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## Establishment of in-vitro models to differentiate macrophage responses to inhaled medicines


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**Table of Contents**

**Abstract**

Inhaled medicines provide a relatively new method to locally treat lung diseases, without the disadvantages of oral and other applications regarding systemic side effects. Given the complexity of lung physiology and homeostasis, this route of application provides many challenges. One of the main obstacles for inhaled drug approval in the pharmaceutical industry is the appearance of vacuolated foamy macrophages, due to drug impact. The aim of this work was to create an in-vitro model of the alveolar epithelial barrier, consisting of A549 cells, mimicking the alveolar epithelial cells type II, J774A.1 cells, representing the alveolar macrophages and *Staphylococcus aureus (S. aureus)* bacteria, in order to investigate the effects of the compounds amiodarone, staurosporine, beclomethasone and fluticasone on cell viability, bacterial growth and possible impact on phagocytic function of alveolar macrophages.

The effects of the drugs of interest on the viability of the cells were evaluated through colorimetric assays (MTT, Resazurin). The tested substances had a clear impact on the viability of A549, J774A.1 and *S. aureus*. A decrease in cell viability was observed for both cell lines, A549 and J774A.1, especially with staurosporine and fluticasone up to 50%. The bacterial *S. aureus* growth was partially highly induced at low dosages, especially under the impact of beclomethasone and fluticasone and suppressed by high dosages. For all four drugs, amiodarone, staurosporine beclomethasone and fluticasone the phagocytic ability of J774A.1 macrophages was induced by low dosages and suppressed again continuously with higher concentrations. The outcomes generated from this project clearly suggest a dose dependant toxicity of the drugs on the tested cell lines, representative of the alveolar layer and a correlation between these compounds and a healthy function of alveolar macrophages.

**Zusammenfassung**

Inhalanda bieten eine relativ neue Methode um Lungenkrankheiten lokal zu behandeln, ohne die Nachteile systemischer Nebenwirkungen, durch orale oder andere Applikationen. Aufgrund der Komplexität der Lungenphysiologie und Homöostase bringt diese Applikationsroute einige Herausforderungen mit sich. Eine der größten

Hürden für die Zulassung von Zubereitungen zur Inhalation in der pharmazeutischen Industrie ist das Auftreten von transformierten Makrophagen (Schaumzellen) nach Drogeneinwirkung. Das Ziel dieser Arbeit war ein in-vitro Model des Alveolar-Epithels bestehend aus A549 Zellen, die die Pneumozyten Typ II nachahmen, J774A.1, repräsentativ für Alveolarmakrophagen und *Staphylococcus Aureus (S. aureus)* zu entwickeln um den Effekt von Amiodaron, Staurosporin, Beclometason und Fluticason auf Zellviabilität und weiteres auf die phagozytäre Funktion von Makrophagen zu untersuchen.

Die Auswirkungen der Testsubstanzen wurden mithilfe von kolorimetrischen Assays evaluiert. Die getesteten Substanzen hatten einen deutlichen Effekt auf die Zelllebensfähigkeit von A549, J774A.1 und *S. aureus*. Eine Reduktion in Zellviabilität wurde sowohl für A549 als auch für J774A.1, unter Einfluss der Drogen, insbesondere bei Staurosporin und Fluticason bis zu 50% beobachtet. Die Vermehrung von *S. aureus* wurde stark induziert bei niedrigen Dosen und deutlich reduziert bei den höheren verwendeten Dosen insbesondere unter Beclomethason und Fluticason Einwirkung. Alle vier Testsubstanzen, Amiodaron, Staurosporin, Beclomethason und Fluticason induzierten bei niedrigen Dosen die phagozytäre Aktivität von J774A.1 Makrophagen und unterdrückten sie bei höheren Konzentrationen. Die erhaltenen Resultate suggerieren eine Konzentration abhängige Toxizität der Testsubstanzen auf die verwendeten Zelllinien die, die alveolare Barriere repräsentieren und eine Beziehung zwischen diesen und einer gesunden Funktion von Alveolarmakrophagen.

**Acknowledgements**

I am very thankful for my supervisor Dr. Magda Swedrowska and the rest of the team for guiding and encouraging me throughout my project and writing process. I would also like to thank Prof. Benjamin Forbes for welcoming me as part of his team and providing me with this amazing opportunity.
Lastly to everyone who supported me throughout my studies, especially my family and friends.

## List of Figures



Ich habe mich bemüht, sämtliche Inhaber*innen der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

## List of Tables

**List of Abreviations:**

**AEC:** Alveolar epithelial cell/s

**AECI:** Alveolar epithelial cell/s type I

**AECII:** Alveolar epithelial cell/s type II

**AMΦ:** Alveolar Macrophage/s

**AM:** Alveolar Macrophagee/s

**CD200**: Cluster of Differentiation 200

**CFU:** Colony forming units

**COPD**: Chronic obstructive pulmonary disease

**DMEM:** Dulbecco's Modified Eagle's Medium

**DMSO:** Dimethyl sulfoxide

**FBS:** Fetal bovine serum

**FMΦ:** Foamy Macrophage/s

**HBSS:** Hank's Balanced Salt Solution

**ICAM-1:** Intracellular adhesion molecule 1

**IL:** Interleukin

**IFN**: Interferon

**LDL:** low-density lipoprotein

**LPS:** Lipopolysaccharide

**MDM:** Monocyte-derived macrophages

**MEM**: Minimum Essential Medium

**MTT:** 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide

**NADH:** Nicotinamide adenine dinucleotide

**NADPH:** Nicotinamide adenine dinucleotide phosphate

**OD:** optical density

**PBMCs**: Peripheral blood mononuclear cells

**PBS:** Phosphate buffered saline

**PK:** Pharmacokinetic

**ROS:** Reactive oxygen species

**RT:** Respiratory Tract

**S. aureus:** Staphylococcus aureus

**TNF α:** Tumour necrosis factor alpha

**VCAM-1:** Vascular cell adhesion molecule

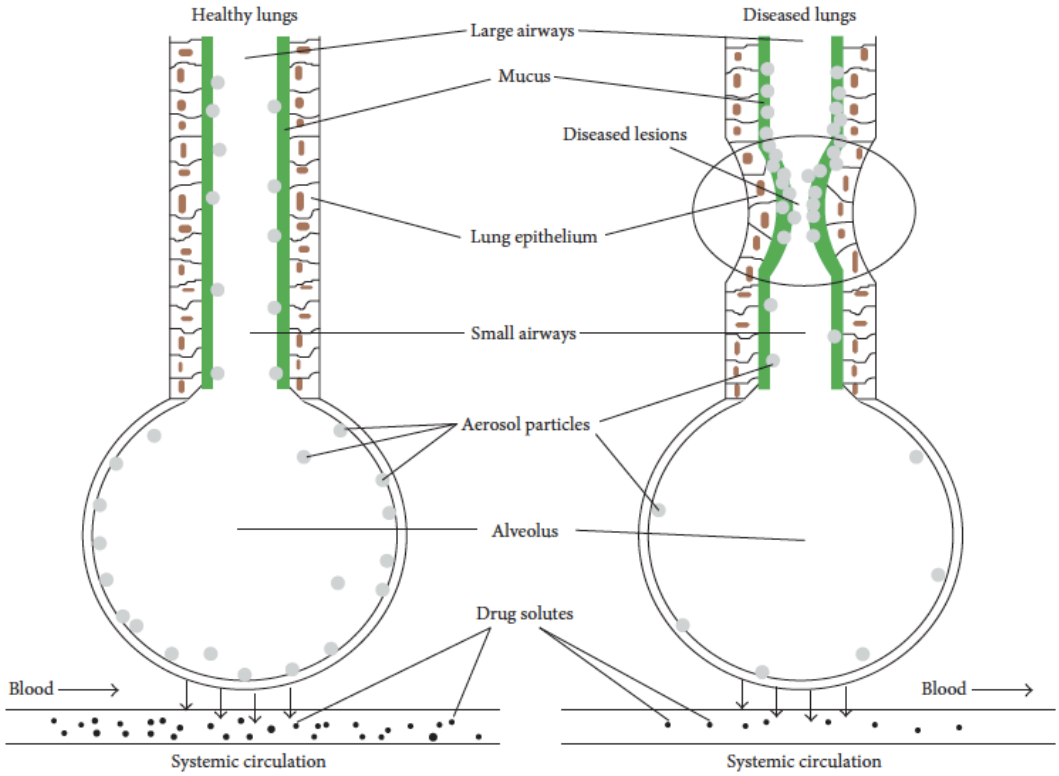
# 1. Introduction

## 1.1 Inhaled Therapy

Being a quite young practice, with e.g. modern inhaled asthma therapy beginning around the 1950's, effective inhalation therapy has been continuously gaining importance over the past decades [1, 2]. Inhaled drugs are the first line therapy for local respiratory diseases such as asthma and COPD (chronic obstructive pulmonary disease). Even though this route faces a lot of obstacles due to its complexity, it is nevertheless also increasingly being considered for systemic therapy, because of several advantages, the main being the prevention of problems regarding plasma-binding and first pass metabolism. Lower respiratory infections and COPD are one of the major death causes worldwide, posing great importance on the development of effective and safe inhalation methods and drugs [3]. For local treatment of respiratory disease compared to other routes of administration, inhalation offers a lot of advantages. The central advantage is the direct delivery of the drug into the target organ where it is needed, ensuring high local and low systemic concentrations, which leads to less systemic side effects. Less than 5% of an oral or intravenous dose reaches the lungs, making inhalation the therapy of choice regarding lung diseases [4]. Therefore, lower local doses can be equivalent or superior, to higher systemic doses. In addition the onset of reaction is more rapid.

However, due to the structural complexity of the lung, multiple pharmacokinetic processes are specific to the pulmonary environment, making the inhalation route more complex than any other administration [5]. Influential aspects are the physicochemical characteristics of the drug, the formulation but also the inhalation device and the patient's competence. Characterizing the pharmacokinetic (PK) of inhaled medicine is challenging, because both systemic and local effects have to be evaluated. PK processes regarding inhalation include several steps: Deposition of the particle in the airway, dissolution of the drug in the lung fluids, ciliary and macrophage clearance in the alveolar tissue, absorption, pulmonary retention and metabolism and drug clearance from the tissue to systemic perfusion [6]. This results in difficulties in interpretation regarding PK. The plasma concentration of an inhaled compound is defined by absorption from several areas of the lung and the gastrointestinal tract, as well as systemic drug disposition kinetics. Another crucial factor is the differentiation between

the PK of a healthy lung and a diseased lung, which has a lower deposition in the alveoli and therefore a lower plasma concentration (Figure 1) [6].




Inhaled drug particle deposition in healthy versus diseased lungs. (Image obtained from Borghardt et al., 2018)

### 1.2 Challenges in pulmonary drug delivery

There are several barriers that a drug needs to pass through during inhalation. The first obstacle is the mechanical barrier. The upper airways, the nasal cavity, pharynx and the trachea, with their narrow angled pathways offer ideal credentials for particle arrest. Likewise, the multiple bifurcations of the lower RT are an obstacle for particles before they reach the huge epithelial surfaces of the alveoli. Therefore, specific particle size and aerodynamics as well as correct usage of the inhaler are required to obtain optimal drug deposition in the peripheral lung. Once the particles have reached the alveolar surface, they are exposed to the next barrier, immune cells, enzymes and chemicals of the surfactant. Furthermore, undissolved particles can be phagocytized by elements of the innate immune system e.g. dendritic cells and macrophages. A crucial

criteria regarding evaluation of inhaled drug delivery and approval of inhaled medicines is the appearance of vacuolated foamy macrophage (FMΦ) phenotypes during in-vitro and clinical development. Distinguishing between adverse and non-adverse vacuolated macrophage appearances poses a challenge, as there is to date a lack of uniform optimized methods [7, 8].

Lastly, as mentioned before, the inhaling technique of the patient is a crucial factor of successful application. Many fail to inhale deeply, slowly and forcefully enough, especially older patients. The primary goal of inhaled drugs is to reduce pulmonary symptoms e.g. prevention of airway inflammation and constriction [9]. The most common inhaled medicines are corticosteroids (Fluticasone, Beclometasone), beta agonists (Salbutamol) and muscarinic antagonists.

### 1.3 Safety concerns in inhaled drug therapy: Alveolar macrophages

A major existing problem in the development of inhaled medicine is safety concerns regarding increased macrophage count and formation of foamy appearance during toxicology studies. The appearance of FMΦ alone is not necessarily and in fact rarely dangerous, often non-adverse and reversible. Thus, it is very important to distinguish between adaptive and adverse alveolar macrophage (AMΦ) response [7, 8]. This currently causes problems in admission processes of inhaled medicines due to lack of standardised guidelines to differentiate between non-adverse and adverse FMΦ formation. Thus, compounds sorted out because of false interpretation of FMΦ regarding safety can be permanently withdrawn [8]. Therefore, advanced knowledge of different macrophage phenotypes and their role in lung biology is required. During toxicological studies of inhaled compounds, especially ones with low solubility an increase in AMΦ number and size is often histopatologically observed. Without further indications of inflammation, it is thought to be an adaptive, reversible reaction due to the presence of particles in the alveoli and is considered non-adverse [10]. Furthermore, it is not known if certain macrophage accumulation or foamy morphology is a primary reaction to the particles. It could also be a secondary response to changes in the alveoli caused by particles [7]. When the disposition of particles in the alveolar region exceeds the alveolar clearance (which most-likely leads to findings of other inflammation markers), the accumulation of AMΦ and FMΦ are considered adverse.

Other pathologies including pulmonary fibrosis, phospholipidosis and alveolar proteinosis, aside from inflammation have clinical relevance and can be an indication for an adverse AMΦ response [11].

### 1.3.1 Alveolar macrophages and development of foamy macrophages

The cellular structure and physiologic functioning of the human RT is extremely complex, responsive, interactive and adaptable. From the point of air entry to the blood-air barrier at the alveolar sacs, where gas exchange occurs, the airway consists of approximately 40 different resident cell types [12]. One of them, the alveolar macrophage, is a major part of the alveolar ecosystem and essential for lung homeostasis and protection of the alveolar epithelial barrier (Figure 2) [13]. Just like other macrophages, AMΦ are mononuclear cells that derive from monocytes from bone marrow. They are transported through the circulatory system into the lungs and adhere onto the endothelium of lung capillaries. In the presence of environmental foreign matters, nanoparticles, toxins or pathogens in general they migrate into the alveoli. Recruitment and migration is achieved through several adhesion molecules such as P-selectin, E-selectin, intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) [14]. Macrophages are the most efficient phagocytes of the innate immune system, known for being able to migrate quickly through capillary endothelia, entering the interstitium to pursue the uptake of pathogens [15]. The AMΦ protect the lower respiratory tract together with the alveolar epithelial cells from insults [16]. Furthermore, they take up excess and old pulmonary surfactant to prevent accumulation in the alveolar cells and thus play a role in stabilizing homeostasis.

