equipment and material

Balance, analytical	ABJ 220 – 4M, 0.01 – 220 g, 0.1 mg minimal weight difference (Kern & Sohn, Balingen – Frommern, Germany); Sartorius portable (Sartorius AG, Göttingen, Germany);
Balance, precision	Mettler P1200, 0.1 – 1200 g, 10 mg minimal weight difference (Mettler – Toledo, Gießen, Germany);
Bloodline	ECC – Bloodline 6.35/1.6 mm (Raumedic AG, Helmbrechts, Germany);
Disposable scalpel	Feather disposable scalpel, sterile (Feather Safety Razor Co. LTD; Osaka, Japan);
Magnetic stirrer	VARIOMAG® Mono (H + P Labortechnink, Oberschleißheim, Germany); IKA – COMBIMAG REO (Janke & Kunke KG, Staufen, Germany);
Magnetic stir bar	cylindric, 60 mm/10 mm, PTFE coating (A. Hartenstein, Würzburg, Germany);
Manometer	Electronic manometer (Hugo Sachs Elektronik, Hugstetten, Germany);

Oxygenator	Jostra Quadrox® (MAQUET Cardiopulmonary AG, Hirrlingen, Germany);
Pipette	Serological pipette 10 and 25 mL, non pyrogenic, sterile (A. Hartenstein, Würzburg, Germany);
Plastic ware	Teflon, Plexiglas® (Pfister & Pfrang technical wholesale, Waldbüttelbrunn, Germany);
Pump	Twinpump PM015-240 (Maquet GmbH & Co. KG, Rastatt, Germany); ISMATEC MCP pump system ISM 726 (ISMATEC SA, Glattbrugg, Switzerland);
Suture material	Prolene® suture cartridges (Ethicon GmbH & Co. KG, Norderstedt, Germany);
Thermostat for water bath	Model Fisons DC3 (Haake, Karlsruhe, Germany);
Three – way valve	Discofix® for infusion and monitoring (B. Braun Melsungen, Melsungen, Germany);
Venous catheter	Vasofix® Braunüle 18 G (B. Braun Melsungen, Melsungen, Germany);

1.3 Performance of human lung perfusion experiments

1.3.1 Ventilation and reperfusion of resected lung lobe preparation

To minimize time loss between lung lobe resection and reperfusion, the ventilation and perfusion system was prepared prior to the patients` surgery. Therefore, the water bath was tempered at 37 °C and 1.5 L of perfusion fluid (see *1.1.2.2*) were filled into the half open system to let it warm up by circulation. The dosing device (see *1.2.2*) was connected to the respirator.

After resection of the lung lobe, the bronchus was sewn to silicone tubing, available arteries were cannulated and the veins were opened. Before starting the experiment the lung lobe was rinsed with 500 mL of perfusion buffer (see *1.1.2.1*) through the cannulated arteries at a flow rate of about 40 – 50 mL/min without recirculation of the fluid. Rinsing fluid was discarded afterwards. The lung lobe was weighed before and after the experiment and the total mass was documented to control for edema formation. By connecting the bronchus to the respirator and the arteries to the fluid circulation the experiment was started. BIPAP (**Bi**phasic **P**ositive **A**irway **P**ressure) was used as ventilation mode allowing a constant maximum inspiration pressure leading in variable respiratory volumes according to the size of the individual lung lobes ventilated. In contrast to a constant respiratory volume ventilation procedure, the BIPAP mode prevents hyperinflation and lung lobe damages. Detailed parameter settings for ventilation and

perfusion procedures in the pharmacokinetic and pharmacodynamic studies see *2.3.2*, *3.3.2* *3.4.2*.

After successfully establishing reperfusion and ventilation, the system was equilibrated for at least five minutes and a 6 mL perfusion fluid sample (blank sample) was drawn before administration of β_2 – agonists. The blank sample was placed on ice immediately and the drawn volume was replaced by fresh perfusion fluid (RT) added to the reservoir. For stability control of the active compounds, drug solution was added to 6 mL of fresh perfusion fluid and kept at 37 ℃ for the duration of experiment, thereafter tr ansported on ice.

To maintain the pH of perfusion fluid within the physiological range of about 7.4, either CO_2 gas was added using a conventional oxygenator (if pH > 7.4) or $NaHCO_3$ solution (10 % m/V) was given into the perfusion fluid if the pH was below 7.4. Thereby, 5 mL $NaHCO_3$ solution (10 % m/V) was added into the reservoir to increase the pH about 0.1.

1.3.2 Application of β_2 – agonists

Subsequent to the equilibration phase of lung lobe perfusion and ventilation, the nebulizer was attached to the dosing device. Application of β_2 – agonists was performed via PARI BOY® nebulizer system. For further information about the administration procedures and the following course of the experiments see *2.3.1* and *3.4.4*.

1.3.3 Termination of lung perfusion experiment

The lung perfusion experiment was terminated individually. The determining factor for termination was a development of a significant edema as judged by the level of the perfusion fluid in the reservoir and the appearance of the specimen. Thus, the experimenter had to decide when to finish in consideration of the decrease in perfusion fluid level in the reservoir, of visual and palpable changes in the constitution of the lung lobe and of the deterioration of ventilation and perfusion pressure. To maintain physiological conditions and not to interfere with later pathological examination of the lung lobe, massive edema formation was to be avoided.

As mentioned above the lung lobe was weighed again and the weight change in percent was documented. The spacer and all connections (tube to respirator, tube to lung lobe) were collected without rinsing for determination of the effectively administered dose (see *2.4.3)* and kept at RT until analysis.

2 Determination of pharmacokinetic properties of inhaled β_2 – agonists using the human lung perfusion model

2.1 Reagents

2.1.1 Substances

Sealed vials containing both pre – weighted GW597901 (cinnamic acid salt) and salbutamol sulphate were prepared by GlaxoSmithKline (Greenford, England). Phenacetin (**i**nternal **s**tandar**d**, *ISD*) was purchased from FLUKA (Buchs, Switzerland). Sources of supply of other used substances were noted previously in *1.1.1.*

2.1.2 Solutions

2.1.2.1 Perfusion fluid

For the pharmacokinetic enabling study a mixture of buffy coats and perfusion buffer (see *1.1.2.1*) was used to extracorporally perfuse lung lobes of six different patients (see *1.2.1*). Prior to combination of buffy coats and perfusion buffer only blood materials with compatible blood groups were pooled, depending on the volumes of each single blood product unit. The haematocrit of the combined blood products was determined by centrifuging 10 mL samples in 15 mL tubes (120 x 17 mm , conical bottom, polypropylene, Sarstedt, Nümbrecht, Germany) in duplicate (3000 rpm, 10 °C, 30 min, see *1.2.3*) [281] and then the blood material was mixed with perfusion buffer resulting in a final haematocrit value of 20 % (= blood cell fraction). Separately, 500 mL plain perfusion buffer were prepared for flushing the lung lobe prior to the experiment. The perfusion fluid and buffer were always prepared one day before lung perfusion experiment and stored at 4 – 8 °C in brown 2.5 and 0.5 L glass bottles, respectively.

2.1.2.2 Solutions for cassette dosing via nebulizer

Lyophilised β_2 – agonist combinations (see *2.1.1*) were reconstituted in Milli - Q® - reagent grade water and diluted if necessary to achieve following drug concentrations:

- *GW597901 base/salbutamol base:* 45/150 µg/mL
- *GW597901 base/salbutamol base:* 180/600 µg/mL

Drug solutions were aliquoted in volumes of 5 mL and stored at -20 °C until usage. Each drug concentration was nebulized in three lung lobe perfusion experiments, respectively.

2.2 Materials and equipment

2.2.1 Human tissue and blood products

Sources and inclusion criteria of resected lung lobes and blood products were described elsewhere (see *1.2.1*). In total, six patients who had to undergo surgery due to bronchial cell carcinoma in the Klinik Schillerhöhe, Gerlingen, Germany, were involved in the pharmacokinetic enabling study.

2.2.2 Other equipment and materials

Centrifuge	Hereus® Megafuge 1.0R (Kendro Laboratory Products, Langenselbold, Germany); Microfuge® 22R Centrifuge (BECKMAN COULTER$_{TM}$, Krefeld, Germany);
Disposable pipette	Polyethylene, non sterile, 7.7 mL, 150 mm (Carl Roth, Karlsruhe, Germany);
Flask 10 mL	Flask, volumetric, 10 mL, ST 10/19, Duran® 50 (WITEG Labortechnik GmbH, Wertheim, Germany);
Nebulizer	PARI Boy® N compressor, PARI LL® nebulizer and PARI LL® Interruptor;
Pipette 10 – 100 µL	Eppendorf Research® variable, 10 – 100 µL (Eppendorf, Hamburg, Germany);
Pipette 100 – 1000 µL	Eppendorf Research® variable, 100 – 1000 µL (Eppendorf, Hamburg, Germany);
Plastic reaction tube	Polypropylene, 1.5 mL, uncoloured (Carl Roth, Karlsruhe, Germany);
Tube 15 mL	Polypropylene, 15 mL, 120 x 17 mm (Sarstedt, Nürmbrecht, Germany);
Tube 50 mL	Polypropylene, 50 mL, 115 x 28 mm (Sarstedt, Nürmbrecht, Germany);
Vortex	Model K – 550 – GE (Bender & Hobein, Zurich, Switzerland)

Further materials and equipment was described previously in *1.2.3*.

2.3 Lung lobe perfusion in the pharmacokinetic enabling study

2.3.1 Prearrangement of the experiment and application of β_2 – agonists

For the pharmacokinetic enabling study six lung lobe perfusion experiments were performed next to the surgery room in the Klinik Schillerhöhe, Gerlingen, Germany. The experimental setup, the preparation steps, perfusion and ventilation settings of the lung lobe *ex vivo* are described elsewhere (see *Figure 28* in *1.2.2* and *1.3*).

Once a successful ventilation and reperfusion of the lung lobe was achieved and the system was equilibrated for at least five minutes the β_2 – agonists were applied via nebulizer. Thus, the drug solution containing GW597901 and salbutamol was nebulized with a MMAD (mass median aerodynamic diameter) of 3.7 µm and a total output rate of 470 mg/min. The PARI Boy® N compressor in combination with the PARI LL® nebulizer provides 64 % by weight below 5 µm of aerosol particle size [282].

Targeted doses of 45/150 µg (n= 3) and 180/600 µg (n= 3) (GW597901/salbutamol, see *2.1.2.2*) were administered. Thereby, the solution was administered by activating the interruptor button during the inspiration phase and releasing it throughout the expiration manoeuvre. To ensure an optimal deposition of the aerosol in the peripheral parts of the airways P_{max} was increased by 5 mbar during application of β_2 – agonists. A change in nebulizing tone during dosing indicated that the targeted dose of the β_2 – agonists had been nebulized and the dosing procedure was stopped. This administration via nebulizer took about 12 minutes. After termination of the dosing procedure P_{max} was returned to the initial value and samples of the perfusion fluid were obtained (for details see *2.3.3*). The nebulizer with remaining solution was closed and carried carefully for later determination of the effectively administered dose (see *2.4*).

2.3.2 Ventilation and perfusions parameters

The settings of different ventilation and perfusion parameters in the pharmacokinetic enabling study are displayed in *Table 11.* The preset maximum respiratory pressure and perfusion flow rate values resulted in a mean minute volume (MV) of 5 L/min (1.4 – 10.0 L/min) and a mean arterial perfusion pressure of 36 mmHg (19 – 120 mmHg). The magnitude of MV strongly depends on the type of ventilated lung lobes, e.g. whether an upper lobe or a whole right lung was available. Thus, the broad range of the MV can be explained. By adjusting the fluid flow rate, the perfusion pressure should be kept in the range of 20 – 40 mmHg to avoid lung tissue damage. In one perfusion experiment an extensive increase of perfusion pressure to 120 mmHg

at the end of the performance was seen, likely due to pulmonary edema formation. Perfusion pressure values in the other experiments were always in the acceptable range.

Table 11: Settings of ventilation and perfusion parameters in the pharmacokinetic enabling study (n= 6).

	Parameter	Mean	Range
Ventilation	P_{Peak}	20.7 mbar	18 – 25 mbar
	PEEP	1.5 mbar	0 – 3 mbar
	Respiratory rate	15/min	-
Perfusion	Fluid flow rate	92 mL/min	60 – 150 mL/min
	Fluid temperature	37 ℃	-

P_{Peak} – maximal airway pressure
PEEP – **p**ositive **e**nd-**e**xpiratory **p**ressure

2.3.3 Sampling

2.3.3.1 Perfusion fluid samples for pharmacokinetic studies of β_2 – agonists

Samples of 6 mL were withdrawn from the venous output with a disposable PE pipette during the dosing procedure 2, 5 and 10 minutes after starting nebulization. Then at time 0 (= termination of dosing procedure), 10, 20, 30, 45 and 60 min 6 mL of perfusion fluid from the venous output and 6 mL from the reservoir were collected. Samples were put on ice immediately. The withdrawn volume was replaced with fresh perfusion fluid (RT). Of each 6 mL sample 2 x 1 mL was withdrawn (= **w**hole **p**erfusion **f**luid samples, WPF) and aliquoted. The remaining 4 mL were centrifuged to obtain the cell – free fraction (3500 rpm, 10 ℃, 10 min) and the supernatants were aliquoted (= **p**erfusion **f**luid **p**lasma samples, PFP). Analysis of the β_2 – agonists in two different types of fluid samples should supplement existing laboratory data concerning the distribution behaviour of GW597901 and salbutamol into blood cells. Samples were stored at -70 ℃ until further analysis.

2.3.3.2 Perfusion fluid samples for evaluation of edema formation

For later analysis of edema development throughout the lung perfusion experiment 1 mL of perfusion fluid was withdrawn from the reservoir in documented time intervals without replacement of perfusion fluid. Samples were immediately put on ice. Before storing at -20 °C, samples were centrifuged (3500 rpm, 10 °C, 10 min), the supernatants were aliquoted in volumes of 100 µL and shock frozen in liquid nitrogen. Already during lung perfusion, it was possible to estimate a formation of edema by observing the perfusion fluid level in the reservoir (see *Figure 30*). With a scale, charted in the reservoir box, the fluid level over time was monitored and documented. A volume decrease in the reservoir indicated a pulmonary edema formation.

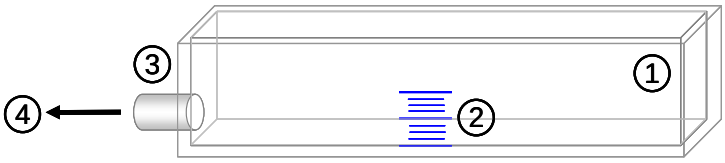

Figure 30: Fluid reservoir in detail that is arranged underneath the lung lobe preparation during the perfusion experiment: 1) block-shaped box 6/20.5/16.5 cm (H/W/D), material: plexiglass, 2) coloured scale for metering the fluid level, 3) aperture connected with an plastic adapter for attaching the blood line, 4) evacuation of perfusion fluid by two peristaltic pumps. The construction was designed and built in an in-house workshop.

2.3.3.3 Lung tissue and bronchoalveolar lavage (BAL)

After termination of the experiment, several tissue samples were obtained. Two tissue samples from peripheral and two from central sites were cut off the lung lobe with a scalpel (see *Figure 31*), each sample weighed about 200 mg. Tissue samples were immediately put on ice. Afterwards the samples were shock frozen in liquid nitrogen and stored at -80 °C.

For the BAL isotonic saline at RT was inserted in the airways with a 10 mL syringe and promptly recovered with a catheter. The volume of the lavage fluid varied from 5 – 10 mL, depending on the total mass of the lung lobe. The recovery ranged from 0.2 – 1.3 mL (4 – 13 %). During transport the samples were kept on ice and stored at -20 °C until further analysis.

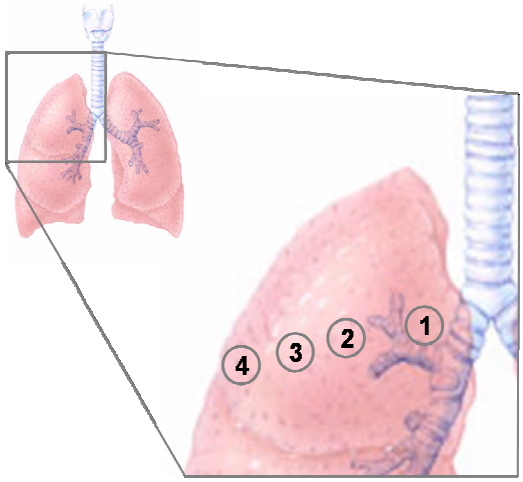

Figure 31: Schematic illustration of four different anatomical positions of lung tissue samplings from site 1 (= central area of the lung lobe) to 4 (= peripheral area of the lung lobe). (Image was modified according to [283])

2.4 Quantification of effectively administered dose of β_2 – agonists

To evaluate the time course and extent of distribution of the β_2 – agonists into the perfusion fluid, the knowledge about the effectively administered dose is essential in order to express the absolute amounts of β_2 – agonists as the percentage of applied doses. Thereby, pooling data of all perfusion experiments was possible, independent of targeted doses of β_2 – agonists. The proportion of the nebulized amount of drug that was actually deposited in the airways was calculated as follows:

Formula 2

$$\text{Dose}_{\text{deposited}} = \text{Dose}_{\text{total}} - \text{Dose}_{\text{retained}} - \text{Dose}_{\text{adsorbed}}$$

To determine the deposited dose of the β_2 – agonists indirectly, required samples (see *2.4.1*, *2.4.2* and *2.4.3*) were analyzed by HPLC (see *4.1.2*, *4.2).*

2.4.1 β_2 – agonist concentration in nebulizing solution (= Dose $_{\text{total}}$)

To calculate the effective β_2 – agonist concentration in the liquid nebulized in the perfusion experiments, solutions with following composition were analyzed by HPLC (see *4.2*). After the termination of the lung lobe experiment, the internal standard (ISD) phenacetin and MeOH HPLC grade were mixed with the inhalation solutions used in the perfusion experiments. The

added volumes of ISD and MeOH complied with targeted doses of GW597901 and salbutamol (see *Table 12*).

Table 12: Composition of analyzed samples for determination of effective β_2 – agonist concentration.

	Targeted dose of GW597901/salbutamol (µg/µg)	
	45/150 (n = 3)	180/600 (n = 3)
Inhalation solution	50 µL	25 µL
Phenacetin V1 (ISD)	50 µL	100 µL
MeOH HPLC grade	400 µL	875 µL

ISD – internal standard

2.4.2 Non applicable solution in PARI nebulizer (= Dose $_{retained}$)

By administering solutions with the PARI LL® nebulizer the fluid level in the nebulizer decreased by time. At a residual volume of about 1.0 – 1.3 mL in the application device, the nebulization stopped. Thus, this amount of dissolved β_2 – agonist in this volume remained in the nebulizer and was not applicable any more. This retained dose was determined by analysing the following samples.

- Perfusion experiments with targeted dose: 45/150 µg GW597901/salbutamol (n= 3):

50 µL of phenacetin stock solution (1 mg/mL) were pipetted in the PARI LL® nebulizer and the remaining solution in the inhalation device was quantitatively transferred into a 10 mL volumetric flask using an Eppendorf pipette (see *1.2.3*). The nebulizer was then washed thrice with 2 mL MeOH and the wash fluid was added into the volumetric flask. At the end, the volume of 10 mL was completed with MeOH and an aliquot was analyzed immediately by HPLC (see *4.2*) by direct injection.

- Perfusion experiments with targeted dose: 180/600 µg GW597901/salbutamol (n= 3):

The amount of internal standard pipetted into the nebulizer reservoir was increased to 200 µL of phenacetin stock solution (1 mg/mL). Again the remaining solution in the inhalation device was quantitatively transferred into a 10 mL volumetric flask. After washing the nebulizer thrice with 2 mL of MeOH the wash fluid was pooled in a volumetric flask and the volume of 10 mL was

completed with MeOH. 250 µL of this solution were diluted with 750 µL MeOH and analyzed directly by HPLC (see *4.2*).

2.4.3 Determination of adsorbed amount of β_2 – agonists to the application device (= Dose $_{adsorbed}$)

Phenacetin stock solution (100 µL of 1 mg/mL) was given into the application device consisting of the glass spacer, the adapter for the nebulizer and connecting silicon tubes to the lung lobe (see *Figure 29*). After the solvent evaporated, the adsorbed β_2 – agonists were carefully washed out twice with 10 mL MeOH, whereas it was important that all inner surfaces of the application device came in contact with the washing fluid to ensure a complete recovery of the adsorbed β_2 – agonists. The wash fluids were combined and an aliquot was analyzed by HPLC (see *4.2*) immediately without further sample preparation.

Analytical methods for quantification of GW597901 and salbutamol in different specimen are described in *4.*

3 Characterization of pharmacodynamic properties of inhaled β_2 – agonists via human lung perfusion model

3.1 Reagents

3.1.1 Substances

Salmeterol base (ISD) was generously provided by GlaxoSmithKline R & D (Stevenage, England). Methacholine chloride (MCh) was purchased from FLUKA (Buchs, Switzerland). Other chemicals and sources of supply are mentioned previously (see *1.1.1*).

3.1.2 Solutions

3.1.2.1 Perfusion fluid

To evaluate the pharmacodynamic and pharmacokinetic qualities of GW597901, a mixture of erythrozyte concentrate and perfusion buffer (see *1.1.2.1*) was used as perfusion fluid in six lung lobe perfusion settings. Due to non-availability of buffy coat products, that had been used as blood cell fraction within the perfusion fluid in the pharmacokinetic enabling study (see *2.1.2.1*), erythrocyte concentrates were chosen as comparable alternative. Preliminary *in vitro* experiments confirmed the consistency of erythrocyte concentrate and buffy coat products concerning the redistribution behaviour of GW597901 into the perfusate and the change in perfusion fluid composition was accepted (see *4.3.1.3*). For each lung lobe perfusion experiment two packed red blood cell concentrates with compatible blood groups (see *1.2.1*) were obtained by a local blood donation service (Institut für Transfusionsmedizin und Hämotherapie, Würzburg, Germany). The blood material was pooled and the haematocrit was determined by centrifuging 10 mL samples in 15 mL tubes in duplicate (3000 rpm, 10 °C, 20 min) [281]. The 2 x 10 mL blood samples serving for the determination of the haemtocrit value were discarded afterwards and not returned into the final perfusion fluid. The pooled erythrocyte concentrate was then mixed with perfusion buffer resulting in a final haematocrit value of 20 %. The handling and mixture procedures of blood containing perfusion fluids were always carried out under aseptic conditions at a laminar airflow work bench. To assess the pharmacodynamic and pharmacokinetic properties of salbutamol, plain perfusion buffer was used in six lung perfusion experiments. The perfusion fluid was always freshly prepared one day prior to experiment and stored at 4 – 8 °C until use.

3.1.2.2 Methacholine solutions

To initiate a defined bronchial challenge (see *3.4.4*) prior to the application of the β_2 – agonist to the lung lobe, methacholine chloride (MCh) was chosen to provoke a bronchoconstriction. On the day before the experiment solid MCh (see *3.1.1*) was dissolved and diluted in Millipore® - water (see *1.1.1*) to achieve concentrations of 50, 25 and 10 mg/mL and 5 mL. Each concentration was either freshly prepared in the morning of the experiment or stored in 15 mL tubes at 4 – 8 °C over night. The concentration of MCh solution used for nebulization was decided individually depending on the total weight and constitution of the lung lobe. In general, the lowest concentration was applied first. If the used MCh solution did not achieve the desired extent of bronchoconstriction the next higher concentration was nebulized (see *3.4.4*).

3.1.2.3 Solutions of β_2 – agonists

In six perfusion experiments GW597901 solution was nebulized at a concentration of 180 µg/mL. Salbutamol solution at a concentration of 600 µg/mL was applied in six perfusion settings. Either GW597901 or salbutamol were nebulized in different perfusion experiments to achieve pharmacodynamic data of each β_2 – agonist separately. The β_2 – agonists were reconstituted in Milli - Q® - reagent grade water to yield clear solutions, aliquoted in volumes of 5 mL in 15 mL tubes and stored at -20 °C until usag e.

3.2 Materials and equipment

3.2.1 Human tissue and blood products

The origin of resected lung lobes was described in detail in *1.2.1*. Inclusion criteria were retained analogous to the previous lung lobe perfusion experiments. Permission of study protocol was obtained from Ethics Committee of the Medizinische Fakultät, Universität Würzburg, Germany. A total of twelve patients who had to undergo surgery due to bronchial cell carcinoma in the Klinik Schillerhöhe, Gerlingen and in the Thoraxzentrum Unterfranken, Münnerstadt, Germany, participated in the pharmacodynamic study.

Packed red blood cell concentrates were obtained by the Institute of transfusion medicine and haemotherapy, Würzburg, Germany. Blood products were only used as components on the perfusion fluid in the experiments (see *3.1.2.1*), if tested according to the guidelines of the Paul-Ehrlich institute [280] and informed consent of the blood donors were present.

3.2.2 Other equipment and materials

Glassware	Glass cases
Multiparameter monitor	Siemens SC 9000, software version VE3 (Siemens Medical Solutions USA Inc., Danvers MA, USA);
Nebulizer	PARI Boy® SX compressor, PARI LC® SPRINT nebulizer and PARI LC® Interruptor (PARI, Weilheim, Germany);
Notebook	Dell Latitude D430 (Dell, Frankfurt, Germany);
Peristaltic pumps and accessories	Ecoline VC-280 II; MCP – Standard pump drive system; MCP/BVP Pro-280 pump head (Ismatec, Wertheim-Mondfeld, Germany);
Sensor for invasive blood pressure monitoring	Pressure transducer with three-way valve, sterile (CODAN pvb Medical GmbH, Lensahn, Germany);
Software	VentView® PC - software (Draeger Medical, Lübeck, Germany)
Tubings	Tygon ST-tubing standard type C, ID 3.2 / WT 1.6 mm (Ismatec, Wertheim-Mondfeld, Germany)
Tubing connectors	Fitting PP for tube ID 6 mm, luer male/female; PP tubing connectors for tube ID 6-8 mm on both sides (neoLab, Heidelberg, Germany)

Further materials and equipment was described previously in *1.2.3* and *2.2.2.*

3.3 Modification of the *ex vivo* human lung perfusion model to monitor pharmacodynamic properties

As instrument for documentation of ventilation parameters during the experiment the VentView® software, provided by Draeger Medical, was used. By connecting an external notebook containing this software to the ventilation machine the experimental setting was modified (see *Figure 32).*

Differing components and materials of equipment used in the pharmacodynamic study of inhaled β_2 – agonists are mentioned in *3.2.2*. All other elements of the IPL setup remained according to the pharmacokinetic enabling study (see *Figure 28* in *1.2.2* and *1.3*).

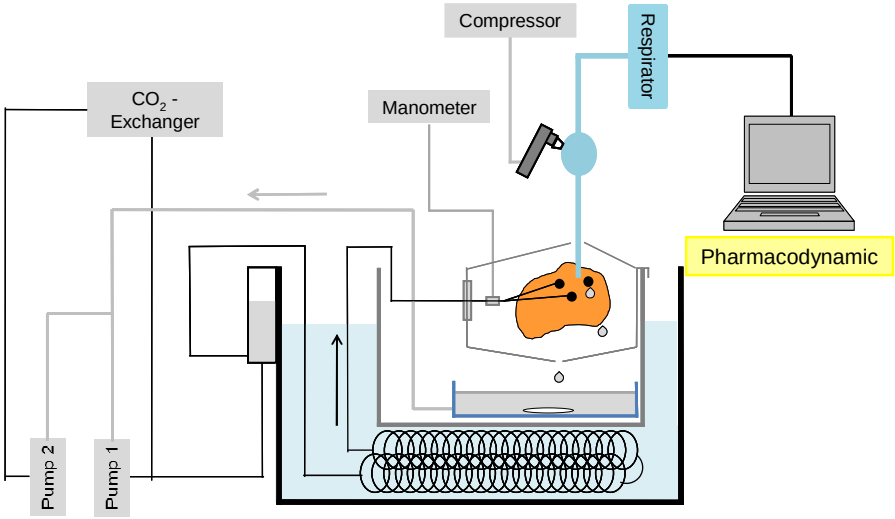

Figure 32: Online documentation of ventilation parameters during lung lobe perfusion. For detailed explanations for single elements in the setup see *1.2.2.*

3.3.1 Software to monitor ventilation data

Permanent online recording of ventilation parameters (see *Table 13)* during the experimental procedure was achieved by connecting a notepad (Dell, Frankfurt, Germany) directly to the COM – port of the respirator using a medibus cable and an USB adapter cable with serial 9 pin interface (HAMA, Monheim, Germany).

