Diplomarbeit

IMPACT OF BUDESONIDE PROPERTIES ON BIOLOGICAL ACTIONS IN THE LUNG

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Zusammenfassung

Budesonid, ein synthetisches Glucocorticoid, findet insbesondere in der Langzeitbehandlung von respiratorischen Erkrankungen wie Asthma bronchiale oder chronisch obstruktiver Lungenkrankheit (COPD) in Form von pulmonaler Verabreichung Anwendung. Direkte Applikation an den Ort des Krankheitsgeschehens bietet den Vorteil eines schnellen Wirkungseintritts, einer Umgehung des First-Pass-Effekts und durch die erniedrigten systemischen Wirkstoffspiegel eine Reduktion etwaiger unerwünschter Wirkungen. Damit der Wirkstoff jedoch die terminalen Bronchiolen und Alveolen (im Folgenden „tiefe Atemwege“ genannt) erreichen kann, muss er zu einer Partikelgröße von unter 5 µm zerkleinert werden. In dieser Arbeit wurde Budesonid durch Sprühtrocknung und durch Prozessierung in einer Luftstrahlmühle modifiziert. Die gewonnenen Partikel wurden bezüglich Größe, Form, Festkörpereigenschaften sowie in ihrer Aufnahme in Zelllinien von Epithelzellen und Makrophagen sowie primären humanen Makrophagen und Permeationsfähigkeit durch einen zellulären Monolayer verglichen. Zur Testung der aerodynamischen Eigenschaften sowie des Löslichkeitsprozesses wurden die Partikel an Laktose als Carrier gebunden (sog. inhalative Mischung). Die inhalativen Mischungen wurden mithilfe eines „Next Generation Impactor“ (NGI) in Partikelfraktionen verschiedener Größe aufgetrennt und die Fraktion < 5 µm in simulierter Lungenflüssigkeit inkubiert. Sprühgetrocknetes, vorwiegend amorphes Budesonid zeichnete sich durch eine glatte Oberfläche und kugelähnliche Form aus, während kristalline, gemahlene Budesonidpartikel eher durch stabförmige, irreguläre Oberflächen und Formen gekennzeichnet waren. Gemahlenes Budesonid wurde von allen getesteten Zelllinien zu einem geringeren Ausmaß aufgenommen, wobei der Unterschied nur für primäre humane Makrophagen signifikant war. Es wurde auch langsamer durch einen zellulären Monolayer transportiert als das sprühgetrocknete. Der Löslichkeitsprozess zeigte ein leicht unterschiedliches Muster und der Unterschied des aerodynamischen Verhaltes erwies sich als statistisch signifikant. Die „Feinpartikelfraktion“ (FPF), der Teil des Wirkstoffes, der die Teile des NGI erreicht, die die tiefen Atemwege repräsentieren, war für die inhalative Mischung mit gemahlenem Budesonid signifikant höher als für die Mischung mit dem sprühgetrockneten Wirkstoff.

Abstract

Budesonide, a synthetic glucocorticoid, is a widely used drug for the long-term treatment of respiratory diseases such as bronchial asthma or chronic obstructive pulmonary disease (COPD). Its pulmonary application enhances a fast onset of action and by avoiding the first pass effect, smaller systemic plasma concentrations and less adverse effects can be beneficial for the patient in comparison to systemic application. The active pharmaceutical ingredient (API) has to be processed to a size of less than 5 µm in order to reach what will be referred to as “the deep lung” (the terminal bronchioles and alveoli). In this study, budesonide was modified by spray drying and jet milling, and the produced particles were compared in terms of size, shape and solid state, as well as their uptake by epithelial and macrophage cell lines and primary human macrophages and their permeability through a monolayer of epithelial cells. For determination of aerodynamic properties particles were bound to lactose carrier molecules (blended) and the detached particles were separated according to their size in the Next Generation Impactor (NGI). Dissolution of the particles in size < 5 µm was assessed by incubation in simulated lung fluid. Spray dried, predominantly amorphous budesonide particles showed smooth surfaces and were spherically shaped, whereas the jet milled, crystalline API had rough surfaces and was rod-like and rather irregular in shape. In all tested cell lines, the uptake and permeability of jet milled budesonide was lower than the respective parameters of the spray dried material. A slightly different dissolution pattern was determined, and there was a notable difference in the aerodynamic performance of the two particles. The fine particle fraction (FPF), the amount of API that reaches the NGI parts that represent the deep lung, was significantly higher for jet milled budesonide compared to the FPF of the blend with the spray dried API. Significantly higher uptake of the spray dried budesonide was seen only in human primary macrophages.

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List of Abbreviations

Abbreviation	Meaning
ALI	Air-Liquid Interface
API	Active Pharmaceutical Ingredient
BCS	Biopharmaceutical Classification System
BUD	Budesonide
COPD	Chronic Obstructive Pulmonary Disease
DMEM	Dulbecco´s Modified Eagle Medium
DPBS	Dulbecco's Phosphate-Buffered Saline
DPPC	1,2-Dipalmitoyl-sn-glycero 3-phosphocholine
DSC	Differential Scanning Calorimetry
ED	Emitted Dose
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
FPD	Fine Particle Dose
FPF	Fine Particle Fraction
GM-CSF	Granulocyte Macrophage – Colony Stimulating Factor
H_3PO_4	Phosphoric acid
HPLC	High Performance Liquid Chromatography
LC	Liquid Cultivation
LH-100	Lactohale-100
MEM	Minimal Essential Medium
MMAD	Mass Median Aerodynamic Diameter
MPS	Mononuclear phagocyte system

NaH_2PO_4	Monosodium phosphate
NGI	Next Generation Impactor
P_{app}	*In vitro* Apparent Permeability Coefficient
PBMC	Peripheral Blood Mononuclear Cells
PET	Polyethylene terephthalate
rpm	Revolutions per minute
RPMI medium	Roswell Park Memorial Institute medium
SD	Standard Deviation
SEM	Scanning Electron Microscopy
SLF	Simulated lung fluid
v/v	Volume per volume
TEER	Transepithelial Electric Resistance

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1 Introduction

Orally inhaled drug formulations have become an important research topic due to their advantages compared to other forms of administration. They are unique in terms of pharmacokinetics and pharmacodynamics. As they reach the lung directly without the need to enter the bloodstream beforehand to reach their target organ, they are most commonly used to treat respiratory diseases. Medication for asthma and chronic obstructive pulmonary disease (COPD) account for more than 70% of inhalable medications (1). The lung with its large surface enables a fast onset of action and avoids the first-pass effect by bypassing the hepatoportal system, which often limits the effect of orally administered drugs by lowering the bloodstream concentration. Consequently, less active agent has to be administered for a respective effect. This also helps to prevent or limit adverse drug reactions.

As a rough model, the human airways can be divided into an upper and a lower portion. While the nasal and oral cavity as well as pharynx and larynx are classified as upper airways, the glottis marks the entrance to the lower airways. The air is conducted from the trachea onwards into the bronchi, which after several bifurcations evolve into bronchioles. The last branches of the bronchioles are called *bronchioli terminales* and represent the end of the solely conductive airways, of which the key task is the warming, humidification and particle clearing of ventilated air. The structures from trachea to *bronchioli terminales* are termed collectively conductive airways to differentiate them from the respiratory airways, where gas exchange takes place. The following *bronchioli respiratorii* end after approximately 3 generations in the *ductus* and *sacculi alveolares* (alveoli), where the majority of the gaseous exchange takes place. According to their main function, the different sections of the airways are not only characterized by a specific wall structure and epithelium, but also a series of different defense mechanisms to maintain lung homeostasis and to prevent dust, microbes and other particles of inhalable size from entering the body via pulmonary route.

The major part of the upper airways as well as the trachea and the upper bronchial structures are lined with typical respiratory epithelium (*ciliated pseudostratified columnar epithelium*). Its main cells are ciliated cells, goblet cells and basal cells.

Goblet cells are columnar epithelial cells that produce mucus, which not only moisturizes the epithelium but also traps particulate material in the inhaled air. Ciliated cells with their specific surface modifications play a key role in mucus transport and contribute to the microciliary clearance (the removal of material from the lung). Basal cells root the epithelium to the basement membrane and have some stem cell properties as they can migrate to injured epithelial sites and differentiate. Certain parts of the respiratory tract such as the oral cavity are exposed to strain that would easily destroy the respiratory epithelium – therefore, they are lined with *squamous stratified epithelium*. All epithelia are polarized, meaning that they have an apical side that faces the lumen of the respective organ, and a basal side that is anchored in the basement membrane. On the outer side of the mucosa, the trachea and the upper bronchi are equipped with seromucous glands and connective tissue that anchors the mucosa to the cartilage. The bronchi have a typical wall structure consisting of luminal mucosa, smooth muscle, cartilage and peribronchial connective tissue. Their seromucous glands are located mostly between the muscle fibers and the cartilage but can also reach into the peribronchial connective tissue. They secrete mucins, IgA, antibacterial molecules and protease inhibitors to limit tissue destruction by immune cell proteases. While the bronchi are lined with typical respiratory epithelium, bronchioles only have a single layer of ciliated epithelium and moreover lack seromucous glands and cartilage. Goblet cells decline in frequency on the way to the deep lung. The *bronchioli respiratorii* and *ductus alveolares* are lined with nonciliated cuboidal cells and club cells. Club cells secrete surfactant proteins (mainly SP-A and SP-D) and *Club cell 10 kDa protein*, which help to reduce excessive inflammation. Furthermore, the surfactant proteins are antimicrobial and operate as opsonins, facilitating phagocytosis (2).

Alveoli are the smallest units in the respiratory tract. Supported by a thin extracellular matrix of elastic fibers and collagen, millions of capillaries maintain close contact to the inhaled air for gaseous exchange. The 3 main cell types of the alveoli are type I and II pneumoctes and alveolar macrophages. Type I alveolar cells are flat and coat approximately 95% of the alveolus. Together with the extracellular matrix they form the interalveolar septum, providing the structural basis of the alveolus. The blood-air barrier of the tissue itself measures on average 0.6 µm and consists of the capillary endothelium, basement membrane and the branches of type I pneumocytes. As the air does not only have to cross the tissue itself but also

the surfactant and the blood plasma, the functional barrier thickness between air and erythrocytes is 1.1 µm on average (2).

Type II alveolar cells are cuboidal and produce pulmonary surfactant that decreases surface tension and enhances the compliance of the tissue during inspiration. Surfactant consists of 90% phospholipids (mainly dipalmitoylphosphatidylcholine) and approximately 10% surfactant proteins and is stored in lamellar bodies of type II alveolar cells. Alveolar macrophages are part of the mononuclear phagocyte system (MPS) and patrol in the alveoli and nonciliated terminal airways. As mobile scavengers, they engulf small particles and debris and once they have ingested enough material, they migrate upwards to the ciliated epithelium and are then transported towards the larynx by the cilia of the bronchial cells (mucociliary clearance). Alternatively, they move to the connective tissue, where they either stay and contribute to what can be seen macroscopically as the lobulus pattern, or they enter lymphatic vessels and are transported to regional lymph nodes.

In order to reach the deep lung, the active pharmaceutical ingredient (API) of an inhalable medicine needs to be processed to a size of 1 to 5 µm. These diameters enable the API to reach the small airways and alveoli. The raw material can be modified differently to get the demanded size by techniques such as milling or spray drying. While jet milling of the raw material generates rather rod-like structures, spray dried API particles are spherical or platelet-shaped (3). However, these small drug particles have a high surface-to-volume-ratio and due to the high surface energies, they stick together and have bad flowability (4). To overcome the van der Waals interactions causing agglomeration, the API is usually administered attached to a carrier, which increases the distance between the API particles and reduces the bonds and forces between them. The carrier and the API normally should disintegrate during inhalation, meaning already in the inhaler or mouth. While the carrier mostly stays in the oropharynx and is swallowed, the API should reach the deep lung and take full effect. When testing new inhalable mixtures, it must be taken into account that different shape, solid state and slightly different particle size of the modified active pharmaceutical ingredient might cause differences not only in particle deposition and dissolution in lung lining fluid, but also in the biological activity of one and the same drug. On the other hand, preferential uptake of APIs by macrophages is intended in the treatment of tuberculosis (5).