In general, three different types of AMΦ and macrophages in general can be distinguished; M1, M2 and regulatory macrophages. M1 macrophages have pro-inflammatory features and are usually activated through interferon gamma (IFN γ). Various bacteria, viruses and especially LPS (lipopolysaccharides) evoke M1 activation which leads to enhanced phagocytic capability and increased levels of pro-inflammatory cytokines for example IFN γ, interleukin 1 (IL-1), IL-6 and tumour necrosis factor alpha (TNF α). Moreover, their production of reactive oxygen species (ROS) increases, which leads to more effective pathogen elimination. M2 activation on the other hand is induced by IL4 and IL13 and results in anti-inflammatory processes. They suppress

inflammation and help tissue repair. Regulatory macrophages, which are in some cases seen as a subtype of M2-activated macrophages, produce large amounts of IL10, a highly anti-inflammatory cytokine [17,18]. AMΦ can be both M1 and M2 activated, depending on stimulating factors. An increase in M1 cells can be an indication of inflammatory diseases and is thought to play a role in pulmonary fibrosis [17]. Although M1 cells have regulatory functions as well, a significant overgrowth can lead to activation of pro-fibrotic factors and airway remodelling typical for chronic asthma [19].

Recent research shows that besides migrating through epithelial barriers, macrophages communicate with the cells of the alveolar epithelium [20], thereby leading to the release of chemokines and cytokines, which modulate inflammatory processes [18]. Nevertheless, even during inflammatory processes the epithelium keeps a certain amount of non-activated, non-inflammatory AMΦ through crosstalk with the Glycoprotein CD200 (Cluster of Differentiation 200). Negative regulation through IL-10 and TGF β released by the epithelium and components of the surfactant released by AECII (for example SP-A, SP-D) also maintain the macrophages in limited anti-inflammatory phenotypes [21]. Epithelial damage and integrity loss is a signal for AMΦ for pro-inflammatory response. The absence of negative regulating factors such as CD200 has been shown to excessively increase the number of AMΦ [22]. In a healthy lung, epithelial barrier and macrophages work in close contact together to maintain optimal barrier function and protection. The presence of apoptotic alveolar cells for example triggers the AMΦ to perform anti-inflammatory modulation to prevent aggressive immune response against own tissue. It also increases the presence of CD200. On the other hand, necrosis leads to the release of proteins associated with pro-inflammatory damage [18].

Alveolar macrophages play a crucial role, regarding inhaled drug delivery, as they are one of the first cells of the innate immune response in the alveolar barrier. They cross-communicate with the alveolar surfactant and epithelial cells and can phagocytize inhaled compounds through the epithelial layer [23]. Through excessive uptake of drug in the lungs and other mechanisms, which will be described in the next chapter, macrophages can form vacuolated phenotypes, which cause obstacles in the administration by inhalation that need to be considered.

The term foamy macrophage (FMΦ) or foam cell, describes a not always pathological, changed morphological state of degenerated macrophages of granular or vacuolated cytoplasmic appearance, which can be viewed by light microscopy. In the classical sense they are M2 Macrophages, which contain high amounts of phagocytized low-density lipoproteins (LDL) and therefore cholesterol, giving them a foamy morphology and appearance [24]. Macrophages, which are the most important cells of the innate immune system with phagocytic ability, are able to internalize lipoproteins through scavenger proteins (e.g. CD36) on the macrophage surface, serving as pattern recognition receptors (PRR's) [14]. Regarding the lung, AMΦ responses are commonly induced by environmental toxins and inhaled medicines. Observed responses can be an excessive increase in cell number or a degeneration of the AMΦ into a foamy morphology. The AMΦ is considered to be the most sensitive to forming vacuolated morphology. In contrast of what is understood under the classical term 'foamy macrophage', which is a definition, causing discrepancy in general, FMΦ does not necessarily refer to macrophages packed with LDL. It must be distinguished between phospholipidosis, caused by excessive accumulation of phospholipids in the lamellar bodies of the AMΦ, neutral lipids present in the cytoplasm, surplus surfactant phagocytized by macrophages, which causes a foamy appearance as well or accumulation of inhaled drug [7, 11].

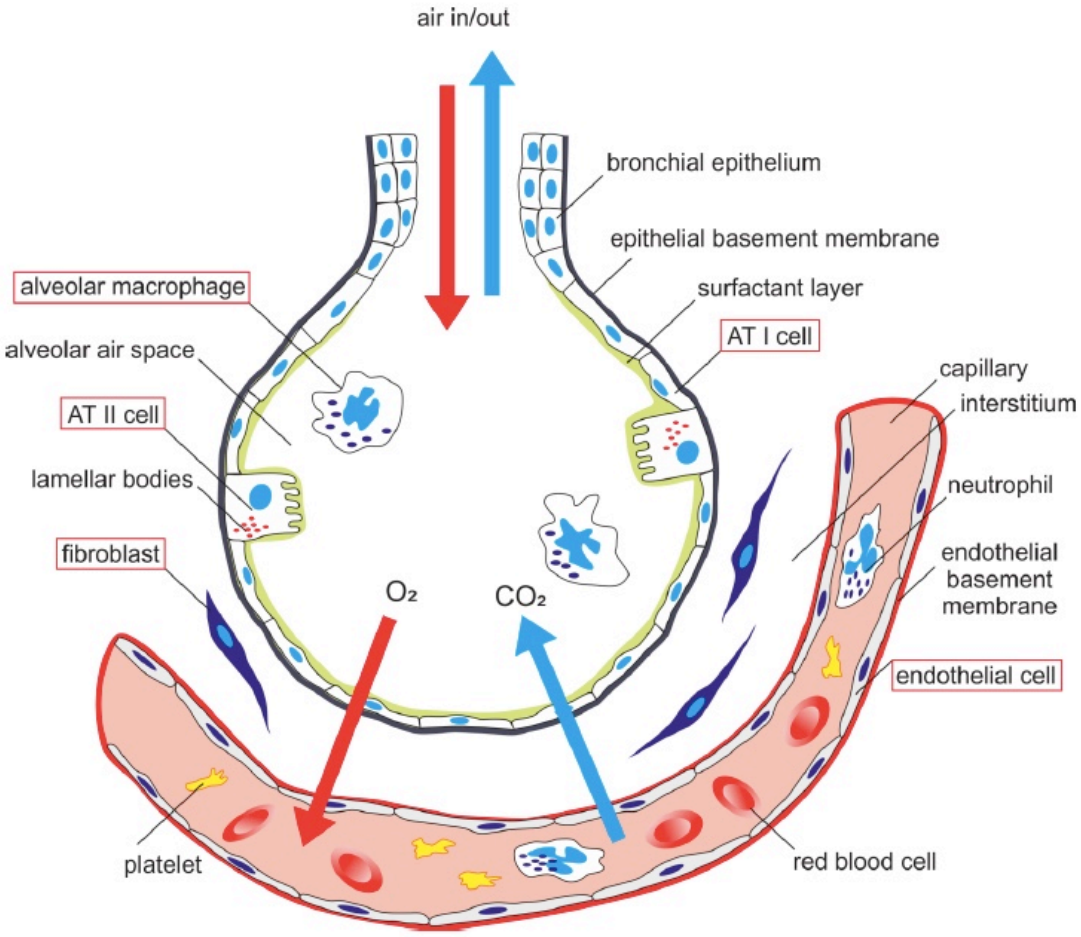



Figure 2: Schematic arrangement of alveolar-capillary membrane-related pulmonary cells. ATI cell—alveolar epithelial type I cell, ATII cell—alveolar epithelial type II cell (Image obtained from Nova et al., 2019) [13].

### 1.3.2 Amiodarone and Staurosporine as inducers of foamy macrophages

Foamy alveolar macrophage formation is commonly observed in lung diseases and in response to infections but can also be induced by drugs directly [7]. There is evidence of inhaled compounds, inducing the formation of foamy macrophage morphologies e.g. amiodarone [25, 26, 27] and staurosporine [28]. Amiodarone toxicity is known to be associated with reversible macrophage phospholipidosis and morphological changes, whereas staurosporine is proven to lead to foamy degeneration followed by the apoptosis pathway [28]. Foamy degradation of AMΦ through phospholipidosis leads to a slower clearance of the inducing drug and results in higher macrophage number and inflammation [11]. Taken together, an observed FMΦ cytoplasm can have different

reasons such as agglomeration of insoluble drug particles, surfactant or phospholipids. Therefore, distinction through light/transmission or electron microscopy is required.
As until to date, the development and consequences of foamy morphology are poorly understood, it remains difficult to interpret. Therefore, further progress in distinguishing between adaptive and adverse responses are required and critical to safety assessment of inhaled medicines.

## 1.4 Models for studying the alveolar barrier

### 1.4.1 In-vitro models for studying pulmonary drug administration and foamy macrophages

As described before, inhalation therapy is becoming increasingly interesting for drug delivery and is the primary choice of administration for local lung diseases [29]. This requires continuous development of models to study the effects of drugs and particles on the lung barriers, particularly cell-drug interactions. To date, there are three main approaches to investigate the impact of inhaled drugs on lung cells: Animal in vivo models, ex vivo and in-vitro cell culture models [30].

There are different ways to perform animal experiments, through whole body exposure, head/mouth exposure or lung exposure, which each has their advantages and disadvantages. The obvious advantage of animal models in general is that the tests are implemented on living species, whereas the disadvantage lies in inter-species variation.
Another method used often is perfused lung experiments, where the rodent lung e.g. is separated from the body and kept in an artificially functioning system. This enables a way to investigate drug delivery through the air-blood barrier. However, isolated organs have only a limited life span [30, 31]. In the past years more complex in-vitro models have been gaining importance throughout different fields of research. Not only do they offer a possibility to reduce animal experiments and its moral aspect, but they also allow studies at cellular and subcellular levels, thus help to understand cell-cell interactions and molecular events [32]. Although, an in-vitro model represents a very simplified model of a tissue or organ, it enables focusing on an exact mechanism of interest. In general, in-vitro models are created to imitate a certain microenvironment or tissue e.g. the epithelial barrier of the lung. They are created using either primary cells or cultured cell lines. Primary cells are isolated cells directly from tissue. They are

specific to the tissue isolated and are not homogenous and reproducible, yet they represent the in vivo situation better. Continuous cell lines are immortalized or cancer-derived cells that are homogenous and stable and therefore better reproducible compared to primary cultures. On the other hand, the disadvantage of cell lines compared to primary cultures is that they are limited in phenotypic differentiation and therefore, less similar to the in vivo situation.

Since cell cultures are isolated from many different factors of the real in-vivo environment, they may not behave exactly how they would in vivo. Nevertheless, in-vitro models offer the possibility to create a reproducible system to investigate what may be similar to in vivo situations [30, 33]. These models can then be used to carry out experiments as for example testing different drugs and investigating cellular reactions.
Thus, due to the standardized environment in-vitro models of the epithelial airway barrier can be used for reproducible research of inhaled drugs and for a wide range of high throughput screenings. There are many different types of in-vitro models to evaluate the human epithelial lung barrier. They can be made of one epithelial cell line or involve several different cell lines in a 3D co-culture system [29, 34].

Monocultures of epithelial airway cells e.g. A459 cells are a simplified representation of the alveolar surface more specifically AECII. A459 cells, originating from lung cell carcinoma, are one of the most well-investigated cell lines and are widely used in in-vitro models of the alveolar barrier [35|. They have a lot of similar characteristics to in-vivo AECII e.g. lamellar bodies, distinct polarization, tight junctions and extensive cytoplasmic extensions and similar trans epithelial resistance values [36]. Even though the A549 cells are a decent representation of the epithelial alveolar surface it does not capture the complexity of the airway barrier containing several cell types. Therefore, 3D Co-Culture models have been created that contain several different cell types such as macrophages on top of the A549 cells and dendritic cells, resulting in a more in-vivo close environment [37, 38].

### 1.4.2 Evaluating macrophage phagocytosis

A decreased macrophage function is associated with a greater susceptibility to bacterial infections [39]. Since AMΦ play a leading role regarding host defence against

pathogens, particular matter and inhaled compounds, it is of great importance to be able to investigate and evaluate their phagocytic function [40].

Consequently, in-vitro exposure of bacteria to macrophages is a suitable method to assess the phagocytic activity of AMΦ [41]. The most commonly used bacteria are *Streptococcus pneumonia* and *S. aureus* [42]. *S. aureus* is a gram-positive bacterium that is present in 20-30% of the populations upper airways and skin as a commensal bacteria. If, however, the immunological defence, e.g. macrophage phagocytic ability is restricted, *S. aureus* can be responsible for several respiratory infections [39]. The bacterium's capability to evade innate defence mechanisms and its growing resistance to antibiotics, makes *S. aureus* a serious threat regarding respiratory infections [43], which no longer are limited to hospitalized patients as also community-acquired *S. aureus* infections are on the rise [43].

## 2. Aim and Objectives

Safety studies for inhaled medicine are designed to meet certain pulmonary safety and regulatory specifications to permit clinical trial. For respiratory drugs, even though in-vitro methods would lead to a reduction of cost and animal experiments, there are currently no validated assays that can replace the in-vivo studies.

The aim of this study was to test the biocompatibility of the different compounds amiodarone, staurosporine (used as control), fluticasone and beclomethasone (test compounds) across the lung epithelial cell line A549, murine macrophages J774A.1 and explore any effect of those compounds on epithelial cell viability and phagocytic function of alveolar macrophages. The obtained results will help to establish an in-vitro 3D co-culture model of the alveolar epithelial barrier consisting of alveolar epithelial cells, macrophages, and bacteria.

## 3. Materials and Methods

### 3.1 Sterile cell culture and medium

To avoid contamination all experiments were performed in a laminar flow cabinet (HERA safe KS18) where air is drawn through a HEPA (High efficiency particulate air) filter and blown in a laminar flow towards the user, ensuring a sterile work surface. The cell culture hood was cleaned with Ethanol 70% before and after every experiment. The cell mediums used were acquired sterile and stored in the refrigerator at 4°C. Before every usage the medium was warmed up in a bead bath at 37°C. The composition of the media used was as following:

- DMEM (Dulbecco's Modified Eagle's Medium), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% nonessential amino acids.
- MEM (Minimum Essential Medium), 10% FBS, 1% penicillin/streptomycin, 1% nonessential amino acids, 1% L-Glutamine

FBS, penicillin/streptomycin, glutamine and nonessential amino acids were added to the medium after being defrosted in the bead bath. Two different cell lines were used: murine J774A.1 monocytes/macrophages and A549, adenocarcinomic human alveolar basal epithelial cells. Both cell types show adherent growth and were cultivated in T25 and T75 flasks and incubated at 37°C and 5% CO2.