Table 13: Selection of ventilation parameters reported and documented by VentView® PC – software.

Parameter	Abbreviation	Unit
Breathing rate	f	1/min
Expiratory Tidal Volume	V_{Te}	mL
Flow	-	L/min
Maximum airway pressure	P_{peak}	mbar
Mean airway pressure	P_{mean}	mbar
Minute Volume	MV	L/min
Positive end – expiratory pressure	PEEP	mbar
Pulmonary compliance	C	mL/mbar
Resistance	R	mbar/L/s

For data communication identical data transfer formats were set in the VentView® programme and the notebook: 19200 baud transfer rate, 8 data bits, one stop bit, no parity.
Several displays of the VentView® PC software displayed either trend data like V_{Te}, MV, C, R etc. or curve data for a maximum of three ventilation parameters like flow, mean airway pressure and respiratory volume (see *Figure 33*). Additionally, there was the possibility to monitor all recorded data listed in a spreadsheet updated every ten seconds for trend data and 16 milliseconds for curve data respectively. The relation of two curve data parameters could be displayed online in a loop diagram. All ongoing ventilation values were saved automatically. Furthermore, by saving reference values of ventilation parameters during the experiment, an immediate comparison of any changes in the respiration procedure in the same setting was enabled [171].

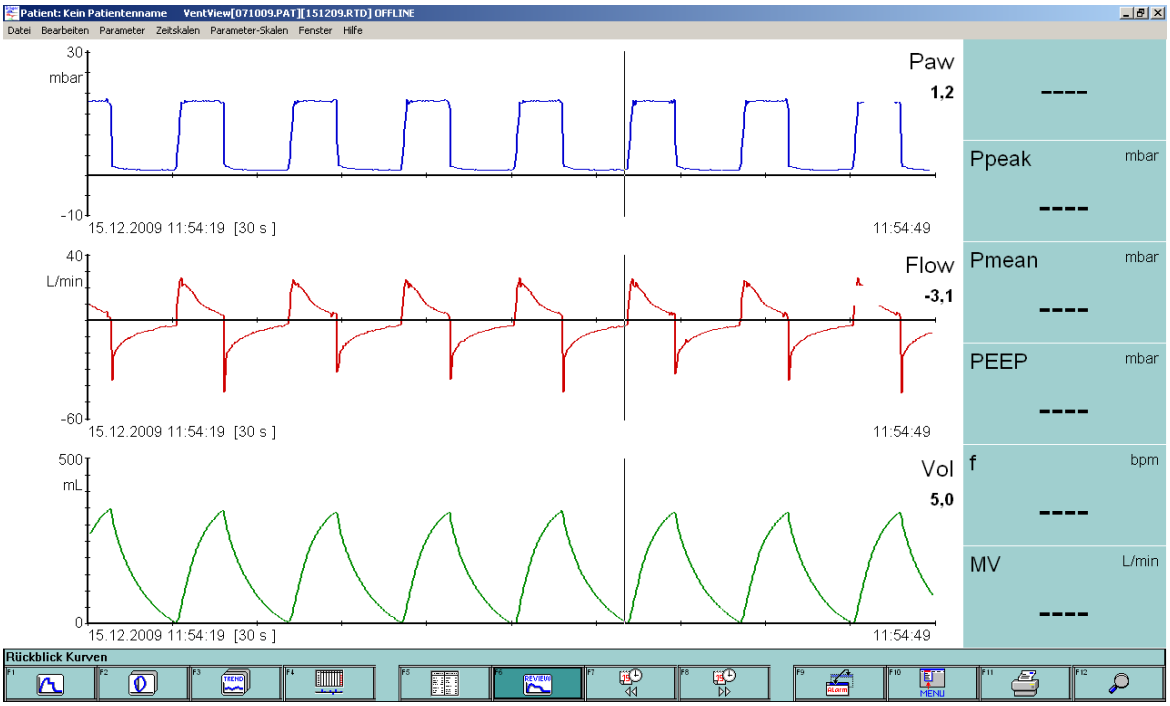

Figure 33: Display of ventilation parameters during a lung lobe perfusion experiment (VentView®).

The breathing rate was kept constant at 15 breaths per minute throughout the experiments, whereas the inspiration time was set at 1.7 s and the expiration phase lasted 2.5 s. Again the BIPAP mode was used for ventilation of the lung lobes, thus the respiratory pressure parameters P_{peak} and PEEP were set, resulting in certain breathing volume values depending on the type and size of the ventilated lung lobe.

3.3.2 Evaluation of the suitability of bronchial challenge and appropriate ventilation parameters

The first tests investigated, whether a bronchial challenge with nebulized MCh led to a measurable bronchoconstrictive effect in the isolated perfused lung. The effective dose of MCh and the duration of a successfully triggered bronchoconstriction were determined. Furthermore, significant ventilation parameters, recorded by VentView® software (see *3.3.1, Table 13*) reflecting pharmacodynamic actions within the airways, were evaluated. The experimental setting, prearrangements, the performance and termination of the lung lobe perfusions were done according to *1.3.1* and *1.3.3* but without application of β_2 - agonists and sampling of perfusion fluids, lung tissue and BAL samples. Summarised settings of these preliminary lung lobe perfusions are shown in *Table 14.* BIPAP mode was used for ventilation and plain perfusion buffer (see *1.1.2.1*) for perfusion of the lung lobes.

Table 14: Settings of ventilation and perfusion parameters in the preliminary lung lobe perfusion experiments (n= 4).

	Parameter	Mean	Range
Ventilation	P_{Peak}	19.8 mbar	15 – 24 mbar
	PEEP	1 mbar	-
	Respiratory rate	15/min	-
Perfusion	Fluid flow rate	95 mL/min	70 – 140 mL/min
	Fluid temperature	37 ℃	-
	Perfusion pressure	23 mmHg	14 – 40 mmHg

P_{peak} – maximal airway pressure
PEEP – **p**ositive **e**nd-**e**xpiratory **p**ressure

After prearrangements and establishment of the perfusion experiment, the lung lobe was carefully ventilated by a slow increase of P_{peak}. When the organ was optimally ventilated, reference values (≡ 100 % value) of ventilation parameters were recorded. The aim was to achieve a bronchoconstriction leading to a reduction (MV, V_{Te}, C etc.) and increase (R) of ventilation parameters by 40 % of reference values, respectively. In four perfusion experiments solutions with increasing concentrations of MCh (see *3.1.2.2*) were nebulized with a PARI LC® SPRINT nebulizer with blue nozzle insert, PARI LC® interruptor and a PARI Boy® SX

compressor (PARI, Weilheim, Germany). This application device had a total output rate of 590 mg/min and delivered aerolised particles with a MMD 2.9 µm and a mass percentage below 5 µm of 75 %. According to the technical data sheet, nebulization with the given device guaranteed an efficient deposition of particles in the central area of the lungs [176].

The dosing device was attached to the spacer (see *1.2.2, Figure 29*) and P_{peak} was enhanced by 5 mbar to improve the deposition of the agent within the airways. Detailed MCh dosing steps of preliminary experiments are described in *Table 15.* Always the least available concentration of MCh was applied first and increased if no bronchoconstriction occurred. Dosing of MCh was carried out in time intervals of 3 – 4 min, between those intervals the application device was removed from the spacer, P_{peak} reduced by 5 mbar and the further course of the ventilation parameters were observed. If the bronchial challenge was successful, depicted by significant deterioration of ventilation parameters (≤ 60 % of reference values), the MCh administration was terminated. In *Table 15,* given application times (t_1 – t_3) for each MCh concentration (c_1 – c_3) corresponded to all added time intervals of nebulization without dosing breaks.

The total administered dose of MCh in each experiment was approximated with

Formula 3:

$$\text{Total dose}_{MCh} = \sum_{i=1}^{n} [(t_i \times f) \times T_{insp}] \times 0.59\ \text{mL/min} \times c_i$$

n = concentration step
t_i = total time of c_i application intervals (min)
f = breathing rate (1/min)
T_{insp} = time of one inspiration cycle (min)
c_i = concentration of MCh solution administered in t_i (mg/mL)

Formula 1 was set up based on different parameters of the dosing procedure for the overall approximation of the actual MCh output during the bronchial challenge. The inspiration time of each respiration cycle T_{insp} was always set at 1.7 s and the breathing rate f was adjusted to 15 breaths per minute. The factor 0.59 mL/min referred to the output rate of the used nebulizer according to the data sheet of the device [176], whereas the density of the applied aqueous MCh solution was assumed to be 1 g/mL (0.59 g/min ≡ 0.59 mL/min).

Table 15: Concentrations and calculated cumulative doses of applied MCh in preliminary experiments and total time of each provocation step.

Preliminary experiment	MCh concentration step n						
	1		**2**		**3**		Total dose (mg)
	c_1 (mg/mL)	t_1 (min)	c_2 (mg/mL)	t_2 (min)	c_3 (mg/mL)	t_3 (min)	
1	0.1	4	0.5	10	1	30	8.9
2	5	10	-	-	-	-	12.5
3	5	18	-	-	-	-	22.6
4	10	13	25	5	-	-	63.9

Prepared MCh concentrations were chosen according to current guidelines and human studies concerning to provocative challenge testing in humans [151, 154, 162]. In the first experiment three different MCh concentrations were applied to the lung lobe starting with a concentration of 0.1 mg/mL for 4 min. Due to no detectable bronchoconstriction, the concentration of applied MCh solution was raised to 0.5 and 1 mg/mL, but no effect of the bronchial challenge was seen and the perfusion experiment was terminated after 102 min. Thus, in further experiments higher concentrations of MCh solutions (5 – 25 mg/mL) were applied to the lung lobes leading to narrowing of the airways in the experiments 2 – 4. The achieved bronchoconstriction in the fourth preliminary lung lobe perfusion lasted for 45 min without additional dosing of MCh. No improvement of ventilation parameters, indicating a sustained action of MCh, was observed in this time interval. The modified setting was therefore suitable for further pharmacodynamic studies of GW597907 and salbutamol.

3.4 Pharmacodynamic and pharmacokinetic (PK/PD) studies of GW597901 and salbutamol using the isolated human perfused lung

3.4.1 Prearrangement of the experiment

Pharmacodynamic and – kinetic experiments were performed next to the surgery room in the Klinik Schillerhöhe, Gerlingen and in the Thoraxzentrum Unterfranken, Münnerstadt, Germany. In total, GW597901 and salbutamol were applied to six lung lobes, respectively. The

experimental setup (see *Figure 28* in *1.2.2* and *Figure 32* in *3.3*), the preparation steps, perfusion and ventilation settings of the lung lobe *ex vivo* are described elsewhere (see *1.3.1*). Contrary to the pharmacokinetic enabling study, 20 mL blank sample were drawn after establishment of perfusion and ventilation for at least 5 min. Blank perfusion fluids later served as matrix for calibration standards (see *4.3.2.1*) in quantitative analysis of β_2 – agonists.

3.4.2 Ventilation and perfusion settings

Again BIPAP mode was chosen for ventilation procedures, a given constant inspiration pressure lead to variable volume values. Summarised settings of ventilation and perfusion parameters in the PK/PD study are shown in *Table 16.* At the begin of the experiment P_{Peak} values were set at 18 or 20 mbar depending on whether the pressure was appropriate for an optimal ventilation of the lung lobe. In average these pressure settings yielded in a mean MV of 4.4 L/min (1.8 – 7.7 L/min). Individual MV values at this time served as 100 % reference values for further experimental proceedings and later pharmacodynamic analysis (see *3.4.4* and *3.5*). The extent of MV was mainly determined by the maximum inspiration pressure and the type of the ventilated lung lobe/s (e.g. upper/lower lung lobe, total lung) and the measured values were therefore varying in a broad range.

Table 16: Settings of ventilation and perfusion parameters in the PK/PD study (n= 12).

	Parameter	Mean	Range
Ventilation	P_{Peak}	19 mbar	16 – 20 mbar
	PEEP	1.1 mbar	1 – 2 mbar
	Respiratory rate	15/min	-
Perfusion	Fluid flow rate	83 mL/min	60 – 130 mL/min
	Fluid temperature	37 ℃	-
	Perfusion pressure	28 mmHg	20 – 48 mmHg

P_{Peak} – maximal airway pressure
PEEP – **p**ositive **e**nd-**e**xpiratory **p**ressure

The mean perfusion fluid flow rate of 82 mL/min resulted in an acceptable mean perfusion pressure of 29 mmHg and was comparable to perfusion settings in the pharmacokinetic enabling study (see *2.3.2*). In each experiment, the perfusion flow rate was adjusted according to the tolerable range of 20 – 40 mmHg.

3.4.3 Recording of ventilation procedure

For collection of ventilation data, the respirator was connected to a notebook via medibus cable. Concomitant to initiation of ventilation and perfusion, VentView® PC – software (see *3.3.1*) was activated to document all ventilation data over the whole duration of experiment. Files were started for recording both trend and curve data, respectively. Thus, all ongoing ventilation values were saved automatically.

3.4.4 Provocation of bronchoconstriction and application of β_2 – agonists

To characterize the pharmacodynamic properties like the extent and onset of bronchodilation produced by β_2 – agonists, a defined initial condition of pulmonary airways was to be achieved by applying a bronchial challenge to the lung lobes prior to administering GW597901 or salbutamol. The target was to decrease eligible ventilation parameter values to 60 % of reference values. Preliminary experiments have shown that MCh concentrations of 5 mg/mL to 25 mg/mL respectively were sufficient to achieve an acceptable extent of bronchoconstriction (see *3.3.2*).

After equilibrating of the lung lobe ventilation (about 10 – 15 min), stable ventilation parameters for at least consecutive 5 min were annotated as reference values and the target values for the targeted bronchoconstriction were calculated. Then application of MCh solution for three minutes was started using a PARI nebulizer with blue nozzle insert that produced aerosol droplets with a median mass diameter (MMD) of 2.9 µm, provided 75 % by weight below 5 µm of aerosol particle size and had a total output rate of 590 mg/min of aerosol (see *3.3.2*). During bronchial provocation, adequate air conditioning of the laboratory was ensured to minimize hazard to the researchers by inhaled MCh. After stopping administration, the ventilation parameters were monitored for several minutes if any sign of bronchoconstriction occurred. If this effect itself or the targeted extent of bronchoconstriction was not shown, the bronchial challenge was continued with the same concentration one more time. A further ineffective provocation led to nebulization of the next higher MCh concentrated solution and so on (see *3.1.2.2*). At maximum, six intervals of bronchial provocation procedures were performed in one lung lobe perfusion experiment. Subsequent application of the β_2 – agonist started

immediately, if a sufficient bronchoconstriction was achieved or the bronchial challenge was without success after six intervals of MCh administration. Used MCh concentrations for bronchial challenges ranged from 10 – 50 mg/mL in 12 experiments resulting in a mean calculated total dose of 90 mg (range 2.5 – 168 mg, see *Formula 1*).

The aim was the administration of 150 µg GW597901 or 600 µg salbutamol (see *3.1.2.3*) in six lung lobe perfusion experiments, respectively. The drug solutions were applied to the lung lobes with a PARI LC® SPRINT nebulizer with red nozzle insert, PARI LC® interruptor and a PARI Boy® SX compressor (PARI, Weilheim, Germany). The given device produced aerosol particles with a MMAD of 2.2 µm and had a total output rate of 450 mg/min. The PARI Boy® N compressor in combination with the PARI LL® nebulizer (red nozzle insert) provided 89 % by weight below 5 µm of aerosol particle size [176].

Thereby, the solution was administered by activating the interruptor button during the inspiration phase (1.7 s) and releasing it throughout the expiration cycle. To ensure an optimal deposition of the aerosol in the peripheral parts of the airways P_{peak} was increased by 5 mbar during application of β_2 – agonists. A change in nebulizing tone during dosing indicated that the targeted dose of the β_2 – agonists had been nebulized and the dosing procedure was stopped. The administration of the β_2 – agonist took about 12 – 13 minutes. After termination of the dosing procedure P_{Peak} was returned to the initial value. The nebulizer with remaining drug solution was closed and carried carefully to avoid loss of solution. The spacer with all adapters used in an experiment and the nebulizer were stored at 4 – 8 °C in the dark until the next day for further analysis of the effectively administered dose (see *3.4.6*). The lung lobe perfusion experiments were stopped when sufficient samples over 60 min after termination of dosing were achieved, obvious massive edema formation occurred and/or the ventilation parameters deteriorated. The termination of the lung lobe perfusion experiments was always according to *1.3.3*.

3.4.5 Sampling

Sampling started during the 12-13 min dosing procedure of GW597901 and salbutamol respectively. Volumes of 6 mL perfusion fluid were drawn from the opened pulmonary vein at 2, 5 and 10 min after start of dosing with a disposable PE pipette. Each sampling step was done with an unused pipette. At the termination of administration (= 0 min) and at times 10, 20, 30, 45 and 60 min samples were collected from the vein and the reservoir. The perfusion fluid in the reservoir was permanently stirred to ensure a homogeneous mixture of the compound within the perfusion fluid. Exactly 2 mL of each sample were transferred into a 15 mL tube (120 x 17 mm, conical bottom, PP, Sarstedt, Nümbrecht, Germany) with an Eppendorf pipette (Research®

variable, 100 – 1000 µL, Hamburg, Germany) for later quantitative analysis of the β_2 – agonist. The remaining fluid samples were aliquoted in volumes of 1 mL in screw cap micro tubes (10.8 x 48 mm, 2 mL, transparent PP, Sarstedt, Nümbrecht, Germany). Samples were put on ice immediately. Withdrawn volumes were subsequently replaced with fresh perfusion fluid (RT). When blood cell containing perfusion fluid was used (perfusions with GW597901 application, see *3.1.2.1*) two aliquots (1 mL) were centrifuged to obtain the cell – free fraction (3500 rpm, 10 °C, 10 min). All perfusion fluid samples were st ored at -25 °C until further analysis.

Collection and storage of perfusion fluid samples for the evaluation of edema formation proceeded according to *2.3.3.2*. Lung tissue samples were obtained after the termination of the experiment. Three separate tissue samples were cut from the peripheral and central tumour free areas of the lung lobes respectively to avoid compromising later pathological examinations of the lung lobe. For the BAL inserted volumes of lavage fluids ranged from 10 – 20 mL and the mean recovery rate of regained lavage fluid was 43 % (15 – 86 %). For further details of tissue and BAL sampling and storage see *2.3.3.3*. Detailed methods for quantitative analysis of β_2 – agonists in obtained specimen are described in *4.3*.

3.4.6 Effectively administered dose of β_2 – agonists

For the quantification of the effective deposited doses of GW597901 and salbutamol, samples were prepared and analyzed according to *2.4* and *4.2*. Instead of phenacetin, equivalent concentrations and volumes of salmeterol solution in MeOH were used as internal standard for each sample and calibration curves (see *4.1.2*).

3.5 Analysis of ventilation data

As described in *3.4.3* special software was used for documentation of ventilation parameters over the duration of the PK/PD experiments. After termination of perfusion and ventilation of the lung lobes all recorded data were saved in separate data files. For data processing, recordings of ventilation parameters were exported from the VentView® PC – software into a csv (**c**omma **s**eparated **v**alues) format file. Before further analysis, data were imported into a Microsoft Excel® data sheet. Tab stop, semicolon and space were specified as tags and inverted commas marked text characters. Then, related data pairs (e.g. time – MV) were displayed in a table and graphically demonstrated in a diagram chart, *see Figure 12* in *2.3.3,* B - Results and Discussion*.* Peaks within the original diagram (application of MCh or the β_2 – agonist) marked the impact of the nebulizer. By attaching the dosing device to the air conducting zone, the system was

expanded by a certain volume and thus, measurements e.g. MV increased at these times indicating the dosing procedures. Hence, the influence of the nebulizer superimposed though without impairing the actual signal of the ventilation parameter. But to detect the onset of action of the pulmonary deposited β_2 – agonist, occurring during the application phase of 12 min, an adjacent curve without the effect of the nebulizer was essential for comparison and further analysis.

To smooth data curves and remove outlier peaks, occurring during dosing procedures, a validated algorithm of Matlab software (The MathWorks, Inc., Natick, United States) was used to edit available data sets of the lung lobe perfusion experiments. Spike removal was done similar to Phillips GR and Eilers P with modifications to detect outliers that consisted of more than one data point [177, 178].

First data pairs were entered as vectors with n elements into the Matlab program and then the resulting curve was smoothed. Next, irregularities within the data set were identified with a quadratic Savitzky – Golay filter, standardised residuals between the original and the smoothed values were formed and the standard deviations calculated. All observations greater than a given threshold (= 3.5 by default) were considered to be outliers deriving from an overlying signal (= influence of the nebulizer). The outliers were then adjusted by a certain degree of smoothing. A width of 5 data points was found to work satisfactorily as a given limit of smoothing.

To analyze and quantify the extent of bronchoconstriction and bronchodilation, consecutive values of ventilation parameters recorded every ten seconds were averaged in the time section of the reference value, after bronchoconstriction and after bronchodilation. Means and standard deviations were calculated from data of at least two minutes intervals (at least n= 12). Averaged values of the reference interval were equated with 100 % and means of sections after pharmacodynamic interventions were expressed as percentage of the reference value. If the bronchial provocation was successful, the difference between the value after bronchoconstriction and after bronchodilation was calculated, demonstrating the extent of the pharmacodynamic effect of the applied $ß_2$ - agonist. If the provocation with MCh failed and no bronchoconstriction was observed, the difference between the value after bronchodilation and 100 % reference value was built.

4 Analytical methods

4.1 Reagents and equipment

4.1.1 Substances

Acetonitrile (MeCN, HPLC – gradient – grade) and diethylether were purchased from VWR Prolabo® (Darmstadt, Germany). Methanol (MeOH) in HPLC – gradient grade quality was taken from an in – house filling system. Salmeterol xinafoate was generously provided by GlaxoSmithKline. Salbutamol-D3 was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Nitrogen in compression gas cylinders (quality 5.0) was purchased from Linde (Munich, Germany). Formic acid and other chemicals in p.a. quality were obtained from E. Merck (Darmstadt, Germany). Water was obtained by an in-house filter system (Milli - Q® - reagent grade water system) from Millipore (Schwalbach, Germany).

4.1.2 Standard solutions for HPLC analysis

- *Working solutions*

1 mg/mL stock solution of each active agent (concentrations are always related to base) in MeOH HPLC grade was used to create working solutions (see *Table 17*) in following concentrations:

Table 17: Concentrations of analytes and ISD in working solutions.

Working solution	c (µg/mL)			
	GW597901 base	Salbutamol base	**ISD** Phenacetin[#]	**ISD** Salmeterol base[†]
V1	50	50	50	50
V2	5	5	5	5

[#] ISD in the pharmacokinetic enabling study
[†] ISD in the PK/PD study

Stock solutions and dilutions were stored protected from light at -20 °C in brown glass vials.

- *Standards for calibration procedure*

Standards for analysis of applied dose were directly produced in HPLC vials by diluting working solutions described above with MeOH HPLC grade (see *Table 18*). Every standard solution consisted of 50 µL V1 phenacetin or salmeterol base (ISD) and the following volumes of working solutions. Then the total volume of 500 µL of each standard was obtained by addition of MeOH, HPLC grade, thoroughly vortex mixed and subsequently analyzed (see *4.2*).

Table 18: Standard concentrations used for HPLC calibration procedure for quantification of applied dose.

Standard	Salbutamol (base)		GW597901 (base)	
	c (µg/mL)	V (µL)	c (µg/mL)	V (µL)
S1	20	200 V1	6	60 V1
S2	15	150 V1	4	400 V2
S3	10	100 V1	2	200 V2
S4	5	50 V1	1	100 V2
S5	2	200 V2	0.2	20 V2
S6	0.5	50 V2	0.1	10 V2

4.1.3 Standard solutions for LC-MS/MS analysis

- *Working solutions*

GW597901 and salbutamol were weighed out and dissolved in MeOH HPLC to create stock solutions with a concentration of 1 mg/mL (concentrations are always related to base) and stored at -25 °C protected from light. Dilutions were produced with Millipore water leading to concentrations of 500 (V1*), 50 (V2*) and 5 ng/mL (V3*). Dilutions were always freshly prepared. The stock solution of salbutamol - D3 (100 µg/mL in acetone, ISD) was diluted in MeOH gradient grade to prepare a working solution with a concentration of 0.1 µg/mL (V1).

- *Matrices for preparation of standard solutions*

Blank fluid samples (see *3.4.1*) drawn at the beginning of each lung lobe perfusion experiment (20 mL) were used as matrices to prepare standards for further calibration procedures. Thus, perfusion buffer was used for the analysis of salbutamol perfusion samples and a mixture of erythrocyte concentrates and perfusion buffer (HCT 20 %) for the quantification of GW597901 in perfusion fluid samples. Until further handling, blank samples were stored at -25 °C analogous to other samples.

- *Standards for calibration procedure*

Standard solutions for LC-MS/MS analysis were always processed as perfusion fluid samples. Certain volumes of the matrix (RT) were taken into 15 mL tubes (see *4.1.5*) and filled up to total volumes of 2 mL with working solutions of GW597901 and salbutamol, respectively (see *Table 19).* 100 µL of ISD was added and the achieved solution mixed on a vortex. 4 mL MeCN was given to the solution and again mixed for 30 s to precipitate proteins. After centrifugation (4000 rpm, 20 min, 10 °C) the supernatants were transferred in 5 mL pear shaped flasks and dried down under a slight stream of nitrogen. The residuals were dissoluted in 50 µL of 0.1 % formic acid. Before analysis with LC-MS/MS (see *4.3.2.2*), standards were centrifuged again to remove undissolved particles.

Table 19: Standard concentrations used for LC - MS/MS calibration procedure.

Standard	Salbutamol (base)			GW597901 (base)		
	c (ng/mL)	V (µL)	Matrix (µL)	c (ng/mL)	V (µL)	Matrix (µL)
S1	25	100 V1*	1900	12.5	50 V1*	1950
S2	15	60 V1*	1940	7.5	30 V1*	1970
S3	7.5	30 V1*	1970	2.5	100 V2*	1900
S4	3.75	150 V2*	1850	1.25	50 V2*	1950
S5	1.25	50 V2*	1950	0.25	100 V3*	1900
S6	0.125	50 V3*	1950	0.125	50 V3*	1950

4.1.4 Analytical equipment

Analytical HPLC column, accessories	Zorbax® SB – C8, 4.6 x 450 mm, 5 µm particle size (Agilent, Santa Clara, USA); XTerra MS – C18, 3.0 x 150 mm, 3.5 µm particle size (Waters, Eschborn, Germany); Security cartridge RP1, 4 mm L x 3 mm ID, with cartridge holder (Phenomenex, Aschaffenburg, Germany);
Autosampler	Model 717plus (Waters); Aquity UPLC sample organizer (Waters);
Control system / data processing	Software Breeze® Version 3.30 (Waters) MassLynx® (Waters);

Degaser	Model 1525 *In – line* vacuum degaser (Waters);
Detector	Dualband – UV – Detector model 8487 (Waters);
	Flourescence – Detector RF – 10AXL (Shimadzu, Duisburg, Germany);
	Quattro - Premier™ XE (Waters);
Pump	Model 1525 for binary gradients with analytical pump head (Waters);
	Aquity UPLC with binary solvent manager

4.1.5 Other equipment

Balance, precision	Mettler P1200, 0.1 – 1200 g, 10 mg minimal weight difference (Mettler – Toledo, Gießen, Germany);
Centrifuge	Hereus® Megafuge 1.0R (Kendro Laboratory Products, Langenselbold, Germany);
	Microfuge® 22R Centrifuge (BECKMAN COULTER$_{TM}$, Krefeld, Germany);
Flasks	Pear – shaped, 5 mL, ST 14/23, acc. to DIN 12383. PRECISO;
	Volumetric, 5 mL Boro 3.3; ST 7/16; 10 mL, ST 10/19, Duran® 50 (WITEG Labortechnik GmbH, Wertheim, Germany);
Pipette 10 – 100 µL	Eppendorf Research® variable, 10 – 100 µL (Eppendorf, Hamburg, Germany);
Pipette 100 – 1000 µL	Eppendorf Research® variable, 100 – 1000 µL (Eppendorf, Hamburg, Germany);
Transferpipette	20 – 200 µL, 500 – 5000 µL, Transferpette® electronic (BRAND, Wertheim, Germany);
Tube 15 mL	Polypropylene, 15 mL, 120 x 17 mm (Sarstedt, Nürmbrecht, Germany);
Vials	Transparent glass vials with PP snap cap, 500 µL;
	Brown glass vials with 300 µL glass insert, 9 mm screw cap with silicone/PTFE seal (Chromacol, YMC Europe GmbH, Dinslaken, Germany)
	Brown glass vials, 4 mL, screw cap with silicone/PTFE seal (VWR International GmbH, Darmstadt, Germany)
Vortex	Model K–550–GE (Bender & Hobein, Zurich, Switzerland)

4.2 HPLC analysis

Samples for the determination of effectively administered dose of β_2 – agonists (see *2.4 and 3.4.6*) and standards (see *4.1.2*) were analyzed immediately. The chromatographic separation of the compounds was carried out on a Zorbax SB – C8 column with a flow rate of 1 mL/min and a gradient elution (see *Table 20*). As mobile phase a mixture of 1 % acetic acid in Millipore® water (*Component A*) and MeCN (*Component B*) was used. The injection volume was 20 µL. The UV – absorption at 280 nm and the fluorescence signal (excitation at 230 nm, emission at 306 nm) were measured in parallel.