Aim of the study

It is well known that milling and spray drying results in particles with distinct properties regarding their shape and solid state. The aim of this diploma thesis was to evaluate whether distinct particle properties, namely shape of a model drug have an impact on biological effects in the lung. Therefore, the effect of jet milled and spray dried budesonide in terms of *in vitro* aerodynamic performance, dissolution into simulated lung fluid, permeability through epithelial cells and uptake into epithelial cells and macrophages was compared. For the evaluation of aerodynamic performance, *in vitro* lung deposition studies were performed with the Next Generation Impactor (NGI). In a more advanced step, the NGI experiments were extended to study the dissolution of API into simulated lung lining fluid over time. Regarding cell studies, it was investigated with different cell types if the rate of transport and accumulation of API differs when the cells are exposed to same concentrations of raw, jet milled and spray dried budesonide. Both permeation over time and uptake of the three budesonide types through a monolayer of Calu-3 cells was assessed. Calu-3 cells were cultivated in two ways – with an air-liquid interface (ALI) and submersed in liquid (LC). As ALI cultivated cells, they produce mucus on the apical side and therefore resemble the respiratory epithelium of the bronchioles, whereas they are an appropriate model for studying alveolar cells when cultivated in liquid (6). The second epithelial cell line for uptake testing were A-549 cells. They form monolayers with type II alveolar cell characteristic morphology. As especially phagocytes are known to ingest spherical particles rather than needle-shaped ones, a series of macrophages, namely DMBM-2, J774 and PBMC, were studied in regard to their ability to accumulate raw, spray dried and jet milled API particles. This might be of special interest as the impairment of alveolar macrophages by an overload of non-clearable fragments is currently researched. Exposure to poorly soluble particles leads to morphologic changes *in vivo*, also known as “foamy macrophages”, which are an indicator for adverse drug reactions on a cellular level (1). Due to the reproducible phenotype established cell lines present advantages for routine testing compared to primary cells. When studying specific cell properties, primary cells may be advantageous. Macrophages isolated from human peripheral blood mononuclear cells were used for a physiologically more relevant testing of particle uptake.

2 Materials and Methods

2.1 In vitro lung studies

2.1.1 Preparation of adhesive mixtures

2.1.1.1 Budesonide

Budesonide, the model API used in this study, is a synthetic glucocorticoid. It is a white crystalline solid with a poor solubility in water (28 µg/mL (7)). Like all glucocorticoids, it acts via modification of protein transcription in cells, enhancing and repressing expression of specific inflammatory mediators. Budesonide is currently used for long-term treatment of asthma and COPD (inhalable forms), allergic rhinitis and nasal polyps (nasal spray) and treatment of irritable bowel diseases (rectally administered).

As it is listed as a category 2 CMR substance, all experiments with a possibility of emerging powder were performed with nitrile gloves and under the aspiration hood or with proper airway protection. The raw API with a purity of 98% was purchased from Thermo Fisher Scientific (Vienna, Austria) and modified in two different ways to generate particle sizes small enough to reach the deep lung – air-jet milling and spray drying.

2.1.1.2 Jet milling

Milling of raw budesonide was performed with an air-jet mill (Spiral Jet Mill 50 AS, Hosokawa Alpine AG, Augsburg, Germany). An inlet pressure of 6 bar and a milling pressure of 3 bar were used. The raw budesonide powder was fed into the vessel at the top of the mill manually. As it is drawn through the mounted machine by a strong airstream, powder pieces collide on the inside and particles of inhalable sizes are generated. While larger particles are deposited at the outer perimeter of the mill, small particles exit centrally and can be collected in a bag at the bottom. The milled API was stored in a desiccator over silica gel at room temperature until later use.

2.1.1.3 Spray drying

Spray drying is another common method to produce small particles for inhalable mixtures. Solutions of the API are fed and dispersed through small nozzles into the chamber of the spraying tower where the droplets are dried with hot gas. After all

the liquid has vaporized, the dry particles can be collected at the collection electrode. For this study, a Nano Spray Drier B-90 (Büchi Labortechnik AG, Flawil, Switzerland) was used.

Size and morphology of the spray dried powder can for example be tuned by adjustment of inlet temperature and addition of organic compounds such as ethanol to the API solution (8). To optimize the yield and to obtain particles of the desired size, the composition of the solvent and process parameters were chosen as described in a recent study of Boarey et al. (9).

Prior to spray drying, 1.5% budesonide solutions in ethanol:purified water 75:25 v/v were prepared. The purified water was taken from “TKA MicroPure UV UltraPure Water System” (TKA Wasseraufbereitungssysteme GmbH, Niederelbert, Germany). The solution was fed into the drying chamber and a 7 µm mesh was used to generate the desired particle size. The following settings were used throughout the spray drying process:

Inlet temperature: 120 °C

Flow: 110 L/min

Pressure: 36 mbar

Spray intensity: 40%

After collection, the spray dried powder was stored in a desiccator over silica gel at room temperature.

2.1.1.4 Particle characterization

2.1.1.4.1 Laser diffraction

Laser diffraction is a widely used method to measure particle sizes from nanometers up to several millimeters. Laser light passes through a stream of particles in a liquid or gaseous medium and depending on the particle size, the light is either reflected, scattered or can pass freely. It is then passed onwards to a detector, and from the light scattering pattern it is possible to calculate the particle size.

In this study, laser diffraction was performed with the HELOS/KR system (Sympatec, Clausthal-Zellerfeld, Germany). The powder particles were dispersed with a dry dispersing unit (Rodos/L, Sympatec, Clausthal-Zellerfeld, Germany) and

a vibrating chute (Vibri, Sympatec, Clausthal-Zellerfeld, Germany). A dispersing pressure of 2 bar was used. Analysis of the collected data was carried out with Windox 5 software (Sympatec). Three characteristic particle diameters were measured: x10, x50 and x90. They indicate the diameter at which the respective percentage of particles is smaller than the measured value, for example 10% of measured particles are smaller than x10 and 90% are larger.

2.1.1.4.2 Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) is a well-established method of thermal analysis. Usually, the sample to test is filled into a small pan and coupled with another empty pan. The pans are heated or cooled and then compared. The difference in temperature in the two pans with the same energy input or energy needed to heat them to the same temperature can be plotted into thermograms. They give information about thermal events such as melting, crystallization or glass transition.

In this study, modulated DSC experiments were carried out with a DSC 204 F1 Phoenix (Netzsch, Selb, Germany) to determine the thermal behavior and solid state of the different engineered particles. About 5-10 mg of samples were weighed into pans and hermetically sealed on a high precision scale (XP205DR, Mettler Toledo GmbH, Vienna, Austria). Samples were heated from 20 °C to 280 °C at a heating rate of 5.0 K/min, using pure nitrogen as purging gas at a flow rate of 50 mL/min. For data analysis the Netzsch Proteus Thermal analysis software version 6.1.1 (Netzsch, Selb, Germany) was used.

2.1.1.4.3 Scanning electron microscopy (SEM)

To study the morphology of the raw and engineered API particles and the blends, a Zeiss Ultra 55 (Zeiss, Oberkochen, Germany) scanning electron microscope was used. The samples were gold palladium sputtered and the operating energy used was 5 kV. All images were taken at FELMI (Austrian Center for Electron Microscopy and Nanoanalysis, Graz, Austria).

2.1.1.5 Blending protocol

Two different adhesive mixtures of 10 g each with a budesonide content of 2% were prepared under similar conditions, differing only in type of modified API (spray dried

vs. jet milled). Lactohale 100 (LH-100, donated from DFE Pharma, Goch, Germany) was used as a carrier.

Before preparing the mixtures in a sandwich method, the jet milled budesonide was initially sieved through a 200 µm sieve and the spray dried API through a 400 µm sieve. Per blend, 4.9 g of LH-100 were then weighed into a specific vessel, followed by 0.2 g of API. For the top layer, another 4.9 g of LH-100 were added. For the preparation of the blend with jet-milled budesonide, a plastic vessel was used, whereas a metallic vessel was used for the spray dried mixture.

Afterwards, the jet milled formulation was mixed for 90 minutes at 62 rpm (revolutions per minute) in a tumble blender (T2F Turbula, Willy A. Bachhofen AG Maschinenfabrik, Muttenz, Switzerland). After sieving the blend at 400 µm, another 30 minutes at 32 rpm were added. The spray dried blend was initially mixed for 60 minutes at 62 rpm, then sieved at 400 µm and put in the tumble blender for 30 more minutes at 32 rpm.

Prior to proceeding, the mixing homogeneity had to be determined for both mixtures. From each blend, 10 samples of approximately 25 mg were taken: 3 from the top of the vessel, 4 from the middle and 3 from the bottom.

Electrostatic forces of the small particles made the collection of the proper amount difficult as some of the powder adhered to the neck of the flask or even to the outside. In the latter case, both flask and spatula were discharged prior to taking a new sample.

The samples were then dissolved in a total of 50 mL solvent (acetonitrile:purified water 70:30 v/v) each by placing them in an ultrasonic bath for approximately 20 minutes. Every few minutes the sample was checked for undissolved powder and if no solid particles were seen, the flask was taken out and shaken gently to make sure that all the powder that had formerly adhered to the neck of the flask could dissolve. Afterwards, a proper volume was transferred to labeled vials for high performance liquid chromatography (HPLC) analysis.

Knowing the API concentration and the mass of each sample, the API content could be calculated. The mixing homogeneity was expressed as the relative standard

deviation of API content of the blend throughout the 10 samples. For this study, a relative standard deviation of below 10% was considered acceptable.

Whenever not in use, the blends were stored in the desiccator over silica gel at room temperature.

2.1.2 The Next Generation Impactor (NGI)

2.1.2.1 Principle of function

Deposition in the lung and dissolution in lung lining fluid are important parameters for the effectiveness of an inhalable mixture.

The *in vitro* studies obtaining predictors for these values were performed via Next Generation Impactor (NGI, Copley Scientific, Nottingham, United Kingdom), which can be seen as a simulated lung. It is a multistage cascade impactor that enables measurement of the distribution of the active ingredient. The NGI consists of a frame that holds a cup tray with collection cups (stages), a lid with seal body containing nozzle pieces and O-rings and a handle to close the impactor. By closing the lid, the nozzle pieces are fixed in the right position above the corresponding cup.

The impactor is connected to a critical flow controller (model TPK, Copley Scientific, Nottingham, United Kingdom) and a vacuum pump. By pressing a specific button of the critical flow controller, the solenoid valve of the device is opened and allows the vacuum pump to suck air through the NGI in a saw tooth pattern. Different amounts of particles deposit in the collection cups as the velocity of the air stream is increased by forcing it through the nozzles with reducing jet diameters.

In order to discharge powder into the NGI, not only the devices mentioned above must be assembled, but also a pre-separator, an induction port, a mouth piece and the inhaler itself have to be attached. The inhaler (Cyclohaler, Pharmachemie, Haarlem, Netherlands) holds the capsules with the powder and is connected to the corresponding mouthpiece which is positioned at the oral end of the induction port. The induction port represents the throat and is put onto the pre-separator, which captures large particles. Fig. 1 shows the fully assembled NGI.