#### 3.1.1 Cell passaging

For cultivation, the cells were taken out of the liquid nitrogen tank, defrosted at room temperature and added to a t25 flask together with 5-8 mL medium to neutralize the DMSO (dimethyl sulfoxide). After defrosting the cells, the medium was changed after one day instead of the usual two days to avoid damage through DMSO. The flasks were kept in the incubator at 37°C and 5% CO2. Before passaging the cells, it was important to check for contamination and confluency with the microscope. The confluency of the cells in the flask should not deceed 70% and exceed 90% on the surface.

For passaging of the cells, the medium in the flask was discarded completely in a container under the hood or removed by aspirating with a pasteur pipette, connected to a pump. After rinsing the flask twice with phosphate buffered saline (PBS), 5 mL of trypsin were added to detach the cells and the flask was incubated for 5 min at 37°C. This method was mainly used with A549. For J774A.1, the cells were detached with a

cell scraper. For that, 5 mL medium was added to the flask and the cell scraper moved with a little pressure horizontally from the end of the flask to the top. After incubating with trypsin for 5 min resp. scraping the cells, 10 mL of medium was added to neutralise the trypsin for A549 and collecting the cells for J774A.1. Then medium was added and the cell suspension was collected in a 20mL tube or 50mL falcon tube and centrifuged for 5 minutes. After centrifugation, the remaining medium above the cell pellet was discarded carefully but in one go. The pellet was re-suspended and dissolved with 1 mL medium by pipetting up and down a few times slightly above the pellet. After that, another 4 mL medium was added. Then, 100 µL were taken from the 5mL cell suspension into an Eppendorf tube to count. The 100 µL were mixed with 100 µL of Trypan Blue to analyse cell viability in the cell counter. The cell counter calculated the viable cells showing the percentage of viable cells and the viable cell count per mL. The number of live cells was determined, in order to calculate the amount of cell suspension needed for a certain experiment.

For the cell viability experiments 100 µL cell suspension per inner well of a 96-well plate was needed, containing 40 000 cells/well. For the 60 inner wells 6 ml cell suspension per plate was required, therefore 7ml was prepared to make sure enough was available. To calculate the amount needed from the cell suspension the following equation was used:

Equation 1:

$$(40.000 \times 7ml) \div \left(X \times 10^{\frac{6}{ml}}\right) = Yml\ cell\ suspension$$

The number of cells needed for example for a 96-Well plate were multiplied with the amount of liquid needed for a plate in mL (7mL), divided by the number of live cells per mL. With the calculated amount of cell suspension, a separate suspension for the certain experiment can be prepared in a separate tube with additional medium. The received amount of cell suspension was suspended in the amount of liquid needed (7ml). To complete the passaging procedure, a certain amount (50 - 300 µL) of the original suspension was added to a new t25 flask with 5-7 mL medium. The flask was then sprayed with ethanol and incubated at 37°C and 4-5% CO2.

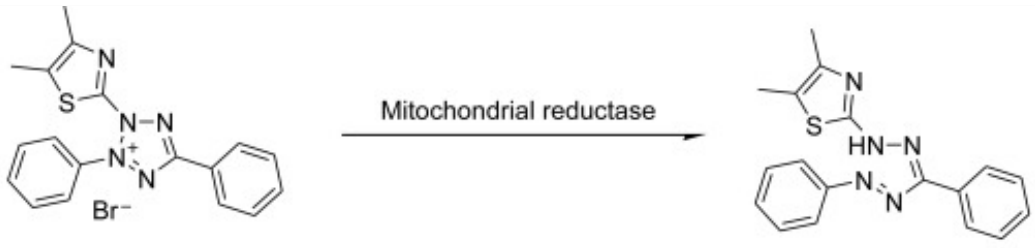
### 3.1.2 Test substances

For the experiments with A549 and J774A.1 the effect of amiodarone (0.01, 0.03, 0.1, 0.3, 1, 3.16, 10, 31.6 µM), staurosporine (0.32, 2, 3.17, 10.03, 31.69, 100.14, 316.46, 500 nM), beclomethasone and fluticasone (same concentrations as amiodarone) were measured. The main methods used to examine the impact of these drugs at different concentrations on the cell viability were the MTT and Resazurin assays.

### 3.2 MTT- Assay

The MTT assay is a colorimetric method for measuring cell metabolic activity. Because of the strong correlation between metabolic activity and cell viability of cells, MTT was used to evaluate these. It was used to measure viable cells in 96-Well plates without the need of elaborate cell counting, as it is the most common method used to determine cytotoxicity of drugs at different concentrations.

The cells were treated with 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), a yellow tetrazolium salt to determine their cell viability compared to a control group. The assay is based on the reduction of MTT, a yellow hydrophilic dye (MTT) into blue-violet hydrophobic formazan-crystals.




3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide **(MTT)**

(E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenyl-formazan **(formazan)**

Reduction of MTT to formazan crystals (Hulya et al.,2018)

The reduction of MTT through NADH and NADPH is contingent of enzymes of the endoplasmic reticulum. The partial reduction of MTT through succinate is contingent of the succinate dehydrogenase in the mitochondria. The principle of the MTT assay is that for most viable cells mitochondrial activity and the activity of the

endoplasmic reticulum is constant and thereby an increase or decrease in the number of viable cells is linearly related to cell activity.

Any increase or decrease in viable cell number can be detected by measuring the formazan concentration reflected in optical density using a plate reader at 570 and 650 nm. The equation for the cell viability assessment is explained in chapter 3.2.4 (Equation 2).

### 3.2.1 Plate setup

All steps have been performed likewise for A549 and J774A.1. Therefore, the cells were split as described above. After calculating the amount of cell suspension needed and adding it into a new 20mL tube, medium was added to receive 14 mL for two 96- Well plates to obtain a cell density of 4 x $10^4$ cells/mL per well. Equation 1 was used to calculate.

Then, the content of the tube was poured into a plastic reservoir and the pipette was set to 100 µL with 6 attachments. Each time, before pipetting into the plate, the cell suspension was mixed a few times. Then, 100µL cell suspension was added row by row, whilst (from B to G, and 2-11) leaving out all outer rows of wells. The outer wells were not used for the experiment due to evaporation and were filled with 100 µL of medium to keep the evaporation of the plate to the minimum. The plates were incubated at 37°C and 4-5% CO2 for 24 hours (A549)/ 48 hours (J774A.1) to give the cells time to attach to the plate.

### 3.2.2 Drug preparation and incubation

For amiodarone, beclomethasone and fluticasone 10 mM stock solutions were prepared. Starting from the stock solution a working solution was made by adding 20 µL stock solution to 980 µL phenolred free medium in a glass vial. 960 µL of that solution were mixed with 2160 µL medium to obtain 3,12 mL working solution II. The obtained concentration was therefore 63,2 µM. For staurosporine a 1mM stock solution was prepared. Working solution I was made by diluting 20 µL of stock with 80 µL of medium. Working solution II was obtained by adding 30 µL of working solution I to 2970 µL of medium. The obtained concentration was therefore 2 µM.

To create a serial dilution, two new 96-Well plates were needed. Each Plate was plated with two drugs. Both plates were filled with 130 µL of medium from B-G and 4-10. In Plate A to B3, C3 and D3 190 µL of amiodarone was added, to E3, F3, G3 190 µL of staurosporine was added. Plate B was prepared in the same manner for beclomethasone and fluticasone. To create the serial dilution, 60 µL were taken from row 3 by the multichannel pipette to row 4 and mixed. Then 60 µL were taken from row 4 to row 5 and so on until row 10. The last 60 µL were discarded, so that every inner well contained 130 µL.

After 24/48 hours of incubation the plates containing cells were taken out of the incubator into the cell culture hood. The old medium was removed, by pouring the plates content upside down into a waste container and drying the plate on a blue tissue, as the cells were attached to the bottom of the wells by then. Then 200 µL of medium were added to the border wells and to positive and negative control (row 2 and 11). To all remaining wells, 100 µL were added. Finally, 100 µL of the corresponding wells containing drugs were added to the plates containing cells (B-G, 3-10).

Herewith, the final plate concentrations for amiodarone, beclomethasone and fluticasone were 31.6, 10, 3.16, 1, 0.3, 0.1, 0.03 and 0.01 µM (row 3-10). Concentrations for staurosporine were 1000, 316.46, 100.14, 31.69, 10.03, 3.17, 1.00 and 0.32 nM (Table 1). The two plates were incubated for 24 hours at 37°C and 5% CO2.

Table 1: Drug concentrations used in cell viability assays

| Drug | Concentrations | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Amiodarone | 31.6 µM | 10 µM | 3.16 µM | 1 µM | 0.3 µM | 0.1 µM | 0.03 µM | 0.01 µM |
| Staurosporine | 1000 nM | 316.46 nM | 100.14 nM | 31.69 nM | 10.03 nM | 3.17 nM | 1.00 nM | 0.32 nM |
| Beclometasone | 31.6 µM | 10 µM | 3.16 µM | 1 µM | 0.3 µM | 0.1 µM | 0.03 µM | 0.01 µM |
| Fluticasone | 31.6 µM | 10 µM | 3.16 µM | 1 µM | 0.3 µM | 0.1 µM | 0.03 µM | 0.01 µM |

### 3.2.3 MTT preparation and incubation

The MTT solution was prepared by dissolving 100mg MTT in 20 mL of PBS. The solution was sterilized through a sterile syringe and filter. The tube was wrapped in aluminium foil, as MTT is light sensitive. After 24 hours of incubation, the two plates were placed in the hood and 200 µL of Triton X were added to row 11 as a negative control. The plates were incubated for 10-15 minutes. After that, the plate's content was discarded into a waste container and 100 µL of medium and 25 µL of MTT were added to each well. This step was performed in the dark with the lights switched off inside of the hood. The plates were incubated for 4 hours, which gave the cells enough time to convert the dye into formazan crystals. Afterwards, the remaining liquid was discarded into a waste container and 100 µL of SDS were added to each well. The SDS was added to dissolve the formazan crystals. The plates were incubated over night at 37°C and 5% CO2.

### 3.2.4 Measurement

The optical density (OD) was measured at 570 and 650 nm via "Spectramax 340PC. 570 nm was detecting the purple formazan crystals, 650 nm the remaining yellow background. First the 650 nm OD background was subtracted from the 570 nm OD total signal, then following formula was used:

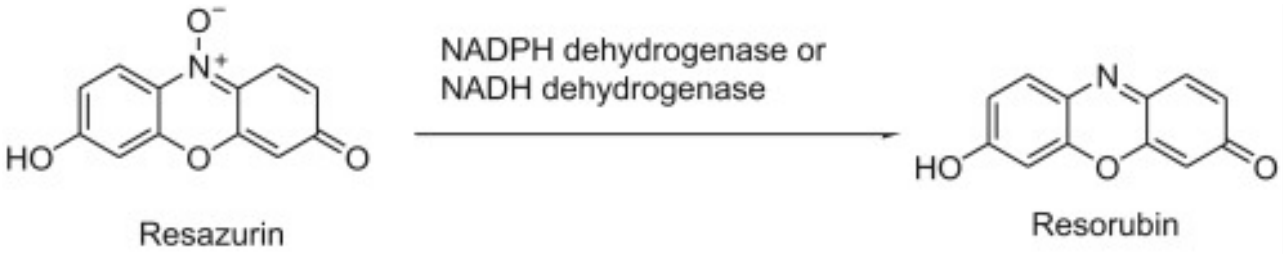
Equation 2:

$$(OD\ treated\ well[-blank]) \div (mean\ OD\ control\ well\ [-blank]) \times 100$$

The OD of the blank border wells (without cells and without drug as well) was subtracted from the average OD of all other wells. Then, the values of the wells containing cells and drugs were divided by the control cells without drug multiplied by hundred. That way, an average percentage of living cells could be determined for each concentration of drug, which was plotted graphically. The experiment was repeated 4 times for each cell line.

### 3.3 Resazurin Assay

The Resazurin assay also known as alamar blue assay is used as an indicator for cell viability of mammalian cells and bacteria. The low toxicity of resazurin allows for long-term studies. Additionally, compared to MTT, resazurin and the assays reduced end product are soluble and the cells do not have to be lysed before measurement. The efficacy is based on the reduction of the purple coloured resazurin to the pink coloured highly fluorescent resorufin through the mitochondrial reductase during cell respiration. Therefore, resorufin is a highly reliable indicator for cell viability.




Reduction of resazurin resorubin/resorufin (Hulya et al.,2018)

In this work, the resazurin assay was used to measure bacterial growth of *S. aureus* after drug treatment. Therefore, the same drug dilutions were prepared as for the MTT assay in 96 well plates. The cell density was slightly different from A459 and J774A.1: 1 x $10^6$ colony forming units (CFU)/mL → 5 x $10^4$ CFU/well

50 µL of drug dilution was added to the wells containing 50 µL of bacteria dilution. The bacteria treated with the drug dilutions were incubated for 24h the same way as for the MTT assay. After incubation, 10 µL of 10% alamar blue was added to each well. Then, the plates were incubated for two hours, and read afterwards, via "Spectramax 340PC" at excitation 570 and emission 600 nm. The experiment was repeated four times for amiodarone, beclomethasone and fluticasone at concentrations 31.6, 10, 3.16, 1, 0.3, 0.1, 0.03 and 0.01 µM.

### 3.4 Microbiology

Broth was prepared by dissolving 15g in 500mL distilled water in a bottle. Agar was prepared by dissolving 7,5 g Agar and 15 g Broth in 500mL distilled water. Both bottles were sterilized by autoclaving. 10 µg Chloramphenicol was added to both bottles after autoclaving. 20-25 mL warm agar was poured into each agar plate under the hood. The plates were closed after the agar became solid to avoid condensation and creation of water drops on the lid.

Red fluorescent *S. aureus* was stored in small vials at -70°C in the freezer. A small aliquot was added into a tube containing 30mL of broth with a plastic inoculating loop. Then, it was kept in a shaking incubator at 37°C for 24 hours together with a 30 mL of broth only as a control. After 24 hours, the broth should be clear and the broth containing bacteria should appear cloudy. If the broth was cloudy, a new broth was prepared and the experiment repeated. The tube containing bacteria was centrifuged for 30 minutes. After that, the broth was discarded into a container containing bleach and the pellet re-suspended in 30 mL of broth. A volume of 1,5 – 2 mL of broth only and bacterial suspension was added to cuvettes and the optical density was measured at 600nm.

The assumption used to create dilutions was that at an OD of 0.1 the amount of bacteria would be $10^8$/mL. The OD value of the cell suspension can be above 0.1. Therefore, the dilution was calculated to obtain an optical density of 0.1. Beginning with the cell suspension of 0.1 OD meaning 10^8 cells per mL, dilutions were created to receive $10^6$, $10^3$ and $10^2$/mL. The different cell suspensions were plated into agar plates by adding 50 µL and distributed with 5-12 glass beads. Then, the plates were laced on the lid and incubated at 37°C.After 24 hours, the colonies (CFU's) were counted for each dilution. The dilution of interest for further experiments was $10^3$, which should create 1000 CFU's.