Table 20: Time course of HPLC gradient elution for determination of effective applied dose of β_2 – agonists.

Time after injection of sample (min)	*Component A (%)*	*Component B (%)*	*Event*
0	*95*	*5*	*Linear gradient elution*
28	*53*	*47*	
29	*0*	*100*	*Condition of analytical column*
40	*0*	*100*	
41	*95*	*5*	*Equilibration of analytical column*
50	*95*	*5*	

4.3 LC-MS/MS analysis

4.3.1 Method development

4.3.1.1 Adsorption effects to two different storage devices

To compare adsorption effects of GW597901 and salbutamol to different materials as storage devices, 3 x 2 mL samples at five different concentration levels for each agent (see *Table 18*, *4.1.2*) were incubated at 37 °C for 1 h in either glass o r PE centrifuge tubes in a heating cabinet (Memmert, Schwabach, Germany). Phenacetin was used as internal standard and added after incubation leading to a final concentration of 5 µg/mL in each sample. A mixture of buffy coat/perfusion buffer *1.1.2.1* (HCT 20%). served as matrix (HCT 20%). 100 µL of each sample were withdrawn, mixed with 400 µL of MeOH HPLC grade for at least 30 s on a vortex and centrifuged for 20 min at 13000 rpm (RT) Obtained supernatants were directly analyzed with

HPLC (see *4.2*). Additionally, standards prepared in MeOH were analyzed in the same batch to calculate the percentaged relative recovery.

4.3.1.2 Sample purification

To optimise purification of perfusion fluid samples prior to analysis, several preliminary experimental approaches were undertaken. Liquid-liquid extraction procedures with different extracting agents were performed. First, extractions were done with samples of different matrices (water, perfusion buffer, perfusion buffer /plasma, perfusion buffer /erythrocyte concentrates), containing GW597901, salbutamol and ISD (phenacetin or salmeterol) in concentrations measurable by fluorescence detection. As extracting agents diethylether, ethylacetate and chloroform were tested. Samples, neutral or alkalised with 0.1 N NaOH, were extracted twice with 2 mL of organic solvent. Extraction was carried out by rotating sample/organic phase containing tubes on a mixing wheel for 20 min. A short centrifugation step (1400 rpm, 5 min) was performed for sufficient separation of the liquid phases. Afterwards, the organic phases were pooled and evaporated under gaseous nitrogen. The residues were dissolved in 50 µL MeOH HPLC gradient grade and analyzed (see *4.2).* Due to insufficient purification of the samples, a protein precipitation step was done prior to the liquid-liquid extraction. To remove proteins, samples were mixed with a threefold volume of MeCN, centrifuged and the supernatants were processed as mentioned above. Thereby the purification of the samples was enhanced. Regarding the recovery rates of the β_2 – agonists, the best results were seen for samples just pretreated with protein precipitation without consecutive liquid – liquid extraction**.**

For further improvement of the method sensitivity, 2 mL samples instead of 500 µL were purified by protein precipitation with 4 mL of MeCN HPLC grade (30 s vortex). Prior to protein precipitation, perfusion buffer (= matrix) was spiked with methanolic working solutions of β_2 – agonists (see *4.1.2*) yielding 2 mL samples with known concentrations. After centrifugation (4000 rpm, 10 °C, 20 min) of precipitated proteins, supernatants were transferred in 5 mL pear shaped flasks and dried under a stream of nitrogen at 35 °C. The residues were reconstituted in 50 µL MeOH HPLC grade (= forty– fold concentration of analytes, see *Table 21*), insoluble particles were separated by centrifugation for 5 min (RT) and the remaining clear samples were injected onto the HPLC system. Samples were analyzed promptly as described in *4.2.* Standards with matching concentrations of GW597901 and salbutamol in MeOH were analyzed as well, to determine the relative recovery (RR %) of the method.

Table 21: Samples with following concentrations of analytes in perfusion buffer were tested

Standard	GW957901		Salbutamol		Salmeterol (ISD)	
	c_p (µg/mL)	c_A (µg/mL)	c_p (µg/mL)	c_A (µg/mL)	c_p (µg/mL)	c_A (µg/mL)
S1	0.15	6.0	0.5	20.0	0.125	5.0
S2	0.10	4.0	0.375	15.0	0.125	5.0
S3	0.05	2.0	0.25	10.0	0.125	5.0
S4	0.025	1.0	0.125	5.0	0.125	5.0
S5	0.005	0.2	0.05	2.0	0.125	5.0
S6	0.0025	0.1	0.0125	0.5	0.125	5.0

c_P – concentration in 2 mL sample prior to protein precipitation

c_A – concentration in 50 µL sample after pre-treatment, prior to analysis

4.3.1.3 Comparison of different matrices as potential perfusion fluids

To evaluate the distribution of β_2 – agonists in different matrices, quantitative analysis were performed in the following media, either as whole matrix sample (blood cells + plasma/perfusion buffer) or matrix plasma sample (supernatant obtained after centrifugation). Each experiment was performed in triplicate.

Matrices to compare:

- Whole blood + perfusion buffer *1.1.2.1*
- Whole blood cells + perfusion buffer
- Fresh erythrocyte concentrate + perfusion buffer
- Expired erythrocyte concentrate + perfusion buffer
- Buffy Coat + perfusion buffer

Target haematocrit was always set at 20 %, determined by centrifugation (3000 rpm, 10 °C, 30 min) for each matrix. Prior to extraction, each 2 mL sample contained concentrations of 0.05 µg/mL GW597901 and salbutamol and salmeterol (ISD). After incubation (37 °C, 1 h), whole matrix samples were frozen and thawed twice for cell lysis and matrix plasma samples were centrifuged (3500 rpm, 10 °C, 10 min) to obtain supernatants for further analysis. 4 mL of MeCN HPLC grade were added to whole medium samples and matrix plasma samples for protein precipitation, vortex mixed for 30 s and centrifuged (4000 rpm, 10 °C, 20 min). Supernatants were dried under a stream of nitrogen at 35 °C and reconstituted in 50 µL MeOH

HPLC grade and analyzed as described in *4.2.* Blank samples of each matrix were identically pretreated and analyzed.

4.3.2 Method

4.3.2.1 Standard and sample preparation

Certain volumes of matrices were taken into 15 mL tubes and spiked with working solutions of GW597901 and salbutamol as described in Table 19 (see *4.1.3*). Matrices for standard preparation were obtained in the perfusion experiments of the PK/PD study (see *3.4.1*). 100 µL of 0.1 µg/mL salbutamol-D3 solution (ISD) were given to the 2 mL spiked samples. After mixing, protein precipitation was performed by adding 4 mL MeCN HPLC grade to each sample (30 s vortex). Samples were centrifuged (4000 rpm, 10 °C, 20 min), the supernatants were transferred into 5 mL pear – shaped flasks and dried down under gaseous nitrogen at 35 °C. Residues were dissolved in 50 µL 0.1 % formic acid. Before LC-MS/MS analysis particulate materials were removed by centrifugation.

Perfusion fluid and blank samples obtained in the PK/PD study (see *3.4.5*) with unknown concentrations of GW597901 and salbutamol were treated equally. Standards were always produced simultaneously to each sample batch. Finally, 50 µL samples were given into brown glass vials with 300 µL insert (see *4.1.5*) and stored at -25 °C until analysis via LC-MS/MS method.

4.3.2.2 LC-MS/MS method

Standards and samples of the PK/PD study were chromatographically analyzed via gradient elution (see *Table 22*). Volumes of 20 µL, tempered on 4 °C in the autosampler, were injected and the separation was carried out on a XTerra MS C18 column with a flow rate of 0.4 mL/min. As mobile phase a mixture of 0.1 % formic acid in Millipore® water (*Component A*) and MeCN HPLC grade (*Component B*) was used.

Table 22: Time course of LC-MS/MS gradient elution.

Time after injection of sample (min)	*Component A (%)*	*Component B (%)*	*Event*
0	*90*	*10*	*Linear gradient elution*
10	*55*	*45*	
10.01	*0*	*100*	*Condition of analytical column*
15	*0*	*100*	
15.01	*90*	*10*	*Equilibration of analytical column*
25	*90*	*10*	

For detection of compounds, the samples were evaporated and ionised in ES+ ion mode with a capillary voltage of 2.50 kV and a gas cell pirani pressure of 3.41 e-3 mbar. The source temperature was set at 120 ℃, the desolvation temp erature at 350 ℃ and the cone gas flow at 48 L/h. MRM mode with the following settings was used as type of detection (see *Table 23*).

Table 23: Settings of MRM mode.

Channel	*Compund*	*Precursor (m/z)*	*Fragment (m/z)*	*Cone (V)*	*Coll (eV)*	*Delay (s)*
1	GW597901	495	232	23.0	22.0	0.02
2			459*	23.0	22.0	0.10
3	Salbutamol	240	148*	14.0	14.0	0.02
4			166	14.0	14.0	0.02
5	Salbutamol – D3 (ISD)	243	151*	14.0	14.0	0.02
6			169	14.0	14.0	0.02

* - Quantifier

4.4 Analysis

4.4.1 Quantification of effectively applied dose

Quantification of applied dose of β_2 – agonists (see *2.4* and *3.4.6*) was determined via flourescence detection using Breeze® software (Waters). Actual concentrations of β_2 – agonists in application solutions, total amounts remaining in the PARI nebulizer after administration and the adsorbed fractions at the spacer device were determined against a calibration curve generated from area ratio of analyte to ISD.

4.4.2 Pharmacokinetic enabling study of β_2 – agonists

Samples of the isolated human lung enabling study (see *2.3.3*) were sent to GlaxoSmithKline (Medical Research Centre, Stevenage, UK) for analysis. Acquired data were analyzed with Excel software. All data were pooled for targeted dosage combinations of GW597901/salbutamol both 45/150 µg and 180/600 µg, respectively, and expressed as time course of release into perfusion fluid as percentage of applied dose (%) over time of perfusion experiment.

4.4.3 PK/PD study

For quantitative analysis, transitions of β_2 – agonists were extracted from the total ion chromatogram and the peaks of the quantifiers (see *Table 23, 4.3.2.2*) were integrated. Area ratios of standard substances to ISD were calculated and calibration curves were built by linear regression. For the determination of unknown concentrations of β_2 – agonists in perfusion fluid samples, area ratios of the chromatograms were referred to calibration lines. Results were then demonstrated as percentage of applied dose over time

5 Validation of analytical methods

Validation of analytical methods used for pharmacokinetic evaluation of β_2 – agonists in biological matrices was done according to the FDA Guidance for Industry, Bioanalytical Method Validation [284]. Type and volumes of matrices used for validation procedures corresponded to standards and quality control (QC) samples produced for the PK/PD analysis mentioned previously, if not otherwise stated. Samples were stored at least overnight at -20 ℃ until further treatment or analysis. Processing of samples for validation procedure was carried out according to standard samples (see *4.3.2*). Received data were analyzed with Microsoft® Excel 2002 and GraphPad™ Prism Version 4.00 for Windows, San Diego, USA.

5.1 Selectivity

In each sample batch, blank samples of used matrices were analyzed to exclude interferences of the results with already present substances and to assure selectivity (e.g. see *Figure 34*). In the course of the validation, three aliquots of independant matrices were analyzed. Instead of the internal standard, 100 µL of MeOH HPLC grade were given to the blank samples.

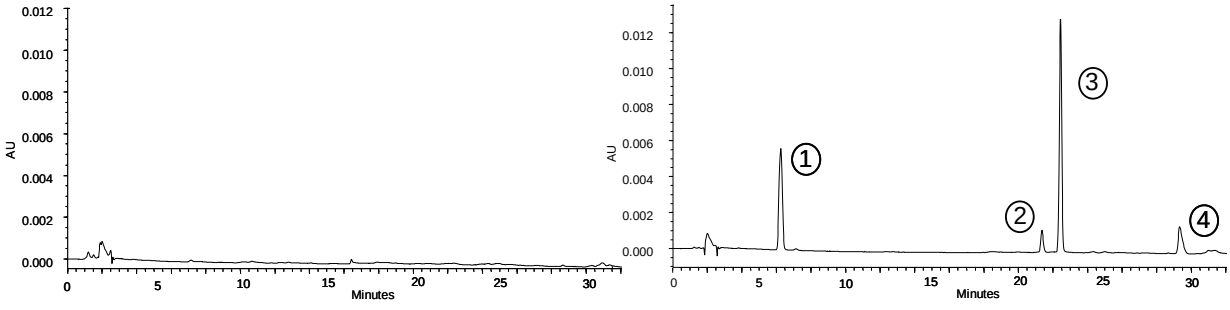

Figure 34: The left chromatogram displays an analysis of blank perfusion buffer via HPLC – UV and the right chromatogram demonstrates a pretreated sample containing 1. salbutamol, 2. GW597901 (3. cinnamic acid) and 4. salmeterol (ISD).

Using a LC-MS/MS method with MRM detection for quantification, selectivity of interesting compounds was generally high. In each sample batch, all blank matrices used for standard and sample preparation were analyzed in parallel. Blank matrices did not produce any significant interferences at the retention times of the analytes [285].

5.2 Linearity

The calibration function was calculated with *Formula 4* assuming a linear relation between the concentrations of the analyte in the sample to the area ratio of analyte to internal standard.

Formula 4: $y = b + a \bullet x$

a – Slope of calibration line
b – Intercept of calibration line
x – Concentration of analyte
y – Area ratio

For the proof of linearity six independent standard samples of six different concentration levels (see *Table 19* in *4.1.3*) were analyzed, data sets obtained and subjected to a linear regression analysis for determination of the slope *a* and the intercept *b* (see *Table 24*). The method of least squares was used without forcing the calibration line going through y= 0, when x= 0. The concentration of the standard was seen as independent and the area ratio as a dependant variable.

Furthermore, a non – linear regression on the basis of polynomial second order function was conducted to confirm linearity of data sets. If the 95 % confidence interval of the calculated coefficient of the quadratic term includes the zero value, the relation is linear [286].

Table 24: Results of regression analysis; data are best fit values with [95 % CI], n= 36

Substance	*Matrix*	*Slope a*	*Intercept b*	*Coefficient of quadratic term*
GW597901	EC/PB	0.1652 [0.1596-0.1709]	0.03038 [-0.004032-0.06478]	-0.0006325 [-0.002524-0.001259]
Salbutamol	PB	0.2623 [0.2560-0.2687]	0.01931 [-0.05903-0.09765]	5.1720×10^{-6} [-0.0009434-0.0009537]

CI – Confidence interval
EC/PB – Mixture of erythrocyte concentrate and perfusion buffer (HCT 20 %)
PB – Perfusion buffer

Linear regression assumes that the distribution of residuals follows Gaussian distribution. To test whether this assumption applies to the data set, the Kolmogorov – Smirnov test was performed. Residuals of analyzed data were normally distributed ($p > 0.05$), and the correlation

of area ratios to concentration was significant and linear with a correlation of R > 0.99 (see *Figure 35).*

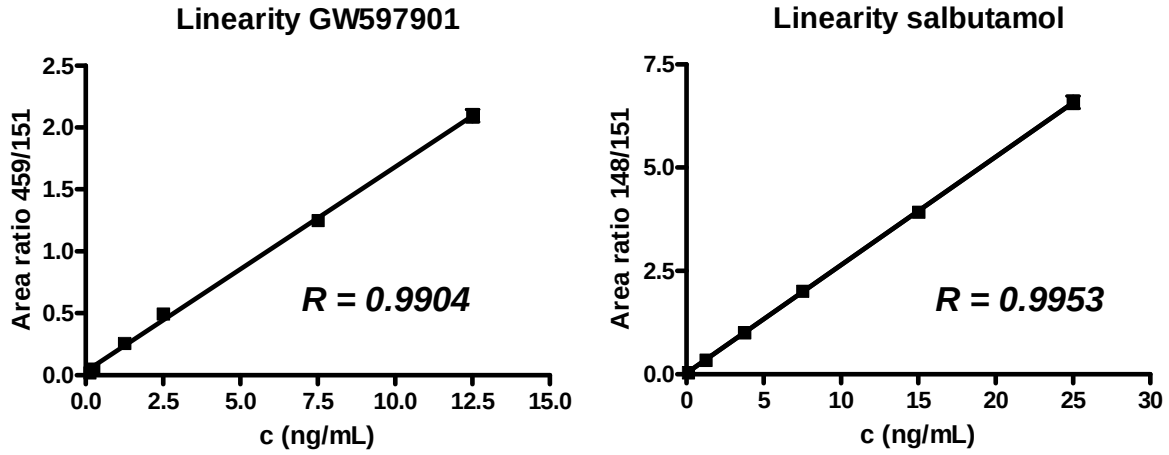

Figure 35: Calibration lines of GW597901 and salbutamol. Six independent samples of six concentration levelsls were analyzed and a regression analysis was performed (n= 36). R – Correlation coefficient.

5.3 Precision and accuracy

For the determination if precision and accuracy of the method, 12 QC samples of three different concentrations levels were analyzed. The lowest concentration level was three times the LoQ of the analyte (see *5.4*), one concentration was chosen in the mid- range of the standard line and the highest concentration level was 80 % of the upper limit of quantification. Every concentration was analyzed six times a day for intra – day precision and six times on consecutive days for the determination of inter-day precision. The relative standard deviations (RSD) in percent were calculated for the assessment of precision and the accuracy was determined by the following formula:

Formula 5

$$\text{Accuracy (\%)} = \frac{\sum_{i=1}^{6} C_n}{6 \bullet C_a} \bullet 100$$

C_n – nominal concentration of i - QC sample

C_a – actual concentration of QC sample

The QC acceptance criteria for intra- and inter-batch precision were a RSD of less than 15 % and an accuracy between 85 % and 115 % [285, 287].

For the preparation of QC samples, working solutions of GW597901 and salbutamol were added to the matrix (analogue to *5.2*) leading to concentrations mentioned in *Table 25* and

purified according to *4.3.2.1*. For each QC batch a set of standard samples was produced and analyzed to control the quality of each run. Intra – and inter – day precision and accuracy values were in the range of acceptance criteria for each concentration level of QC samples.

Table 25: Precision and accuracy (n= 6) of QC samples

Substance	*Intra - day*		*Inter - day*	
	Precision (% RSD)	*Accuracy* (%)*	*Precision (% RSD)*	*Accuracy* (%)*
GW597901 (ng/mL)				
10	7.71	94.37	9.51	92.84
6.25	11.1	108.68	11.26	101.78
0.375	6.15	108.47	12.1	96.53
Salbutamol (ng/mL)				
20	8.94	92.26	5.63	101.15
12.5	3.91	101.32	5.17	99.67
0.375	8.58	88.31	11.08	88.9

* - Accuracy calculated with Formula 5

5.4 Limit of quantification (LoQ)

The LoQ corresponded to the lowest analyzed concentration of the standard samples, 0.125 ng/mL for both GW597901 and salbutamol. Six independent LoQ samples were prepared in EC/PB for GW597901 and PB for salbutamol and analyzed according to the sample preparation and LC – MS/MS method. In parallel, standard samples were prepared and calibration lines for the β_2 – agonists were calculated. Area ratios of the six LoQ samples were related to calibration lines and the actual concentrations were determined. The LoQ was accepted, if the actual concentrations did not deviate to the nominal concentrations more than 20 % and the RSD were less than 20 % [287]. With an accuracy of 108.5 % and a RSD of 12.3 %, the LoQ of 0.125 ng/mL for GW597901 in EC/PB was confirmed. A concentration of 0.125 ng/mL of salbutamol extracted from PB could be quantified with an accuracy of 85.5 % and a RSD of 5.3 %.

5.5 Stability

To control the long – term stability of GW597901 and salbutamol in biological matrices, QC samples with two different concentrations each were produced and stored at -25 ℃ for 4 month according to the longest storage time of perfusion fluid samples prior to further analysis. As matrix for GW597901 a mixture of EC/PB and for salbutamol PB was used. For the stability sample set, every concentration was prepared and analyzed independently in triplicate (see *Table 26*). At the time of stability sample preparation, fresh calibration standards were produced and analyzed. Again resulting area ratios of stability samples were related to the given calibration line and the actual concentrations were calculated. The substance was considered as chemically stable in the relevant biological matrix, if the actual concentrations of the β_2 – agonists in the stability samples met the acceptance criteria according in section *5.3*.

Table 26: Precision and accuracy (n= 3) of stability control samples after storage for 4 months at -25 ℃ in biol ogical matrices (GW597901: erythrocyte concentrate/perfusion buffer, HCT 20 %; salbutamol: perfusion buffer)

Substance	*Precision %*	*Accuracy (%)*
GW597901 (ng/mL)		
10	2	93.8
0.375	3.6	89.7
Salbutamol (ng/mL)		
20	14.9	110.85
0.375	1.3	100.87

The precision values were within the acceptable range of RSDs of less of 15 %, and the accuracies of the actual concentrations were always between 85 and 115 %. Thus, GW597901 and salbutamol in biological matrices were considered to be stable under the described inappropriate storage conditions.

6 Evaluation of significant markers of edema formation during lung perfusion experiment

6.1 Quantification of angiotensin converting enzyme (ACE) in perfusion fluid samples

6.1.1 Standard solutions and sample preparation

All components of Quantikine® Human ACE Immunoassay were used at room temperature (RT). ACE standard solutions for calibration were prepared in plastic reaction tubes by appropriate diluting the ACE stock solution with calibrator diluent RD 6 – 45, a buffered protein base with preservatives. By diluting a 100 ng/mL ACE stock solution the following standard concentrations were obtained:

Standard	ACE (ng/mL)
S1	25.0
S2	6.25
S3	3.12
S4	1.56
S5	0.78
S6	0

According to the manufacturer's instructions the assay kit was validated to quantify ACE in human serum and plasma samples. Therefore, perfusion fluid samples containing blood components were centrifuged (3500 rpm, 10 °C, 10 min), the supernatants were aliquoted in volumes of 100 µL, shock frozen in liquid nitrogen and stored at -20 °C.

6.1.2 Materials and equipment

ACE Elisa Kit	Quantikine® Human ACE Immunoassay (R&D – Systems, Minneapolis, USA);
Microplate Reader	Multiskan Ascent® (Thermo Electron Corporation, Vantaa, Finland);
Microplate Autowasher	MultiWash Advantage® (TriContinent™, Grass Valley; CA, USA);
Transferpipette 20 – 200 µL	Transferpette® electronic (BRAND, Wertheim, Germany);

Plastic reaction tube	Polypropylene, 1.5 mL, uncoloured (Carl Roth, Karlsruhe, Germany);
Horizontal microplate shaker	Unimax 1010 (Heidolph, Kelheim, Germany);
Timer	Digital timer (Carl Roth, Karlsruhe, Germany);
Vortex	Model K – 550 – GE (Bender & Hobein, Zurich, Switzerland);

6.1.3 Method

100 µL assay diluent and 50 µL standard or perfusion fluid sample (+ blank sample) were pipetted in each well, covered with an adhesive strip and agitated on a plate shaker at 250 rpm for 2 h at RT. Perfusion fluid samples were diluted with perfusion fluid if necessary prior to the analysis. After the first incubation, the samples were aspirated and the wells were washed four times with 250 µL washing buffer per well. 200 µL ACE conjugate solution were given in each well, the plate was covered and shaked for 2 h at 250 rpm. The wells` contents were aspirated and washed again four times with washing buffer. To induce the colour reaction, 200 µL substrate solution were added to each well and incubated for 30 min at RT protected from light before adding stop solution. Immediately the absorptions were measured with a microplate reader at 450 nm and a correction wavelength of 560 nm after shaking the microplate for 10 s. For each test series absorption intensities of standards were determined on the same plate.

6.1.4 Analysis

For the calibration procedure the absorption intensity of S6 (see *6.1.1*) was subtracted from those of the standards. Resulting values were plotted against corresponding contents of ACE. Net absorption intensities of perfusion fluid samples were referred to calibration line to determine unknown ACE contents.

Results were calculated as maximum absolute and percent differences of first measured values of perfusion fluid samples (= reference sample) over time whereas the start of perfusion experiment was defined as time 0 min.

6.2 Quantification of lactate dehydrogenase (LDH) in perfusion fluid samples

6.2.1 Substances

LDH stock solution 550 U/mg (10 mg/mL) was purchased from Roche Diagnostics (Mannheim, Germany). Sources of other chemicals in p.a. quality were mentioned in *1.1.1.*

6.2.2 Solutions

- *Perfusion buffer pH 7.4* (see *1.1.2.1*)
- *Standards:*

LDH stock solution was diluted with perfusion buffer to produce following standard concentrations:

Standard	c (U/L)
S1	11
S2	8.25
S3	4.125
S4	2.75
S5	1.375

6.2.3 Material and equipment

LDH colorimetric assay	Cytotoxicity Detection Kit^{PLUS} (Roche Diagnostics, Mannheim, Germany);
Microplate reader	Bio – Rad Multiplate Reader (Bio – Rad Laboratories, Hercules, USA);
96 – well plate	Disposable, flat bottom 96 – well plate, transparent, Falcon 3912 MicroTest III^{TM} (Becton Dickinson, CA, USA)

Every other material is mentioned in 6.1.2.

6.2.4 Method

After allowing all samples, background control (= perfusion buffer) and standards to equilibrate at RT, 100 µL each were given into a well of a 96 – well plate. Shortly before use, reconstituted catalyst and dye solution were mixed in a ratio of 1:45 and 100 µL were pipetted in each well.

Incubation took place at RT for 30 min before discontinuing the colour reaction with 50 µL stop solution. After shaking the microplate for 10 s the absorbance was measured at 490 nm.

6.2.5 Analysis

By subtracting absorbance of the blank sample from absorption intensities of standards, the calibration line was calculated using linear regression by plotting net absorptions against corresponding LDH activities. The maximum changes of LDH activity in perfusion fluid samples were calculated as absolute and percent difference of first measured value in perfusion fluid sample series (= reference sample) over time whereas the start of perfusion experiment was defined as time 0 min, according to *6.1.4.*

6.3 Quantification of urea in perfusion fluid samples

6.3.1 Substances

Urea, urease 1.3 U/mg, glutamate dehydrogenase (GDH) 40 U/mg, $NADH_2$ and α – ketoglutarate were purchased from E. Merck (Darmstadt, Germany).

6.3.2 Solutions

- Phosphate buffer 0.33 M pH 7.5:

3.12 g Na_2HPO_4 and 1.50 g KH_2PO_4 were dissolved in 90 mL destilled water, the pH value was adjusted to 7.5 either with NaOH or H_3PO_4 and the volume of 100 mL was completed with destilled water.

- Enzyme solution:

GDH	25 mg
α – Ketoglutarate	20 mg
$NADH_2$	15 mg
Urease	40 mg
Phosphate buffer 0.33 M pH 7.5	ad 100 mL

- Perfusion buffer pH 7.4 (see *1.1.2.1*)
- Standards

A 1 mg/mL stock solution of urea in perfusion buffer pH 7.4 was used to produce following standard concentrations:

Standard	c (µg/mL)
S1	250
S2	50
S3	25
S4	2.5

6.3.3 Materials and equipment

Balance, precision	Mettler P1200, 0.1 – 1200 g, 10 mg minimal weight difference (Mettler – Toledo, Gießen, Germany);
Flask	Volumetric, 100 mL, ST 10/19, Duran® 50 (WITEG Labortechnik GmbH, Wertheim, Germany);
Pipette	Transferpette® electronic (BRAND, Wertheim, Germany); Eppendorf Research®, 0.10 – 10 µL (Eppendorf AG, Hamburg, Germany);

Other materials are described in *6.1.2* and *6.2.3*.