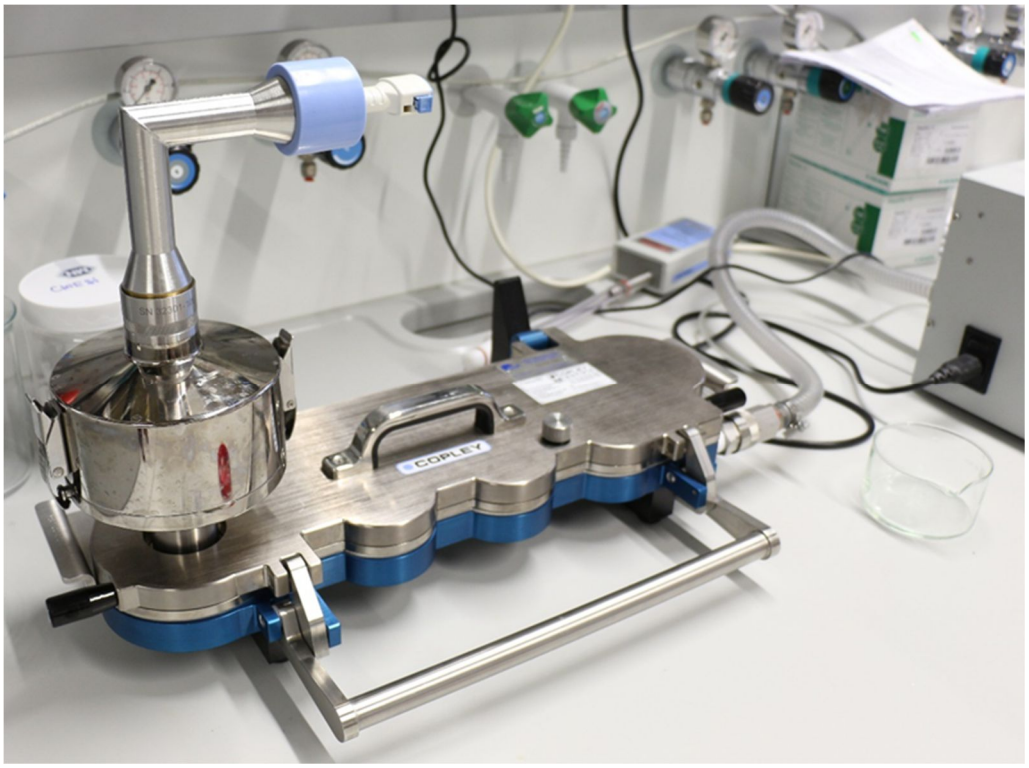


Fig. 1: Fully assembled NGI

2.1.2.2 Aerodynamic performance testing

The vacuum pump was switched on approximately 30 minutes prior to the first NGI experiment for warming up. All collection cups were coated with 2% TWEEN 20 in absolute ethanol and dried under the hood for 15 to 30 minutes. The coating solution helps to retain the API particles once they have deposited on the stage during the experiment. After drying, the tray with collection cups was put into the NGI body and the system was closed by pushing down the handle. The pre-separator was connected and filled with 10 mL of solvent prior to each experiment, followed by attaching the induction port, mouth piece and inhaler. For all NGI experiments, acetonitrile:purified water 70:30 v/v was used as a solvent. The purified water was taken from “TKA MicroPure UV UltraPure Water System” (TKA Wasseraufbereitungssysteme GmbH, Niederelbert, Germany).

Before the first experiment, the NGI must be calibrated. For the calibration, the mouthpiece and the inhaler are put aside. The timer of the critical flow controller is set to a high value and pressing the “timer” button opens the solenoid valve. Air is sucked through the NGI as long as the timer is counting. The flow meter (model

DFM3, Copley Scientific, Nottingham, United Kingdom) is inserted into the induction port as shown in Fig. 2 and measures the flow rate, which is adjusted to exactly 100,0 L/min by rotating the flow control valve. When the right amount of air is set, the P2 and P3 pressure values are obtained by pressing the representative buttons. The measurement of the absolute pressures ensures that the flow through the NGI is constant and independent from fluctuations of the pump. The P3/P2 ratio needs to be less than 0.5. If this requirement is met, a leak test is performed. A rubber stopper is inserted into the induction port and the tightness of the system is checked by setting the timer to 60 seconds and opening the solenoid valve. The rise of P1 pressure must not exceed 2.0 kPa during this period.

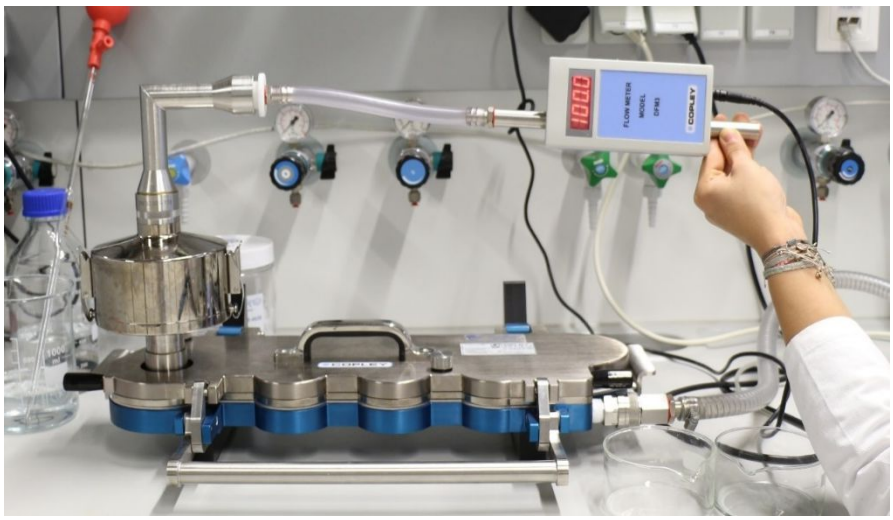


Fig. 2: Calibration of the NGI to a flow of 100 L/min

After a successful calibration, the experiments were performed and repeated 3 times (n = 3) for both blends. For each experiment, 4 capsules (Capsugel gelatin capsules size 0, stored in the desiccator before use) were filled manually with approximately 25 mg of the required mixture. The capsules were then put into the inhaler one by one. In order to allow a proper discharge of the power into the NGI, they were pierced twice before opening the solenoid valve. The powder in each capsule was given 2.4 seconds to be deposited. After discharging the 4 capsules consecutively, the NGI parts were dissembled and each one was rinsed with a specific amount of solvent. All discharged capsules were put into a beaker after use, and at the end of the experiment, the inhaler was added to the same beaker. This vessel was rinsed with 10 mL of solvent. The induction port was rinsed with 10 mL of solvent by closing its edges with parafilm and shaking the piece, the liquid was

then put into a beaker in which also the mouthpiece was stored after use. The pre-separator was rinsed with 50 mL of solvent and proper dissolution was maintained by closing the ends with parafilm and gently shaking the device. By dismounting the pre-separator, it could be seen that a significant amount of powder still adhered to the metallic vessel. As in the other parts of the NGI no solubility problems of the API were present, it was figured that the adhering powder could be the carrier lactose. Different techniques were tried to dissolve the powder during the 3 NGIs with the jet milled blend, including storing parts of the pre-separator in the ultrasonic bath for up to 20 minutes. However, no significant differences in the results were seen compared to rinsing the powder off as best as possible with the solvent of the pre-separator. Therefore, this approach was continued.

All of the 8 collection cups were rinsed with 10 mL solvent each.

Proper amounts of the API solution were taken from each compartment, resulting in a total of 11 vials per experiment for HPLC quantification of the drug content. They were stored in the freezer at -20 °C until HPLC processing.

From the NGI results, several important parameters to characterize new inhalable formulations can be obtained. The fine particle dose (FPD) is the amount of API with an aerodynamic diameter of below 5 µm. The emitted dose (ED) is the total mass of API found in the whole impactor (mouthpiece, induction port, pre-separator and impaction stages). Knowing these parameters, the fine particle fraction (FPF) can be calculated (FPD/ED). It is defined as the percentage of API that is < 5µm in size and represents the proportion of administered mixture that separates from the carrier and can therefore reach the deep lung. The FPF is by far the most common parameter to describe and compare the performance of different formulations. The MMAD (mass median aerodynamic diameter) is the diameter at which 50% of the particles by mass are larger and 50% are smaller. It provides insight into the expectable particle deposition pattern in the lungs.

The two mixtures were compared in terms of FPD, ED, FPF and MMAD.

2.1.2.3 Dissolution studies

In experiments performed with a special modification of the NGI collection cups, the dissolution of budesonide into simulated lung fluid (SLF) over time was observed. Per blend, the dissolution studies were performed in triplicate (n = 3).

The stage that had shown the highest API content in the NGI deposition studies (stage 2 for the spray dried, stage 3 for the jet milled blend) was equipped with a removable impaction insert.

Simulated lung fluid was produced directly before the dissolution experiment and consisted of DPBS (Dulbecco´s Phosphate Buffered Saline without Calcium and Magnesium, Lonza, Verviers, Belgium), DPPC (1,2-Dipalmitoyl-sn-glycero-3-phosphocholine, TCI Deutschland GmbH, Germany) and absolute ethanol. For each dissolution experiment, 13 mg of DPPC were dissolved in 1 mL of absolute ethanol by stirring on the heating plate. In parallel, 65 mL of DPBS were pre-heated to approximately 37 °C. The DPPC-ethanol solution was then added slowly, drop by drop, to the PBS puffer and stirred properly for about 30 minutes. Afterwards, 55 mL of the SLF were transferred into a crystallizing dish, and the rest of the solution was poured into a smaller beaker which was sealed with parafilm to avoid vaporization. Both dishes were placed on a laboratory shaker at 37 °C and 60 rpm.

Proceeding with the NGI, a 0.1 µm membrane filter (Sterlitech Corporation, Kent, United States) was adjusted in size to fit the impaction insert, and the corresponding membrane holder was put out ready. The NGI was prepared and the experiment performed as described in the section above. After rinsing the capsules and inhaler, mouthpiece and throat and the pre-separator with solvent, the seal body was opened, and the impaction insert was removed from the corresponding collection cup as shown in Fig. 3.

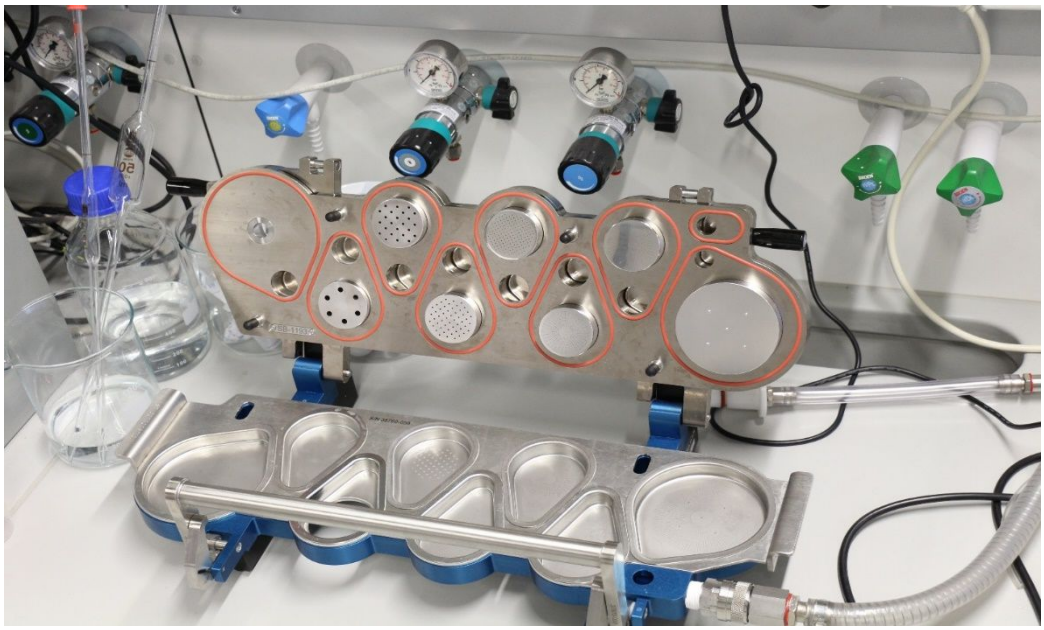


Fig. 3: NGI - Open seal body with deposited powder in the collection cups and removed impaction insert of stage 3

The membrane filter was then pre-soaked with SLF and placed over the impaction insert with the shining side up. After sealing the filter in place by screwing the membrane holder onto the insert, it was put into the crystallizing dish with 55 mL of SLF. An amount of 500 µL SLF was transferred into HPLC vials with glass inserts (Agilent Technologies, Vienna, Austria) at the beginning of the dissolution and then after 2, 5, 10, 20, 40, 60, 120 and 180 minutes. Every time a sample had been taken, the volume was replaced with 500 µL of SLF from the small beaker immediately. Per experiment, in total 20 samples were taken and stored in the freezer until HPLC measurement.

The extent of dissolution of budesonide over time was described as the percentage of dissolved API mass in SLF at each time point in relation to the total dissolved API content determined after 180 minutes in the same NGI experiment.