### 3.5 Establishment of J774A.1 and *S. aureus* co-culture and investigation of bacteria uptake after drug treatment

The J774A.1 cells were grown to confluency and split from passage 3 to 4, as described previously. Each 1 mL of the cell suspension was added to each well of a 24-well plate. 2 x $10^5$ / well was seeded. After incubation of 24h, drug dilutions were added as shown in figure 5.

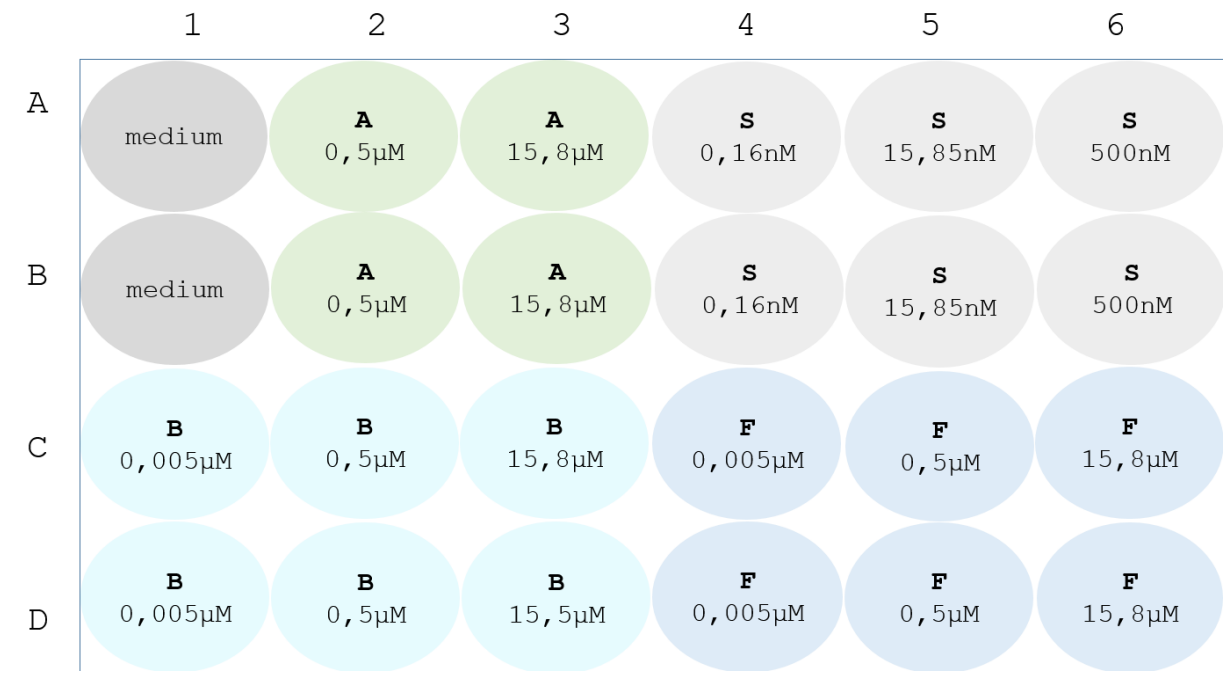



Plate setup of J774A.1 treated with drug dilutions.

A=Amiodarone; S=Staurosporine; B=Beclometasone; F=Fluticasone

After investigation of cell confluency and attachment to the bottom of the wells through microscopy, the remaining medium was aspirated above the confluent cell layer. The wells were washed 3 times with PBS and 1 mL of bacteria was added on top to each well. The cell density of bacteria added was 2 x $10^5$ CFU/mL. After plate incubation for 2h at 37°C, the wells were washed again 3 times with PBS. 50 µg/mL of Gentamycin in PBS was then added and the plate incubated for 15 min. After that, the wells were washed again with PBS. Finally, 1 mL of cold H2O was added to the wells for 15 min. The bottom of the wells was then scraped with a pipette tip and transferred into 24 Eppendorf tubes separately.

The Eppendorf tubes were subsequently centrifuged at maximum speed for 10 minutes. The supernatant was transferred into separate tubes and each pellet was

resuspended in 500 µL Hank's Balanced Salt Solution (HBSS). Of the received dilution in each Eppendorf tube, each 50 µL was added onto agar plates and distributed via beads as described previously. Another 50 µL of a 1:10 dilution was each seeded onto agar plates as well. The experiment was continued only with the plates containing the 1-10 diluted suspension, as the colonies that grew of the undiluted suspension were too many to count. The amount of CFU counted serves as a representation of the bacteria uptake by J774A.1 macrophages with and without drug exposure after 2 hours of incubation with *S. aureus*. The steps described ensure that only *S. aureus* that had been taken up by the J774A.1 macrophages were received and plated at the end. Cold H2O was used to release the bacteria from the cells. *S. aureus* is known to survive intracellular of macrophages for quite a while and since the incubation hour was 2 hours, it was ensured that the bacteria were not killed.
The final seeded agar plates were incubated for 24h and counted manually afterwards and the results were analysed and plotted via Graph Pad Prism v.9. The experiment was repeated 3 times.

### 3.6 Isolation and differentiation of monocyte derived macrophages

Part of the initial aim was to generate monocyte-derived macrophages (MDM) isolated from blood samples obtained from volunteers within the group. The method used was magnetic cell separation MACS by Miltenyi Biotec and was carried out in three steps.

#### 3.6.1 PBMCs isolation

The first step acquired to obtain MDM was to isolate peripheral blood mononuclear cells (PBMCs) from blood. 60 mL freshly collected blood from a volunteer was citrated and diluted with the equal amount of Hanks' balances salt solution (HBSS). Each 40 mL of the 120 mL dilution were added to three 50 mL falcon tubes. Then, 30mL were added to each four SepMate™ tubes containing 15 mL of LymphoPrep. The tubes were centrifuged for 10 min at 1200 g at room temperature, leaving the red blood cells at the bottom and the PBMCs on top. The top layer was then poured each in four 50mL falcon tubes, which were then centrifuged for 10 min at 600 g.

After that, the supernatant was discarded and each pellet was resuspended with 1 mL red blood cell lysis. All four were then combined into one tube and vortexed for 5 seconds. The volume was then completed to 50 mL with 2% FBS-HBSS and vortexed again for 5 seconds and kept in a ice box for 10 min. Subsequently, the tube was centrifuged at 300 g for 10 min at 2-8 °C. The supernatant was discarded, the pellet re-suspended in 1mL 2% FBS-HBSS and completed to 20 mL. After that, the cells were counted in a cell counter. The total number of cells were 9 x $10^5$/mL.

### 3.6.2 CD14 cells isolation

The goal of the second step was to separate CD14+ cells from other. For that, the tube containing the 20 mL PBMCs suspension was centrifuged at 300g for 10 min at 2-8 °C. The supernatant was discarded and re-suspended with 7,2 µL MAC's buffer and 1,8 µL CD14 micro beads by Miltenyi Biotech. Then, the tube was vortexed and incubated in the fridge (2-8 °C) for 15 min. After incubation 90 µL MAC's buffer was added and again centrifuged at 300 g for 10 min at 2-8 °C. After that, another 45 µL was added and the pellet was re-suspended.

### 3.6.3 Magnetic separation

The last step to receive the final monocytes was magnetic separation with MAC's quadro separation unit. The quadro separation unit the LS column by Miltenyi Biotech and plunger were placed in the laminar airflow hood. The LS column was rinsed with 3mL MAC's buffer, which was discarded afterwards. The cell suspension was then added to the column on the magnet. The cell suspension, passing the column was collected. After that the column was washed 3 times with 1 mL MAC's buffer. Then, the suspension collected containing non-CD14+ cells was discarded. Subsequently, the LS column was removed from the magnet and held in an upright position. 5 mL MAC's buffer was then used to wash down the CD14+ cells in the column with the plunger into a sterile falcon tube that was labelled with CD14+. The volume was completed to 20 mL with 2% FBS-HBSS and centrifuged for 10 min at 300 g. Finally, the pellet was re-suspended in 10 mL RPMI medium containing phenol red and the cells were counted.

The cells obtained were too low in number to count and due to time reasons the experiment could not be continued during the project. Further experiments were therefore completed with the J774A.1 macrophage cell line.

# 4. Results

## 4.1 Cell viability

### 4.1.1 Cell viability assay for evaluating compound toxicity, A549: MTT Assay

The cell viability of the alveolar cell line A549, after treatment with the different concentrations of the four test substances (amiodarone, staurosporine, beclomethasone, fluticasone), was investigated through MTT assays in the cell culture lab. The MTT Assay was performed as described in detail in chapter 3.2 of the materials and methods section.

The cell viability in % (x-axis) was investigated after treatment with different concentrations (y-axis) of amiodarone, staurosporine, beclomethasone and fluticasone for 24 hours, as shown in Figure 6. Data points represent the mean cell viability at the respective concentrations, each done in triplicate.

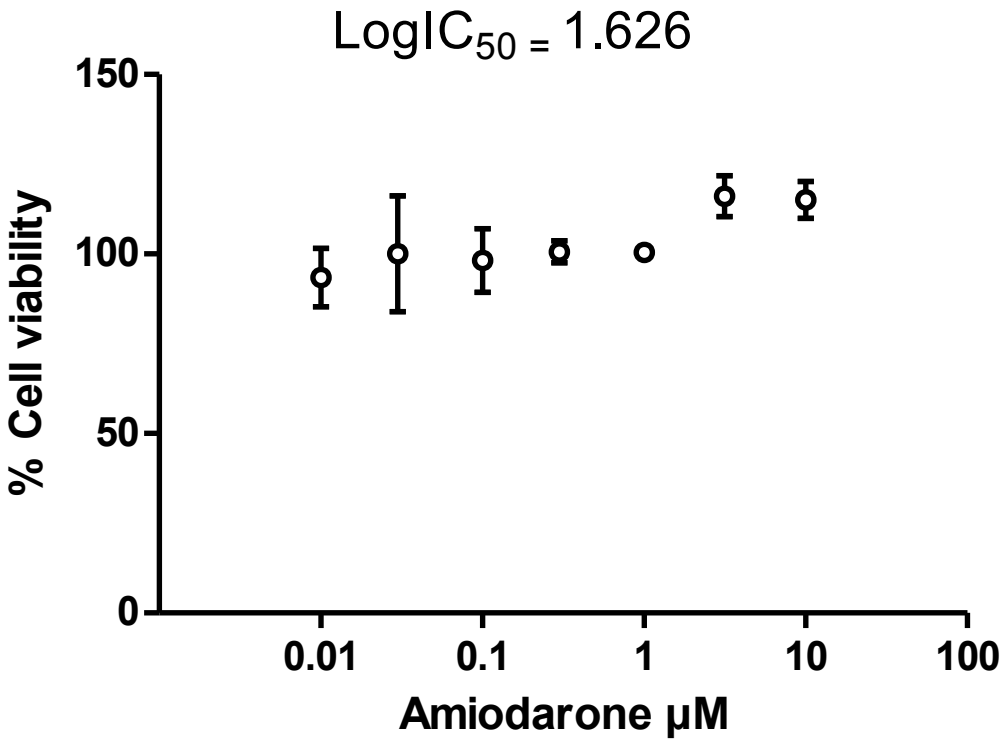
The cell viability of untreated cells was set as 100%. The cells treated with amiodarone remained at around 100% viability at dosages between 0.01 µM and 3.16 µM. At concentrations 10 µM and 31.6 µM an increase of 10% in cell viability above 100% was documented (Figure 6a). The half maximal inhibitory concentration (IC50) of the A549 cells treated with amiodarone was 1.63 µM.

Besides amiodarone, the three other drugs, staurosporine, beclomethasone and fluticasone, caused a loss in A549 viability. The decrease in cell viability was perceived most apparent for the cells treated with staurosporine, despite the low dosages in the nanometer range. Staurosporine showed a clear dose-dependent toxicity profile (Figure 6b). At 3.17 nM the cell viability was reduced to 97%, at 100.14 nM to 75.6%. The highest concentration of staurosporine, 1000 nM, decreased the viability to 45.4%, which was the highest A549 viability reduction of all tested drugs and concentrations. The IC50 of the A549 cells treated with staurosporine was 55.24 nM.

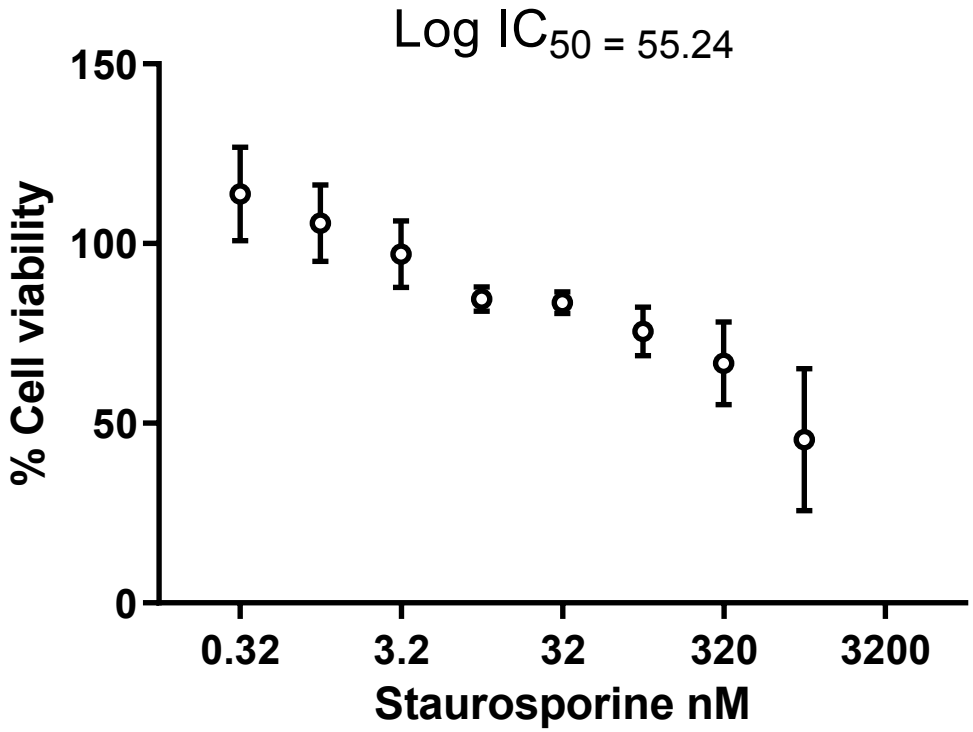
The corticosteroids beclomethasone and fluticasone had a clear effect of A549 viability reduction as well. Especially at dosages above 1 µM, a decrease was observed (Figure 6c, d). → The cell viability of A549 against beclomethasone amounted to 125% at 0.01 µM. Cell viability decreased 2.5% (per 1 µM dosage increase) towards 100% between 0.01 µM and 10 µM. At 31.6 µM the cell viability decreased to 77.7%. The IC50 for the A549 cells treated with beclomethasone was 0.29 µM.