6.3.4 Method

The method applied for the quantification of urea in perfusion fluid samples was based on the enzymatic urea determination in blood and serum developed by Talke. and Schubert [270]. Each 5 µL of standard, sample or blank sample (= fresh perfusion buffer, see *1.1.2.1*) were added to 200 µL enzyme solution. All samples were analyzed in triplicate and previously diluted if necessary. After incubation at RT for 1 h and shaking for 10 s, decrease of absorption intensities due to consumption of NADH, signals was measured at 360 nm.

6.3.5 Analysis

Measured absorption intensities of samples and standards were subtracted from absorbance of the blank sample. For calibration, calculated differences were plotted against concentrations of standard and analyzed via linear regression. Unknown urea contents (µg/mL) were determined by calculating the mean differences of absorption intensities of samples and the maximum changes in the concentrations over time were determined whereas the first measured value in perfusion fluid sample series served as reference value.

6.4 Enzyme – linked immuno sorbent assay (ELISA) for the quantification of human surfactant protein A (SP-A) in perfusion fluid samples

6.4.1 Substances

SP – A stock solution was generously provided by Prof. Dr. Cordula Stamme (Cellular Pneumology, Research center, Borstel, Germany). Goat anti-human SP – A polyclonal antibody, mouse anti-human SP – A monoclonal antibody and donkey anti-goat IG-G HRP-conjugated secondary antibody were obtained from Millipore (Schwalbach, Germany). 3, 5, 3`, 5`- Tetramethylbenzidine (TMB) was purchased from Fluka (Buchs, Switzerland). Tween® 20 Ph. Eur. was acquired by Carl Roth GmbH (Karlsruhe, Germany) and BSA Type H1 was purchased from Gerbu (Gaiberg, Germany). Source of glycerol for molecular biology, minimum 99 % was Sigma Aldrich (Steinheim, Germany). All other salts and chemicals in p.a. quality were obtained by Grüssing GmbH (Filsum, Germany) and Merck (Darmstadt, Germany).

6.4.2 Solutions and buffers

6.4.2.1 Phosphate buffered saline (PBS)

The composition and molarties of salts used for 1000 mL of PBS were specified as follows:

KH_2PO_4	1.76 mM	0.24 g
Na_2HPO_4	10.1 mM	1.44 g
NaCl	136.9 mM	8.0 g
KCl	2.7 mM	0.2 g

Electrolytes were dissolved in 900 mL Millipore water, the pH was adjusted to 7.4 with 0.5 N HCl and the volume of 1000 mL was completed with ultra pure water. The buffer was prepared in 1 L volumetric flasks and stored in transparent screw cap glass bottles at 4 ℃ for a maximum of four weeks.

6.4.2.2 Carbonate / bicarbonate buffer

1 L of the 50 mM carbonate/ bicarbonate buffer consisted of:

Na_2CO_3	15.0 mM	1.59 g
$NaHCO_3$	31.2 mM	2.93 g

After dissolution of Na_2CO_3 and $NaHCO_3$ in 900 mL Millipore water, the pH was adjusted to 9.6 with 10 % $NaHCO_3$ solution (m/V) and the total volume of 1000 mL was complemented with Miilipore water. The buffer was stored at 4 – 8 °C.

6.4.2.3 Other solutions used for ELISA

- Washing solution: 0.05 % Tween® 20 in PBS (see *6.4.2.1*)
- Blocking buffer: 1 % BSA, 0.05 % Tween® 20 in PBS
- Stop solution: 1 M H_2SO_4

Solutions were stored at 4 – 8 °C.

6.4.2.4 Antigen and antibody solutions

- Human SP – A stock solution (c= 1 mg/mL) was aliquoted in volumes of 100 µL, shock frozen in liquid nitrogen and stored at -80 °C. For preparing of standard solutions, the SP-A stock solution was diluted 1:4000 in blocking buffer (see *6.4.2.3*).
- Monoclonal mouse anti human SP – A in ascites (*capture antibody):*
  Aliquots were stored in volumes of 10 µL at -20 °C. According to manufacturer's instructions the stock solution had an approximate concentration of 2-5 mg/mL. The capture antibody was diluted 1:1000 in carbonate buffer pH 9.6 (see *6.4.2.2*) before use.
- Polyclonal goat anti human SP – A in PBS (*detection antibody):*
  The absolute concentration of detection antibody in PBS was unknown. Volumes of 10 µL were aliquoted and stored at -20 °C. Before use, the polyclonal antibody was diluted 1:100 in blocking buffer.
- Donkey anti goat HRP conjugate (*secondary antibody):*
  The lyophilized secondary antibody was reconstituted in 500 µL sterile destilled water leading to a stock solution with a concentration of 1 mg/mL. For long-term storage, the stock solution was mixed 1:1 with glycerol (see *6.4.1*), aliquoted in volumes of 20 µL and kept at -25 °C. Shortly before use the HRP conjugate was diluted 1:20000 in blocking buffer.

On day of the ELISA experiment, antigen and antibody dilutions (RT) were always freshly prepared as mentioned above. Repeated freeze and thaw cycles were avoided.

6.4.2.5 Stability samples

To assess the long – term stability of human SP – A in perfusion fluid samples, quality control samples with two different concentrations of SP – A (125 and 5 ng/mL) were produced in triplicate. Perfusion buffer and a mixture of erythrozyte concentrate and perfusion buffer (HCT

20 %) served as matrices. Stability samples in EC/PB matrix were incubated 1 h at 37 ℃, centrifuged (3500 rpm, 10 ℃, 10 min) and the super natants were transferred in plastic reaction tubes. Samples were then shock frozen in liquid nitrogen and stored at -25 ℃ for 3 months.

6.4.2.6 Substrate solution

Components:

A. TMB: stock solution 10 mg/mL (41.6 mM; 1 % w/v) in 96 % EtOH
B. 1 % H_2O_2 (p.a. quality) solution in H_2O
C. Acetate buffer pH 5.5: 100 mM Na-acetate in H_2O, pH was adjusted with acetic acid

For the preparation of 5 mL substrate solution (RT), 50 µL **A.** and 20 µL **B.** were given to **C.** resulting in 0.42 mM TMB/0.004 % H_2O_2 and mixed thoroughly. The substrate solution was always prepared less than 15 minutes before use in the ELISA and protected from light to avoid degradation of the constituents. Single components were stored at 4 – 8 ℃ in the dark.

6.4.3 Material and equipment

Incubator	Type BM 400, nominal temperature +70 ℃ (Memmert, Schwabach, Germany).
Microplate Reader	Multiskan Ascent® (Thermo Electron Corporation, Vantaa, Finland);
Microplate washer	MultiWash Advantage® (TriContinent™, Grass Valley; CA, USA);
Pipettes	Eppendorf Research®,, 8 – channel and single channel pipette 10 – 100 µL (Eppendorf AG, Hamburg, Germany);
Plastic reaction tube	PP, 1.5 mL, uncoloured (Carl Roth, Karlsruhe, Germany);
Reagent reservoir	PP, high clarity, nonsterile, autoclavable (BRAND GmbH, Wertheim, Germany);
Shaker	Model mini shaker, 7.5 – 30/min, 200 x 200 mm (A. Hartenstein GmbH, Würzburg, Germany);
Timer	Digital timer (Carl Roth, Karlsruhe, Germany);
Vortex	Model K – 550 – GE (Bender & Hobein, Zurich, Switzerland);
96 – well plate	Disposable, nonsterile flat bottom 96 – well plate, transparent, BD Falcon MicroTest™ (BD Bioscience, Bedford, MA, USA)

6.4.4 Development of an ELISA method for the quantification of SP-A

An indirect sandwich ELISA technique was the method of choice (see *Figure 21* in *3.2.3,* B - Results and Discussion) since an enzyme-conjugated antibody against human SP-A was not available. The optimal working concentrations of the antibodies leading to a sensitive performance of the ELISA method were determined in via chessboard titration according to the instructions of Crowther [272] (see *Figure 22* in *3.2.3,* B - Results and Discussion).

In the first preliminary test, the concentration of the mouse anti-human SP-A monoclonal antibody (= *capture antibody*) for coating the wells (5 µg/mL in carbonate buffer *6.4.2.2*) and the dilution of the horseradish peroxidase (HRP) – conjugate (= *secondary antibody,* recommended dilution 1:10 000 in blocking buffer *6.4.2.3*) were kept constant. For maximal binding, 50 µL of capture antibody solution were added to each well corresponding to an absolute amount of 0.25 µg per well. 50 µL of carbonate buffer alone were given into the wells of column 12 to determine the non – specific binding properties of the detecting antibody. After incubation at 37 C for 2 h (= coating process) on a plate shaker, the wells were washed 4 x with 250 µL washing solution (*6.4.2.3*). Binding solution was saved and used again. Next, 200 µL blocking buffer were given to each well to avoid later non-specific binding of SP-A. After incubation at 37℃ for 30 min on the shaker, the wells were washe d again 4 x with 250 µL washing solution and blotted dry.

To determine the optimal working concentrations of SP-A standard solutions and the detecting antibody, a chessboard titration was performed. First, 50 µL of blocking buffer were given into each well. The stock solution of human SP-A was diluted with blocking buffer leading to a concentration of 10 µg/mL and 50 µL were added to the wells A1 – H1 with a multichannel pipette. The antigen solutions in the wells were mixed by pipetting up and down eight times. Then 50 µL of column 1 were transferred to column 2 and mixed again and so on. Thus, SP-A was double diluted across the plate from column 1 to 11, and the last 50 µL from column 11 were discarded. Therefore, column 12 contained no antigen (= control). The plate was covered and incubated at 37 ℃ on the shaker. After 1 h the wells were washed 4 x with 250 µL washing solution and blotted dry.

Again 50 µL of blocking buffer were given to each well. The detecting antibody was diluted 1/100 in blocking buffer and 50 µL were added to row A. Serial double dilution of the detecting antibody across the rows A-H followed. After incubation at 37 ℃ for 1 h the wells were washed 4 x with 250 µL washing solution and blotted dry. For the last working step the HRP-conjugate stock solution was diluted 1:10000 in blocking buffer, according to the manufacturer's instructions, 50 µL were added to each well and incubated at RT on the shaker for 1 h. Before 50 µL of the substrate solution were given into the wells, the plate was washed three times with washing solution. The substrate solution consisted 3, 5, 3´,5´- tetramethylbenzidine and H_2O_2.

Then the plate was covered and incubated at RT in the dark so that the colour reaction proceeded (see *Figure 20* in *3.2.2*). To stop the colour reaction, 50 µL 1 M H_2SO_4 were added and the absorption intensity at 450 nm was read immediately. For further tests, a concentration of the detecting antibody was chosen that produced a maximum in absorption intensity (= saturated system) in column 1 with the highest SP-A content and showed the lowest optical density in column 12 (= background signal).

In the next preliminary test, concentrations of SP-A (0.625 µg/mL) and detecting antibody (dilution: 1:800) were kept constant and the amount of capture antibody was varied. For coating the wells, 50 µL of carbonate buffer were given to columns 1-4, rows B-H. Then, 100 µL of capture antibody dilution (1:1000 in carbonate buffer) were pipetted into the wells A1-A4 and then double diluted and mixed across the rows B-H. The further course of the experiment proceeded as described above. The concentration of the capture antibody solution for coating showing the highest signal intensity was used in the final ELISA method.

To determine the optimal working dilution of the conjugate antibody, again a chess board titration was performed. For coating procedure, 100 µL of capture antibody solution in carbonate buffer (20 µg/mL) were added to A1-H1 and 50 µL of carbonate buffer were given to the wells in the columns 2 and 3 (A-H) followed by double dilution of the capture antibody across the columns 2 and 3. After coating, SP-A and the detecting antibody concentrations were kept constant and the working steps occurred according to prior experiments. For conjugation, 50 µL of blocking buffer were given to the columns 1-3. 50 µL of HRP-conjugate (dilution 1:2500) were added to the blocking buffer in A1-A3 and mixed followed by a serial dilution across the rows B-G. The 50 µL obtained from row G were discarded so row H contained just 50 µL blocking buffer (= background control). An optical density of around 1.2 was regarded as an optimal result. In the last preliminary experiment the ELISA was tested for linearity. All components, except human SP-A, were used in their optimal concentrations. The concentration range of SP-A solution ranged from 500 – 0.49 ng/mL, achieved by double dilution from A1-C2. Linearity in signal intensity was observed from 125 – 1.95 ng/mL.

6.4.5 Indirect sandwich ELISA method for the determination of unknown concentrations of human SP-A in perfusion fluid samples

The final assay procedure is shown as a flow diagram:

Coating: Pipette 50 µL capture antibody (2-5 µg/mL in carbonate buffer) into the wells. Cover the plate followed by incubation for 2 h at 37 °C on a shaker. Wash the wells 4 x with 250 µL washing solution per well and blot dry.

⬇

Blocking: Give 200 µL of blocking buffer to each well and cover the plate. Incubate at 37 °C for 30 min on a plate shaker.

⬇

Standards and samples: For preparation of standard samples give 50 µL of blocking buffer to A1-H1. Well A1 serves as blank sample. Add 50 µL of SP-A standard solution (0.25 µg/mL) to B1 and mix thoroughly. Transfer 50 µL from B1 to C1 and mix and so on. Discard the last 50 µL from H1. For the determination of unknown SP-A concentrations in perfusion fluid samples add 50 µL of undiluted samples to the wells. Cover plate and incubate at 37 °C for 1 h on the shaker. Wash the wells 4 x with washing solution and blot dry.

⬇

Detecting antibody: Dilute detecting antibody 1/800 in blocking buffer. Add 50 µL of the dilution to each well. Cover the plate and incubate the plate at 37 °C for 1 h. Wash the wells 4 x with 250 µL washing solution and blot dry.

⬇

Secondary antibody: Dilute the HRP-conjugate 1:20000 in blocking buffer and add 50 µL to each well. After incubation at RT for 1 h on the shaker, wash 4 x with 250 µL washing solution per well and blot dry. Give 50 µL of substrate solution into the wells, cover the plate and incubate 30 min in the dark. Add 50 µL of 1 M H_2SO_4 to stop the colour reaction and read the absorption intensities immediately at 450 nm.

All solutions and reagents were allowed to equilibrate at RT before use. Standards of at least six different concentrations were always analyzed in parallel for calibration. Perfusion fluid samples with unknown SP – A concentrations showing absorption intensities beyond the calibration range, were reasonably diluted and reanalyzed.

6.4.6 Analysis

Measured absorption intensities of samples and standards were subtracted from absorbance of blank samples. For calibration, calculated differences were plotted against concentrations of standard on a log-log scale, assuming a linear correlation between absorption and concentration of human SP – A. R^2 was always higher than 0.97. Unknown SP – A concentrations (ng/mL) in perfusion fluid samples were determined by corresponding absorption densities to the calibration line produced in the same assay. Results were expressed as the content of SP – A in samples over the time of the relevant perfusion experiment. Furthermore, the maximum in increase of SP-A in percent referring to the first measured value (= reference sample) was calculated as a measurement of the extent of pulmonary edema formation.

D. Appendix

1 The pharmacokinetic enabling study

1.1 Conditions of human lung lobe perfusion experiments

Lung lobe perfusion experiment	Haematocrit in perfusion fluid (%)	Weight of the perfused human lung tissue (g)		
		Prior to experiment	After termination of the experiment	Weight gain in %
1	20.5	217	262	20.7
2	19.9	406	393	-
3	22.6	405	542	33.8
4	21.2	706	721	2.1
5	19.4	325	335	3.1
6	19.2	331	358	8.2

- Data were not collected

Amounts of GW597901 nebulized, adsorbed and actually deposited in the ventilated airways.

Lung lobe perfusion experiment	Amount of nebulized drug (µg)	Adsorbed amount of nebulized drug (%)	Actually applied dose (µg)	Percentage of targeted dose* (%)
1	49.7	8.0	45.7	101.6
2	55.7	13.7	48.1	106.9
3	41.0	18.0	33.6	74.7
4	133.4	19.4	107.5	59.7
5	187.9	16.7	156.5	86.9
6	123.3	28.8	87.8	48.8
Mean	**78.5**	**17.4**	**79.9**	**79.8**

* Targeted doses of GW597901 were 45 µg in the perfusion experiments 1 - 3 and 180 µg in the experiments 4 - 6

Amounts of salbutamol nebulized, adsorbed and actually deposited in the ventilated airways.

Lung lobe perfusion experiment	Amount of nebulized drug (µg)	Adsorbed amount of nebulized drug (%)	Actually applied dose (µg)	Percentage of targeted dose* (%)
1	157.0	12.5	137.4	91.6
2	170.2	16.2	142.6	95.1
3	160.6	13.9	138.3	92.2
4	459.3	18.5	374.2	62.4
5	631.5	17.5	520.9	86.8
6	418.0	26.8	306.1	51.0
Mean	**332.8**	**17.6**	**269.9**	**79.8**

* Targeted doses of salbutamol were 150 µg in the perfusion experiments 1 - 3 and 600 µg in the experiments 4 - 6

1.2 Analyzed concentrations of GW597901 and salbutamol in perfusion fluid and lung tissue samples

1.2.1 Lung lobe perfusion experiment 1

Concentration in samples withdrawn from venous output

Time of sampling (min)	*Concentration in (ng/mL)*					
	Whole perfusion fluid		Perfusion fluid plasma		PFP to WPF ratio (%)	
	Salbutamol	GW957901	Salbutamol	GW957901	Salbutamol	GW957901
-6	2.7	0.5	10.0	2.2	363.1	433.0
0	7.8	n.d.	n.d.	n.d.	-	-
10	16.3	2.6	9.0	n.d.	55.4	-
20	20.0	2.9	12.9	n.d.	64.3	-
30	18.0	2.7	11.0	n.d.	61.0	-
45	16.1	2.7	10.8	n.d.	67.0	-
60	21.2	3.2	12.0	n.d.	56.7	-

n.d. not detectable; - data were not collected
PFP - perfusion fluid plasma; WPF - whole perfusion fluid

Concentration in samples withdrawn from the reservoir

Time of sampling (min)	*Concentration in (ng/mL)*					
	Whole perfusion fluid		Perfusion fluid plasma		PFP to WPF ratio (%)	
	Salbutamol	GW957901	Salbutamol	GW957901	Salbutamol	GW957901
0	6.6	1.4	n.d.	n.d.	-	-
10	16.0	2.8	11.0	n.d.	68.8	-
20	17.3	3.2	10.8	n.d.	62.3	-
30	16.9	2.7	11.5	n.d.	68.2	-
45	20.0	2.7	12.9	n.d.	64.5	-
60	15.9	3.0	12.4	n.d.	77.7	-

n.d. not detectable; - data were not collected
PFP - perfusion fluid plasma; WPF - whole perfusion fluid

Concentration in samples from lung tissue

Site of sampling	*Concentration (ng/g tissue)*	
	Salbutamol	GW957901
1	46.2	23.6
2	184.0	200.0
3	122.0	50.0
4	35.7	29.8
Mean	**97.0**	**75.9**
Amount found in tissue (µg)*	25.4	19.9
Percentage of applied dose (%)	18.5	43.5

* Related to absolute weight of perfused lung lobe (262 g)

1.2.2 Lung lobe perfusion experiment 2

Concentration in samples withdrawn from venous output

Time of sampling (min)	*Concentration in (ng/mL)*					
	Whole perfusion fluid		Perfusion fluid plasma		PFP to WPF ratio (%)	
	Salbutamol	GW957901	Salbutamol	GW957901	Salbutamol	GW957901
-6	5.2	0.6	20.0	1.5	383.7	241.7
0	5.9	0.3	1.8	1.1	29.7	420.0
10	18.6	3.6	8.7	n.d.	46.6	-
20	45.7	8.8	28.8	0.2	62.9	2.3
30	56.4	11.1	51.4	0.2	91.0	1.8
45	50.4	10.1	48.2	n.d.	95.6	-
60	48.8	10.1	58.2	n.d.	119.2	-
80	48.8	11.4	56.1	n.d.	114.9	-

n.d. not detectable; - data were not collected
PFP - perfusion fluid plasma; WPF - whole perfusion fluid

Concentration in samples withdrawn from the reservoir

Time of sampling (min)	*Concentration in (ng/mL)*					
	Whole perfusion fluid		Perfusion fluid plasma		PFP to WPF ratio (%)	
	Salbutamol	GW957901	Salbutamol	GW957901	Salbutamol	GW957901
0	26.7	5.5	23.6	2.3	88.4	41.8
10	65.0	12.4	67.1	3.9	103.3	70.0
20	58.6	11.7	52.6	1.3	89.8	10.1
30	63.3	11.2	49.4	n.d.	78.0	-
45	54.5	15.0	47.3	n.d.	86.9	-
60	52.6	11.3	48.4	6.4	91.9	42.5
80	49.9	11.5	54.1	1.4	108.5	12.1

n.d. not detectable; - data were not collected
PFP - perfusion fluid plasma; WPF - whole perfusion fluid

Concentration in samples from lung tissue

Site of sampling	*Concentration (ng/g tissue)*	
	Salbutamol	GW957901
1	168.3	124.4
2	39.5	23.9
3	41.8	25.5
4	34.9	29.1
Mean	**71.1**	**50.7**
Amount found in tissue (µg)*	28.0	19.9
Percentage of applied dose (%)	19.6	41.4

* Related to absolute weight of perfused lung lobe (393 g)

1.2.3 Lung lobe perfusion experiment 3

Concentration in samples withdrawn from venous output

Time of sampling (min)	*Concentration in (ng/mL)*					
	Whole perfusion fluid		Perfusion fluid plasma		PFP to WPF ratio (%)	
	Salbutamol	GW957901	Salbutamol	GW957901	Salbutamol	GW957901
-6	11.6	0.7	17.1	2.8	147.6	430.0
0	29.3	1.2	18.9	n.d.	64.4	-
10	33.9	2.0	25.9	n.d.	76.3	-
20	21.4	1.5	17.0	n.d.	79.6	-
30	16.8	2.3	16.8	n.d.	100.3	-
45	15.6	2.6	13.9	n.d.	89.1	-
60	12.8	2.4	18.0	n.d.	140.6	-

n.d. not detectable; - data were not collected
PFP - perfusion fluid plasma; WPF - whole perfusion fluid

Concentration in samples withdrawn from the reservoir

Time of sampling (min)	*Concentration in (ng/mL)*					
	Whole perfusion fluid		Perfusion fluid plasma		PFP to WPF ratio (%)	
	Salbutamol	GW957901	Salbutamol	GW957901	Salbutamol	GW957901
0	6.0	1.4	4.1	n.d.	67.5	-
10	5.8	1.0	4.2	n.d.	72.2	-
20	9.3	1.4	7.1	n.d.	75.8	-
30	8.3	1.6	8.4	n.d.	100.6	-
45	10.2	1.1	8.8	n.d.	86.7	-
60	10.1	1.4	6.6	n.d.	65.3	-

n.d. not detectable; - data were not collected
PFP - perfusion fluid plasma; WPF - whole perfusion fluid

Concentration in samples from lung tissue

Site of sampling	*Concentration (ng/g tissue)*	
	Salbutamol	GW957901
1	228.6	120.0
2	119.6	44.6
3	65.2	54.3
4	30.6	25.5
Mean	**111.0**	**61.1**
Amount found in tissue (µg)*	60.2	33,1
Percentage of applied dose (%)	43.5	98.5

* Related to absolute weight of perfused lung lobe (542 g)

1.2.4 Lung lobe perfusion experiment 4

Concentration in samples withdrawn from venous output

Time of sampling (min)	*Concentration in (ng/mL)*					
	Whole perfusion fluid		Perfusion fluid plasma		PFP to WPF ratio (%)	
	Salbutamol	GW957901	Salbutamol	GW957901	Salbutamol	GW957901
-6	12.3	1.6	44.3	9.8	360.2	613.1
0	26.3	5.0	17.6	n.d.	66.7	-
10	81.5	17.1	21.8	n.d.	26.7	-
20	89.9	19.4	27.7	n.d.	30.8	-
30	80.3	17.7	23.8	n.d.	29.6	-
45	91.9	24.8	25.1	n.d.	27.3	-
60	108.2	33.1	28.1	n.d.	26.0	-

n.d. not detectable; - data were not collected
PFP - perfusion fluid plasma; WPF - whole perfusion fluid

Concentration in samples withdrawn from the reservoir

Time of sampling (min)	*Concentration in (ng/mL)*					
	Whole perfusion fluid		Perfusion fluid plasma		PFP to WPF ratio (%)	
	Salbutamol	GW957901	Salbutamol	GW957901	Salbutamol	GW957901
0	13.5	3.3	4.9	n.d.	36.1	-
10	78.7	15.9	16.8	n.d.	21.4	-
20	105.9	26.7	23.1	n.d.	21.8	-
30	106.7	29.8	26.3	n.d.	24.6	-
45	99.7	29.0	31.2	n.d.	31.3	-
60	96.7	30.8	33.7	n.d.	34.9	-

n.d. not detectable; - data were not collected
PFP - perfusion fluid plasma; WPF - whole perfusion fluid

Concentration in samples from lung tissue

Site of sampling	*Concentration (ng/g tissue)*	
	Salbutamol	GW957901
1	33.4	23.8
2	310.8	56.7
3	143.4	45.4
4	55.4	49.4
Mean	**135.8**	**43.8**
Amount found in tissue (µg)*	97.9	31.6
Percentage of applied dose (%)	26.2	29.4

* Related to absolute weight of perfused lung lobe (721 g)

1.2.5 Lung lobe perfusion experiment 5

Concentration in samples withdrawn from venous output

Time of sampling (min)	*Concentration in (ng/mL)*					
	Whole perfusion fluid		Perfusion fluid plasma		PFP to WPF ratio (%)	
	Salbutamol	GW957901	Salbutamol	GW957901	Salbutamol	GW957901
-6	6.8	n.d.	7.3	n.d.	107.9	-
0	22.1	3.6	11.6	n.d.	52.3	-
10	47.5	9.0	22.3	n.d.	46.9	-
20	59.1	12.6	23.4	n.d.	39.6	-
30	55.0	12.7	18.1	n.d.	32.8	-
45	62.6	14.6	12.9	n.d.	20.5	-
60	54.7	13.5	12.5	n.d.	22.8	-

n.d. not detectable; - data were not collected
PFP - perfusion fluid plasma; WPF - whole perfusion fluid

Concentration in samples withdrawn from the reservoir

Time of sampling (min)	*Concentration in (ng/mL)*					
	Whole perfusion fluid		Perfusion fluid plasma		PFP to WPF ratio (%)	
	Salbutamol	GW957901	Salbutamol	GW957901	Salbutamol	GW957901
0	63.5	15.4	32.1	n.d.	50.6	-
10	57.9	13.5	22.7	n.d.	39.2	-
20	64.2	14.1	15.3	n.d.	23.8	-
30	74.8	15.5	13.8	n.d.	18.4	-
45	63.3	12.7	13.2	n.d.	20.8	-
60	62.7	17.1	12.5	n.d.	20.0	-

n.d. not detectable; - data were not collected
PFP - perfusion fluid plasma; WPF - whole perfusion fluid

Concentration in samples from lung tissue

Site of sampling	*Concentration (ng/g tissue)*	
	Salbutamol	GW957901
1	89.6	57.4
2	106.5	64.9
3	165.8	84.6
4	919.5	75.1
Mean	**320.4**	**70.5**
Amount found in tissue (µg)*	107.3	23.6
Percentage of applied dose (%)	20.6	15.1

* related to absolute weight of perfused lung lobe (335 g)

1.2.6 Lung lobe perfusion experiment 6

Concentration in samples withdrawn from venous output

Time of sampling (min)	*Concentration in (ng/mL)*					
	Whole perfusion fluid		Perfusion fluid plasma		PFP to WPF ratio (%)	
	Salbutamol	GW957901	Salbutamol	GW957901	Salbutamol	GW957901
-6	20.9	n.d.	39.8	7.6	190.4	-
0	50.3	3.3	28.2	n.d.	56.0	-
10	47.2	4.8	27.7	n.d.	58.7	-
20	41.5	3.6	28.0	n.d.	67.4	-
30	43.9	3.9	35.7	n.d.	81.3	-
45	44.2	4.6	33.4	n.d.	75.5	-
60	46.6	5.3	30.9	n.d.	66.4	-

n.d. not detectable; - data were not collected
PFP - perfusion fluid plasma; WPF - whole perfusion fluid