2.2 Cell studies

2.2.1 Essentials of cell culture

All cells in this study were cultivated either in 75 cm^2 or 175 cm^2 flasks (Greiner Bio-One GmbH, Rainbach, Austria). In general, the cells adhere to the bottom of the flask and are covered with a specific medium that contains the necessary nutrients to enable cell growth. 75 cm^2 flasks must be filled with at least 15 mL of medium,

whereas the 175 cm^2 flasks need at least 25 mL to have their surface properly covered. Approximately every second to third day, the medium has to be changed. Therefore, it is removed with a glass pipette from the angle of the flask and new medium is added. To avoid detaching of the cells, the new medium is not put directly onto the cells but on the side wall of the flask. It is important to keep track of cell growth as proliferation is inhibited if the cell layer becomes too tight (contact inhibition). Depending on the duplication rate of each cell line, subculture of the cells had to be performed from time to time as described in 2.2.1.2 (Subculturing of cells).

All flasks were stored in an incubator (Galaxy R CO_2 Incubator, RS Biotech, Scotland, Great Britain) at 37 °C in humidified air enriched with 5% CO_2.

Whenever manipulation of the cells was necessary, it was done under a Herasafe KSP class II Biological Safety Cabinet (Thermo Scientific, Thermo electron GmbH, Mannheim, Germany). Moreover, sterile equipment was used for the experiments and all media and solutions were pre-warmed to 37°C before use.

The used centrifuge model was a Heraeus Multifuge 3 L-R.

2.2.1.1 Thawing of cells

In this diploma thesis, both established cell lines and cells of primary culture (= isolated from native tissue) were studied. Established cell lines can be purchased from different providers and are stored frozen in small vials in the vapor phase of a liquid nitrogen tank. Calu-3 cells were purchased from the American Type Culture Collection (ATCC, HTB-55, LGC Standards GmbH, Wesel, Germany). DMBM-2 and A-549 cells were obtained from Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The J774 cells were kindly donated by the Clinical Department of Hematology of the LKH-Universitätsklinikum Graz (Graz, Austria).

Whenever new cells were needed, they were thawed by swiveling the cell vial for approximately 1 minute in a water bath at 37 °C. The still partly frozen cell pellet was suspended in 15 mL of specific medium and then centrifugated at 800 rpm for 5 minutes. The supernatant was removed by sucking it off with a glass pipette and the cell pellet resuspended in 1 mL of new medium and filled into a 75 cm^2 flask,

which had been filled with 14 mL of medium previously, or a 175 cm^2 flask with 24 mL medium to achieve the necessary end volume.

2.2.1.2 Subculturing of cells

As mentioned above, every second or third day, the medium in the cell culture flaks was renewed. Microscopy was done with each medium change and as the cell lines were used frequently in the lab, it was possible to estimate appropriate timing for their splitting. Generally, the cells were subcultured when approximately 80% of the surface of the flask was covered. For this step, they must be detached from the flask. The medium was discarded and as all media contained fetal bovine serum (FBS), which inhibits the following step, the 75 cm^2 flasks were washed with 7-10 mL and the 175 cm^2 flasks with approximately 15 mL of DPBS buffer (Dulbecco´s Phosphate Buffered Saline without Calcium and Magnesium, Lonza, Verviers, Belgium). After removing the buffer, 1 mL of trypsin solution (0.05% trypsin-EDTA, Gibco) was added to the small flasks and 2 mL to the large flasks. The flasks were then kept in the incubator for a specific time depending on the cell line. DMBM-2, J774 and A-549 were only stored for approximately 3-5 minutes, whereas the PBMC and Calu-3 cells needed more time. Detachment can be enhanced by tapping on the side surface of the flask. As the trypsin solution should not be in contact with cells more than 30 minutes because it can damage them, a cell scraper was used if the cells (mainly Calu-3) had not yet detached after half an hour. An inverted microscope (Olympus CKX41, Olympus Austria GmbH, Wien, Austria) was used to follow the process of the detachment. Moreover, it can be seen with the naked eye: when turning the cultivation flask, white liquid representing the cell suspension runs down the bottom of the flask. The trypsin reaction was stopped by adding cell-specific medium (see 2.2.2. Used cell lines and their cultivation), depending on flask size (5 mL for the 75 cm^2 flasks and 10 mL for the 175 cm^2 flasks) and the cells resuspended by pipetting the suspension up and down for approximately 10 times. A specific amount was filled into a test tube (CASY cup) for counting and 10 mL of CASYton were added. The number of cells was obtained with the CASY cell counter, model TT (Innovatis AG, Reutlingen, Germany). The counter has different modes for different cell types. For counting the Calu-3 and DMBM-2 cells, the EA.hy926-mode was used, for A-549 the A-549 mode and for

J774 and PBMC cells the THP-1 mode. In all listed modes except the THP-1 mode an amount of 50 µL of cell suspension was added to the CASY cup for counting. From the J774 and PBMC cell suspension 100 µL were taken and transferred into the counting tube. The sample was shaken carefully to disperse the cells in CASYton and put into the counter, which after 3 measuring cycles displays the number of total and viable cells per mL as well as the viability. For the permeation and uptake studies, a certain number of cells had to be seeded into inserts. With the CASY values it was possible to determine if the number of cells was enough for that purpose. When the number of cells exceeded the necessary number, an appropriate cell suspension was prepared. After centrifugation of the centrifuge tube with the cell suspension (800 rpm, 5 min) the supernatant was discarded, and the cell pellet resuspended in an appropriate volume to get a suspension of the needed concentration for seeding. The rest was usually transferred into a new flask with an appropriate amount of new medium adding up to the necessary end volume of the flask.

2.2.1.3 Seeding

For both permeation and uptake experiments, 12-well plates (Greiner Bio-One GmbH, Rainbach, Austria) with translucent ThinCert cell culture PET inserts with a pore size of 0.4 µm (Greiner Bio-One GmbH, Rainbach, Austria) were used. Depending on the cell line, suspensions with a specific number of cells per mL were generated after detachment of the cells. 500 µL of the cell suspension were transferred into each insert, whereas the well itself was filled with 1.5 mL of the appropriate cell medium. To disperse the cells on the membrane and to avoid air bubbles underneath the insert, the plate was moved gently after seeding. As illustrated in Fig. 4, the insert-well-constitution with the cells that have settled down on the membrane generates an apical (upper) and basolateral (lower) compartment, which not only enables different types of cultivation but is also fundamental for the experimental setup, which will be described in section 2.2.3.

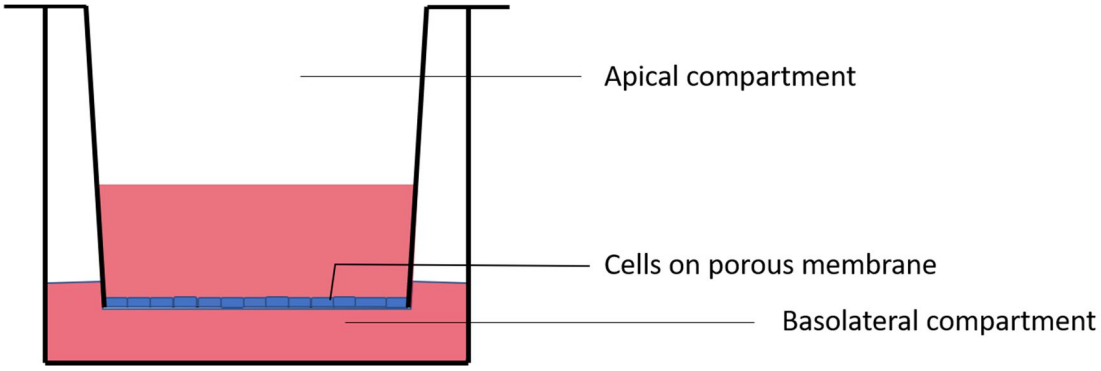


Fig. 4: Schematic overview of the insert-well structure

2.2.2 Used cell lines and their cultivation

2.2.2.1 Calu-3

Calu-3 cells are human epithelial cells derived from an adenocarcinoma of the lung. As they form tight monolayers, they can be used perfectly to study transport of budesonide over time.

They were cultured in a 75 cm^2 flask and cultivated with what will be referred to as “Calu-3 complete medium” (90% minimal essential medium (MEM) with Earle´s salts, enriched with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 2 mM L-glutamine, v/v, Gibco). Every second or third day, the medium was changed and after approximately 1 week, the number of cells was high enough for seeding. After detachment, a cell suspension of 1 million cells/mL was prepared. Two 12-well plates were equipped with inserts as described above. Each insert was filled with 500 µL of the cell suspension, except for one blank in each plate, which was filled with 500 µL of Calu-3 complete medium. Each well was filled with 1.5 mL of Calu-3 complete medium.

Calu-3 cells seeded into inserts can be cultivated in two different ways – ALI (air-liquid interface) and LC (liquid cultivation). The LC mode is characterized by medium on both sides of the cell layer, whereas ALI cultivated cells are exposed to medium on the basal side and air on the upper side (representing basal and apical side of epithelium in the lungs.)

Regarding ALI cultivated Calu-3 cells, the medium of the apical compartment was removed one day after seeding and the volume of the basolateral compartment reduced to 500 µL to avoid hydrostatic pressure on the cells from below.

Every second to third day, the medium was changed. On the plate with ALI cultivated cells, the medium from the basolateral compartment was removed with a glass pipette. The well was filled up again with 500 µL of Calu-3 complete medium. As for the Calu-3 cells in liquid cultivation, primarily the medium from the basolateral side was sucked off, then the medium from the insert itself was gently removed with a large pipette tip without damaging the cell layer or porous membrane.

One week after seeding, the first measurements of the transepithelial electric resistance (TEER) were performed. The TEER value is a parameter that provides information about cell connection and interlinkage. As the cell junctions tighten, TEER values increase. It was measured with an EVOM STX-2-electrode (World Precision Instruments, Berlin, Germany) with 2 arms of different length. The short arm always reaches into the insert, the long one into the well. The electrode was connected to a Millicell ERS Voltohmmeter (World Precision Instruments, Berlin, Germany). The electrodes were moisturized with distilled water prior to the first measurement, which was always the blank value. As the blank insert does not contain any cells, its TEER value represents the resistance of the translucent membrane. Assuming that each well has an area of 1.13 cm^2, the resistance can be calculated according to the following equation (Eq. 1):

$$TEER = (TEER\ measured\ (\Omega) - TEER\ blank(\Omega)) * 1.13\ cm^2 \quad (\Omega*cm^2) \quad \text{Eq.1}$$

The measuring electrodes were cleaned after use by placing them in 70% ethanol for 15 minutes and then taken out of the liquid to dry for the next use.

The TEER was measured every second to third day and coupled with medium changes. When the values calculated from the equation reached 300 $\Omega*cm^2$, the cell monolayer was considered tight enough to perform the experiments.

2.2.2.2 A-549

A-549 cells, like Calu-3 cells, are human cells derived from an adenocarcinoma of the lungs. In this study, they were used to explore budesonide uptake but not permeation as they do not form tight intercellular junctions.

The cells were cultivated in a 175 cm^2 flask with A-549 complete medium (90% Dulbecco´s Modified Eagle Medium (DMEM) with 4.5 g/L D-glucose and L-glutamine, enriched with 10% FBS, 2mM L-glutamine and 1% penicillin/streptomycin, v/v, Gibco).

The medium was changed every second to third day until the number of cells was high enough to fill the inserts. 500 µL of a 0.5 million cells/mL suspension were transferred into each of 11 inserts. As no TEER measurements were necessary, there was no need for a blank. One day after seeding, the cultivation type was switched to ALI, meaning that with the first medium change, only the basolateral compartments were filled up again with 500 µL A-549 complete medium each, and the apical compartments remained empty.

The medium in the lower compartment was changed every 3 days and after 7 days of cultivation, the uptake experiment was performed.

2.2.2.3 DMBM-2

DMBM-2 cells are macrophages that have been derived from the bone marrow of a female C3H/HeJ mouse. They were cultured in a 175 cm^2 flask and DMEM supplemented with 20% horse serum, 2 mM L-glutamine and 1% penicillin/streptomycin v/v (Gibco) was used as medium.

500 µL of a 1 million cells/mL suspension were filled into each insert. Like A-549 cells, DMBM-2 cells do not form intercellular junctions and the TEER does not have to be measured, therefore there was no need for a blank insert. After one day of letting the cells attach to the translucent membrane, the uptake experiment could be performed.