The A549 viability against fluticasone was above 100% for concentrations 0.1 μM (121.2%) and 0.3 μM (109.9%). At 3.16 μM, the cell viability decreased clearly to 78.6% and at the highest dosage, 31.6 μM a reduction over 46.1% was documented. The IC50 of the A549 cells treated with fluticasone was 3.085 μM.

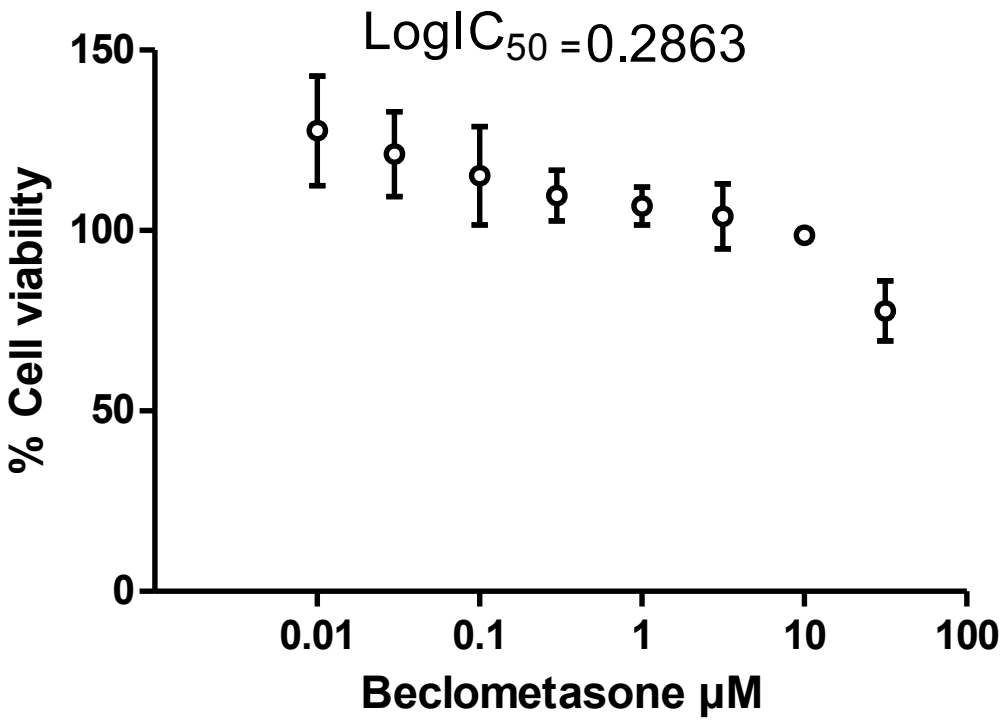
**a)**


LogIC50 = 1.626
150
100
50
0
% Cell viability
0.01
0.1
1
10
100
Amiodarone µM

**b)**


Log IC50 = 55.24
150
100
50
0
% Cell viability
0.32
3.2
32
320
3200
Staurosporine nM

**c)**


LogIC50 =0.2863
150
100
50
0
% Cell viability
0.01
0.1
1
10
100
Beclometasone µM

**d)**

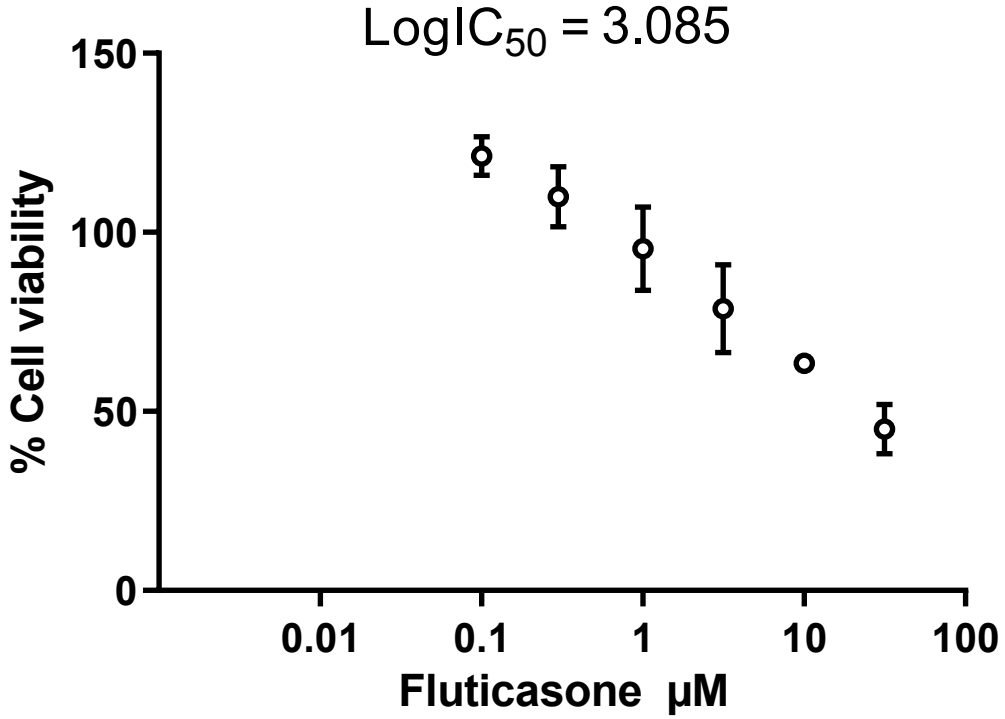

LogIC50 = 3.085
150
100
50
0
% Cell viability
0.01
0.1
1
10
100
Fluticasone µM

Figure 6: Assessment of the effects on the cell viability of A549 at concentrations stated in Table 1; a) Amiodarone; b) Staurosporine; c) Beclomethasone; d) Fluticasone. Data points represent the mean cell viability at the respective concentrations, each done in triplicate; SD presented as error bars; IC50 is specified in the unit of the x-axis.

### 4.1.2 Cell viability assay for evaluating compound toxicity, J774A.1: MTT Assay

The MTT Assay with the macrophage cell line J774A.1 was performed in the same manner as for A549. The only difference was the cell density used and the drug incubation time, which was 48 hours instead of 24 hours as described in chapter 3.2 of the materials and methods section. The cell viability of untreated cells was set as 100%.

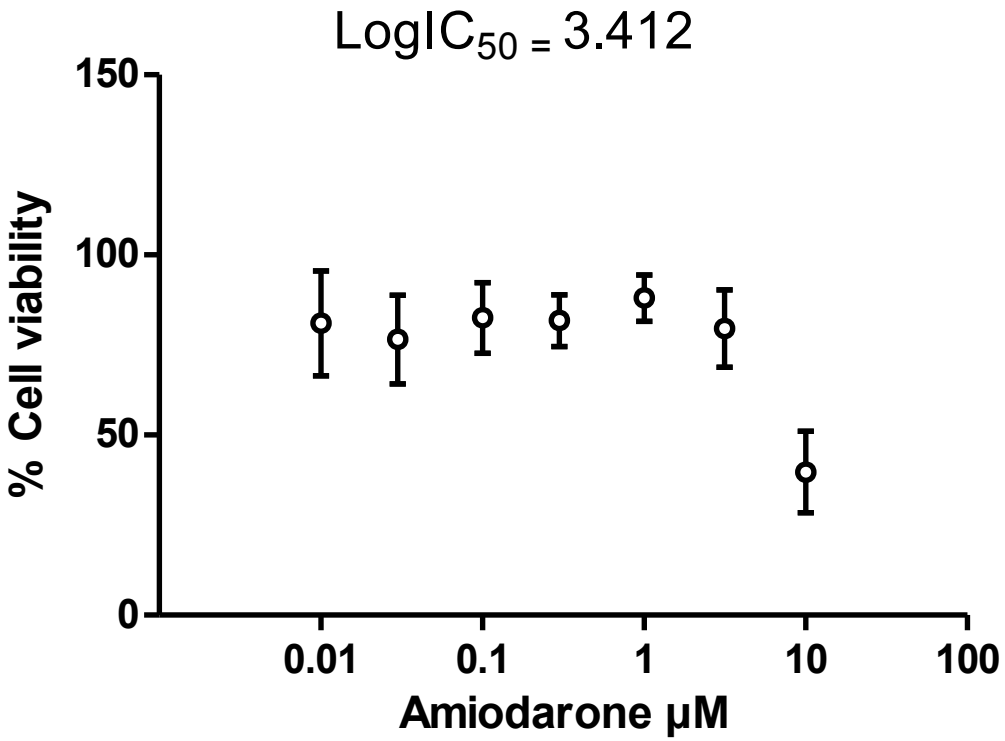
The cell viability of J774A.1 remained relatively steady around 75% from dosages of 0.01 µM to 10 µM, after amiodarone treatment as seen in Figure 7a. Only at the highest concentration, 10 µM, a distinct decrease to 39.7% compared to control was observed. The IC50 of the J774A.1 cells treated with amiodarone was 3.41 µM.

Similar to the MTT assay of A549, staurosporine showed to have the strongest effect on the cell viability of J774A.1 (Figure 7b). The viability decreased drastically with higher drug concentrations and fell below 50% at concentrations above 3.2 nM. At concentrations 100.14 nM, 316.46 nM and 1000 nM, the cell viability remained at only 7-13% compared to untreated cells. The IC50 of the J774A.1 cells treated with staurosporine was 119 nM.

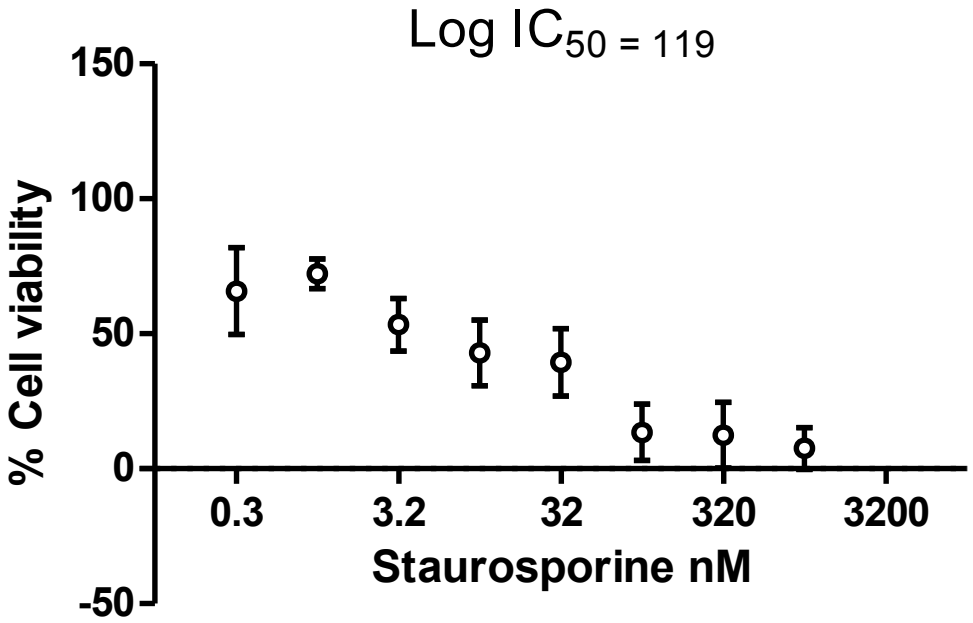
Beclomethasone showed a relatively constant decrease in J774A.1 cell viability (Figure 7c). At 0.01 µM, the cell viability was reduced to 55.6%, at the higher dosages the cell viability remained constant around 75%. The IC50 of the J774A.1 cells treated with beclomethasone was 0.29 µM.

The cells treated with fluticasone only clearly decreased in cell viability starting at concentrations above 1 µM (Figure 7d). The cell viability was reduced by half at 1 µM, and hovered around 20% at 10 µM and 31.6 µM. The IC50 of the J774A.1 cells treated with fluticasone was 0.87 µM.

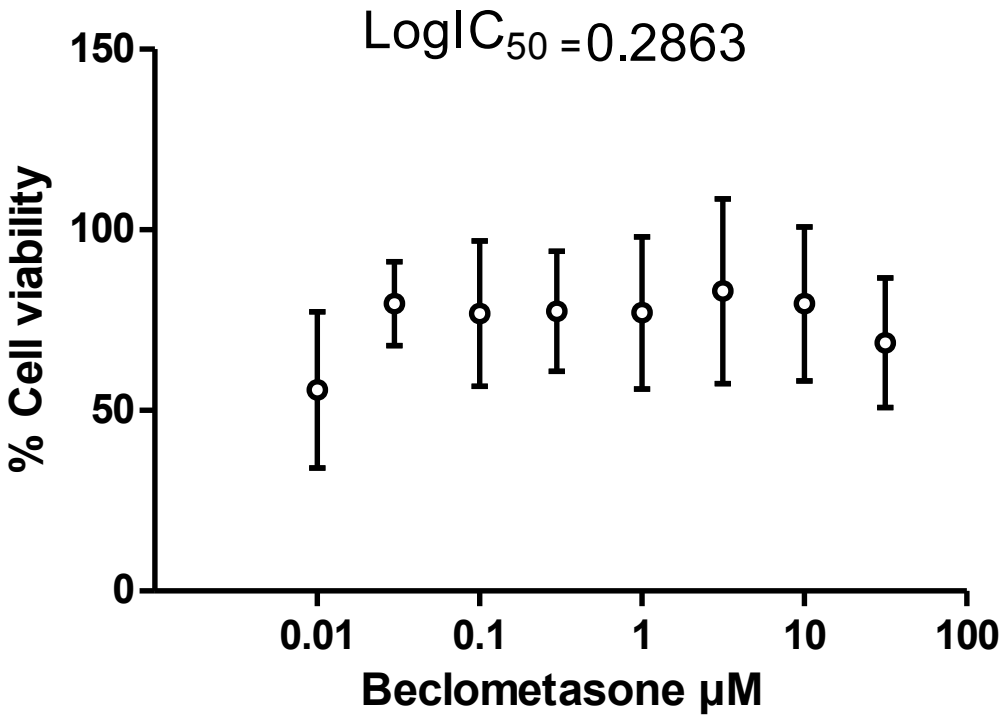
**a)**


LogIC50 = 3.412
150
100
50
0
% Cell viability
0.01
0.1
1
10
100
Amiodarone µM

**b)**


Log IC50 = 119
150
100
50
0
-50
% Cell viability
0.3
3.2
32
320
3200
Staurosporine nM