Concentration in samples withdrawn from the reservoir

Time of sampling (min)	*Concentration in (ng/mL)*					
	Whole perfusion fluid		Perfusion fluid plasma		PFP to WPF ratio (%)	
	Salbutamol	GW957901	Salbutamol	GW957901	Salbutamol	GW957901
0	25.2	3.2	14.3	n.d.	56.7	-
10	29.3	2.4	15.4	n.d.	52.5	-
20	37.4	3.3	26.7	n.d.	71.4	-
30	43.0	3.5	23.7	n.d.	55.2	-
45	46.6	4.8	20.5	n.d.	44.0	-
60	52.0	6.0	16.2	n.d.	31.2	-

n.d. not detectable; - data were not collected
PFP - perfusion fluid plasma; WPF - whole perfusion fluid

Concentration in samples from lung tissue

Site of sampling	*Concentration (ng/g tissue)*	
	Salbutamol	GW957901
1	2592.9	351.2
2	152.8	64.7
3	158.9	38.2
4	47.3	42.3
Mean	**738.0**	**124.1**
Amount found in tissue (µg)*	264.2	44.4
Percentage of applied dose (%)	86.3	50.6

* Related to absolute weight of perfused lung lobe (358 g)

1.3 Data summary

1.3.1 Time course of GW597901 in perfusion fluid

1.3.1.1 Samples withdrawn from *venous output*

Release into *whole perfusion fluid* as percentage of applied dose (%)

Time of sampling (min)	*Lung lobe perfusion experiment (No)*						*Mean*	*SD*
	1	*2*	*3*	*4*	*5*	*6*		
-6	0.5	0.6	0.7	0.6	0	0	0.4	0.3
0	0	0.8	5.1	7.0	3.5	5.6	3.7	2.8
10	8.4	11.3	8.8	23.9	8.7	8.2	11.5	6.1
20	9.6	27.4	6.8	27.3	12.2	6.2	14.9	9.9
30	9.0	34.8	10.2	25.1	12.3	6.8	16.4	11.1
45	8.9	32.1	11.6	35.2	14.2	8.0	18.3	12.1
60	10.8	32.3	11.1	47.1	13.3	9.3	20.7	15.6

SD standard deviation

Release into *perfusion fluid plasma* as percentage of applied dose (%)

Time of sampling (min)	*Lung lobe perfusion experiment (No)*						*Mean*	*SD*
	1	*2*	*3*	*4*	*5*	*6*		
-6	2.4	1.5	3.0	3.9	0	3.7	2.4	1.5
0	0	3.3	0	0.1	0	0.1	0.6	1.3
10	0	0.0	0	0.1	0	0.1	0	0
20	0	0.7	0	0.1	0	0.1	0.1	0.3
30	0	0.7	0	0.1	0	0.1	0.1	0.3
45	0	0.1	0	0.1	0	0.1	0	0
60	0	0.1	0	0.1	0	0.1	0	0

SD standard deviation

1.3.1.2 Samples withdrawn from the *reservoir*

Release into *whole perfusion fluid* as percentage of applied dose (%)

Time of sampling (min)	*Lung lobe perfusion experiment (No)*						*Mean*	*SD*
	1	*2*	*3*	*4*	*5*	*6*		
0	4.6	17.2	6.2	4.5	14.8	5.4	8.8	5.6
10	9.2	38.7	4.5	22.2	13.0	4.1	15.3	13.2
20	10.5	36.9	6.1	37.5	13.7	5.7	18.4	14.9
30	9.1	35.7	7.0	42.1	15.1	6.1	19.2	15.7
45	9.1	47.6	5.1	41.3	12.6	8.3	20.7	18.7
60	10.0	36.6	6.3	44.1	16.9	10.4	20.7	15.8

SD standard deviation

Release into *perfusion fluid plasma* as percentage of applied dose (%)

Time of sampling (min)	*Lung lobe perfusion experiment (No)*						*Mean*	*SD*
	1	*2*	*3*	*4*	*5*	*6*		
0	0	7.2	0	0	0	0	1.2	2.9
10	0	12.1	0	0	0	0	2.0	4.9
20	0	4.1	0	0	0	0	0.7	1.7
30	0	0.2	0	0	0	0	0.0	0.1
45	0	0.2	0	0	0	0	0.0	0.1
60	0	20.0	0	0	0	0	3.3	8.2

SD standard deviation

1.3.2 Time course of salbutamol in perfusion fluid

1.3.2.1 Samples withdrawn from *venous output*

Release into *whole perfusion fluid* as percentage of applied dose (%)

Time of sampling (min)	*Lung lobe perfusion experiment (No)*						*Mean*	*SD*
	1	*2*	*3*	*4*	*5*	*6*		
-6	3.0	5.5	12.5	4.9	2.0	10.2	6.4	4.2
0	8.5	6.2	31.8	10.6	6.4	24.7	14.7	10.8
10	17.8	19.6	37.1	32.8	13.7	23.4	24.1	9.1
20	22.1	48.3	23.8	36.4	17.4	20.7	28.1	11.9
30	20.0	59.9	19.0	32.8	16.1	22.1	28.1	14.9
45	18.1	54.0	17.8	38.0	18.7	22.6	28.2	14.8
60	23.8	52.8	14.9	44.6	16.3	23.7	29.4	15.6

SD standard deviation

Release into *perfusion fluid plasma* as percentage of applied dose (%)

Time of sampling (min)	*Lung lobe perfusion experiment (No)*						*Mean*	*SD*
	1	*2*	*3*	*4*	*5*	*6*		
-6	10.9	21.0	18.5	17.8	2.1	19.5	15.0	7.2
0	0	1.9	20.5	7.1	3.3	13.9	7.8	7.9
10	9.9	9.2	28.3	8.9	6.5	13.8	12.7	8.0
20	14.2	30.4	18.9	11.3	6.8	14.0	15.9	8.1
30	12.2	54.4	18.8	9.8	5.3	17.9	19.8	17.7
45	12.1	51.5	15.8	10.4	3.9	16.9	18.4	16.9
60	13.5	62.4	20.4	11.7	3.8	15.8	21.3	20.9

SD standard deviation

1.3.2.2 Samples withdrawn from the *reservoir*

Release into *whole perfusion fluid* as percentage of applied dose (%)

Time of sampling (min)	*Lung lobe perfusion experiment (No)*						*Mean*	*SD*
	1	*2*	*3*	*4*	*5*	*6*		
0	7.2	28.1	6.5	5.4	18.3	12.4	13.0	8.8
10	17.5	68.5	6.3	31.6	16.8	14.4	25.9	22.5
20	19.0	62.4	10.2	42.7	18.8	18.5	28.6	19.8
30	18.7	67.8	9.2	43.4	22.0	21.4	30.4	21.5
45	22.3	59.1	11.3	40.9	18.8	23.3	29.3	17.6
60	18.0	57.6	11.3	40.0	18.8	26.2	28.7	17.2

SD standard deviation

Release into *perfusion fluid plasma* as percentage of applied dose (%)

Time of sampling (min)	*Lung lobe perfusion experiment (No)*						*Mean*	*SD*
	1	*2*	*3*	*4*	*5*	*6*		
0	0	24.8	4.4	1.9	9.2	7.0	7.9	8.9
10	12.0	70.8	4.5	6.8	6.6	7.6	18.0	26.0
20	11.8	56.0	7.7	9.3	4.5	13.2	17.1	19.3
30	12.7	53.1	9.2	10.7	4.1	11.8	16.9	18.0
45	14.4	51.4	9.8	12.7	4.0	10.4	17.1	17.2
60	13.9	52.9	7.4	13.8	3.8	8.3	16.7	18.2

SD standard deviation

1.3.3 Perfusate concentration to tissue concentration ratio

Perfusion fluid* concentration to tissue concentration ratio of *GW597901 (%)*

Site of sampling	*Lung lobe perfusion experiment (No)*						*Mean*	*SD*
	1	*2*	*3*	*4*	*5*	*6*		
1	13.6	9.1	2.0	139.1	23.4	1.5	31.4	53.3
2	1.6	47.5	5.4	58.4	20.7	8.1	23.6	23.8
3	6.4	44.5	4.4	72.9	15.9	13.8	26.3	27.0
4	10.7	39.0	9.4	67.0	17.9	12.4	26.1	22.8

* Concentration in whole perfusion fluid sample from venous output drawn at 60 min

Perfusion fluid* concentration to tissue concentration ratio of *salbutamol (%)*

Site of sampling	*Lung lobe perfusion experiment (No)*						*Mean*	*SD*
	1	*2*	*3*	*4*	*5*	*6*		
1	45.8	29.0	5.6	324.0	61.0	1.8	77.9	122.7
2	11.5	123.5	10.7	34.8	51.4	30.5	43.7	42.0
3	17.3	116.6	19.6	75.5	33.0	29.3	48.6	39.2
4	59.2	139.8	41.8	195.3	5.9	98.4	90.1	69.2

* Concentration in whole perfusion fluid sample from venous output drawn at 60 min

1.3.4 Total fraction of applied doses recovered in lung tissue

Lung lobe perfusion experiment	Mean percentage of applied dose (%)*	
	Salbutamol	GW597901
1	18.5	43.5
2	19.6	41.4
3	43.5	98.5
4	26.2	29.4
5	20.6	15.1
6	86.3	50.6

* For detailed data of each lung lobe perfusion experiments see *1.2*.

1.3.5 Concentrations of GW597901 and salbutamol in BAL fluid samples

Lung lobe perfusion experiment	Volume of BAL fluid (mL)	Recovered volume of BAL fluid (%)
1	5.0	20
2	5.0	12
3	5.0	14
4	10.0	9
5	5.0	26
6	5.0	4

Lung lobe perfusion experiment	Concentration (ng/mL) analyzed in BAL fluid		Total amount (µg) recovered in BAL fluid		Percentage of applied dose recovered in BAL fluid (%)	
	Salbutamol	GW597901	Salbutamol	GW597901	GW597901	Salbutamol
1	21.6	8.2	0.1	0.04	0.08	0.09
2	37.2	7.6	0.2	0.04	0.13	0.08
3	27.1	4.6	0.1	0.02	0.10	0.07
4	1390.0	312.0	13.9	3.12	3.71	2.90
5	3060.0	708.0	15.3	3.54	2.94	2.26
6	2030.0	478.0	10.15	2.39	3.32	2.72

2 PK/PD study of GW597901 and salbutamol

2.1 Results of lung perfusion experiments for the PK/PD characterization of GW597901

2.1.1 Conditions of human lung lobe perfusion experiments

Lung lobe perfusion experiment	Haematocrit in perfusion fluid (%)	Weight of the perfused human lung tissue (g)		
		Prior to experiment	After termination of the experiment	Weight gain in %
1	20.7	146	208	42.5
2	19.5	310	378	21.9
3	22.6	154	162	5.2
4	18.2	427	495	15.9
5	20.5	455	480	5.5
6	19.5	310	319	2.9

Lung lobe perfusion experiment	Amount of nebulized drug (µg)	Adsorbed amount of nebulized drug (%)	Actually applied dose (µg)	Percentage of targeted dose* (%)
1	166.2	21.6	130.2	72.3
2	154.1	16.4	128.9	71.6
3	189.0	17.4	156.1	86.7
4	117.9	9.6	106.6	59.2
5	135.5	13.8	116.9	64.9
6	179.1	22.3	139.1	77.3
Mean	**157.0**	**16.9**	**129.6**	**72.0**

* Targeted dose was 180 µg

2.1.2 Lung lobe perfusion experiment GW597901-1

- **Pharmacokinetic data**

Time of sampling (min)	*Concentration in (ng/mL)*	
	Venous output	Reservoir
-10	0.3	-
-7	0.3	-
-2	0.3	-
0	0.2	0.3
10	0.3	0.3
20	0.4	0.5
30	0.5	0.6
45	0.7	0.8
60	0.9	0.8

- Data were not collected

- **Pharmacodynamic data**

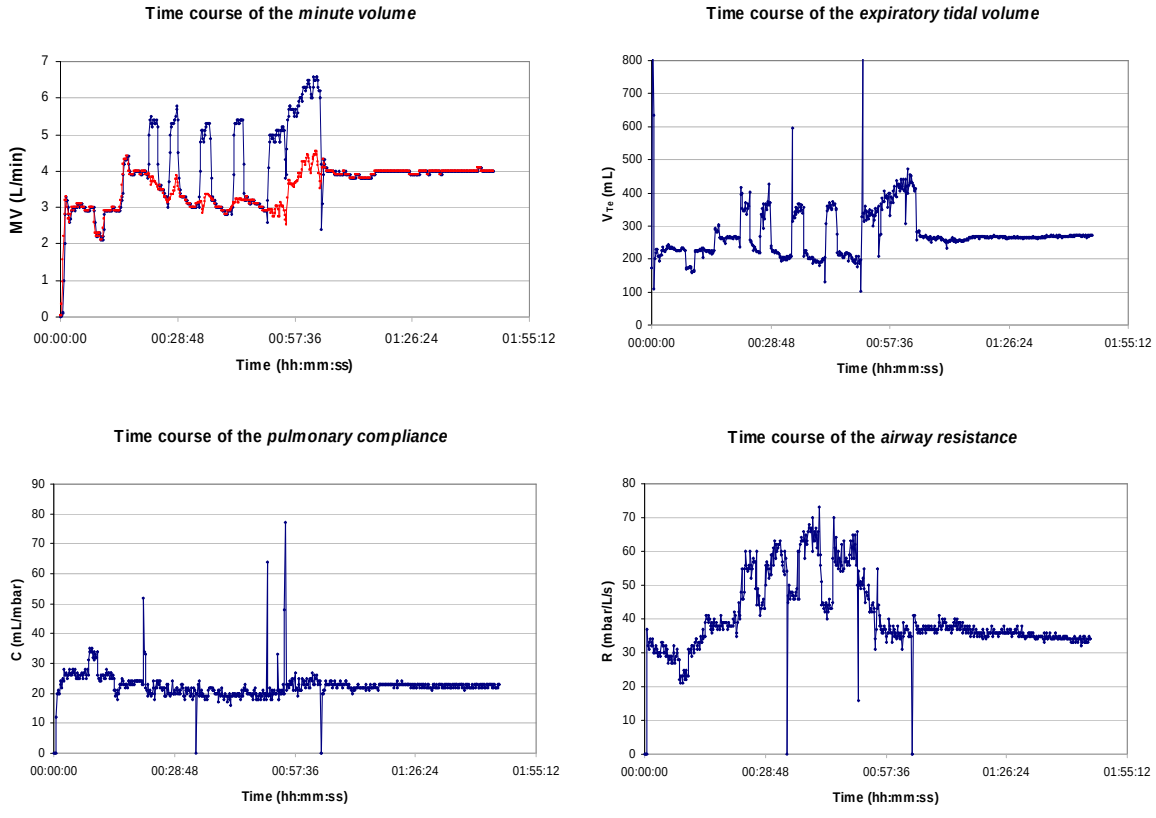

Figure 36: Online recordings of ventilation data for the PD characterization of GW597901 in lung perfusion experiment 1. Blue data points are raw data, red data points represent normalised data curve of the minute volume without the influence of the nebulizer.

Table 27: Summary of ventilation data in perfusion experiment GW597901 - 1

Parameter	*Reference value*		*After bronchoconstriction*		*After bronchodilation*	
	Mean (SD) (n= 16)	Reference (%)	Mean (SD) (n= 12)	Reference (%)	Mean (SD) (n= 16)	Reference (%)
MV (L/min)	4.0 (0.1)	100	2.9 (0.1)	70.9	4.0 (0)	101.0
V_{Te} (mL)	262.5 (5.0)	100	185.5 (29.6)	70.7	266.8 (1.9)	100.7
C (mL/mbar)	23.3 (0.7)	100	19.3 (1.0)	82.6	23.0 (0)	98.7
R (mbar/L/s)	38.4 (1.0)	100	60.5 (4.6)	157.5	35.9 (1.0)	93.4

MV – minute volume; V_{Te} – expiratory tidal volume; C – pulmonary compliance; R – airway resistance
SD – standard deviation

2.1.3 Lung lobe perfusion experiment GW597901-2

- **Pharmacokinetic data**

Time of sampling (min)	*Concentration in (ng/mL)*	
	Venous output	Reservoir
-10	0.3	-
-7	0.2	-
-2	0.8	-
0	1.6	0.3
10	0.8	0.2
20	0.6	0.9
30	2.8	0.6
45	5.2	0.8
60	4.8	0.6

- Data were not collected

- **Pharmacodynamic data**

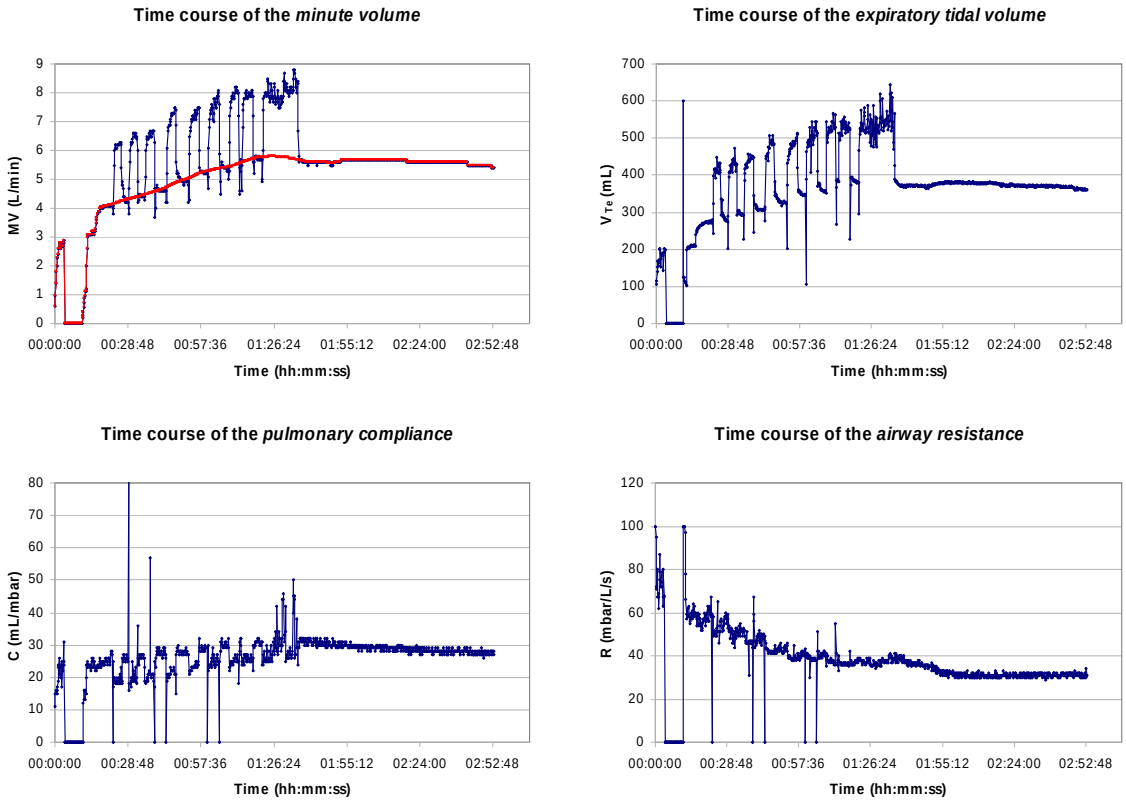

Figure 37: Online recordings of ventilation data for the PD characterization of GW597901 in lung perfusion experiment 2. Blue data points are raw data, red data points represent normalised data curve of the minute volume without the influence of the nebulizer.

Table 28: Summary of ventilation data in perfusion experiment GW597901 - 2

Parameter	*Reference value*		*After bronchoconstriction*		*After bronchodilation*	
	Mean (SD) (n= 16)	Reference (%)	Mean (SD)	Reference (%)	Mean (SD) (n= 16)	Reference (%)
MV (L/min)	4.1 (0)	100	-	-	5.6 (0)	136.3
V_{Te} (mL)	274.5 (2.2)	100	-	-	371.8 (2.1)	135.4
C (mL/mbar)	26.0 (1.3)	100	-	-	31.1 (0.7)	119.7
R (mbar/L/s)	60.2 (3.0)	100	-	-	36.8 (1.1)	61.0

MV – minute volume; V_{Te} – expiratory tidal volume; C – pulmonary compliance; R – airway resistance
SD – standard deviation; - data were not collected (no successful bronchoconstriction)

2.1.4 Lung lobe perfusion experiment GW597901-3

- **Pharmacokinetic data**

Time of sampling (min)	*Concentration in (ng/mL)*	
	Venous output	Reservoir
-10	0.2	-
-7	0.2	-
-2	0.3	-
0	0.3	0.4
10	0.5	0.9
20	4.2	0.5
30	0.7	0.5
45	0.7	0.5
60	0.6	0.7

- Data were not collected

- **Pharmacodynamic data**

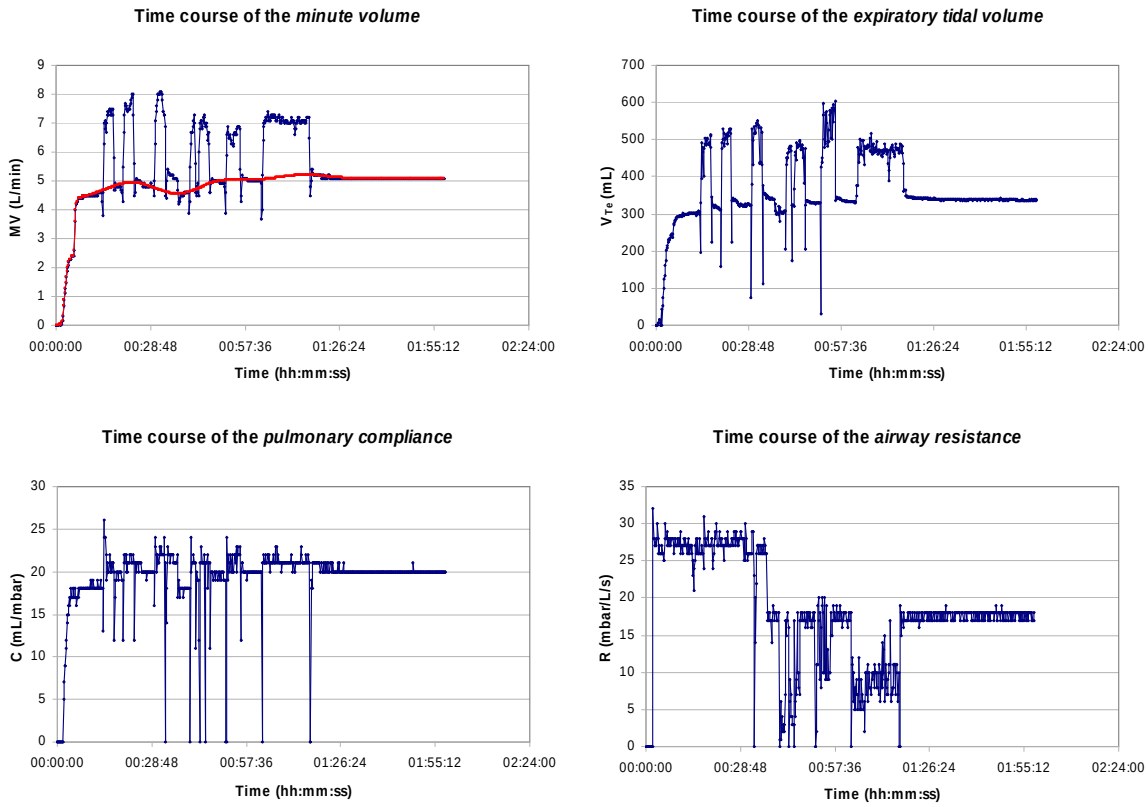

Figure 38: Online recordings of ventilation data for the PD characterization of GW597901 in lung perfusion experiment 3. Blue data points are raw data, red data points represent normalised data curve of the minute volume without the influence of the nebulizer.

Table 29: Summary of ventilation data in perfusion experiment GW597901 - 3

Parameter	*Reference value*		*After bronchoconstriction*		*After bronchodilation*	
	Mean (SD) (n= 16)	Reference (%)	Mean (SD)	Reference (%)	Mean (SD) (n= 16)	Reference (%)
MV (L/min)	4.5 (0.1)	100	-	-	5.2 (0.1)	115.0
V_{Te} (mL)	298.0 (27.1)	100	-	-	344.3 (1.2)	115.5
C (mL/mbar)	18.3 (2.4)	100	-	-	20.8 (0.4)	113.8
R (mbar/L/s)	26.2 (1.8)	100	-	-	17.4 (0.5)	66.4

MV – minute volume; V_{Te} – expiratory tidal volume; C – pulmonary compliance; R – airway resistance
SD – standard deviation; - data were not collected (no successful bronchoconstriction)

2.1.5 Lung lobe perfusion experiment GW597901-4

- **Pharmacokinetic data**

Time of sampling (min)	*Concentration in (ng/mL)*	
	Venous output	Reservoir
-10	3.3	-
-7	3.7	-
-2	5.8	-
0	5.4	3.2
10	5.1	4.3
20	3.8	4.4
30	5.9	4.1
45	5.8	5.0
60	-	-

- Data were not collected

- **Pharmacodynamic data**

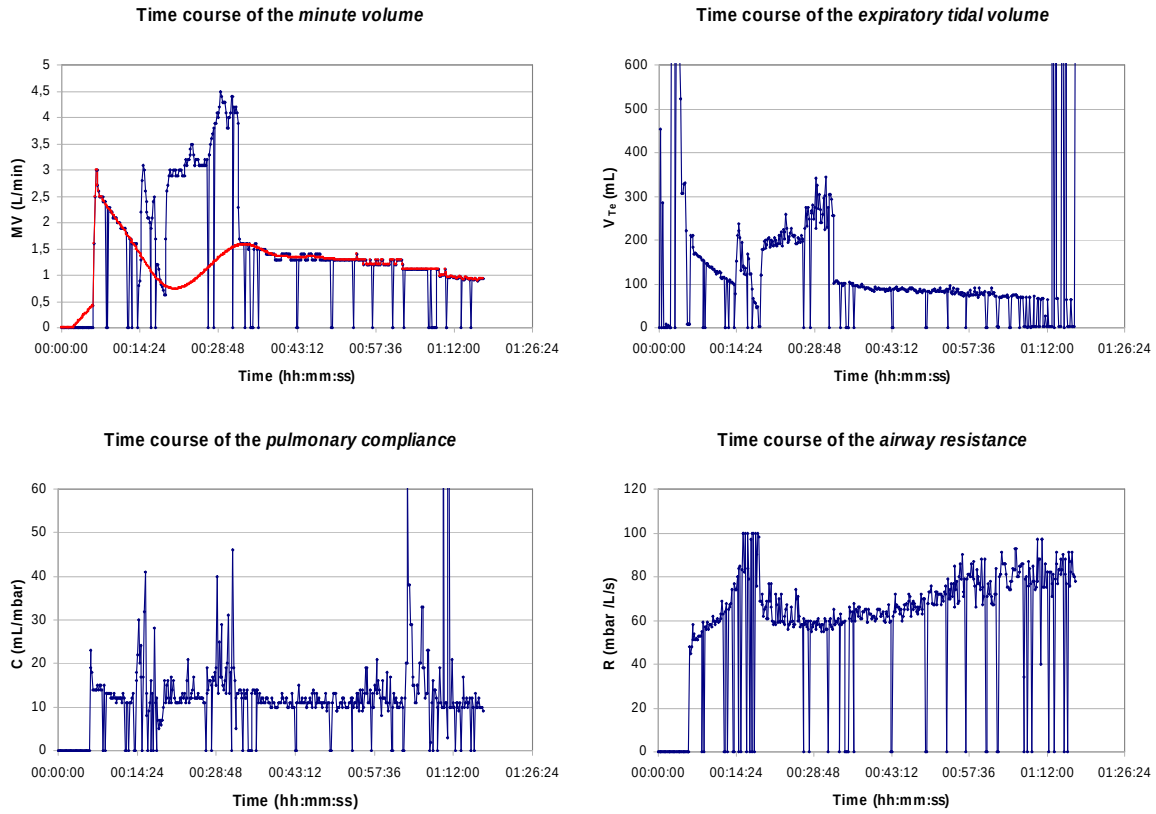

Figure 39: Online recordings of ventilation data for the PD characterization of GW597901 in lung perfusion experiment 4. Blue data points are raw data, red data points represent normalised data curve of the minute volume without the influence of the nebulizer.