2.2.2.4 J774

J774 cells are another well-established line of murine BALB/C macrophages. They were cultivated in a 175 cm^2 flask with DMEM (with 4.5 g/L D-glucose and L-glutamine), enriched with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin, v/v (Gibco).

500 µL of a 1 million cells/mL suspension were transferred into each of the prepared inserts. No TEER measurements were necessary and as J774 cells grow

loosely and do not form monolayers, the uptake experiment was carried out one day after seeding.

2.2.2.5 PBMC

In order to study the effect of API particle size and shape on human immune cells from a primary culture as well, peripheral blood mononuclear cells (PBMC) were isolated from fresh human blood. The blood was provided by the Department of Blood Group Serology and Transfusion Medicine of the LKH-Universitätsklinikum Graz (Graz, Austria) and transferred to the laboratory in a leukocyte reduction chamber.

In total, five 175 cm^2 flasks were coated with 15 mL of 10 µg/mL rat collagen (collagen type I solution from rat, Sigma-Aldrich, Vienna, Austria) each. The flasks were stored at 37 °C for 1.5 hours, then washed with approximately 15 mL of DPBS and dried in the incubator.

For isolation of the PBMC, Ficoll-Paque PLUS (GE Healthcare Bio-Sciences, Uppsala, Sweden) was used. It is a density gradient media consisting of polysaccharides that enables easy collection of PBMC. The blood of the reduction chamber (approximately 15 mL) was dripped into 25 mL of DPBS buffer. The suspension was then gently and slowly added to 15 mL of Ficoll-Paque PLUS to prevent mixing. While the density gradient media created a layer at the bottom of the centrifuge tube, the blood/DPBS was supposed to stay above. The centrifuge tube was centrifugated at 400 × g for 35 min. By avoiding use of the brake of the centrifuge, it was made sure that the layering created by centrifugal forces and the ficoll molecules remained intact.

As shown in Fig. 5, the following layers from top to bottom could be observed in the centrifuge tube after centrifugation: blood plasma, PBMC ring, Ficoll-Paque PLUS, sediment of erythrocytes and granulocytes

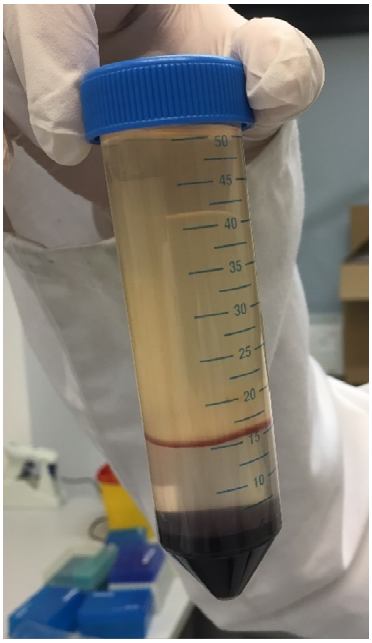
. 

Fig. 5: Layering of blood components generated by Ficoll-Paque PLUS after centrifugation

The reddish PBMC ring was transferred to two 15 mL centrifuge tubes with a Pasteur pipette that had been rinsed with 70% ethanol and DPBS buffer. The centrifuge tubes were filled up to the 10 mL mark and afterwards centrifugated for 5 minutes at 400 × g without brake. The supernatant was discarded, and the cell pellet resuspended in 10 mL of DPBS each and centrifugated again for 5 minutes at 400 × g. After discarding the DPBS, each cell pellet was resuspended in 10 mL of RPMI complete medium (90% RPMI supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% non-essential amino acids, 1% L-glutamine and 1% sodium pyruvate, v/v, Gibco) and the cell suspensions were transferred into a new 50 mL centrifuge tube, to which 30 mL of RPMI complete medium were added. The number of cells was counted with the TC20 Automated Cell Counter (Bio-Rad Laboratories GmbH, München, Germany). 75 million cells were added to each flask and RPMI complete medium was filled in until a proper volume of 25 mL was reached in the flasks.

The cells were given 3 hours to grow on the coated flask, then the medium was changed to a differentiation promoting medium (RPMI complete with 10 ng/mL GM-CSF (recombinant human GM-CSF, Peprotech, purchased from Eubio, Vienna, Austria)). Later calculations showed that from the cells that initially were filled into the flasks approximately 6% actually were adherent, while the rest was removed by the first medium change. On the third day, the differentiation promoting medium was

renewed. On the 6th day, the differentiation medium was replaced by RPMI complete without GM-CSF and the cells cultivated one more day until they were seeded into inserts. After detachment from the flasks, a cell suspension of 2 million cells/mL was generated and 500 µL were transferred to each insert. No TEER measurements were performed. One day after seeding, the cells were considered ready to test the uptake of budesonide.

2.2.3 Experimental setup for cellular uptake studies

To evaluate the uptake and accumulation of differently modified budesonide particles, the cells on the inserts were exposed to suspensions of raw, jet milled and spray dried budesonide of the same molarity. In several pilot experiments, different approaches were tried to find a suitable concentration for cell treatment. As the samples were measured via HPLC, the amount of detectable budesonide had to be high enough to meet the HPLC limit of quantification (0.1 µg/mL), but exposure doses should not be too unrealistic. A concentration of 60 µM/mL was considered acceptable to meet both requirements.

Before each experiment, 300 µM stock suspensions of raw, jet milled and spray dried budesonide in a specific medium (Minimal Essential Medium (MEM) + 1% L-glutamine without phenol red, Gibco) were prepared. Homogenization was maintained by placing the centrifuge tubes in the water bath at 37 °C for approximately 30 minutes and mixing them every 5-10 minutes. Afterwards, a proper amount was diluted in the specific medium to generate suspensions of 60 µM/mL.

The experiments were performed under light protected conditions in a microplate incubator (THERMOstar, BMG LAB TECH GmbH, Ortenberg, Germany) at 37 °C and a shaking intensity of 200 rpm. In general, all uptake studies were performed in triplicate. Per cell line, 3 cell-filled inserts were treated with the 60 µM suspensions containing raw, jet milled or spray dried budesonide. As per cell line 11 inserts had been seeded, 2 inserts were used as blank and treated with medium only. They served to determine the number of cells per insert later. All samples were transferred to HPLC vials with glass inserts (Agilent Technologies, Vienna, Austria) and frozen at -20 °C immediately.

For each experiment, a new 12-well plate was prepared, and 11 wells were filled with exactly 1500 µL of MEM+L-glutamine without phenol red. Afterwards, any liquid in the apical compartments of the cell-filled inserts to test was removed gently with a large pipette tip. In the next step, 600 µL of treatment suspension or medium were added to the apical (donor) compartment of the inserts, which were then placed in the previously filled wells. Fig. 6 illustrates the experimental setup. Table 1 shows the treatment for all cell lines schematically.

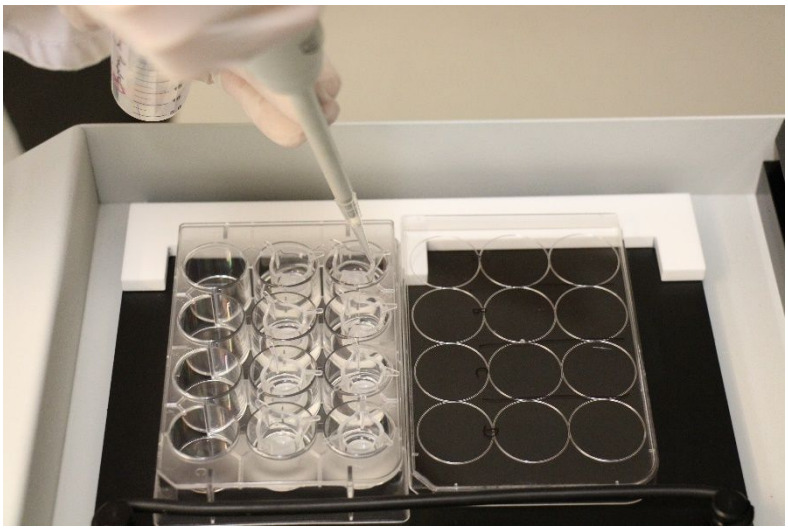


Fig. 6: Experimental setup of one of the pilot experiments.
The main studies were performed in triplicate.

A sample of 100 µL was taken from the upper compartment immediately to obtain the starting concentration. After 120 minutes, a 100 µL sample was taken from the basolateral (receiver) compartment and a 100 µL sample from the apical compartment again.

Table 1: Schematic overview of insert treatment for all cell lines

Insert	A	B	C	D
1	raw	jet milled	spray dried	MEM+L-glutamine without phenol red
2	raw	jet milled	spray dried	MEM+L-glutamine without phenol red
3	raw	jet milled	spray dried	empty

After the last sample had been taken, the content of the donor and receiver compartments was removed and separately frozen in labeled Eppendorf Tubes.

In the nine inserts that had been treated with budesonide suspensions, the cellular uptake had to be measured, whereas the two inserts treated with medium were once more supplied with 500 µL of MEM+L-glutamine without phenol red from above and 1.5 mL from below and stored in the incubator until they were used to determine the number of cells.

From the budesonide-exposed inserts, the cells were removed by adding 300 µL of Aqua dest. (Aqua bidest. “Fresenius”, Fresenius Kabi Austria GmbH, Graz, Austria) to each upper compartment and scratching the cell layer off gently with a large pipette tip. The cell suspension was then transferred to properly labeled CRYO.S cryovials (Greiner Bio-One GmbH, Rainbach, Austria) that had been filled with 700 µL of acetonitrile previously. The cryovials were put into liquid nitrogen for 30 minutes. In the meantime, the two inserts for cell counting were processed: The medium was removed from the apical compartment and 100 µL of trypsin solution were added. Depending on the cell line, the inserts were stored in the incubator for

a specific time. Whereas DMBM-2 and J774 only needed a few minutes, Calu-3 cells and PBMC were given 15 minutes. A-549 cells were stored in the incubator for 10 minutes for detachment. Then, the trypsin reaction was stopped by adding 500 µL of the cell-specific complete medium. The cells were scratched off the membrane with a large pipette tip and well resuspended. The number of cells was measured with the CASY counter.

After 30 minutes, the cryovials were taken out of the liquid nitrogen and thawed for 30 minutes at room temperature as shown in Fig. 7. Due to the treatment with Aqua dest., acetonitrile and the freezing, it was ensured that the cell membranes were destroyed, and the accumulated budesonide had been released. The samples were centrifugated for 10 minutes at 4000 rpm and 800 µL of the supernatant were transferred into HPLC vials for quantification.

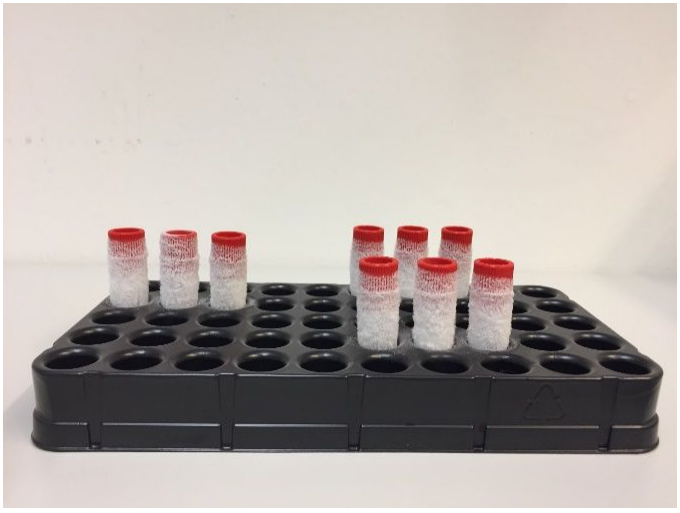


Fig. 7: Thawing of cryovials

From the detected budesonide concentration and the number of cells obtained with the CASY counter, it was possible to determine and compare the extent of accumulation of the respective API. For each cell line, the mean number of cells of the two measured inserts was calculated and the uptake expressed as µg detected budesonide in the supernatant per 1 million cells. Moreover, the recovery rate in % was calculated according to the following equation (Eq. 2):

$$Recovery\ rate = \frac{\sum m}{m_0} \quad (\%) \quad \text{Eq.2}$$

∑m amount of budesonide in the donor and receiver compartment together 120 minutes after start (µg)

m_0 amount of budesonide in the donor compartment at start

2.2.4 Experimental setup for cellular permeation studies

Not only the uptake, but also the permeation of the 3 API types was studied in Calu-3 cells and an extended version of the experimental setup for uptake testing was used.