**c)**


LogIC50 =0.2863
150
100
50
0
% Cell viability
0.01
0.1
1
10
100
Beclometasone µM

**d)**

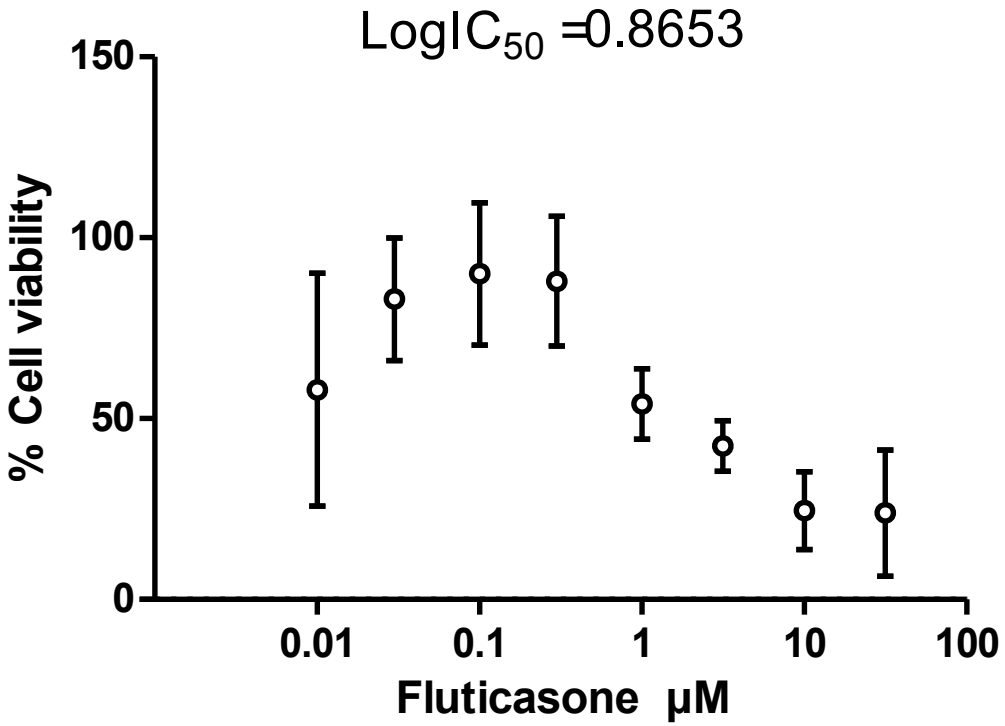

LogIC50 =0.8653
150
100
50
0
% Cell viability
0.01
0.1
1
10
100
Fluticasone µM

Figure 7: Assessment of the effects on the cell viability of J774A.1 at concentrations stated in Table 1; a) Amiodarone; b) Staurosporine; c) Beclomethasone; d) Fluticasone. Data points represent the mean cell viability at the respective concentrations, each done in triplicate; SD presented as error bars; IC50 is specified in the unit of the x-axis.

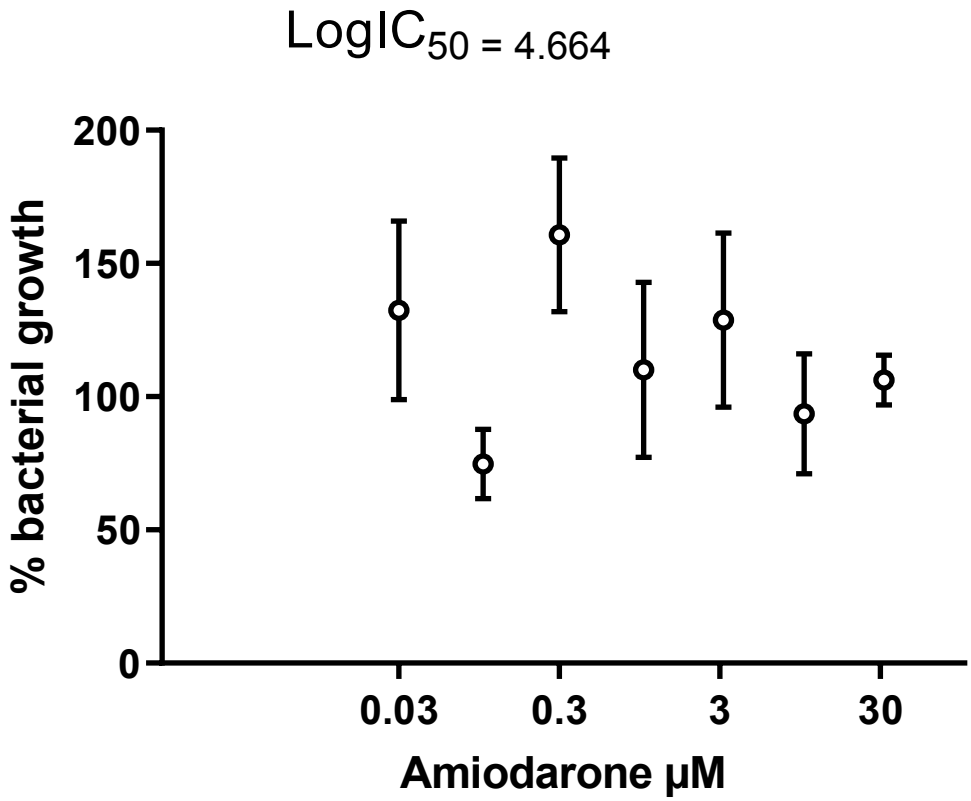
### 4.1.3 Bacterial growth assay for evaluating compound toxicity, *S. aureus*: Resazurin Assay

The Resazurin assay was performed as described in chapter 3.3 of the materials and methods section. Different from the cell viability assays for A549 and J774A.1, *S. aureus* was treated with amiodarone, beclomethasone and fluticasone only. The bacterial growth of untreated cells was set as 100%.

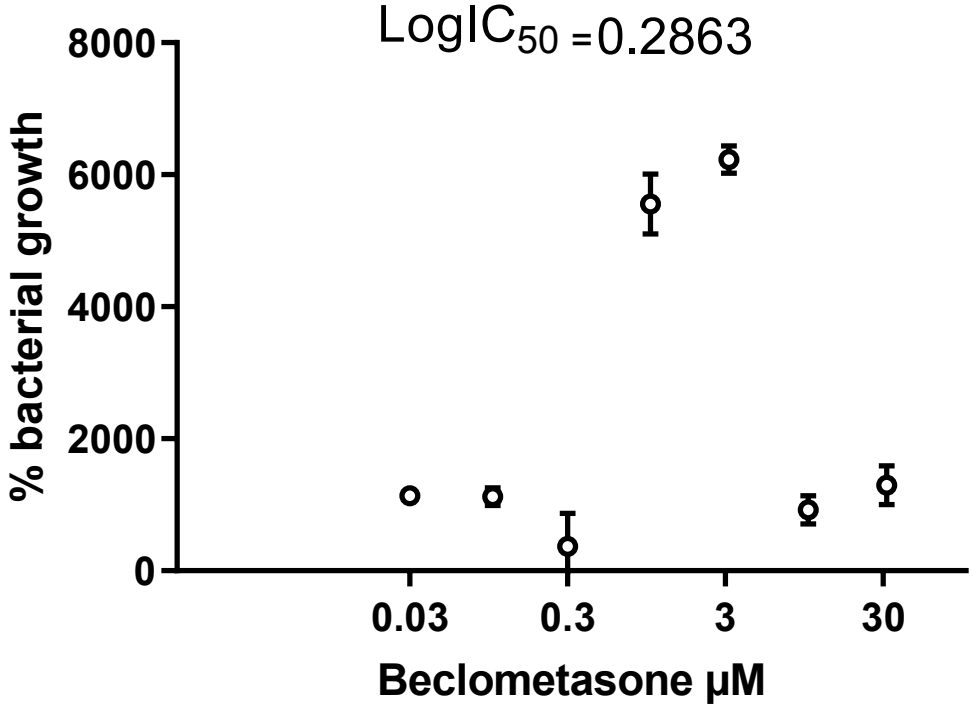
The *S. aureus* bacteria treated with amiodarone did not show a clear trend regarding the effect on bacterial growth depending on drug concentration, although overall a small increase is seen at low and high dosages except at 0.1 µM, where a reduction of bacterial growth is shown to approximately 74.8% (Figure 8a). The IC50 of staphylococcus treated with amiodarone was 4.664 µM.

In contrast however a very strong increase of bacterial growth was monitored for the corticosteroids beclomethasone and fluticasone. As portrayed in Figure 8b and 8c the cell viability went up to 6229,8% with beclomethasone treatment and up to 4447.4% for fluticasone. For both corticosteroids, it can be observed that the largest increase in bacterial growth occurred at concentrations above 1 µM and a large decrease again above 10 µM. The IC50 of staphylococcus treated with beclomethasone was 0.29 µM and with fluticasone 1.58 µM.

**a)**


LogIC50 = 4.664
% bacterial growth
200
150
100
50
0
0.03
0.3
3
30
Amiodarone µM

**b)**


LogIC50 =0.2863
% bacterial growth
8000
6000
4000
2000
0
0.03
0.3
3
30
Beclometasone µM

**c)**

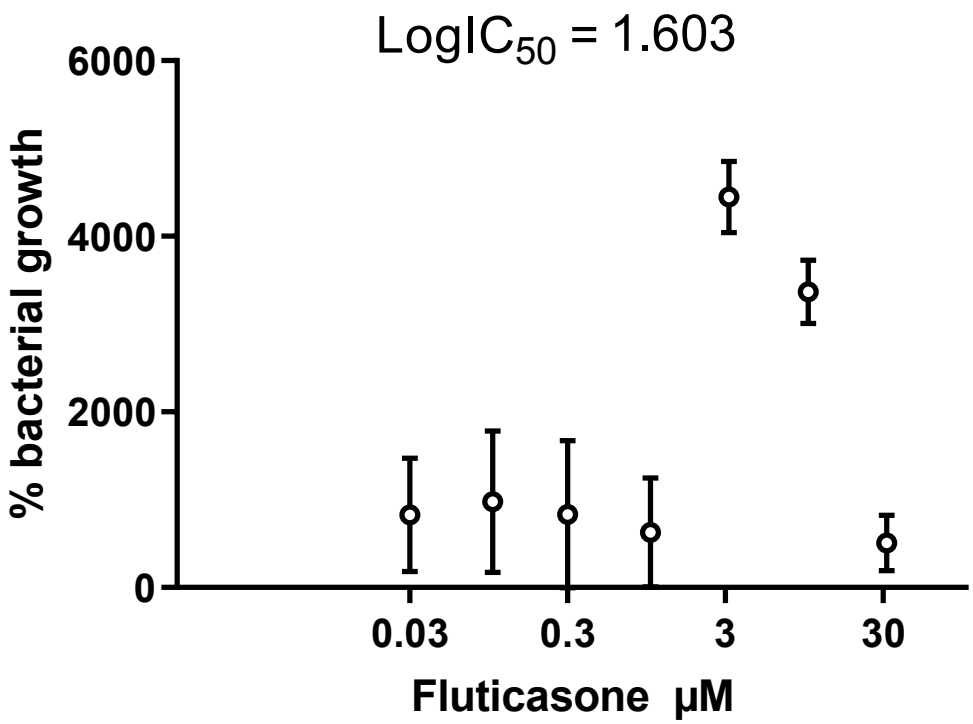



Figure 8: Assessment of the effects on the bacterial growth of *S. aureus* at concentrations stated in Table 1; a) Amiodarone; b) Beclomethasone; c) Fluticasone; Data points represent the mean bacterial growth at the respective concentrations, each done in triplicate; SD presented as error bars; IC50 is specified in the unit of the x-axis.

## 4.2 Bacteria uptake of J774A.1 Macrophages after drug treatment

The colony forming units (CFU) of the final agar plates of the three repeated experiments, as described in materials and methods chapter 3.5 were counted manually and the results displayed in the following tables. The CFU count represents the initial bacteria uptake by J774A.1.

As seen in Figure 9, the manually counted CFU's were plotted against the concentrations of drugs used to treat the macrophages before bacteria uptake compared to J774A.1 in medium only, without drug exposure. The average CFU count of the untreated control in medium only was 76 and is seen on the y-axis of Figure 9 at 0 drug concentration. The CFU count of the phagocytized bacteria by untreated J774A.1 macrophages at 0 µM drug, represents the unimpaired physiological ability of macrophages to take up bacteria and was therefore set to 100% and serves as control (Figure 10). CFU data are presented as mean standard deviation.

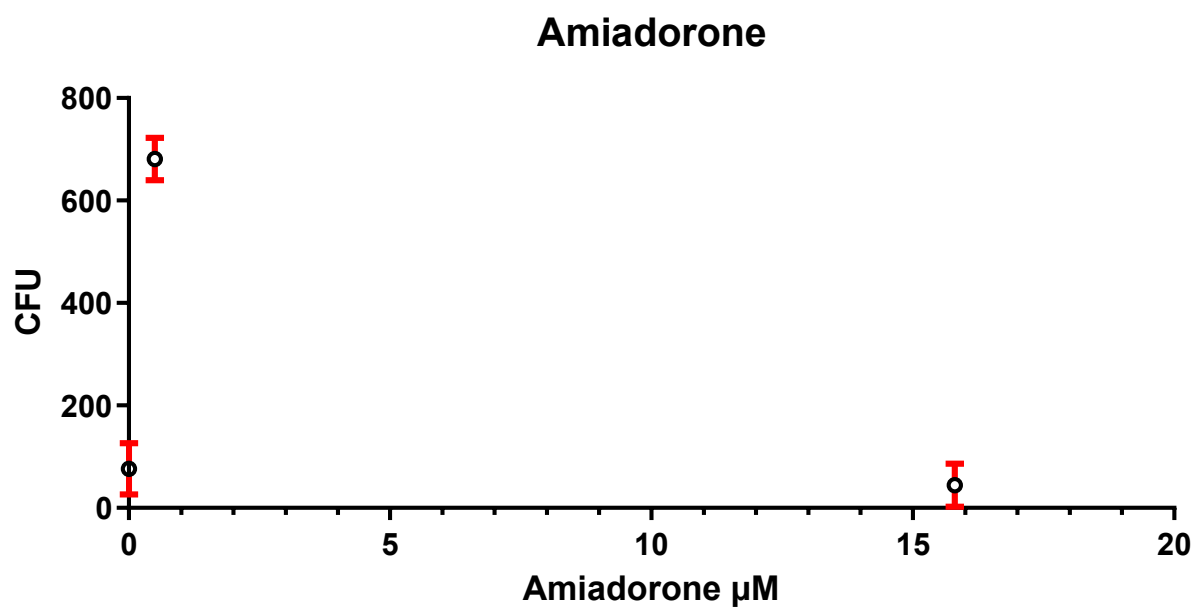
For 0.5 µM amiodarone, the mean CFU count was 497 +/- SD 41.7 and for 15.8 µM 45 +/- SD 41.9 (Figure 9a). The mean J774A.1 uptake potential at 0,5 µM compared to untreated J774A.1 was 654%, which marks a large increase in bacteria phagocytosis. At the highest concentration (15.8 µM), a significant decrease to 60% compared to control was observed (Figure 10a).