Table 30: Summary of ventilation data in perfusion experiment GW597901 - 4

Parameter	*Reference value*		*After bronchoconstriction*		*After bronchodilation*	
	Mean (SD) (n= 16)	Reference (%)	Mean (SD) (n= 10)	Reference (%)	Mean (SD) (n= 16)	Reference (%)
MV (L/min)	2.0 (0.1)	100	0.9 (0.2)	45.0	1.6 (0.1)	80.6
V_{Te} (mL)	126.1 (8.6)	100	53.8 (8.9)	42.7	101.9 (0.8)	80.8
C (mL/mbar)	12.1 (0.6)	100	7.3 (2.2)	60.4	13.3 (3.1)	109.8
R (mbar/L/s)	61.1 (2.9)	100	89.1 (13.9)	145.7	60.5 (2.5)	98.9

MV – minute volume; V_{Te} – expiratory tidal volume; C – pulmonary compliance; R – airway resistance
SD – standard deviation

2.1.6 Lung lobe perfusion experiment GW597901-5

- **Pharmacokinetic data**

Time of sampling (min)	*Concentration in (ng/mL)*	
	Venous output	Reservoir
-10	0.9	-
-7	0.3	-
-2	0.4	-
0	0.4	0.7
10	1.1	0.4
20	1.3	1.7
30	2.0	4.1
45	2.1	2.3
60	2.3	2.7

- Data were not collected

- **Pharmacodynamic data**

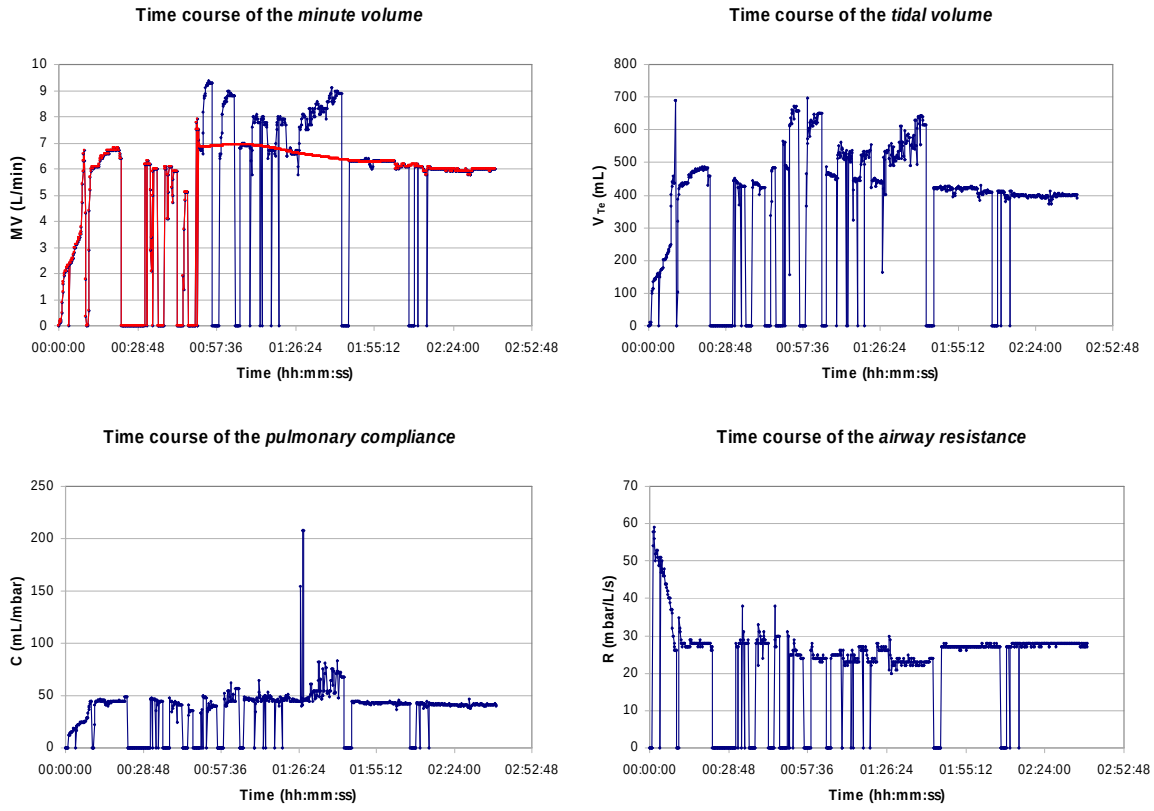

Figure 40: Online recordings of ventilation data for the PD characterization of GW597901 in lung perfusion experiment 5. Blue data points are raw data, red data points represent normalised data curve of the minute volume without the influence of the nebulizer.

Table 31: Summary of ventilation data in perfusion experiment GW597901 - 5

Parameter	*Reference value*		*After bronchoconstriction*		*After bronchodilation*	
	Mean (SD) (n= 16)	Reference (%)	Mean (SD)	Reference (%)	Mean (SD) (n= 16)	Reference (%)
MV (L/min)	6.7 (0)	100	-	-	6.3 (0)	93.8
V_{Te} (mL)	481.1 (3.2)	100	-	-	422.4 (2.6)	87.8
C (mL/mbar)	44.6 (0.6)	100	-	-	43.8 (0.7)	98.0
R (mbar/L/s)	27.9 (0.3)	100	-	-	27.1 (0.3)	96.9

MV – minute volume; V_{Te} – expiratory tidal volume; C – pulmonary compliance; R – airway resistance
SD – standard deviation; - data were not collected (no successful bronchoconstriction)

2.1.7 Lung lobe perfusion experiment GW597901-6

- **Pharmacokinetic data**

Time of sampling (min)	*Concentration in (ng/mL)*	
	Venous output	Reservoir
-10	0.2	-
-7	0.2	-
-2	0.4	-
0	0.7	0.4
10	0.9	0.4
20	1.4	1.4
30	1.2	1.3
45	1.7	1.1
60	1.7	1.6

- Data were not collected

- **Pharmacodynamic data**

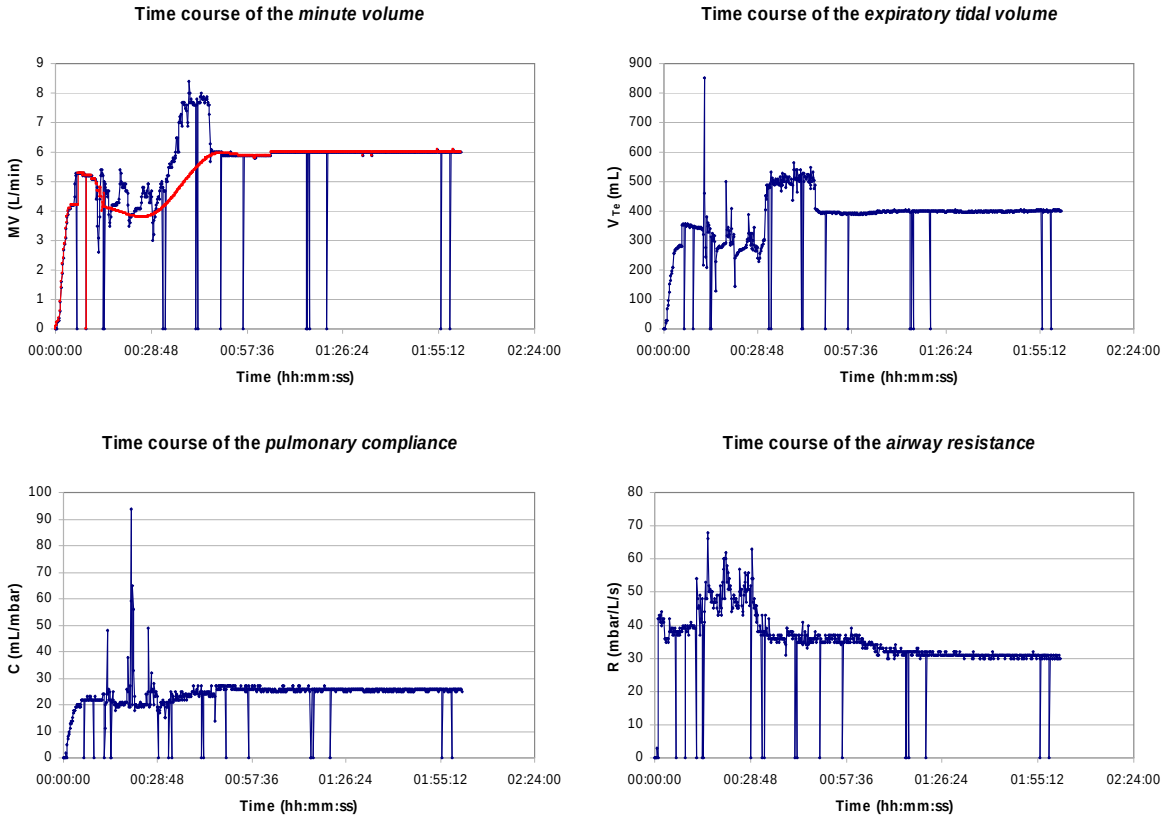

Figure 41: Online recordings of ventilation data for the PD characterization of GW597901 in lung perfusion experiment 6. Blue data points are raw data, red data points represent normalised data curve of the minute volume without the influence of the nebulizer.

Table 32: Summary of ventilation data in perfusion experiment GW597901 - 6

Parameter	*Reference value*		*After bronchoconstriction*		*After bronchodilation*	
	Mean (SD) (n= 16)	Reference (%)	Mean (SD) (n= 5)	Reference (%)	Mean (SD) (n= 18)	Reference (%)
MV (L/min)	5.3 (0)	100	3.5 (0.3)	65.6	6.0 (0)	113.9
V_{Te} (mL)	350.8 (3.7)	100	240.0 (11.4)	68.4	396.7 (3.0)	113.1
C (mL/mbar)	22.2 (0.4)	100	18.3 (1.0)	82.2	26.7 (0.5)	120.1
R (mbar/L/s)	38.6 (1.0)	100	54.0 (5.8)	139.9	36.2 (0.9)	93.8

MV – minute volume; V_{Te} – expiratory tidal volume; C – pulmonary compliance; R – airway resistance
SD – standard deviation

2.2 Results of lung perfusion experiments for the PK/PD characterization of salbutamol

2.2.1 Conditions of human lung lobe perfusion experiments

Lung lobe perfusion experiment	Weight of the perfused human lung tissue (g)		
	Prior to experiment	After termination of the experiment	Weight gain in %
1	130	163	25.4
2	199	401	101.5
3	286	658	130.1
4	239	289	20.9
5	838	792	-
6	314	531	69.1
7	1225	1450	18.4

- Data were not collected

Lung lobe perfusion experiment	Amount of nebulized drug (µg)	Adsorbed amount of nebulized drug (%)	Actually applied dose (µg)	Percentage of targeted dose* (%)
1	492.1	8.1	452.1	75.4
2	636.0	15.0	540.7	90.1
3	505.5	5.3	478.9	79.8
4	419.0	22.0	326.8	54.5
5	311.1	25.1	232.8	38.8
6	328.1	24.6	247.3	41.2
Mean	**448.6**	**16.7**	**379.8**	**63.3**

* Targeted dose was 600 µg

2.2.2 Lung lobe perfusion experiment salbutamol-1

- **Pharmacokinetic data**

Time of sampling (min)	*Concentration in (ng/mL)*	
	Venous output	Reservoir
-10	0.5	-
-7	0.5	-
-2	0.6	-
0	0.5	0.3
10	1.0	0.5
20	0.7	0.6
30	0.7	0.8
45	0.8	0.8
60	1.2	1.7

- Data were not collected

- **Pharmacodynamic data**

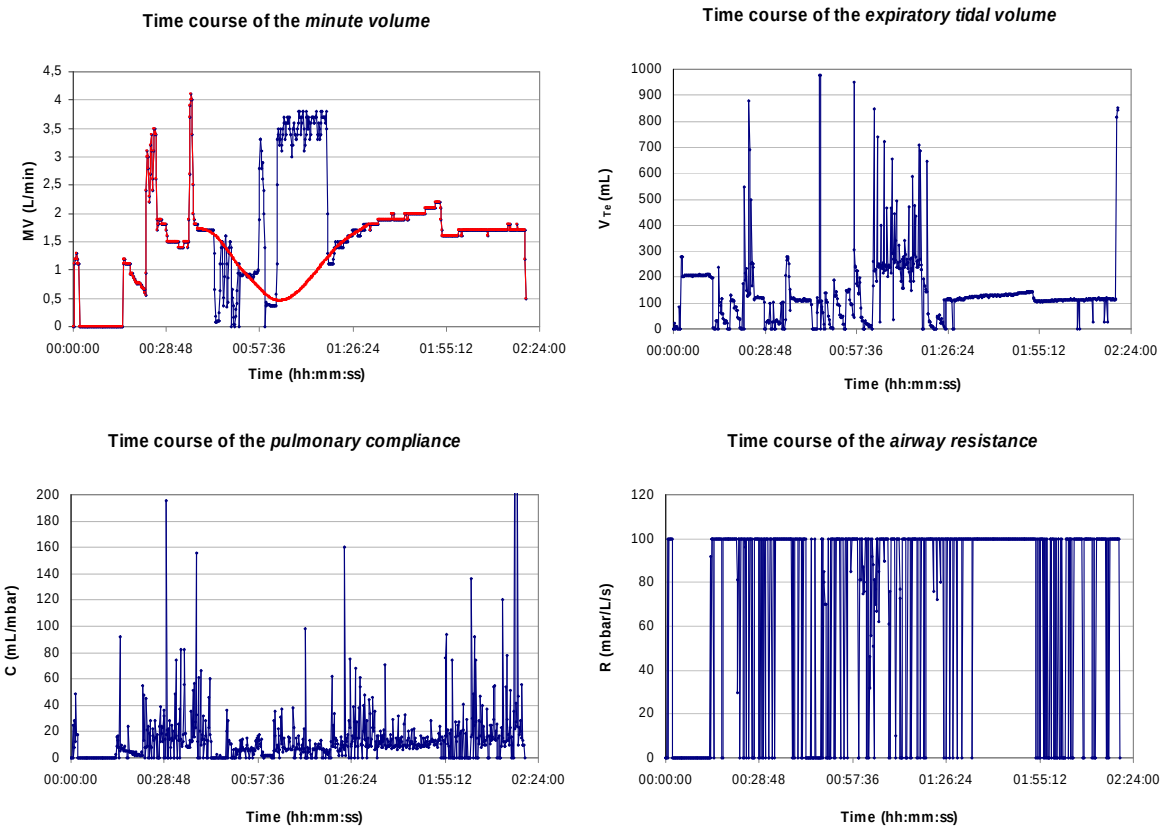

Figure 42: Online recordings of ventilation data for the PD characterization of salbutamol in lung perfusion experiment 1. Blue data points are raw data, red data points represent normalised data curve of the minute volume without the influence of the nebulizer.

Table 33: Summary of ventilation data in perfusion experiment salbutamol - 1

Parameter	*Reference value*		*After bronchoconstriction*		*After bronchodilation*	
	Mean (SD) (n= 16)	Reference (%)	Mean (SD) (n= 12)	Reference (%)	Mean (SD) (n= 16)	Reference (%)
MV (L/min)	1.7 (0)	100	0.4 (0)	22.5	1.7 (0)	100.0
V_{Te} (mL)	111.1 (2.6)	100	17.9 (6.4)	16.1	110.4 (0.6)	99.4
C (mL/mbar)	14.4 (2.6	100	9.3 (0)	64.4	20.2 (12.3)	139.8
R (mbar/L/s)	100.0 (0.0)	100	89.9 (11.1)	89.9	100.0 (0)	100.0

MV – minute volume; V_{Te} – expiratory tidal volume; C – pulmonary compliance; R – airway resistance
SD – standard deviation

2.2.3 Lung lobe perfusion experiment salbutamol-2

- **Pharmacokinetic data**

Time of sampling (min)	*Concentration in (ng/mL)*	
	Venous output	Reservoir
-10	1.1	-
-7	2.5	-
-2	4.8	-
0	8.0	4.6
10	8.6	7.1
20	11.5	8.6
30	11.2	12.2
45	-	-
60	-	-

- Data were not collected

- **Pharmacodynamic data**

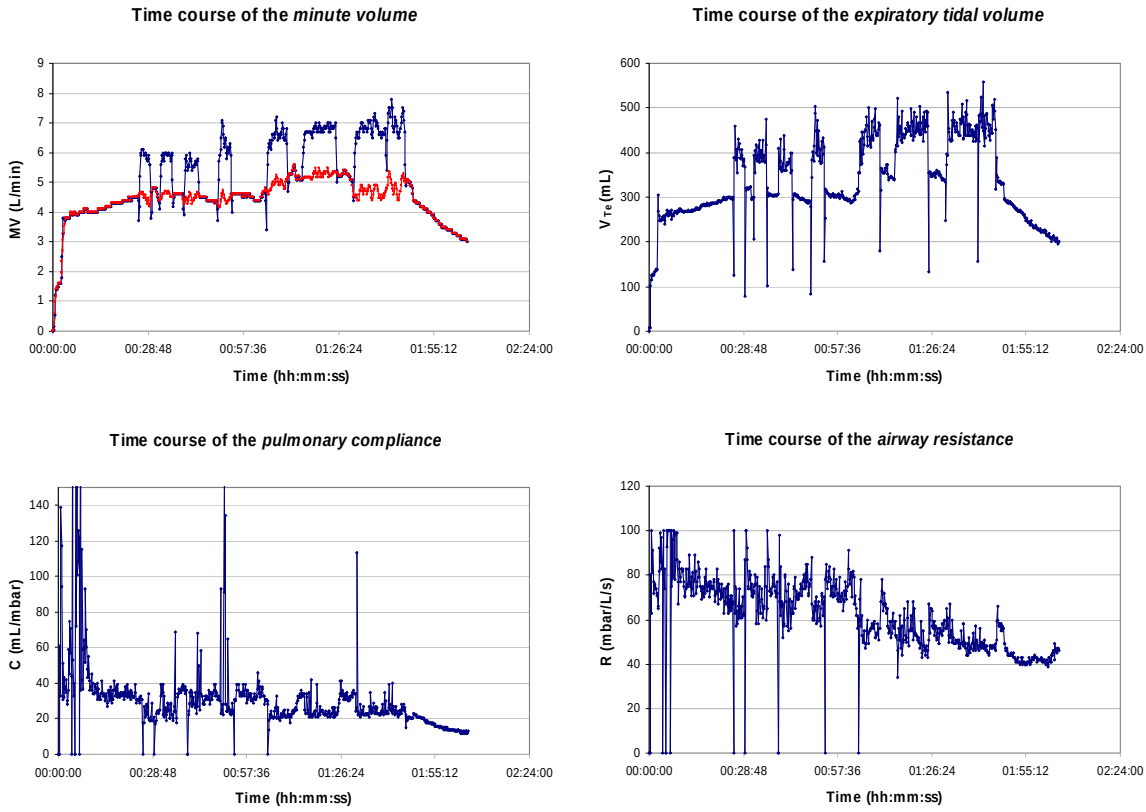

Figure 43: Online recordings of ventilation data for the PD characterization of salbutamol in lung perfusion experiment 2. Blue data points are raw data, red data points represent normalised data curve of the minute volume without the influence of the nebulizer.

Table 34: Summary of ventilation data in perfusion experiment salbutamol - 2

Parameter	*Reference value*		*After bronchoconstriction*		*After bronchodilation*	
	Mean (SD) (n= 16)	Reference (%)	Mean (SD)	Reference (%)	Mean (SD) (n= 16)	Reference (%)
MV (L/min)	4.4 (0.1)	100	-	-	4.3 (0.1)	98.7
V_{Te} (mL)	291.4 (3.3)	100	-	-	286.5 (4.0)	98.3
C (mL/mbar)	34.2 (2.5)	100	-	-	20.9 (0.7)	61.1
R (mbar/L/s)	73.4 (3.9)	100	-	-	44.5 (1.1)	60.6

MV – minute volume; V_{Te} – expiratory tidal volume; C – pulmonary compliance; R – airway resistance
SD – standard deviation; - data were not collected (no successful bronchoconstriction)

2.2.4 Lung lobe perfusion experiment salbutamol-3

- **Pharmacokinetic data**

Time of sampling (min)	*Concentration in (ng/mL)*	
	Venous output	Reservoir
-10	4.0	-
-7	4.3	-
-2	6.1	-
0	9.0	6.6
10	11.2	9.3
20	13.4	11.0
30	14.0	11.9
45	13.1	14.3
60	15.9	14.4

- Data were not collected

- **Pharmacodynamic data**

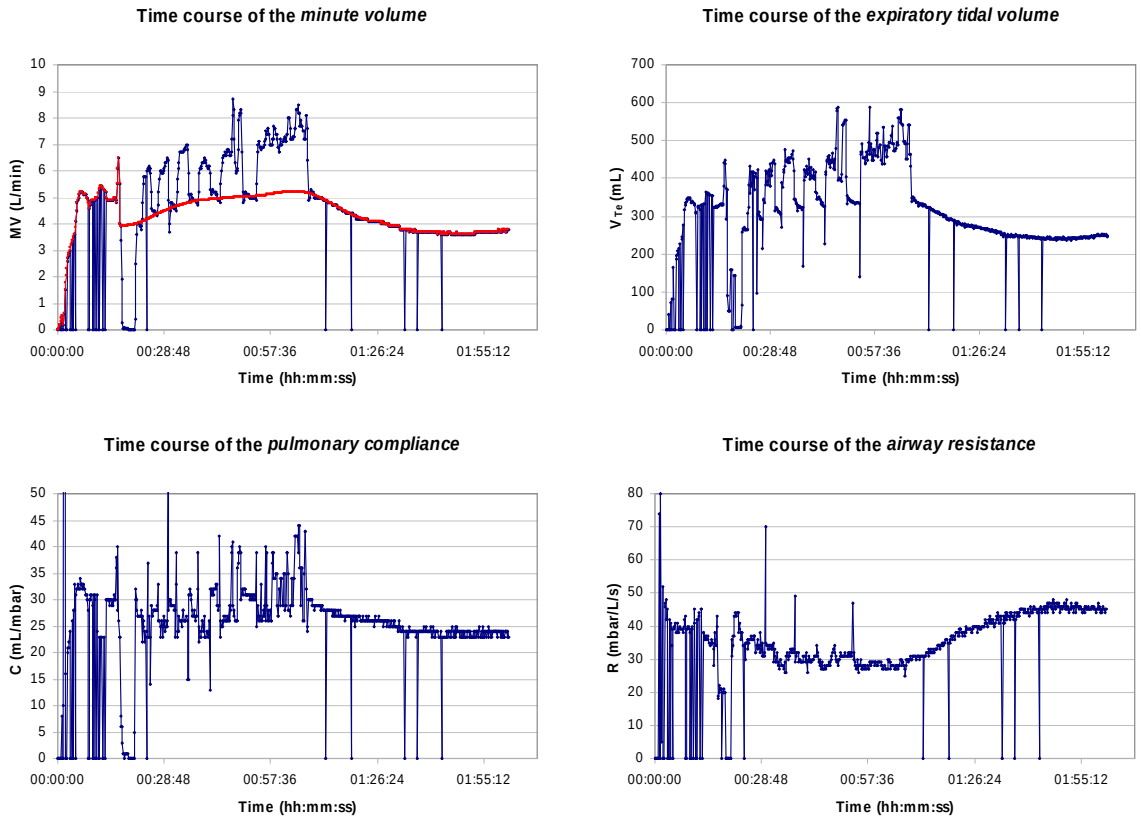

Figure 44: Online recordings of ventilation data for the PD characterization of salbutamol in lung perfusion experiment 3. Blue data points are raw data, red data points represent normalised data curve of the minute volume without the influence of the nebulizer.

Table 35: Summary of ventilation data in perfusion experiment salbutamol - 3

Parameter	*Reference value*		*After bronchoconstriction*		*After bronchodilation*	
	Mean (SD) (n= 16)	Reference (%)	Mean (SD)	Reference (%)	Mean (SD) (n= 16)	Reference (%)
MV (L/min)	4.9 (0.1)	100	-	-	5.0 (0.1)	100.9
V_{Te} (mL)	343.4 (37.0)	100	-	-	327.3 (2.9)	95.3
C (mL/mbar)	31.1 (3.3)	100	-	-	28.4 (0.5)	91.2
R (mbar/L/s)	35.2 (2.2)	100	-	-	30.9 (0.3)	87.9

MV – minute volume; V_{Te} – expiratory tidal volume; C – pulmonary compliance; R – airway resistance
SD – standard deviation; - data were not collected (no successful bronchoconstriction)

2.2.5 Lung lobe perfusion experiment salbutamol-4

- **Pharmacokinetic data**

Time of sampling (min)	*Concentration in (ng/mL)*	
	Venous output	Reservoir
-10	4.5	-
-7	5.7	-
-2	11.7	-
0	14.0	13.9
10	16.8	15.3
20	-	17.5
30	-	-
45	-	-
60	-	-

- Data were not collected

- **Pharmacodynamic data**

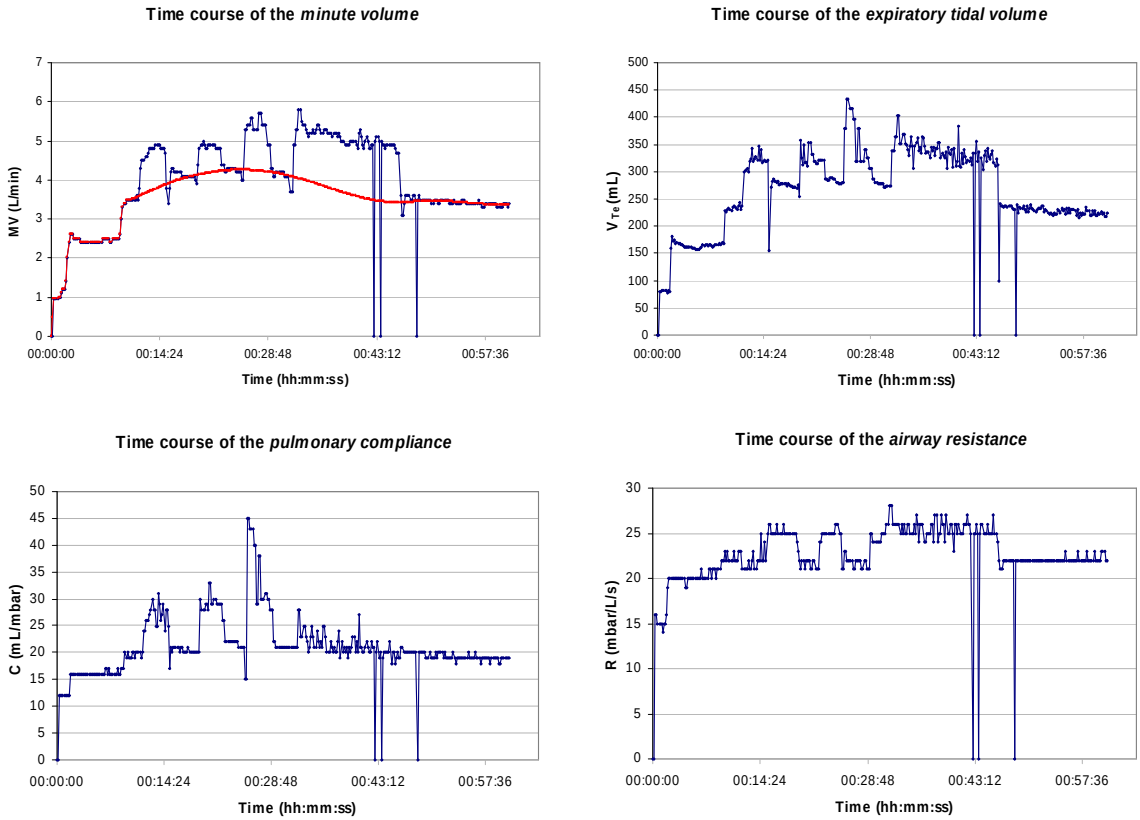

Figure 45: Online recordings of ventilation data for the PD characterization of salbutamol in lung perfusion experiment 4. Blue data points are raw data, red data points represent normalised data curve of the minute volume without the influence of the nebulizer.