First, the 300 µM stock suspensions and the 60 µM treatment suspensions were prepared as described in the section above. Afterwards, the TEER was measured first in Calu-3 complete medium, then in MEM+L-glutamine without phenol red. The two inserts with the lowest TEER were treated with medium only, whereas the other inserts were treated with the respective budesonide suspension (as described in 2.2.3). At the beginning of the experiment, a sample of 100 µL was taken from the donor compartment to obtain the starting concentration. From the receiver compartments, 100 µL samples were taken at the beginning and after 30, 60, 90 and 120 minutes. The loss of volume was substituted with pre-warmed 100 µL of MEM+L-glutamine without phenol red each time a sample had been taken. After 120 minutes, another 100 µL were taken from the donor compartment. Afterwards, the content of the upper and lower compartments was removed and separately frozen in labeled Eppendorf Tubes.

The apical compartment was once more filled with 500 µL of MEM+L-glutamine without phenol red and the basolateral one with 1500 µL to perform the last TEER measurement which should ensure that neither the cell layer nor the membrane had been damaged throughout the experiment. Afterwards, both the inserts treated with budesonide and the two inserts for cell counting were processed according to the description of the uptake experiment.

Regarding Calu-3 cells, the different API and cultivation types (ALI vs. LC) were not only compared in terms of budesonide accumulation, but also the permeability for

the respective API. Therefore, the *in vitro* apparent permeability coefficient (P_{app}) was calculated using Eq. 3:

$$P_{app} = \frac{\Delta Q}{\Delta t * A * c_0} \quad \text{(cm/s)} \quad \text{Eq.3}$$

ΔQ difference of budesonide amount in the receiver compartment at time point 120 min and 30 min after start (µg)

Δt duration of experiment (s)

A area of insert (cm^2)

c_0 starting concentration measured in the donor compartment at the beginning of the experiment ($µg/cm^3$)

Furthermore, for each time point the percentage of API that had already permeated to the receiver compartment in relation to the starting point and the recovery rate were calculated. The API accumulation was expressed as µg detected budesonide/million cells as described above.

2.3 HPLC settings and measurement of samples

The budesonide concentration of all samples was measured via a Waters Alliance 2695 HPLC instrument (Waters Corporation, Milford, USA) and a photodiode array detector (Waters 2996), based on a validated method previously established.

As mobile phase, the following was used: 70% acetonitrile + 30% buffer (0.02 M NaH_2PO_4, adjusted to a pH value of 3.0 with H_3PO_4). As stationary phase a Waters Atlantis dC18 3µm, 3.9 x 150 mm column was utilized. A flowrate of 1 mL/min and a column temperature of 40 °C were chosen. Per measurement, 50 µL of the sample were injected into the HPLC system by an autosampler. For the preparation of the standards, acetonitrile:purified water 70:30 v/v was used as a diluent.

3 Results and Discussion

3.1 In vitro lung studies

3.1.1 Particle characterization

3.1.1.1 Laser diffraction

Raw, jet milled, and spray dried budesonide particles were characterized by their x10, x50 and x90 diameters as summarized in Table 2.

Table 2: Characteristic particle diameters of raw and processed budesonide

API type	x10 (µm)	x50 (µm)	x90 (µm)	SPAN (µm)
Raw	0.57	3.82	12.07	11.92
Jet milled	0.43	1.75	4.25	4.00
Spray dried	0.69	1.63	3.04	2.61

As x10, x50 and x90 represent the 10^{th}, 50^{th} and 90^{th} percentiles of the particle diameter, the values provide insight into the particle size pattern. Raw budesonide particles are generally larger than the processed API particles, which are, with x50 values of 1.75 µm for jet milled and 3.04 µm for spray dried budesonide, small enough to reach the deep lung.

The span of the raw API diameter is the largest, indicating big differences in particle size, which is considered normal for the non-processed material. With jet milling, a span of 4 µm can be achieved, and spray dried particles differ the least in their diameter with a span of 2.61 µm. This indicates that the spray dried particles are most homogenous in size.

3.1.1.2 Differential scanning calorimetry (DSC)

With DSC analysis it was observed that jet milled and spray dried budesonide differ in solid state. The thermograms are presented in Fig. 8 and Fig. 9. As the DSC thermogram of the jet milled API shows only one endothermic peak at 253.3 °C, the crystalline nature of the powder was confirmed. This is in good agreement with Velaga et al., who reported that the melting point of crystalline budesonide is 258.7 °C (10). As for the spray dried budesonide, two peaks were seen, one recrystallization exotherm with a peak at 126.1 °C and one endothermic event with a peak at 249.7 °C, which indicates the amorphous nature of the powder. The results are in a good agreement with the conclusion of Tajber et al. (3), who observed an exotherm peak at 130 °C and an endotherm peak at 262 °C for spray dried predominantly amorphous budesonide.

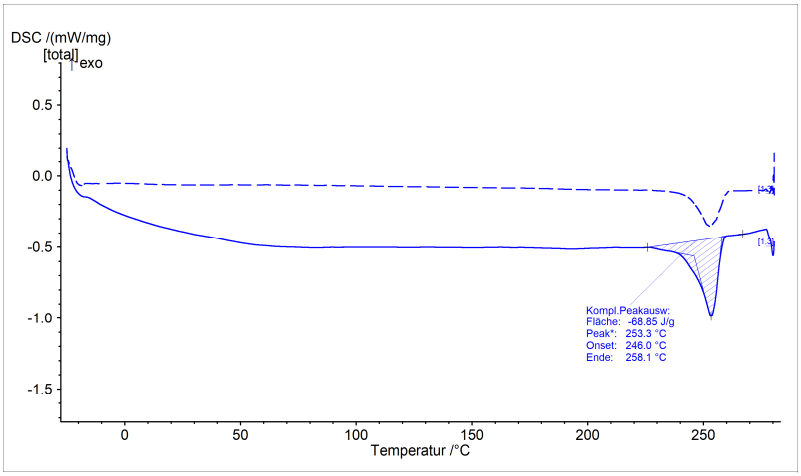


Fig. 8: DSC thermogram of jet milled budesonide

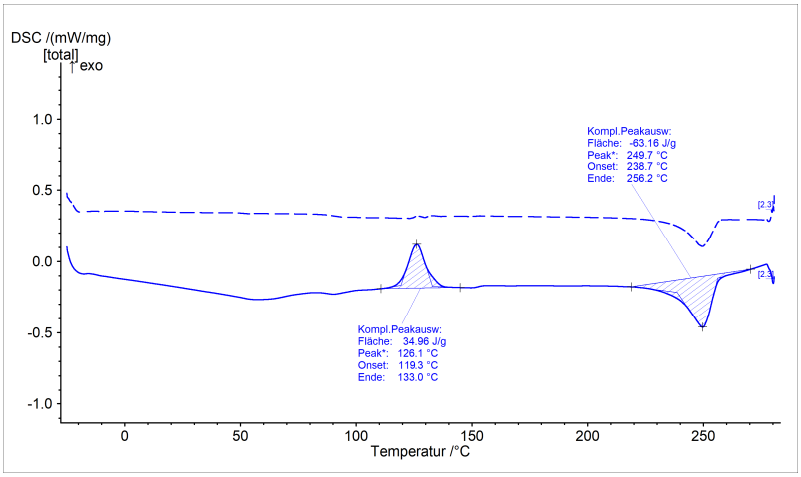


Fig. 9: DSC thermogram of spray dried budesonide

3.1.1.3 Scanning electron microscopy (SEM)

With SEM analysis, a difference in particle shapes was observed. The raw budesonide material was composed of rough, rod-shaped and irregular particles of larger size (Fig. 10a), whereas the spray dried powder particles were spherical with a smooth surface (Fig. 10c). The jet milled budesonide resembled the raw material but was smaller and more homogenous in particle size (Fig. 10b).

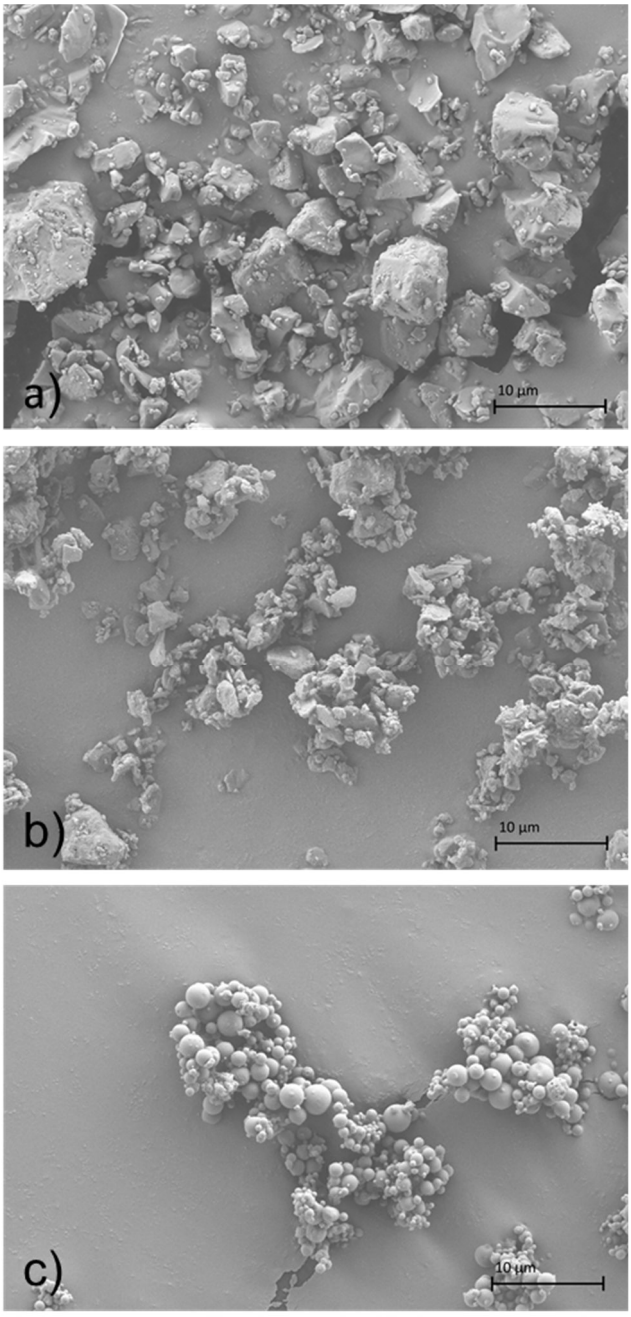


Fig. 10: SEM micrographs of a) raw b) jet milled and c) spray dried budesonide

In Fig. 11, the particle configuration of API attached to LH-100 particles of the two different blends is presented. Once more, it could be clearly distinguished between spherical spray dried (Figure 11b) and more irregularly shaped jet milled particles (Figure 11a) distributed over the carrier surface.

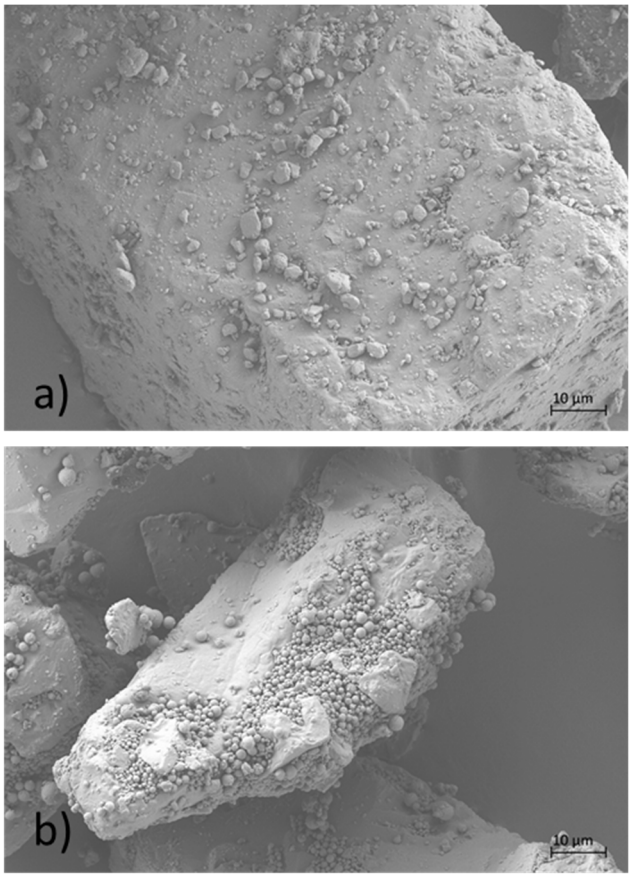


Fig. 11: SEM micrographs of a) jet milled and b) spray dried budesonide particles attached to LH-100

3.1.2 NGI results

3.1.2.1 Aerodynamic performance testing

After blending for both mixtures, the deviation from the mean API content was 6.85% for the blend with the jet milled and 6.68% for the blend with the spray dried API. According to Hassan et al., the two blends can therefore be considered homogenous (11).