For 0.16 nM staurosporine the mean CFU count was 687 +/- SD 51.1 and for 500 nM 16 +/- SD 1.2 (Figure 9b). Therefore, the calculated mean J774A.1 uptake potential was 892% compared to untreated at the lowest concentration 0.16 nM. At the highest concentration 500 nM, a significant decrease in J774A.1 uptake occurred to 21%, which marked the strongest loss in phagocytosis compared to all tested drugs (Figure 10b).

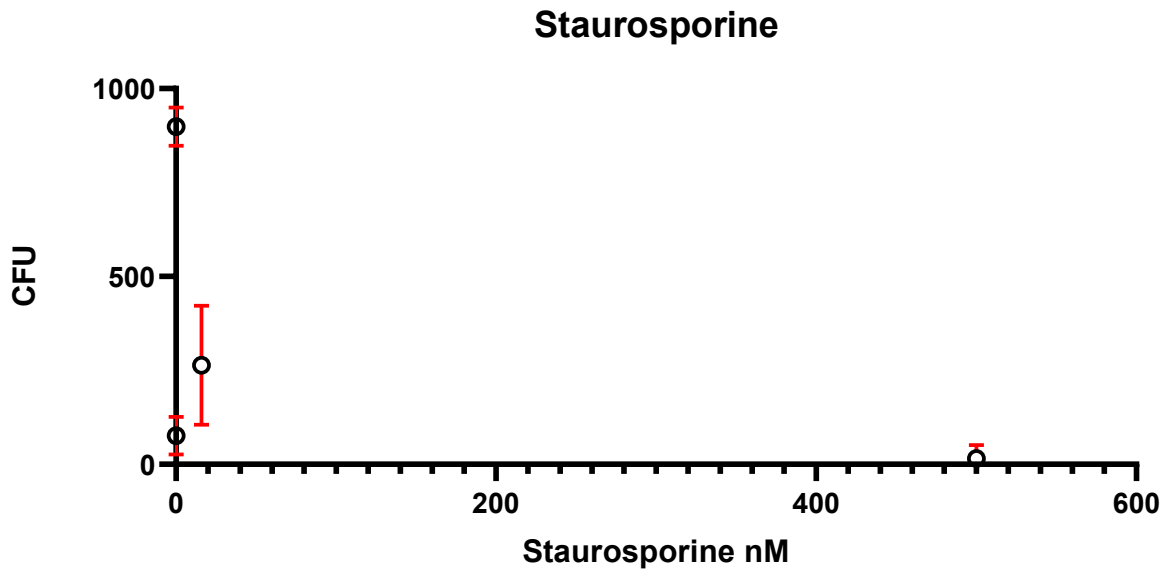
For 0.005 µM beclomethasone the mean CFU count was 547 +/- SD 79 and for 15.8 µM 439 +/- SD 45.3 (Figure 9c). Therefore, for the lowest dosage 0.005 µM, the mean J774A.1 uptake potential compared to control was 720%, and for the highest 15.8 µM 578%, which shows a small dose-dependent decrease in J774A.1 phagocytosis potential in contrast to the other test substances (Figure 10c). And lastly for 0.005 µM fluticasone, 429 +/- 10 SD CFU's were counted and for 15.8 µM fluticasone 121 +/- 19.19 SD (Figure 9d). At the lowest dosage 0.005 µM the mean J774A.1 uptake potential was 564%. The phagocytosis potential decreased at the highest concentration 15.8 µM to 159% compared to the lowest but not compared to the control (Figure 10d).

A general pattern could be observed for all four drugs, amiodarone, staurosporine, beclomethasone and fluticasone likewise, where drug exposure at low dosages lead to clear increase in CFU's compared to the untreated control. It can also be seen that at very high dosages, 15.8 μM for amiodarone, beclomethasone, fluticasone and 500 nM for staurosporine, the CFU's decrease strongly compared low dosages. Although compared to the untreated control only staurosporine and amiodarone decreased. By plotting the J774A.1 uptake potential in % relative to the drug concentrations a clear increase at lower drug concentrations and a consciously descending potential the higher the dosages, becomes evident for all 4 drugs (Figure 10).

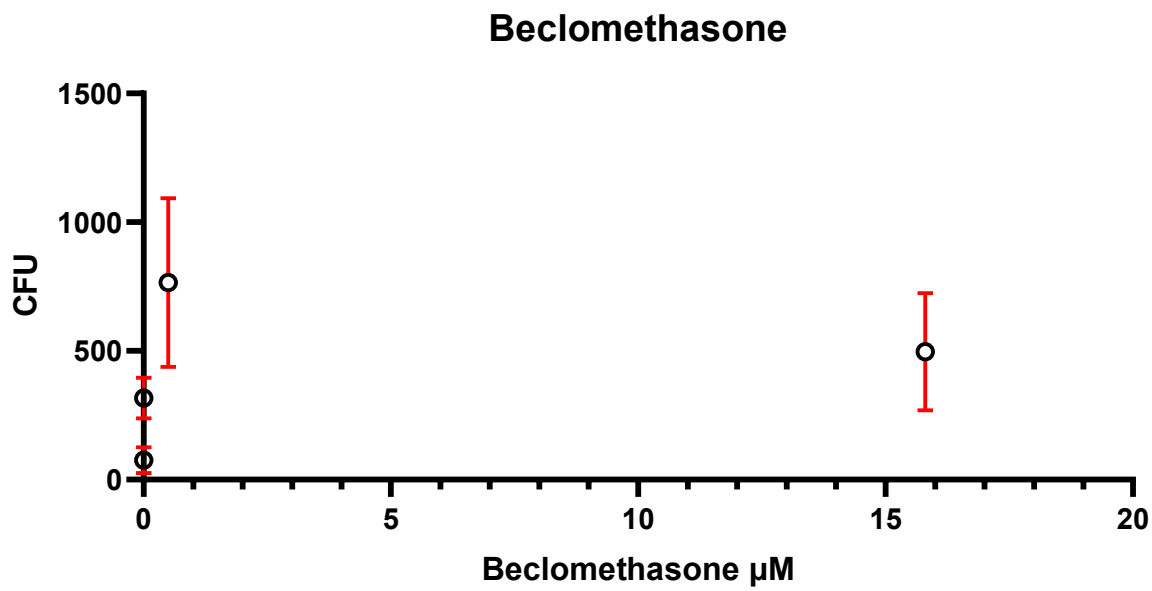
**a)**


Amiadorone
800
600
400
200
0
CFU
0
5
10
15
20
Amiadorone µM

**b)**


Staurosporine
1000
500
0
CFU
0
200
400
600
Staurosporine nM

**c)**




**d)**

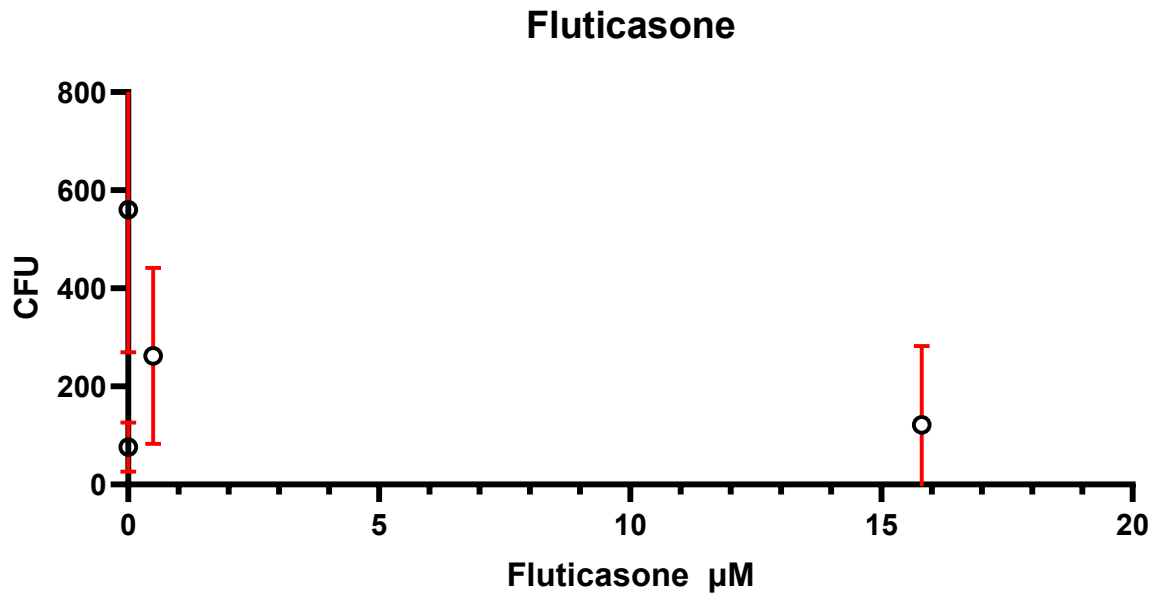
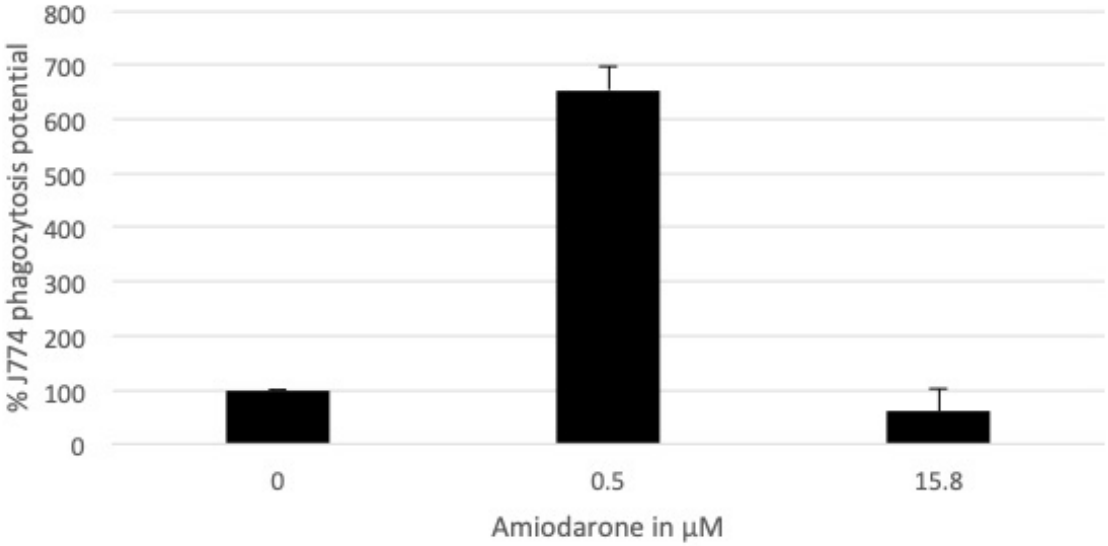
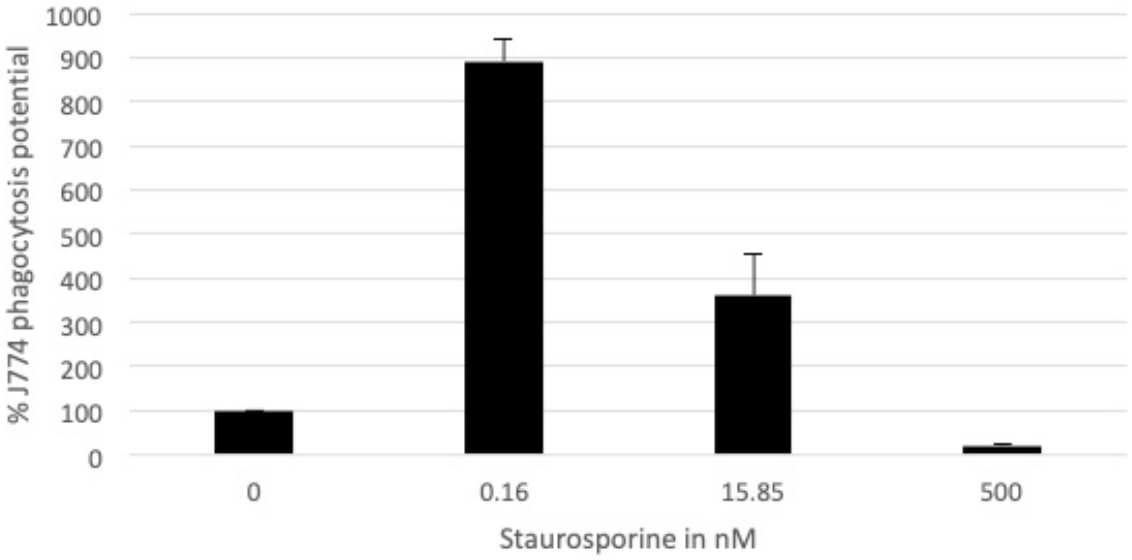



Figure 9: Assessment of *S. aureus* CFU uptaken by macrophages incubated with drug concentrations staded in figure 5; a)Amiodarone; b) Staurosporine; c) Beclomethasone; d) Fluticasone compared to untreated, done in triplicate.