Table 36: Summary of ventilation data in perfusion experiment salbutamol - 4

Parameter	*Reference value*		*After bronchoconstriction*		*After bronchodilation*	
	Mean (SD) (n= 16)	Reference (%)	Mean (SD)	Reference (%)	Mean (SD) (n= 16)	Reference (%)
MV (L/min)	3.6 (0.3)	100	-	-	3.5 (0.1)	97.2
V_{Te} (mL)	246.6 (27.4)	100	-	-	234.3 (4.1)	95.0
C (mL/mbar)	20.6 (2.1)	100	-	-	20.0 (0)	97.3
R (mbar/L/s)	22.3 (0.6)	100	-	-	21.9 (0.3)	98.0

MV – minute volume; V_{Te} – expiratory tidal volume; C – pulmonary compliance; R – airway resistance
SD – standard deviation; - data were not collected (no successful bronchoconstriction)

2.2.6 Lung lobe perfusion experiment salbutamol-5

- **Pharmacokinetic data**

Time of sampling (min)	*Concentration in (ng/mL)*	
	Venous output	Reservoir
-10	1.2	-
-7	4.9	-
-2	5.2	-
0	5.7	4.8
10	6.3	6.7
20	6.4	7.2
30	6.4	7.3
45	7.5	6.9
60	6.9	8.2

- Data were not collected

- **Pharmacodynamic data**

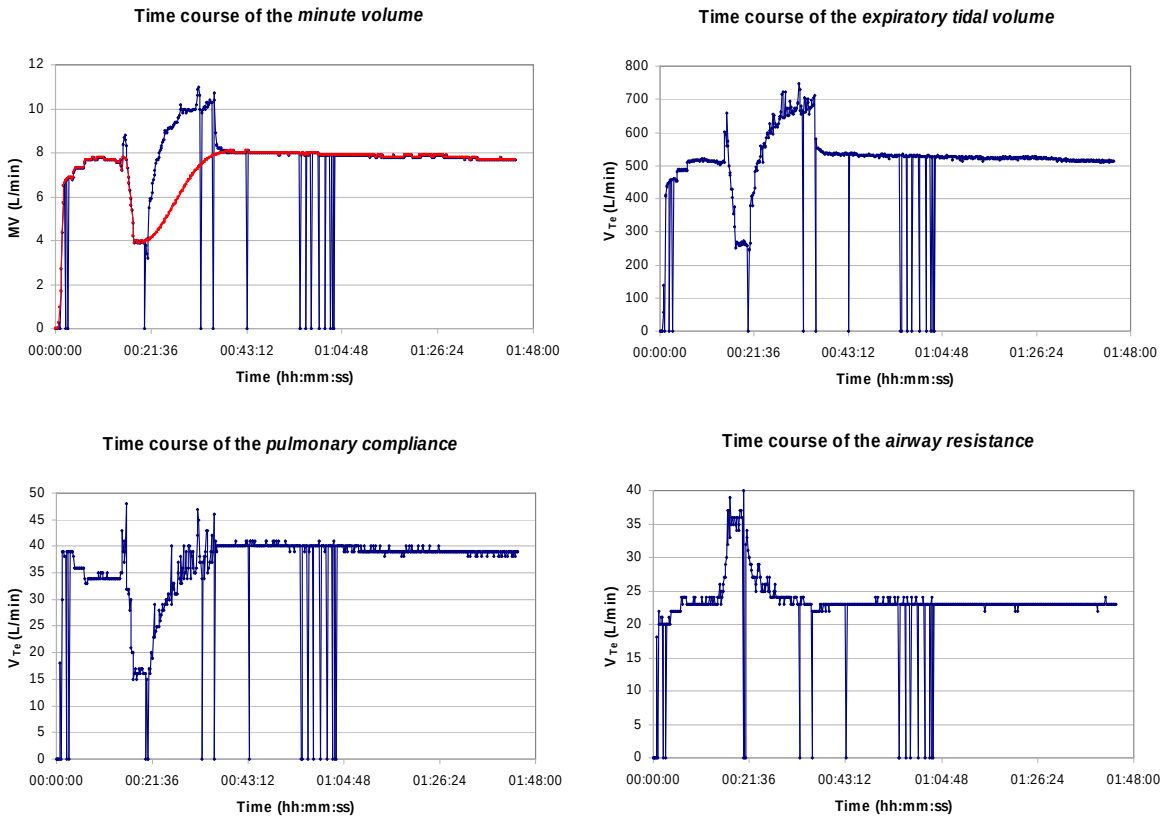

Figure 46: Online recordings of ventilation data for the PD characterization of salbutamol in lung perfusion experiment 5. Blue data points are raw data, red data points represent normalised data curve of the minute volume without the influence of the nebulizer.

Table 37: Summary of ventilation data in perfusion experiment salbutamol - 5

Parameter	*Reference value*		*After bronchoconstriction*		*After bronchodilation*	
	Mean (SD) (n= 18)	Reference (%)	Mean (SD) (n= 18)	Reference (%)	Mean (SD) (n= 18)	Reference (%)
MV (L/min)	7.7 (0)	100	3.9 (0.1)	50.7	8.0 (0)	104.3
V_{Te} (mL)	511.4 (3.8)	100	261.4 (6.7)	51.1	534.7 (2.0)	104.5
C (mL/mbar)	34.1 (0.2)	100	16.1 (0.6)	47.4	40.1 (0.2)	117.6
R (mbar/L/s)	23.3 (0.5)	100	35.9 (1.4)	154.4	22.9 (0.2)	98.6

MV – minute volume; V_{Te} – expiratory tidal volume; C – pulmonary compliance; R – airway resistance
SD – standard deviation

2.2.7 Lung lobe perfusion experiment salbutamol-6

- **Pharmacokinetic data**

Time of sampling (min)	*Concentration in (ng/mL)*	
	Venous output	Reservoir
-10	1.6	-
-7	5.3	-
-2	9.8	-
0	12.4	10.8
10	16.5	13.2
20	16.7	15.8
30	17.2	15.3
45	18.8	18.7
60	18.4	17.1

- Data were not collected

- **Pharmacodynamic data**

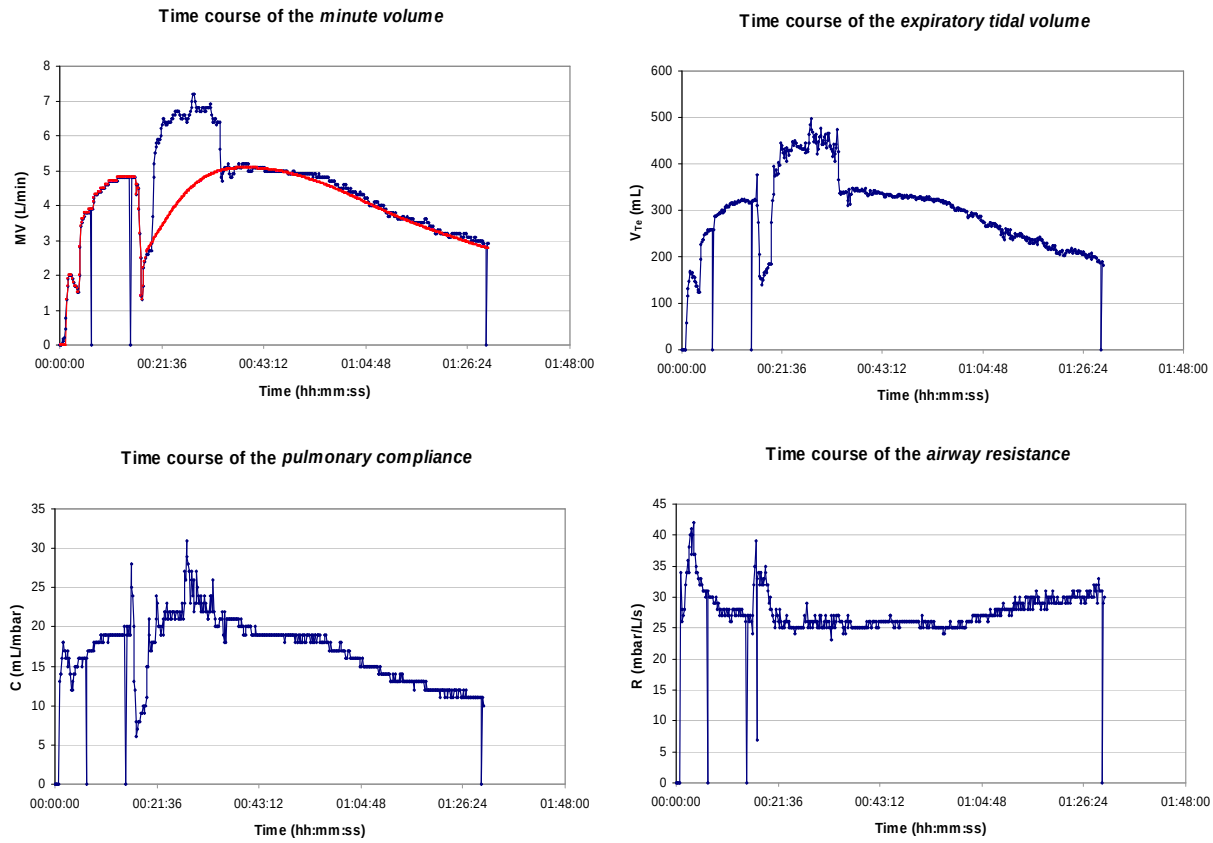

Figure 47: Online recordings of ventilation data for the PD characterization of salbutamol in lung perfusion experiment 6. Blue data points are raw data, red data points represent normalised data curve of the minute volume without the influence of the nebulizer.

Table 38: Summary of ventilation data in perfusion experiment salbutamol – 6

Parameter	*Reference value*		*After bronchoconstriction*		*After bronchodilation*	
	Mean (SD) (n= 22)	Reference (%)	Mean (SD) (n= 16)	Reference (%)	Mean (SD) (n= 16)	Reference (%)
MV (L/min)	4.8 (0)	100	2.4 (0.5)	50.4	5.1 (0.1)	105.8
V_{Te} (mL)	320.0 (2.0)	100	183.7 (47.1)	57.4	339.2 (9.9)	106.0
C (mL/mbar)	19.1 (0.4)	100	9.3 (2.1)	48.7	20.6 (1.0)	107.7
R (mbar/L/s)	27.2 (0.7)	100	31.7 (6.8)	116.6	25.2 (0.6)	92.7

MV – minute volume; V_{Te} – expiratory tidal volume; C – pulmonary compliance; R – airway resistance
SD – standard deviation

2.3 Data summary

2.3.1 Time course of GW597901 in perfusion fluid

Release into perfusion fluid as percentage of applied dose (%)
Samples withdrawn from venous output

Time of sampling (min)	*Lung lobe perfusion experiment (No)*						*Mean*	*SD*
	1	*2*	*3*	*4*	*5*	*6*		
-10	0.4	0.4	0.2	4.6	1.1	0.2	1.1	1.1
-7	0.3	0.2	0.2	5.3	0.4	0.3	1.1	1.4
-2	0.4	1.0	0.3	8.2	0.5	0.5	1.8	2.1
0	0.2	1.9	0.3	7.7	0.6	0.7	1.9	1.9
10	0.3	1.0	0.5	7.4	1.5	1.0	1.9	1.8
20	0.5	0.8	4.0	5.6	1.7	1.6	2.4	1.7
30	0.6	3.3	0.7	8.6	2.6	1.3	2.8	2.1
45	0.8	6.1	0.7	8.6	2.8	1.9	3.5	2.6
60	1.0	5.7	0.7	9.0	3.0	1.9	3.5	2.6

SD - standard deviation

Release into perfusion fluid as percentage of applied dose (%)
Samples withdrawn from the reservoir

Time of sampling (min)	*Lung lobe perfusion experiment (No)*						*Mean*	*SD*
	1	*2*	*3*	*4*	*5*	*6*		
0	0.3	0.4	0.4	4.5	0.9	0.4	1.1	1.1
10	0.4	0.2	0.9	6.1	0.5	0.4	1.4	1.6
20	0.6	1.0	0.5	6.3	2.2	1.5	2.0	1.5
30	0.7	0.8	0.5	5.9	5.3	1.4	2.4	2.1
45	0.9	1.0	0.5	7.1	3.0	1.2	2.3	1.9
60	0.9	0.7	0.7	6.0	3.5	1.8	2.3	1.7

SD - standard deviation

2.3.2 Time course of salbutamol in perfusion fluid

Release into perfusion fluid as percentage of applied dose (%)
Samples withdrawn from venous output

Time of sampling (min)	*Lung lobe perfusion experiment (No)*						*Mean*	*SD*
	1	*2*	*3*	*4*	*5*	*6*		
-10	0.2	0.3	1.3	2.0	0.8	1.0	0.9	0.7
-7	0.2	0.7	1.4	2.6	3.2	3.2	1.9	1.3
-2	0.2	1.3	1.9	5.4	3.4	5.9	3.0	2.3
0	0.2	2.2	2.9	6.5	3.8	7.6	3.9	2.8
10	0.3	2.4	3.6	7.8	4.1	10.1	4.7	3.6
20	0.2	3.2	4.3	-	4.3	10.3	4.5	3.7
30	0.3	3.2	4.5	-	4.3	10.6	4.6	3.8
45	0.3	-	4.2	-	5.0	11.7	5.3	4.7
60	3.8	-	5.1	-	4.7	11.6	6.3	3.6

SD - standard deviation

Release into perfusion fluid as percentage of applied dose (%)
Samples withdrawn from the reservoir

Time of sampling (min)	*Lung lobe perfusion experiment (No)*						*Mean*	*SD*
	1	*2*	*3*	*4*	*5*	*6*		
0	0.1	1.3	2.1	6.4	3.1	6.5	3.2	2.7
10	0.1	2.0	2.9	7.1	4.3	8.1	4.1	3.0
20	0.1	2.4	3.5	8.1	4.7	9.7	4.8	3.6
30	0.2	3.4	3.8	-	4.8	9.4	4.3	3.3
45	0.2	-	4.6	-	4.6	1.5	5.2	4.7
60	0.3	-	4.6	-	5.4	10.6	5.2	4.2

SD - standard deviation

2.3.3 Summary of ventilation data of GW597901 and salbutamol

- *Minute volume*

Table 39: Minute volume in percentage of reference value in 6 perfusion experiments.

Exp. (No)	*GW597901*			*Salbutamol*		
	% Reference value (= 100 %)		Δ	% Reference value (= 100 %)		Δ
	After broncho-constriction	After broncho-dilation		After broncho-constriction	After broncho-dilation	
1	70.9	101.1	30.2	22.5	100.0	77.5
2	-	136.3	36.3*	-	98.7	-1.3*
3	-	115.0	15.0*	-	100.9	0.9*
4	45.0	80.6	35.6	-	97.2	-2.8*
5	-	93.8	-6.2*	50.7	104.3	53.6
6	65.6	113.9	48	50.4	105.8	55.4
Mean			**26.5**			**30.6**
SD			**19.3**			**35.7**

Exp. (No) – number of experiment; - no successful bronchoconstriction; SD – standard deviation;
Δ – percental change after bronchodilation relating to value after bronchoconstriction or 100 % reference value*

- *Expiratory tidal volume*

Table 40: Expiratory tidal volume in percentage of reference value in 6 perfusion experiments.

Exp. (No)	*GW597901*			*Salbutamol*		
	% Reference value (= 100 %)		Δ	% Reference value (= 100 %)		Δ
	After broncho-constriction	After broncho-dilation		After broncho-constriction	After broncho-dilation	
1	70.7	100.7	30.0	16.1	99.4	83.3
2	-	135.4	35.4	-	98.3	-1.7*
3	-	115.5	15.5	-	95.3	-4.7*
4	42.7	80.8	38.1	-	95.0	-5.0*
5	-	87.8	-12.2	51.1	104.5	53.4
6	68.4	113.1	44.7	57.4	106.0	48.6
Mean			**25.3**			**29.0**
SD			**20.8**			**37.8**

Exp. (No) – number of experiment; - no successful bronchoconstriction; SD – standard deviation;
Δ – percental change after bronchodilation relating to value after bronchoconstriction or 100 % reference value*

- *Pulmonary compliance*

Table 41: Pulmonary compliance in percentage of reference value in 6 perfusion experiments.

Exp. (No)	*GW597901*			*Salbutamol*		
	% Reference value (= 100 %)		Δ	% Reference value (= 100 %)		Δ
	After broncho-constriction	After broncho-dilation		After broncho-constriction	After broncho-dilation	
1	82.6	98.7	16.1	64.4	139.8	75.4
2	-	119.7	19.7*	-	61.1	-38.9*
3	-	113.8	13.8*	-	91.2	-8.8*
4	60.4	109.8	49.4	-	97.3	-2.7*
5	-	98.0	-2.0*	47.4	117.6	70.2
6	82.2	120.1	37.9	48.7	107.7	59.0
Mean			**22.5**			**25.7**
SD			**18.4**			**48.4**

Exp. (No) – number of experiment; - no successful bronchoconstriction; SD – standard deviation;
Δ – percental change after bronchodilation relating to value after bronchoconstriction or 100 % reference value*

- *Airway resistance*

Table 42: Airway resistance in percentage of reference value in 6 perfusion experiments.

Exp. (No)	*GW597901*			*Salbutamol*		
	% Reference value (= 100 %)		Δ	% Reference value (= 100 %)		Δ
	After broncho-constriction	After broncho-dilation		After broncho-constriction	After broncho-dilation	
1	157.5	93.4	-64.1	89.9	100.0	10.1
2	-	61.0	-39.0	-	60.6	-39.4*
3	-	66.4	-33.6	-	87.9	-12.1*
4	145.7	98.9	-46.8	-	98.0	-2.0*
5	-	96.9	-3.1	154.4	98.6	-55.8
6	139.9	93.8	-46.1	116.6	92.7	-23.9
Mean			**-38.8**			**-20.5**
SD			**20.3**			**21.4**

Exp. (No) – number of experiment; - no successful bronchoconstriction; SD – standard deviation;
Δ – percental change after bronchodilation relating to value after bronchoconstriction or 100 % reference value*

3 Determination of potential markers for edema formation in perfusion fluid samples

3.1 Weight gain of perfused lung lobes

3.1.1 Edema class I

Lung lobes perfused with *perfusion buffer* showing a weight gain of 0 - 29 % (edema class I).

Perfusion experiment	Weight of perfused lung lobes (g)		Weight gain (%)	Decrease of the fluid level in the reservoir (mm)
	Before	After		
IPB-1	314	324	3	-
IPB-2	348	410	18	-
IPB-3	273	325	19	-
IPB-4	278	330	19	4
IPB-5	373	349	-6	2
IPB-6	624	643	3	2
IPB-7	276	299	8	2
IPB-8	547	534	-2	0.5
IPB-9	130	163	25	1
IPB-10	239	289	21	4.5
IPB-11	838	792	-5	2

PB - perfusion buffer, - not recorded

Lung lobes perfused with *perfusion buffer and blood components* showing a weight gain of 0 - 29 % (edema class I).

Perfusion experiment	Weight of perfused lung lobes (g)		Weight gain (%)	Decrease of the fluid level in the reservoir (mm)
	Before	After		
IPB+B-1	217	262	20	3
IPB+B-2	406	393	-3	2
IPB+B-3	706	721	2	1
IPB+B-4	325	335	3	2
IPB+B-5	331	358	8	3
IPB+B-6	418	426	2	2
IPB+B-7	612	618	1	1
IPB+B-8	311	306	-2	2
IPB+B-9	310	378	22	6
IPB+B-10	134	162	21	1
IPB+B-11	427	495	16	2
IPB+B-12	455	480	5	2
IPB+B-13	310	319	3	2

PB+B - perfusion buffer was a mixture of perfusion buffer and blood components.

3.1.2 Edema class II

Lung lobes perfused with *perfusion buffer* showing a weight gain of 30 - 59 % (edema class II).

Perfusion experiment	Weight of perfused lung lobes (g)		Weight gain (%)	Decrease of the fluid level in the reservoir (mm)
	Before	After		
IIPB-1	207	270	30	-
IIPB-2	214	298	39	2
IIPB-3	258	336	30	3
IIPB-4	158	219	38	3
IIPB-5	151	218	44	2
IIPB-6	204	279	37	4

PB - perfusion buffer, - not recorded

Lung lobes perfused with *perfusion buffer and blood components* showing a weight gain of 30 - 59 % (edema class II).

Perfusion experiment	Weight of perfused lung lobes (g)		Weight gain (%)	Decrease of the fluid level in the reservoir (mm)
	Before	After		
IIPB+B-1	349	496	42	5
IIPB+B-2	405	542	34	3.5
IIPB+B-3	146	208	42	4

PB+B - perfusion buffer was a mixture of perfusion buffer and blood components.

3.1.3 Edema class III

Lung lobes perfused with *perfusion buffer* showing a weight gain of > 60 % (edema class III).

Perfusion experiment	Weight of perfused lung lobes (g)		Weight gain (%)	Decrease of the fluid level in the reservoir (mm)
	Before	After		
IIIPB-1	419	692	65	-
IIIPB-2	221	356	61	-
IIIPB-3	204	349	71	-
IIIPB-4	199	401	102	9
IIIPB-5	286	658	130	13
IIIPB-6	314	531	69	6

PB - perfusion buffer, - not recorded

3.2 Analyzed concentrations/activities of potential edema markers in samples obtained from human IPL experiments

3.2.1 Edema class I

Buffer perfusions

Lung lobe perfusion experiment IPB-1

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
5	23.9	n.d.	n.d.	236.0
11	22.7	3.5	19.2	233.0
21	24.6	5.4	8.0	168.7
31	30.1	7.3	6.5	214.3
41	38.8	5.3	14.1	205.5
56	23.0	5.3	7.2	202.9
71	33.0	1.3	18.6	246.4

Lung lobe perfusion experiment IPB-2

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
5	22.0	1.0	n.d.	67.9
9	23.4	n.d.	18.3	50.2
19	30.1	0.8	18.0	84.9
29	35.0	1.9	16.3	62.7
39	45.5	2.5	15.1	76.8
54	37.3	2.4	n.d.	94.5

Lung lobe perfusion experiment IPB-3

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
8	24.9	n.d.	12.5	93.0
13	10.5	0.9	12.5	93.3
23	24.8	2.0	20.0	82.7
33	23.3	2.0	17.8	82.7
43	20.1	3.0	16.6	90.5
58	21.8	4.7	22.4	99.8
78	26.1	4.4	26.1	104.1

Lung lobe perfusion experiment IPB-4

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
22	10.0	6.3	26.7	56.8
46	10.2	6.4	16.5	67.4
61	10.9	4.7	12.4	70.3
78	23.4	2.5	15.1	68.8
93	17.0	7.2	11.5	55.9
108	13.1	0.5	14.0	82.1

Lung lobe perfusion experiment IPB-5

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
21	50.5	n.d.	6.1	28.9
42	16.5	n.d.	n.d.	36.4
60	37.3	n.d.	n.d.	27.3
75	64.7	1.5	5.9	48.7
90	22.2	1.8	n.d.	56.9
105	58.4	1.0	9.5	62.7

Lung lobe perfusion experiment IPB-6

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
20	3.9	1.3	n.d.	104.2
35	2.6	2.8	6.7	115.7
45	4.1	1.1	5.5	89.0
65	11.7	2.6	6.7	94.1
80	12.5	2.8	n.d.	146.5
100	17.8	4.3	n.d.	172.8
115	17.6	3.7	6.1	170.5

Lung lobe perfusion experiment IPB-7

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
25	10.3	0.9	31.8	76.1
40	15.4	5.2	34.2	36.5
60	16.8	2.8	40.4	50.6

Lung lobe perfusion experiment IPB-8

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
20	6.7	n.d.	24.8	81.9
35	7.5	n.d.	16.4	90.3
45	10.8	1.2	8.9	95.1

70	6.9	3.3	48.3	66.2	60	9.2	2.5	11.8	100.0
85	13.7	2.9	64.3	60.7	80	4.4	2.8	21.9	101.3
100	15.3	3.3	62.2	62.7	109	11.3	3.8	21.9	106.4
120	17.5	5.7	40.0	104.5	120	14.9	6.5	33.6	106.1

Lung lobe perfusion experiment IPB-9

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
8	6.9	n.d.	-	-
15	6.3	n.d.	-	-
33	8.7	1.1	-	-
62	15.6	1.5	-	-
75	13.9	3.2	-	-
94	13.7	4.0	-	-
125	17.8	5.0	-	-

Lung lobe perfusion experiment IPB-10

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
0	n.d.	-	-	-
15	4.3	-	-	-
30	6.2	-	-	-
45	8.3	-	-	-
60	35.5	-	-	-
70	42.5	-	-	-

Lung lobe perfusion experiment IPB-11

Time (min)	SP-A c (ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
0	n.d.	-	-	-
2	20.2	-	-	-
12	41.3	-	-	-
25	47.8	-	-	-
36	56.9	-	-	-
48	48.7	-	-	-
66	66.8	-	-	-
77	75.5	-	-	-
91	76.2	-	-	-
101	75.2	-	-	-
112	77.5	-	-	-

SP-A - surfactant protein A, ACE - angiotensin converting enzyme, LDH - lactate dehydrogenase, A (U/L) - enzyme activity in unit/litre, PB - perfusion buffer, - not analyzed, n.d. not detected or concentration below limit of quantification.

Blood/buffer perfusions

Lung lobe perfusion experiment IPB+B-1

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
10	26.3	46.1	130.7	149.4
30	38.0	42.7	148.8	141.7
45	35.2	44.6	148.4	134.2
60	36.3	46.9	133.6	130.5
80	37.9	50.0	151.3	131.8

Lung lobe perfusion experiment IPB+B-2

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
5	56.8	104.7	111.8	286.1
15	49.5	95.8	111.8	313.0
30	52.3	80.4	86.1	313.0
50	54.6	66.7	104.3	344.9
60	45.7	77.9	95.2	344.2
80	44.1	72.5	65.3	355.6
100	60.7	87.3	57.0	353.7

Lung lobe perfusion experiment IPB+B-3					Lung lobe perfusion experiment IPB+B-4				
Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)	Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
5	42.2	43.9	94.4	-	5	35.7	94.3	130.6	-
15	50.2	43.0	100.2	-	15	38.9	93.5	137.5	-
30	42.6	42.5	98.5	-	30	41.4	66.6	123.0	-
45	58.1	41.2	111.0	-	45	50.4	82.2	140.3	-
60	44.8	30.3	137.1	-	60	52.4	76.1	107.3	-
75	71.3	42.9	161.2	-	80	63.9	92.3	146.7	-

Lung lobe perfusion experiment IPB+B-5					Lung lobe perfusion experiment IPB+B-6				
Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)	Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
5	54.5	101.1	184.2	-	15	40.9	60.7	-	-
15	91.9	72.2	170.5	-	22	7.1	67.6	-	-
30	95.3	76.0	166.6	-	36	12.7	62.4	-	-
45	68.4	65.0	113.0	-	51	40.1	63.9	-	-
60	72.7	69.2	128.7	-	66	16.4	67.7	-	-
75	41.3	70.3	78.2	-	81	29.4	67.1	-	-
85	94.7	71.8	139.4	-	96	24.2	68.5	-	-

Lung lobe perfusion experiment IPB+B-7					Lung lobe perfusion experiment IPB+B-8				
Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)	Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
17	39.9	48.0	-	-	10	126.0	143.1	-	-
33	54.0	54.5	-	-	20	90.4	83.6	-	-
49	53.4	47.5	-	-	35	83.4	89.8	-	-
63	46.7	52.7	-	-	45	81.7	128.5	-	-
78	50.1	55.7	-	-	60	76.4	138.4	-	-
87	58.4	56.2	-	-	70	54.6	98.6	-	-
					80	19.4	93.6		
					90	39.5	149.7		
					100	42.0	122.0		

Lung lobe perfusion experiment IPB+B-9					Lung lobe perfusion experiment IPB+B-10				
Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)	Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
5	11.5	6.5	-	-	9	7.2	5.5	-	-
20	16.0	7.3	-	-	16	8.4	6.8	-	-
31	17.4	6.8	-	-	36	6.7	8.3	-	-
41	11.9	12.4	-	-	48	13.2	4.6	-	-
51	22.3	10.2	-	-	59	12.0	5.2	-	-
62	20.1	11.3	-	-	77	12.4	7.0	-	-
83	22.5	7.8	-	-	88	17.1	7.1	-	-
97	21.6	8.6	-	-					

124	22.4	10.1	-	-					
136	21.8	12.3	-	-					
150	22.8	12.4	-	-					

Lung lobe perfusion experiment IPB+B-11

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
15	18.5	12.4	-	-
37	25.2	16.8	-	-
57	38.2	23.4	-	-
68	39.9	26.4	-	-

Lung lobe perfusion experiment IPB+B-12

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
0	4.6	-	-	-
20	12.5	-	-	-
40	10.5	-	-	-
80	27.3	-	-	-
100	28.3	-	-	-
110	14.6	-	-	-

Lung lobe perfusion experiment IPB+B-13

Time (min)	SP-A c (ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
0	16.9	-	-	-
6	8.8	-	-	-
16	15.9	-	-	-
30	9.7	-	-	-
45	10.4	-	-	-
60	11.9	-	-	-
75	15.1	-	-	-
90	17.4	-	-	-
105	8.3	-	-	-
120	20.4	-	-	-
128	23.2	-	-	-

SP-A - surfactant protein A, ACE - angiotensin converting enzyme, LDH - lactate dehydrogenase, A (U/L) - enzyme activity in unit/litre, PB+B - perfusion buffer was a mixture of perfusion buffer and blood components, - not analyzed, n.d. not detected or concentration below limit of quantification.