Per blend, 6 NGI experiments were performed and the FPD, ED, FPF and MMAD were calculated. The average respective parameters and corresponding standard deviations are summarized in Table 3.

Table 3: Results of aerodynamic performance testing (n = 6, mean ± SD)

Blend type	FPD (µg)	ED (µg)	ED (%)	FPF (%)	MMAD (µm)
Jet milled	243.07 ± 36.19	1682.35 ± 167.03	84.15 ± 2.95	14.45 ± 1.72	3.15 ± 0.24
Spray dried	183.72 ± 52.41	1806.44 ± 195.55	85.69 ± 5.75	10.36 ± 3.47	4.61 ± 0.51

As for the ED, no notable difference could be observed, whereas the results show that the FPF for the jet milled blend with a value of 14.45% is significantly higher than the FPF for the spray dried blend with a value of 10.36% ($p < 0.05$, 2-tail t-test for equal variances). As the respirable fraction is higher for the blend with jet milled budesonide, more of the jet milled API reaches the part of the NGI that represents the deep lung. It is therefore likely that under the same circumstances of inhalation, more jet milled than spray dried API might be able to reach the alveoli. One possible explanation of the different FPD and FPF between the blends in the NGI studies might be the particle size. The mass median aerodynamic diameter (MMAD) can be used to describe the average particle size of an inhalable API. While the mean MMAD of the spray dried blend is 4.61 µm, the mean MMAD of the jet milled blend is noticeably smaller with a value of 3.15 µm. The larger MMAD of the spray dried particles could be a result of the formation of particle agglomerates that are not dispersed or not entirely dispersed during inhalation.

3.1.2.2 Dissolution studies

For both blends 3 NGI experiments coupled with dissolution tests were performed and the results expressed in % of dissolved budesonide at the respective time points as described in section 2.1.2.3. For both blends, the average percentage at each time point and the standard deviation were calculated, and the results were plotted into a chart (Fig. 12) to visualize the trend.

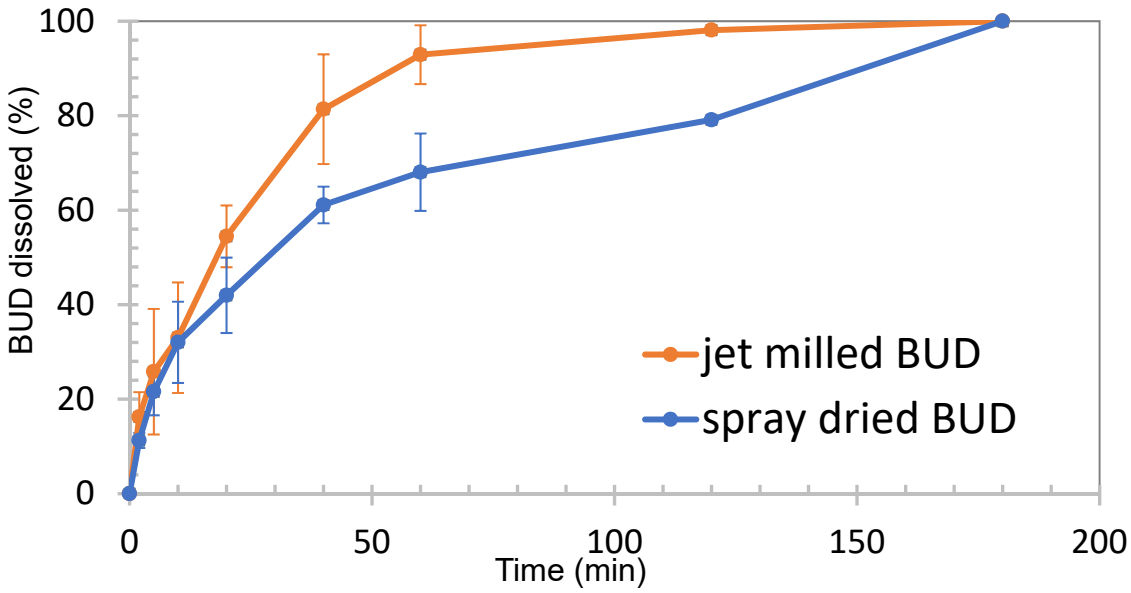


Fig. 12: Dissolution of jet milled and spray dried budesonide into SLF over time (n = 3, mean ± SD)

The results of the dissolution showed slightly different dissolution behavior between the two formulations. The equilibrium solubility in SLF of budesonide is 25.85 µg/mL and it was assumed that everything deposited is dissolved after 180 min. So, the final API content dissolved after 180 min was similar. Looking at the dissolution over time, the dissolution graph for jet milled budesonide shows a steep gradient of the curve from the beginning and the percentage of dissolved powder reaches an almost stable value after 60 minutes, whereas the dissolution of spray dried budesonide into SLF appears to be slower and does not reach a plateau so early. It seems that the dissolution has not yet reached its equilibrium. At all time points, the percentage of dissolved jet milled budesonide was higher than the percentage of dissolved spray dried API with significant differences at 20, 60, and 120 min ($p < 0.05$, 2-tail t-test for equal variances). Given that the experiments were carried out under conditions that can be considered equal, this indicates that shape and size of jet milled budesonide particles promote faster dissolution into simulated lung fluid, which might be of special interest for the estimation of time to the onset of action in the lung.

3.2 Cell studies

The setup of the permeation and uptake experiments was challenging because many factors had to be taken into consideration. Firstly, it was required that the liquid to prepare the budesonide suspensions was not harmful but nourishing for the cells. It should not alter their physiology so that *in vitro* budesonide transport and accumulation could be researched in a setting as close as possible to *in vivo* conditions. Secondly, the budesonide concentration had to be kept as low as possible to imitate *in vivo* conditions, but still high enough to enable HPLC detection. At the same time, the amount of dissolved API should be as small as possible because the aim of the thesis was to see if there were different outcomes in the cell studies that were due to the different particulate – not molecular – properties of processed budesonide, as jet milling and spray drying are not known to alter the molecular structure of the API. If the cells were treated with equimolar budesonide solutions, no difference in the investigated parameters would be detectable. Therefore, the cells had to be treated with a liquid that had, to a large extent, the properties of a suspension, and to a small extent the properties of a budesonide solution. Lastly, it had to be ensured that the fluid to treat the cells with did not interfere with budesonide detection and showed no peaks around the budesonide spikes in the chromatograms. The cell medium without phenol red met these criteria and could therefore be used to perform the experiments.

3.2.1 Cellular permeation studies

For each suspension and cultivation type, the experiment was performed in triplicate. The transported amount of budesonide at each time point was expressed in % of the starting concentration, from which values the average cumulative amount of permeated API and its standard deviation were calculated and plotted in Fig. 13 and Fig. 14. Finally, the average *in vitro* apparent permeability coefficient (P_{app}) and its standard deviation were determined and compared for each budesonide type as described in section 2.2.4 and summarized in Table 4.

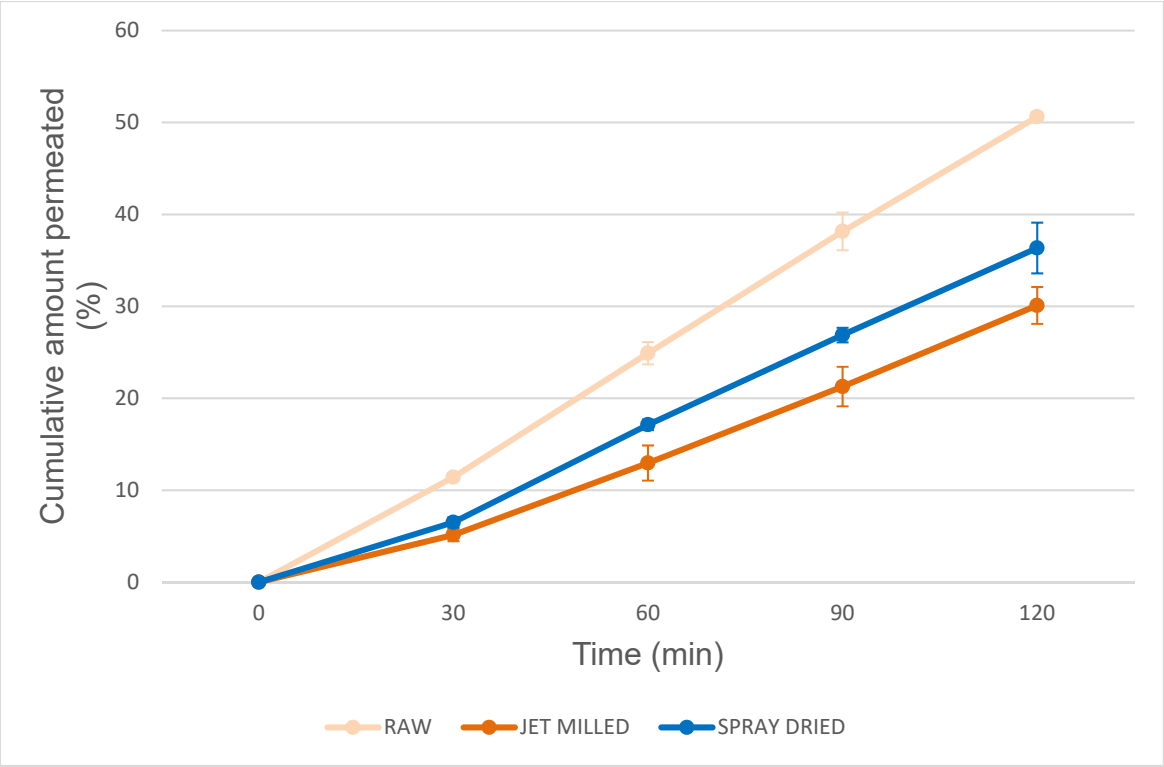


Fig. 13: Permeation of budesonide types over time through an ALI cultivated monolayer of Calu-3 cells