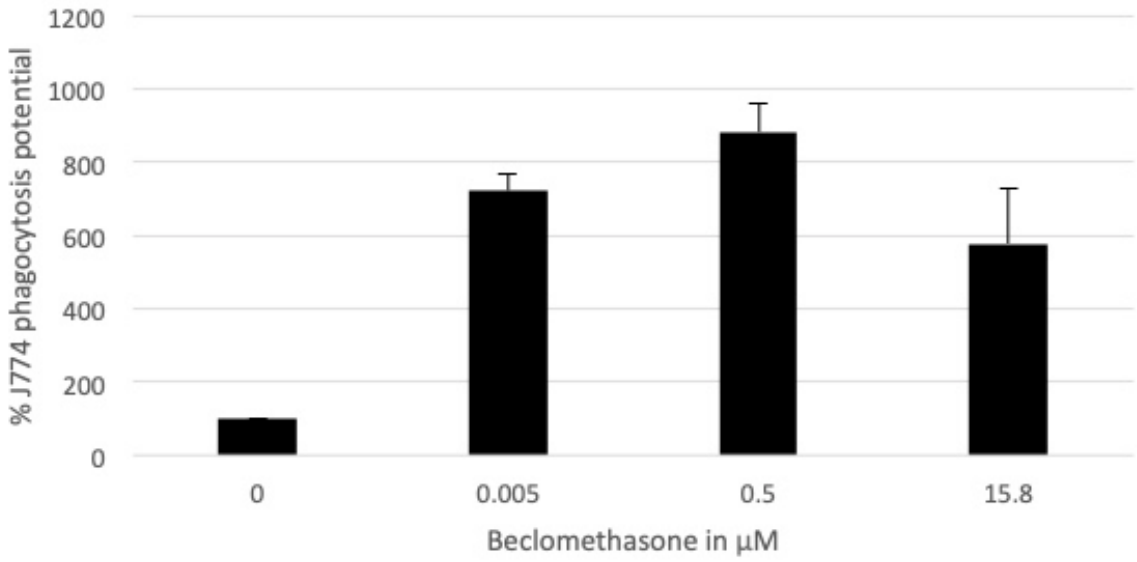
**a)**


800
700
600
500
400
300
200
100
0
% J774 phagozytosis potential
0
0.5
15.8
Amiodarone in µM

**b)**


1000
900
800
700
600
500
400
300
200
100
0
% J774 phagocytosis potential
0
0.16
15.85
500
Staurosporine in nM

**c)**




**d)**

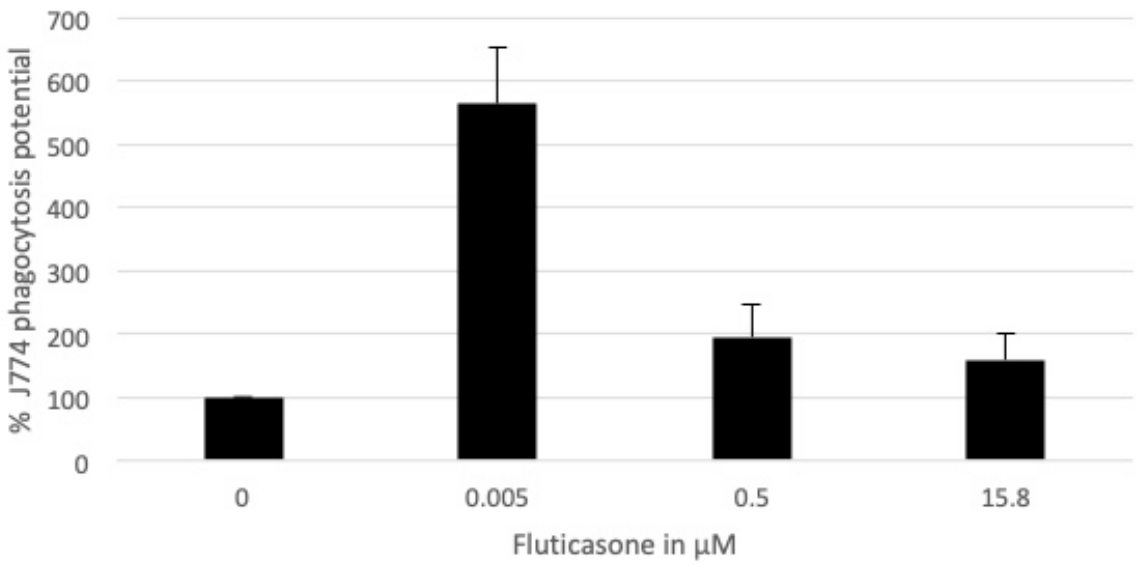



Figure 10: Assessment of J774 bacteria uptake potential in % incubated with drug concentrations compared to untreated

## 5. Discussion

The main motivation for this project was provided by the work of Noel et al., which aimed to establish a macrophage-enteroid co-culture model to investigate cross-communication between macrophages and epithelial cells, with the goal to receive an in-vitro system to study gut physiology and responses to enteric pathogens [45].

The initial aim was to create a similar in-vitro model consisting of the alveolar epithelial layer, macrophages and bacteria in order to investigate the effect of inhaled compounds on the model, specifically on the macrophages and their potential to form a foamy phenotype. As mentioned before in chapter 1.3.1, there is evidence of foamy AMΦ induction by inhaled medicines e.g. amiodarone [7, 11, 46], while there is to date a lack of methods to distinguish between adverse and non-adverse foamy macrophage appearance. To avoid false interpretation of FMΦ during inhaled drug development and production in the pharmaceutical industry, which lead to early permanent withdrawals during in-vitro and nonclinical studies, there is a need of new methods and further knowledge of AMΦ phenotypes, their ability to form a foamy composition and most importantly their cross communication with the alveolar barrier and other cells of the lung homeostasis [11, 8]. Therefore, it was it important to create an in-vitro tripple-culture model, which represents the alveolar epithelial barrier, with A549 type II alveolar epithelial cells, J774A.1 macrophages and staphylococcus bacteria, similar to the work published by Rothen-Rutishauser and team [37, 38]. It is a representation in a simplified matter but also allows investigating cell-cell interactions and influence of the different lung cell types on each other with and without pathogen or drug exposure.

In order to be able to establish a 3D-tripple-cell culture, the three different involved cell types, A459 representing the alveolar epithelial barrier, J774A.1 representing the AMΦ and *S. aureus*, were investigated after treatment with our test substances separately first, as a basis to further procedures that would lead to the aspired model.

Amiodarone is an often lifesaving, highly effective class III antiarrhythmic medication, which however is associated with severe pulmonary side effects in approximately 5% of treated patients. It is known for causing acute and chronic pulmonary fibrosis [25]. The exact mechanisms of amiodarone-caused pulmonary insult are unclear, although several studies have shown that amiodarone accumulation in

macrophages is often observed [26]. Therefore, amiodarone was the main test substance of interest during these investigations on its effects on the in-vitro model components. As seen in chapter 4.1.1 and Figure 6a, amiodarone showed to have only little effect on A549, which represents the AECII of the epithelial lung barrier. The reason for that is the usage of low dosages. At the concentrations 10 µM and 31.6 µM, even an increase of 10% in cell viability above 100% was documented. The IC50 was 1.62 µM. Other studies have shown a decrease in cell viability only starting at concentrations higher than the highest concentration 31.6 µM [47]. At an incubation time of 24h, Seki et al. stated in their cell viability results an IC50 of 50.1 +/- 4.5 µM [47]. Moreover, it has been shown that N-monodesethylamiodarone (DEA), the major metabolite of amiodarone has a stronger effect on the viability of A549 than amiodarone itself. Other reports have shown that amiodarone can cause apoptosis of human and rat epithelial cells in-vitro and induces epithelial-mesenchymal transition in A549 cells, which is a significant factor in pulmonary fibroses [48, 49].

J774A.1 treated with amiodarone remained at a cell viability of 75% for concentrations 0.01 µM to 3.16 µM, although a significant loss of 39.7% in cell viability at 10 µM, was documented (chapter 4.1.2, Figure 7a). The IC50 was 3.41 µM. This is consistent with previous reports [50, 51], as for example Quaglino et al. reported a dose-dependent inhibition in AMΦ cell viability by amiodarone and analogues starting at concentrations of approx. 10 µM [50].

Previous studies have shown that amiodarone accumulates in macrophages, induces superoxide release and inhibits fibronectin release. Macrophages treated with amiodarone also showed increased lamellar inclusions and appeared vacuolated [52]. The effects of amiodarone on macrophages observed in this work and previous studies indicates a strong correlation between amiodarone and induced pulmonary fibroses and the macrophages seem to be effected the most compared to other lung cells.

As seen in Figure 8a of chapter 4.1.3 amiodarone did not have a clear effect on the bacterial growth of *S. aureus*, although a decrease up to 70% at 0.1 µM can be seen at an IC50 of was 4.664 µM. This is consistent with previous studies that suggest an antibacterial effect of amiodarone at an MIC of 0.5-32 µg/ml. [53]

At low dosages, amiodarone increased the uptake-ability of J774A.1 up to 654% at 0.5 µM. At high concentrations however, amiodarone caused a significant decrease in J774A.1 uptake of *S. aureus* of 60% (chapter 4.2, Figure 9a). Previous studies have

shown clear effects of amiodarone on macrophages as described before [51, 52], although there have not been many published studies investigating its effect on phagocytosis. As seen in Figure 9a, we were able to document a clear decrease in J774A.1 bacteria uptake at 15,8 μM of amiodarone. On the basis of these and previous investigations, a main role of AMΦ and their vacuolated form in the process of amiodarone-induced firbrosis and lung toxicity seems very likely.

The second test substance, staurosporine, showed the most significant effects in the MTT cell-viability experiments, for both A549 and J774A.1 cells. As seen in Figure 6b of chapter 4.1.1, staurosporine reduced the cell viability of A549 clearly to 75.6% at 100.24 nM and continuously stronger at higher concentrations up to under 45.4% at 1000nM. The IC50 was 55.24 nM. It has been reported previously that staurosporine and similar analogues inhibit cell growth and cause alterations in cell shape and morphology, which underline the outcomes, which show a high toxicity of staurosporine on the cell viability of A549. Moreover, signs of apoptosis, vacuole fragmentation and decrease in microvilli, causing reduced adhesion and mobility of A549 after staurosporine treatment have been observed [54, 55]. A similar, even higher toxicity of staurosporine could be observed for J774A.1 cells (chapter 4.1.2, Figure 7b). At concentrations 100.14 nM, 316.46 nM and 1000 nM, the cell viability remained at only 7-13% compared to untreated cells. The IC50 was 119 nM. Similar toxicity has been reported previously for a different macrophage like cell line P388D1, where caspase-8 activation and increased TNF-alpha levels lead to apoptosis [56]. In the co-culture experiment (chapter 4.2), staurosporine had the highest influence on J774A.1's bacteria uptake especially at high doses of 500 nM the mean uptake potential was reduced to 21 % compared to untreated J774A.1 macrophages (Figure 10b). This could be caused by dysfunctional macrophages under the treatment of high staurosporine doses, although there are not many useful previously published studies investigating the effect of staurosporine on macrophage phagocytosis.

Representative of the group of glucocorticoids, the two remaining test substances were beclomethasone and fluticasone. Beclomethasone and fluticasone treatment both led to a decrease in cell viability at high dosages for A549 as well as for J774A.1. Although fluticasone had a stronger effect than beclomethasone on the viability

for both cell lines. The cell viability of A549/J774A.1 against 31.6 µM beclomethasone caused a decrease of 75/77.7% in cell viability. The IC50 for both A549 and J774A.1 was 0.29 µM. For treatment with fluticasone, the A549 cell viability at 31.6 nM was clearly lower at 46.1% (IC50 3.085 µM) and for J774A.1 as low as 20% (IC50 0.87 µM). Underlining the outcomes, previous research has shown glucocorticoid-induced inhibition of A549 cell growth, correlated to prostaglandin E2 (PGE2) inhibition [57], were A549 growth was inhibited by beclomethasone by 70% and by fluticasone by 82.2%. Further, an inhibition of pro-inflammatory macrophage M1 phenotypes has been observed under glucocorticoid influence [58].

Compared to amiodarone, beclomethasone and fluticasone led to a high increase of *S. aureus* cell growth at concentrations between 1 µM and 10 µM. As portrayed in Figure 8b and 8c, the bacterial growth went up to 6229.8% with beclomethasone treatment and up to 4447.4%% for fluticasone. Supporting these results, previous studies show a clear correlation between the risk of community-aquired *S. aureus* bacteremia (CA-SAB) and glucocorticoid use [59]. The study requests that glucocorticoid users experienced considerably increased risk of CA-SAB compared with nonusers (adjusted OR=2.48; 95% CI, 2.12-2.90), which could be explained by the increased bacterial growth under glucocorticoid influence.

Regarding the co-culture bacteria uptake experiment, both beclomethasone and fluticasone increased the J774A.1 phagocytosis of *S. aureus* at low dosages, continuously reducing at higher dosages. At 0.005 µM beclomethasone, the mean J774A.1 uptake potential was 720%, for 0.5 µM 882% and for 15.8 µM 578% (Figure 10c). At 0.005 µM fluticasone, the mean J774A.1 uptake potential was 564%, for 0.5 µM 195% and for 15.8 µM 159% (Figure 10d). There are contradicting publications on this topic. A study has shown guinea pig AMΦ phagocytosis stimulation after beclomethasone treatment [60]. On the other hand Belchamber et al. denied a correlation between monocyte derived macrophage phagocytosis and fluticasone treatment [61].

The outcomes of the experimental work of this project, suggest that amiodarone, staurosporine, beclomethasone and fluticasone have influence on cell growth and viability on both alveolar epithelial cells and AMΦ. Through the thereby received data of the different cell lines separately, preparations have been made, to be able to investigate the effects of the drugs on alveolar epithelial cells and macrophages together

in a co-culture system in the future. This is important, since several previous studies confirm the probability of cross communication between AEC and АМФ, which could have crucial effects on drug-response, phagocytosis and formation of foamy alveolar phenotypes [7, 8, 37, 38].

Further, evidence has been given, that the tested compounds have a substantial effect on the ability of macrophages to phagocytize bacteria.

## 6. Conclusion

In this work, preperational steps have been taken towards establishing an in-vitro model that allows the investigation of the effect of inhaled compounds on the alveolar epithelial barrier, including the physiologically relevant alveolar macrophages. In order to create an in-vitro co-culture model that mimics the reality of the in-vivo epithelial barrier, observations on the alveolar epithelial cells alone are not sufficient. As described in this thesis the alveolar epithelial layer is a complex structure, consisting of two different cell types (AECI, AECII) and is in contact with a variety of different cell types completing human physiological lung homeostasis. One of the most important part of lung homeostasis are immune cells, in particular alveolar macrophages. Since AMΦ are thought to be closely related to several pathological alterations induced by inhaled medicine [8, 25, 26, 52], the main aim of this work was to create a model that simultaneously mimics the epithelial airway barrier as a whole, with consideration of different cell type and also allows to focus on the effects on AMΦ.

Even though in-vitro models do not represent the in-vivo situation fully accurate, they provide excellent possibilities to combine different cell types and focus on specific mechanisms and cells of interest at the same time. Evidence on the restriction in cell viability for A549 cells, mimicking the alveolar epithelial cells type II and J774A.1, mimicking the AMΦ, under influence of amiodarone, staurosporine, beclomethasone and fluticasone were given in this thesis. Moreover, a clear influence of drug dosage on the bacteria uptake of J774A.1 was documented.

Taken together, the investigations accomplished in this project can serve as an overview and starting point to combine A549, J774A.1 and *S. aureus* in a triple co-culture system that allow to observe reactions to drug treatment that mimic the actual in vivo situation more accurate and allow for observation of cross communication and lastly better investigation of FMΦ formation. Although, these in-vitro models cannot replace in-vivo and clinical studies as a whole, they provide an excellent opportunity to establish sophisticated models that allow screening of inhaled compounds in order to reduce false rejections in the pharmaceutical industry.

In the future an in-vitro combination of A549, J774A.1 and bacteria could provide valuable answers on the effects of inhaled compounds in the alveolar space. A549 could be compared with a similar model containing the novel TT1 cell line which

represents the AEC I which cover most of the alveolar surface and J774A.1 could be replaced by monocyte derived macrophages. These adaptions could provide a more accurate comparison to the actual in-vivo situation.

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