3.2.2 Edema class II

Buffer perfusions

Lung lobe perfusion experiment IIPB-1

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
6	46.0	4.1	n.d.	322.9
11	45.3	4.3	6.4	320.9
21	40.0	5.3	14.5	324.4
31	31.1	4.9	19.9	215.7
41	22.0	6.1	24.3	198.0
56	36.6	10.2	28.9	203.2

Lung lobe perfusion experiment IIPB-2

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
14	26.2	n.d.	19.8	75.2
40	40.2	n.d.	15.9	93.6
57	36.0	n.d.	4.9	103.3
67	54.5	14.37	16.2	135.9
77	135.8	14.91	9.6	150.2

Lung lobe perfusion experiment IIPB-3					Lung lobe perfusion experiment IIPB-4				
Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)	Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
7	11.8	n.d.	17.3	-	8	7.9	n.d.	17.1	-
37	11.9	0.8	4.3	-	15	5.4	0.8	18.6	-
52	20.2	2.1	25.7	-	30	19.3	4.3	25.6	-
67	14.2	1.4	31.5	-	45	29.2	2.9	24.5	-
82	19.5	2.7	31.5	-	59	28.9	3.8	29.6	-
97	11.8	3.7	27.3	-					

Lung lobe perfusion experiment IIPB-5					Lung lobe perfusion experiment IIPB-6				
Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)	Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
5	2.1	1.2	14.4	-	5	n.d.	n.d.	13.6	-
15	2.5	1.6	14.0	-	16	5.1	n.d.	7.8	-
30	6.7	n.d.	5.3	-	31	7.9	1.1	2.7	-
48	8.1	1.3	14.4	-	47	11.9	1.8	7.6	-
60	7.3	1.6	14.0	-	60	11.9	2.0	10.5	-
77	10.7	3.5	6.4	-	75	23.4	3.1	15.0	-
82	9.5	3.3	3.6	-	91	25.8	2.0	26.2	-
102	16.6	4.1	5.9	-	105	20.0	3.3	25.2	-
					118	24.3	4.3	14.3	-
					136	23.7	5.5	22.1	-
					151	33.2	4.8	30.9	-

SP-A - surfactant protein A, ACE - angiotensin converting enzyme, LDH - lactate dehydrogenase, A (U/L) - enzyme activity in unit/litre, PB perfusion buffer, - not analyzed, n.d. not detected or concentration below limit of quantification.

Blood/buffer perfusions

Lung lobe perfusion experiment IIPB+B-1					Lung lobe perfusion experiment IIPB+B-2				
Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)	Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
30	59.3	41.5	178.4	388.4	4	30.2	55.5	148.3	446.1
50	91.9	43.5	161.9	432.7	15	62.7	57.8	76.3	413.8
65	11.0	45.1	169.0	434.7	30	52.6	55.7	77.7	468.3
80	73.2	69.5	156.4	419.2	45	64.8	56.2	82.9	527.1
90	89.1	50.7	165.4	505.8	60	49.0	76.3	87.0	535.0
					75	75.9	64.2	162.7	
					90	43.3	63.9	186.8	

Lung lobe perfusion experiment IIPB+B-3				
Time (min)	SP-A c (ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
10	2.3	4.9	-	-
22	3.7	7.3	-	-

28	16.8	23.7	-	-
38	8.3	9.3	-	-
46	17.3	9.0	-	-
66	15.1	17.3	-	-
84	7.7	16.8	-	-
112	13.2	8.5	-	-
124	12.1	8.1	-	-

SP-A - surfactant protein A, ACE - angiotensin converting enzyme, LDH - lactate dehydrogenase, A (U/L) - enzyme activity in unit/litre, PB+B - perfusion buffer was a mixture of perfusion buffer and blood components, - not analyzed, n.d. not detected or concentration below limit of quantification.

3.2.3 Edema class III

Buffer perfusions

Lung lobe perfusion experiment IIIPB-1

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
6	24.5	n.d.	n.d.	147.7
10	26.1	1.1	5.6	88.5
20	31.3	0.9	14.3	80.5
30	49.0	2.8	10.2	64.5
40	45.2	2.4	12.0	124.2
55	67.6	5.3	70.1	73.7
70	36.9	10.7	44.0	81.3
90	40.0	4.4	49.0	200.7
110	53.0	6.1	65.5	341.9

Lung lobe perfusion experiment IIIPB-2

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
5	12.7	3.4	8.2	94.1
10	11.4	4.5	n.d.	96.2
20	7.0	6.0	n.d.	157.3
30	4.1	5.8	n.d.	136.6
40	29.3	7.5	n.d.	168.7
55	15.3	7.0	6.3	154.2

Lung lobe perfusion experiment IIIPB-3

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
6	17.0	6.7	50.2	94.1
10	n.d.	7.1	16.7	60.5
20	n.d.	4.8	38.0	52.7
30	n.d.	5.2	7.9	32.0
40	23.5	8.1	23.7	50.6
55	n.d.	6.8	26.7	55.8

Lung lobe perfusion experiment IIIPB-4

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
10	1.9	0.8	-	-
20	6.1	1.3	-	-
31	10.4	4.1	-	-
44	11.1	1.8	-	-
62	14.6	2.8	-	-
83	27.9	5.9	-	-
115	54.7	12.5	-	-
125	63.5	10.2	-	-

Lung lobe perfusion experiment IIIPB-5

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
6	2.0	-	-	-
10	7.0	-	-	-
30	22.4	-	-	-

Lung lobe perfusion experiment IIIPB-6

Time (min)	SP-A c(ng/mL)	ACE c(ng/mL)	Urea c(µg/mL)	LDH A (U/L)
10	2.0	-	-	-
15	3.7	-	-	-
28	35.2	-	-	-

68	36.0	-	-	-	40	69.4	-	-	-
84	28.3	-	-	-	50	68.2	-	-	-
97	37.9	-	-	-	56	55.5	-	-	-
116	42.8	-	-	-				-	-
128	51.1	-	-	-				-	-

SP-A - surfactant protein A, ACE - angiotensin converting enzyme, LDH - lactate dehydrogenase, A (U/L) - enzyme activity in unit/litre, PB - perfusion buffer, - not analyzed, n.d. not detected or concentration below limit of quantification.

3.3 Change of edema marker in perfusion fluid samples

3.3.1 Edema class I

Maximum change in edema marker analyzed in perfusion fluid samples in IPL experiments over time (edema class I). Used perfusion fluid was plain perfusion buffer.

Perfusion experiment	Change in concentration (c) or activity (A) over time								Weight gain (%)
	SP-A		**ACE**		**Urea**		**LDH**		
	c (ng/mL)	%	c (ng/mL)	%	c (µg/mL)	%	A (U/L)	%	
IPB-1	14.9	62.4	3.7	106.2	-0.6	-3.2	10.4	4.4	3
IPB-2	23.5	106.8	1.5	155.2	5.7	31.1	26.6	88.4	18
IPB-3	1.2	4.8	3.9	839,8	13.5	108.1	11.1	11.9	19
IPB-4	13.4	133.7	0.9	14.9	-15.3	-57.1	25.3	44.5	19
IPB-5	7.9	15.6	0.2	16.4	3.2	53.0	38.0	131.5	-6
IPB-6	13.9	352.7	3.0	237.5	-0.6	-61.4	66.2	63.6	3
IPB-7	7.2	69.6	4.8	522.6	28.4	89.5	28.5	37.5	8
IPB-8	8.2	121.4	5.3	426.6	8.8	35.4	24.2	29.5	-2
IPB-9	10.9	157.3	3.9	346.5	-	-	-	-	25
IPB-10	27.2	412.2	-	-	-	-	-	-	21
IPB-11	57.2	282.7	-	-	-	-	-	-	-5
Mean	**16.9**	**156.3**	**3.0**	**296.2**	**4.3**	**18.4**	**28.8**	**51.4**	**10.5**

SP-A - surfactant protein A, ACE - angiotensin converting enzyme, LDH - lactate dehydrogenase, A (U/L) - enzyme activity in unit/litre, PB - perfusion buffer, - not analyzed, % - maximum percental change.

Maximum change in edema marker analyzed in perfusion fluid samples in IPL experiments over time (edema class I). Used perfusion fluid was a mixture of *perfusion buffer and blood components*.

Perfusion experiment	Change in concentration (c) or activity (A) over time								Weight gain (%)
	SP-A		**ACE**		**Urea**		**LDH**		
	c (ng/mL)	%	c (ng/mL)	%	c (µg/mL)	%	A (U/L)	%	
IPB+B-1	11.6	44.3	3.9	8.4	20.6	15,8	-17.6	-11.8	20
IPB+B-2	3.8	6.7	-17.4	-16.6	-54.8	-47,0	69.5	24.3	-3
IPB+B-3	29.2	69.2	-1.0	-2.2	66.9	70,9	-	-	2
IPB+B-4	28.2	79.1	-2.0	-2.1	16.1	12,3	-	-	3

IPB+B-5	40.1	73.6	-29.3	-29.0	-44.8	-42,2	-	-	8
IPB+B-6	-16.7	-40.8	7.8	12.9	-	-	-	-	2
IPB+B-7	18.5	46.4	8.3	17.2	-	-	-	-	1
IPB+B-8	-84.0	-66.6	6.6	4.6	-	-	-	-	-2
IPB+B-9	11.3	97.7	6.0	92.3	-	-	-	-	22
IPB+B-10	9.8	135.8	2.8	51.3	-	-	-	-	21
IPB+B-11	21.3	115.1	14.0	112.3	-	-	-	-	16
IPB+B-12	23.6	509.4	-	-	-	-	-	-	5
IPB+B-13	6.3	37.3	-	-	-	-	-	-	3
Mean	**7.9**	**85.2**	**0**	**22.6**	**0.8**	**2.0**	**26.0**	**6.3**	**7.5**

SP-A - surfactant protein A, ACE - angiotensin converting enzyme, LDH - lactate dehydrogenase, A (U/L) - enzyme activity in unit/litre, PB+B - perfusion buffer was a mixture of perfusion buffer and blood components, - not analyzed, % - maximum percental change.

3.3.2 Edema class II

Maximum change in edema marker analyzed in perfusion fluid samples in IPL experiments over time (edema class II). Used perfusion fluid was plain perfusion buffer.

Perfusion experiment	Change in concentration (c) or activity (A) over time								Weight gain (%)
	SP-A		**ACE**		**Urea**		**LDH**		
	c (ng/mL)	%	c (ng/mL)	%	c (µg/mL)	%	A (U/L)	%	
IIPB-1	-9.4	-20.5	6.1	148.8	22.5	354.0	-119.7	-37.1	30
IIPB-2	109.6	418.6	14.9	-	-10.2	-51.4	75.0	99.7	39
IIPB-3	8.4	71.3	2.9	381.5	14.2	82.4	-	-	30
IIPB-4	21.3	269.0	3.5	437.5	12.5	72.7	-	-	38
IIPB-5	14.5	697.2	2.9	254.5	-8.5	-59.1	-	-	44
IIPB-6	28.1	546.4	4.4	403.0	17.3	127.0	-	-	37
Mean	**28.7**	**330.3**	**5.8**	**270.9**	**8.0**	**87.6**	**-22.4**	**31.3**	**36,3**

SP-A - surfactant protein A, ACE - angiotensin converting enzyme, LDH - lactate dehydrogenase, A (U/L) - enzyme activity in unit/litre, PB - perfusion buffer, - not analyzed, % - maximum percental change.

Maximum change in edema marker analyzed in perfusion fluid samples in IPL experiments over time (edema class II). Used perfusion fluid was a mixture of *perfusion buffer and blood components*.

Perfusion experiment	Change in concentration (c) or activity (A) over time								Weight gain (%)
	SP-A		**ACE**		**Urea**		**LDH**		
	c (ng/mL)	%	c (ng/mL)	%	c (µg/mL)	%	A (U/L)	%	
IIPB+B-1	32.6	23.4	28.0	67.5	-12.9	-7,3	117.4	30.2	42
IIPB+B-2	45.7	151.3	8.4	15.1	38.5	26,0	88.9	19.9	34
IIPB+B-3	15.0	637.8	18.8	380.6	-	-	-	-	42
Mean	**31.1**	**270.8**	**22.5**	**161.9**	**12.8**	**9.4**	**103.1**	**25.1**	**39.3**

SP-A - surfactant protein A, ACE - angiotensin converting enzyme, LDH - lactate dehydrogenase, A (U/L) - enzyme activity in unit/litre, PB+B - perfusion buffer was a mixture of perfusion buffer and blood components, - not analyzed, % - maximum percental change.

3.3.3 Edema class III

Maximum change in edema marker analyzed in perfusion fluid samples in IPL experiments over time (edema class III). Used perfusion fluid was plain perfusion buffer.

Perfusion exp.	Change in concentration (c) or activity (A) over time								Weight gain (%)
	SP-A		**ACE**		**Urea**		**LDH**		
	c (ng/mL)	%	c (ng/mL)	%	c (µg/mL)	%	A (U/L)	%	
IIIPB-1	43.1	176.0	9.8	1093.6	64.5	1140.8	194.2	131.5	65
IIIPB-2	16.6	131.0	4.2	123.5	-2.0	-23.9	74.6	79.2	61
IIIPB-3	6.5	38.6	1.4	21.3	-23.5	-46.8	-38.3	-40.7	71
IIIPB-4	61.6	3199.0	11.7	1562.5	-	-	-	-	102
IIIPB-5	49.1	2456.7	-	-	-	-	-	-	130
IIIPB-6	67.4	3370.0	-	-	-	-	-	-	69
Mean	**40.7**	**1561.9**	**6.8**	**670.6**	**13.0**	**356.7**	**76.8**	**56.7**	**83.0**

SP-A - surfactant protein A, ACE - angiotensin converting enzyme, LDH - lactate dehydrogenase, A (U/L) - enzyme activity in unit/litre, PB - perfusion buffer, - not analyzed, % - maximum percental change.

E. Summary

1 Summary

The inhaled pharmacotherapy is fundamental in the management of obstructive lung diseases such as asthma bronchiale or chronic obstructive pulmonary disease. In this context short- and long-acting β_2-agonists play a prominent role as relieve and control medication. Deposition of drugs within the airways requires lower doses and achieves higher local concentrations in compared to the oral route. Regarding the risk-benefit profile of an inhaled drug, the pattern of pulmonary deposition and the rate and extent of absorption into systemic circulation are essential parameters. New developments of drugs are characterized by high lung retention and improved efficacy. The aim of the present thesis was the parallel evaluation the pharmacokinetic (PK) and -dynamic (PD) properties of inhaled β_2-agonists employing an isolated human lung perfusion model (IPL). The short-acting β_2-agonist salbutamol and the newly developed ultra long-acting β_2-agonist GW597901 were chosen for the analysis of pulmonary drug absorption and bronchodilation.

In a pharmacokinetic enabling study an established human IPL setting was modified to monitor the pharmacokinetics of the β_2-agonists by measuring the concentrations in perfusion fluid, lung tissue and BAL samples obtained during and after the experiments. For this purpose solutions containing both agents GW597901 and salbutamol were nebulized and the aerosols were administered to the human lung lobes. A mixture of blood components and buffer served as perfusion fluid.

The IPL model revealed differences in the pulmonary absorption behaviour of GW597901 and salbutamol. The lipophilic compound GW597901 was distributed to a lower extent into the perfusion fluid compared to the more hydrophilic compound salbutamol. Since the agents were administered as solutions the observed differences were solely due to the tissue retention behavior/ absorption rate of the β_2-agonists. The analyzed time profiles of nebulized salbutamol in the perfusate were consistent to with a clinical study if considering experimental conditions as the actual deposited doses and the differing volume of distribution. Thus, the suitability of the IPL model for the PK analysis of inhaled β_2-agonists was confirmed.

In a PK/PD study the human *ex vivo* model was employed for the first time for the evaluation of the clinical relevant bronchodilating effect induced by inhaled β_2-agonists in addition to the analysis of their pharmacokinetics. Thereby the focus was to determine the onset and extent of bronchodilation. A new method was established to monitor changes in lung function parameters due to pharmacodynamic interventions over the duration of the experiment that allowed permanent online recording of the ventilation volume and lung mechanic parameters. Prior to the application of either 180 µg GW597901 or 600 µg salbutamol to the

lung lobes the airways were challenged by nebulized methacholine (MCh) to achieve a defined bronchoconstriction.

Bronchial challenges with aerolised MCh were performed successfully in isolated ventilated human lung lobes, even though the responder rate was lower than expected despite high administered doses. The administration of the short acting agent salbutamol led to an immediate onset of action recognized as a sudden increase of the ventilation volumes. The bronchodilation following the application of GW597901 was observed delayed after about 6 min. Monitored lung function parameters considerably improved by both β_2 - agonists in the IPL setting but not significantly different. Thus, in regard of the different applied doses GW597901 had a higher intrinsic activity and bronchodilating potency than salbutamol.

The concentrations of salbutamol and GW597901 in the perfusate determined in the PK/PD study were significantly lower than those observed in the pharmacokinetic enabling study, while the t_{max} values and the course of the distribution profiles remained similar. Most likely, the application of nebulized MCh prior to the administration of the β_2 - agonists had a substantial influence on their pharmacokinetic behaviour. It is yet not clear whether pharmacodynamic effects or molecular competition processes for the passage to the systemic circulation or both influenced the redistribution of the β_2 - agonists as seen in the PK/PD study. The potential clinical relevance of this observation has to be further investigated.

The development of pulmonary edema during the experiment was one limitation of the IPL model. For the determination of the onset of edema formation four potential biochemical markers, specifically surfactant-protein A (SP-A), angiotensin-converting enzyme (ACE), urea and lactate dehydrogenase, were measured in perfusion fluids. In this context, an ELISA method for the quantification of human SP-A in biological matrices was successfully established. The investigations showed that the concentrations of SP-A and ACE in the perfusate increased over time as a sign for lung tissue damage and correlated with the degree of edema formation. For the first time the IPL model was used for the evaluation of potential pulmonary edema marker and the results have shown that it is valuable tool for further investigations in this field.

In conclusion, the pharmacokinetic and pharmacodynamic characterization of GW597901 and salbutamol was successfully achieved using the IPL model. This *ex vivo* methodology may contribute to further insights and understanding of the complex pharmacokinetic processes of inhaled β_2 – agonists in the lung.

2 Zusammenfassung

Die Inhalationstherapie stellt den grundlegenden Baustein in der Behandlung obstruktiver Lungenerkrankungen wie Asthma bronchiale oder chronisch obstruktiver Lungenerkrankung dar. Eine essentielle Rolle spielen dabei kurz- und langwirksame β_2 – Agonisten, die sowohl als Bedarfs- und Kontrollmedikation eingesetzt werden. Durch die Deposition von Arzneistoffen in den Atemwegen werden im Vergleich zur oralen Einnahme geringere Dosen benötigt und es werden lokal höhere Konzentrationen erreicht. Bei Betrachtung des Nutzen-Risiko-Verhältnisses eines inhalativen Arzneistoffes sind das pulmonale Depositionsmuster und die Geschwindigkeit und das Ausmaß, mit welcher der Arzneistoff in die systemische Zirkulation gelangt, von zentraler Bedeutung. Die Entwicklung neuer inhalativer Arzneistoffe strebt daher eine höhere Verweildauer der Substanzen in der Lunge bei gleichzeitiger verbesserter Wirksamkeit an. Das Ziel dieser Arbeit war die Bestimmung pharmakokinetischer (PK) und -dynamischer (PD) Eigenschaften inhalativer β_2 – Agonisten anhand des isolierten humanen Lungenperfusionsmodells (IPL). Der kurzwirksame β_2 – Agonist Salbutamol und der neue langwirksame Vertreter GW597901 dienten dabei als Modellsubstanzen für die Analyse der pulmonalen Absorption und den Effekt der Bronchodilatation.

In einer ersten Versuchsreihe wurde das etablierte IPL Modell derart modifiziert, um mittels gemessener Konzentrationen in der Perfusions- und Lavageflüssigkeit sowie in Lungengewebsproben, die während und nach den Experimenten entnommen wurden, die Pharmakokinetik von β_2 – Agonisten zu bestimmen. Zu diesem Zweck wurde eine Lösung, die GW597901 und Salbutamol enthielt, als Aerosol an humane Lungenlappen verabreicht. Als Perufsat diente eine Mischung aus Blutbestandteilen und salinem Puffer.

Das IPL Modell zeigte Unterschiede im pulmonalen Absorptionsverhalten von GW597901 und Salbutamol. Der lipophile Vertreter GW597901 wurde in geringerem Ausmaß in die Perfusionsflüssikeit umverteilt als das hydrophile Salbutamol. Da sich beide Arzneistoffe bereits bei der Verabreichung in Lösung befanden, sind die demonstrierten Unterschiede allein auf deren Retentions- Absorptionsverhalten zurückzuführen. Das analysierte Absorptionsprofil von Salbutamol korrelierte dabei sehr gut mit Daten einer Humanstudie, wenn man die experimentellen Bedingungen wie die tatsächlich deponierte Dosis und das abweichende Verteilungsvolumen berücksichtigte. Dadurch war die Eignung des IPL Modells zur PK Analyse inhalativer β_2 – Agonisten bestätigt.

Zusätzlich zur Betrachtung der PK wurde das humane IPL Modell nun zum ersten Mal zur Untersuchung des klinisch relevanten Effekts der Bronchdilatation der β_2 – Agonisten herangezogen. Dabei sollten vor allem der Beginn und das Ausmaß der induzierten Bronchodilatation bestimmt werden. Es konnte erfolgreich eine neue Methode etabliert werden,

die es erlaubte durch permanente online Aufzeichnung von Atemvolumina und lungenmechanischer Parameter Änderungen in der Lungenfunktion darzustellen, die durch pharmakodynamische Interventionen ausgelöst wurden. Vor der pulmonalen Verabreichung von entweder 180 µg GW597901 oder 600 µg Salbutamol wurden hierzu die Atemwege mit vernebeltem Methacholin (MCh) provoziert, um eine definierte Bronchokonstriktion zu erzeugen.

Erstmals wurde erfolgreich eine bronchiale Provokation an einem *ex vivo* ventilierten humanen Lungenlappen durchgeführt. Jedoch war trotz hoher verabreichter MCh Dosen die Responderrate niedriger als erwartet. Die Applikation des kurzwirksamen Vertreters Salbutamol führte zu einem sofortigen Effekt, was an einem unmittelbaren Anstieg der Atemvolumina zu erkennen war. Der Wirkeintritt der Bronchodilatation verursacht durch GW597901 war hingegen um ungefähr 6 Minuten verzögert. Alle aufgezeichneten Lungenfunktionsparameter haben sich durch die Applikation beider β_2 – Agonisten entscheidend und in gleichem Ausmaß verbessert. Unter Berücksichtigung der unterschiedlichen verabreichten Dosen zeigen diese Ergebnisse eine höhere intrinsische Aktivität und atemwegserweiternde Wirksamkeit von GW597901 im Vergleich zu Salbutamol.

Die analysierten Konzentrationen von GW597901 und Salbutamol im Perfusat waren in der PK/PD Studie signifikant niedriger als diejenigen, die in der vorherigen PK Versuchsreihe gemessen wurden, während die jeweiligen T_{max} Werte und Umverteilungsprofile unverändert blieben. Die wahrscheinlichste Erklärung hierfür ist, dass die Applikation von MCh vor der Vernebelung der β_2 – Agonisten einen erheblichen Einfluß auf deren pharmakokinetischen Verhalten hatte. Gegenwärtig kann nicht mit Sicherheit eine Aussage darüber getroffen werden, ob pharmakodynamische Effekte, Konkurrenzmechanismen um die Aufnahme in den systemischen Kreislauf auf molekularer Ebene oder beides zu einem veränderten Umverteilungsverhalten der β_2 – Agonisten in der PK/PD Studie geführt haben. Auch die klinische Relevanz dieser Beobachtung sollte weiter untersucht werden.

Die Entwicklung von Lungenödemen im Verlauf der Experimente war eine Einschränkung des IPL Modells. Um den Beginn einer Ödembildung besser bestimmen zu können, wurden vier potenzielle biochemische Marker in Perfusatproben untersucht, im Einzelnen das Surfactant Protein-A (SP-A), das Angiotensin-konvertierende Enzym (ACE), Harnstoff und das Enzym Lactatdehydrogenase (LDH). In diesem Zusammenhang wurde erfolgreich eine ELISA Methode zur Quantifizierung von SP-A in biologischen Matrices entwickelt. Die Untersuchungen haben gezeigt, dass die Konzentrationen von SP-A und ACE im Perfusat über die Zeit als Anzeichen für eine Lungengewebsschädigung in Korrelation zum Ausmaß der Ödembildung anstiegen. Zum ersten Mal wurde das IPL Modell für die Bestimmung von biologischen Markern für pulmonale Ödementstehung herangezogen und die

Ergebnisse haben gezeigt, dass dieses *ex vivo* Modell einen vielversprechenden Ansatz für weitere Untersuchungen in diesem Bereich bietet.

Damit konnten erfolgreich Methoden entwickelt werden, um mit Hilfe des humanen IPL Modells die pharmakokinetischen und -dynamischen Eigenschaften von GW597901 und Salbutamol zu charakterisieren. Die angewandte *ex vivo* Methodik kann hierbei einen wertvollen Beitrag und weiterführende Erkenntnisse zum besseren Verständnis der komplexen pharmakokinetischen Vorgänge inhalativer β_2 – Agonisten leisten.

F. Abbreviations in alphabetical order

ACE	Angiotensin converting enzyme
AUC	Area under the curve
BAL	Broncho-alveolar lavage
BC	Buffy coat
BIPAP	Biphasic Positive Airway Pressure
BSA	Bovine serum albumine
C	Pulmonary compliance
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
csv	comma separated values
DAB	Deutsches Arzneibuch
eEC	Expired erythrozyte concentrate
f	Breathing rate [1/min]
fe	Female
fEC	Fresh erythrozyte concentrate
FEV1	Forced expiratory volume in one second
GDH	Glutamate dehydrogenase
HCT	Hematocrit
HPLC	High pressure liquid chromatography
H/W/D	Height/ Width/ Depth
IPL	Isolated perfused lung
ISD	Internal standard
LABA	Long-acting β_2 – agonist
LDH	Lactate dehydrogenase
m	Male
MCh	Methacholine chloride
MDM	Mean deviation of the mean
MeCN	Acetonitrile
MeOH	Methanol
MMAD	Median mass aerodynamic diameter
MV	Minute volume
m/z	Ratio of molecular mass to charge
Na	Sodium
NADH	Nicotinamide adenine dinucleotide

PB	Perfusion buffer
PE	Polyethylene
PEEP	Positive endexpiratory pressure
PEF	Peak expiratory flow
Pen/Strep	A solution containing the antibiotics penicillin G and streptomycin
PFP	Perfusion fluid plasma samples
Ph.Eur.	European pharmacopoeia
P_{max}	Maximal airway pressure
pMDI	pressurized metered-dose inhaler
P_{mean}	Mean airway pressure
PP	Polypropylene
R	Airway resistance
rpm	Rounds per minute
RR	Relative recovery
RT	Room temperature
SABA	Short-acting β_2 – agonist
SP-A	Surfactant protein A
ST	Standard taper ground joint
UK	United Kingdom
UV	Detection by measuring the absorption of the ultraviolet rays
V_{Te}	Expiratory Tidal Volume
WBC	Whole blood cells
WPF	Whole perfusion fluid samples

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