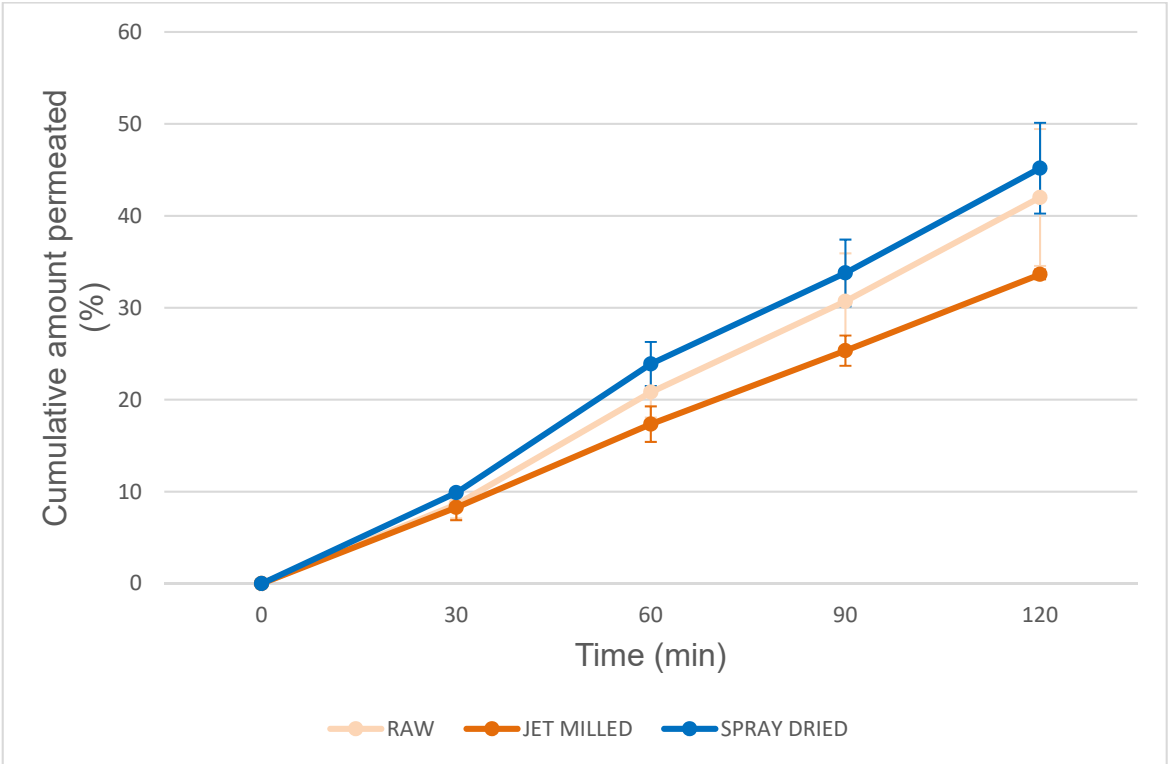


Fig. 14: Permeation of different budesonide types over time through a LC cultivated monolayer of Calu-3 cells

Table 4: Average P_{app} (cm/s) of raw, jet milled and spray dried budesonide suspensions permeating through a monolayer of ALI and LC cultivated Calu-3 cells (n = 3 per cell line, mean ± SD)

	Raw	Jet milled	Spray dried
ALI	3.2×10^{-5} $\pm 2.0 \times 10^{-7}$	2.0×10^{-5} $\pm 1.1 \times 10^{-6}$	2.4×10^{-5} $\pm 2.0 \times 10^{-6}$
LC	2.7×10^{-5} $\pm 4.9 \times 10^{-6}$	2.1×10^{-5} $\pm 9.4 \times 10^{-7}$	2.9×10^{-5} $\pm 3.9 \times 10^{-6}$

In both ALI and LC cultivated Calu-3 cells, the permeation of budesonide follows an approximately linear pattern and appears to be the slowest for jet milled particles. The linear transport right from the start of the experiment suggests the absence of prominent transporter action. This finding is not unexpected because budesonide is not a substrate of transporters, except P-glycoprotein (12).

In the ALI cultivated cells, the fastest permeation was seen for the raw particles, whereas in the LC cultivated cells, the P_{app} of spray dried particles exceeds the one of the raw material by little. Permeability of jet milled budesonide through an epithelial monolayer is slightly lower than the permeability of spray dried API. It is unlikely that this will have a marked influence on the onset of therapeutic action and effectiveness of budesonide in the lung because it is assumed that budesonide is intracellularly stored and released from the cells at a later time point (13).

3.2.2 Cellular uptake studies

The cellular uptake of raw, jet milled, and spray dried budesonide was determined for A-549, PBMC, J774, DMBM-2 as well as ALI and LC cultivated Calu-3 cells and all experiments were performed in triplicate. The amount of uptake was expressed in µg/million cells and the average uptake as well as the corresponding standard deviations calculated for each cell and treatment type. Moreover, the average recovery rates were calculated. All the data are summarized in Table 5 to Table 10 and Fig. 15

Table 5: Average budesonide uptake in A 549 cells (n = 3, mean ± SD) and recovery rate (mean)

	Uptake (µg/million cells)	**Recovery rate (%)**
Raw	2.72 ± 0.31	129
Jet milled	0.29 ± 0.15	90
Spray dried	1.61 ± 1.14	88

Table 6: Average budesonide uptake in PBMC cells (n = 3, mean ± SD) and recovery rate (mean)

	Uptake (µg/million cells)	**Recovery rate (%)**
Raw	3.68 ± 1.46	141
Jet milled	0.33 ± 0.07	105
Spray dried	8.02 ± 1.62	125

Table 7: Average budesonide uptake in J774 cells (n = 3, mean ± SD) and recovery rate (mean)

	Uptake (µg/million cells)	**Recovery rate (%)**
Raw	4.78 ± 0.78	164
Jet milled	0.63 ± 0.21	100
Spray dried	2.15 ± 1.58	117

Table 8: Average budesonide uptake in DMBM-2 cells (n = 3, mean ± SD) and recovery rate (mean)

	Uptake (µg/million cells)	**Recovery rate (%)**
Raw	0.45 ± 0.05	124
Jet milled	0.04 ± 0.01	100
Spray dried	0.27 ± 0.01	116

Table 9: Average budesonide uptake in ALI cultivated Calu 3 cells (n = 3, mean ± SD) and recovery rate (mean)

	Uptake (µg/million cells)	**Recovery rate (%)**
Raw	1.02 ± 0.03	104
Jet milled	0	69
Spray dried	0.02 ±0.01	79

Table 10: Average budesonide uptake in LC cultivated Calu 3 cells (n = 3, mean ± SD) and recovery rate (mean)

	Uptake (µg/million cells)	**Recovery rate (%)**
Raw	0.10 ± 0.02	76
Jet milled	0.05 ± 0.01	76
Spray dried	0.06 ± 0.01	94

As for the recovery rates, recovery > 80% provides an acceptable approximation of P_{app} values for the classification of compounds in the Biopharmaceutical Classification System (BCS), while lower mass balance results in underestimation of P_{app} values. A lower recovery than 80% is acceptable for high permeability compounds (14). With a P_{app} value of > 10^{-5} cm/s, budesonide is a highly permeable compound. Furthermore, recovery > 80% applies only for testing in solution.

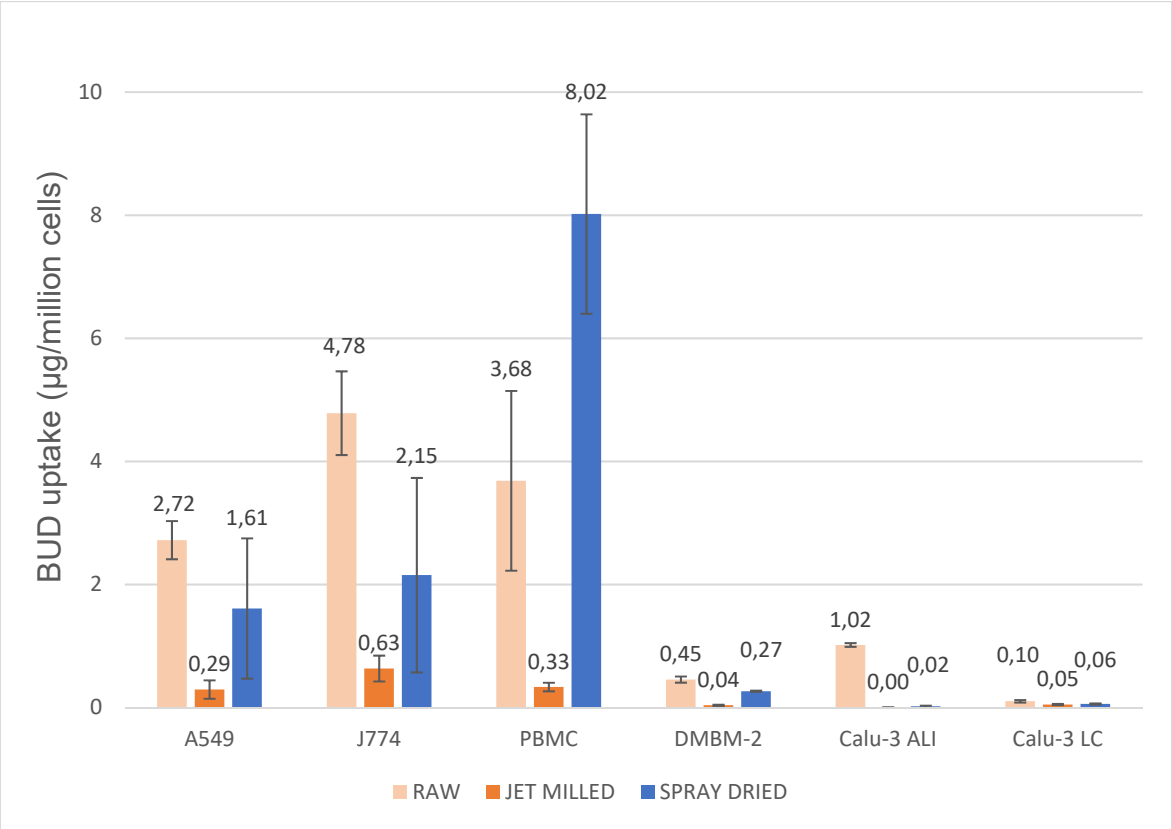


Fig. 15: Uptake of different budesonide types into tested cell lines

As shown in Fig. 15, the uptake of jet milled budesonide was the lowest in all used cell types with values as low as below detection limit in ALI cultivated Calu-3 cells. All cell types ingested and stored raw budesonide to the highest extent, with one exception of the PBMC cells, which stored an extraordinarily high amount of spray dried API. However, the variation of spray dried budesonide uptake between the samples of the same cell line was also remarkably higher than the variation of uptake of raw and jet milled API. To obtain more homogenous data on the uptake of the spray dried material, it would be necessary to repeat the experiment with a larger number of samples.

As non-processed budesonide is too big to reach the cell types that are present in the terminal bronchioles and alveoli, the main conclusion that can be drawn from the uptake studies is that jet milled budesonide is ingested and stored in both the tested macrophages and epithelial cells to a smaller extent than the spray dried particles. In PBMC cells, the uptake of jet milled material is even significantly lower than the uptake of spray dried API. The spherical shape and slightly different size of the latter seem to make them somewhat more appealing to the investigated cells,

which must be considered when designing and testing new formulations for pulmonary application. Thinking ahead, preferential budesonide uptake into epithelial cells might mean more effect and a higher effectiveness of the drug in a given disease. However, budesonide is used mainly in chronic diseases such as asthma and COPD and the target audience of inhalable medications benefit mostly from its long-term use, meaning that possibly more powder is deposited in the lungs over time that cannot be cleared. Non-degradable particles in the small airways and alveoli are normally removed by alveolar macrophages, and only particles smaller than 200 nanometers can also be ingested by alveolar epithelial cells (1). If the lung, however, gets overloaded by material that cannot be cleared by the pulmonary defense mechanisms, adverse effects may take place. In the case of inhaled corticoids, for instance, the antibacterial defense may be reduced. Accumulation in macrophages, on the other hand, may be advantageous for specific APIs, for instance for the treatment of tuberculosis (5). Compared to jet milled particles, the preferential uptake in macrophages of spray dried particles is higher. It may be suggested that spray drying could be a better strategy to deliver tuberculostatic drugs to the lungs (5).

4 Summary and Conclusion

Concluding, it can be stated that spray dried spherical budesonide particles showed a lower respirable fraction and slower dissolution into simulated lung fluid compared to the jet milled rod-shaped API. In order to attribute a difference in the FPF of the blends just to the shape of the particles, it would be necessary to generate jet milled and spray dried API fragments of the same size and solid state.

The permeation pattern of budesonide through a Calu-3 monolayer appears linear, with the slowest permeation rate observed for the jet milled API. The P_{app} values, however, were not significantly different between the 3 tested budesonide types.

Both epithelial cells and macrophages showed preferential uptake of spray dried budesonide in comparison to the jet milled material, and macrophages in general stored more API than Calu-3 and A-549 cells, which is not an unexpected finding. In order to obtain more homogenous data on the uptake, however, it would be necessary to repeat the uptake studies with more replicates.

In a previous yet unpublished study, preferential cellular uptake of spray dried API was observed also for a highly water-soluble drug, where particle properties are expected to play a minor role because dissolution is very fast. There was, however, no marked difference between epithelial cells and macrophages. Formulation dependent differences in preferential uptake by epithelial cells or macrophages might be a strategy to design inhalable blends for targeted delivery.

Evaluation of other APIs is needed to find out whether preferred macrophage uptake of spray dried particles is correlated to poor water solubility.

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