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In-vitro Assay Establishment to Assess the Influence of Inhaled Medicine on Cell Viability and Antimicrobial Activity

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ABSTRACT

Background: The pulmonary route is an efficient way for the administration of medicine as the lung possesses the perfect preconditions for both, local and systemic drug delivery. Unfortunately, due to challenges in inhaled drug development, pulmonary drug administration is only limited to a small group of compounds. The main difficulty is the evaluation of the toxicity in preclinical stages of drug development due to the limitations of *in-vitro* models to predict the *in-vivo* situation. Besides physical barriers, the alveolar macrophages are decisive for the protection of the lung, which is why an excessive particulate burden can result in the formation of foamy macrophages (FM). The interpretation of those vacuolated macrophages (adaptive response or adverse event) poses a major challenge to the science industry. The aim of this study was therefore to establish a method to assess the safety of a new inhaled compounds in the lung. The objective was to identify a correlation between the cytotoxicity of a FM-inducer and its impact on the phagocytic function of alveolar macrophages. The assay was established with amiodarone, an antiarrhythmic drug and known inducer of FM, on the murine cell line J774A.1 and *Staphylococcus aureus* (*S. aureus*).

Methods: The cell viability assay was performed using different concentrations of amiodarone on the J774A.1 cell line. The activity of varying concentrations of amiodarone was also assessed against *S. aureus* to determine the MIC (minimal inhibitory concentration) and the effect on the bacterial growth curve. Non-toxic concentrations of amiodarone were further evaluated in the assay to determine the phagocytic function of the macrophages.

Results: Amiodarone showed a dose-dependent toxicity profile on the J774A.1 cells with an EC_{50} of 11.87 µM. The cell viability and the phagocytic function decreased dose-dependently. The lag phase of the growth curve (timepoint when the bacteria were added to the macrophages) was not affected by any concentration of amiodarone, and the MIC was >56.2 µM.

Conclusion: The results from the cytotoxicity assay was similar to the one reported previously.

1. Introduction

1.1 The Pulmonary Route: An Efficient Pathway for Drug Administration

1.1.1 Pulmonary Drug Administration

Inhalation therapy has been used for over 3500 years and has experienced increasing attention in recent times as a lot of research is being done in medicine development for topical lung delivery [1]. Especially for the treatment of respiratory diseases, such as asthma or chronic obstructive pulmonary disease (COPD), inhaled drug delivery has become fundamental. The inhalation of bronchodilators is part of almost every therapy against obstructive lung disease where inhaled antibiotics are used as a remedy for cystic fibrosis (CF) and pulmonary arterial hypertension (PAH). Even though the pulmonary route is mostly being used for β_2-agonists, antimuscarinic drugs and inhaled corticosteroids in the treatment of morbid condition of the lungs, there are many other examples that inhaled drug delivery could be favourable (antibiotics, antiviral agents, mucoactive agents, antituberculotic drugs, inhaled gene therapy etc.) [2].

For targeted drug administration to the lungs, one of the biggest advantages of the pulmonary route compared to oral drug administration is the delivery of the medicine directly to the site of action, whereas some orally taken medications may partly be inactivated by the liver due to the first-pass effect. It is thus understandable that inhaled drug delivery is the ideal way for drugs with a distinct first-pass metabolism as well as a low gastrointestinal absorption rate. Consequently, the direct delivery to the lungs enables a reduction in dosages with the same effectiveness and also leads to much lower blood levels. As a result, the risk of side effects is being reduced significantly, which becomes crucial in the treatment with corticosteroids or prostacyclin-analogues. Another advantage is an almost immediate onset of the therapy, which

is a significant factor for the treatment of acute asthma with inhaled bronchodilators [2]. In addition to treating the inflammation, some of the inhaled medicines (eg. dornase alfa, mannitol) are able to reduce the viscosity of the extensive mucus layer present in most forms of respiratory disease [1].

Besides targeted drug delivery, the lung also provides the possibility for systemic drug administration. The large epithelial surface in addition to the high perfusion and the thin layer of alveolar epithelium make the lung perfectly suitable to achieve substantial blood levels of a certain drug [3].

Unfortunately, up to this day there are only few compounds that are being used for systemic delivery, such as Ralneza against the influence virus, Flumist, a nasal delivery system for a vaccination against the influence virus, or Malacalin against osteoporosis [1]. The development of new medicines for systemic delivery to the lungs is still challenging and requires more research. Drugs, which prove to be safe for other routes need not necessarily be safe for pulmonary administration which is why specific toxicity assays of the component on the lungs have to be performed in order to be able to make a statement about its harmful potential [3]. This is the reason why inhaled therapy is, as mentioned above, mainly reserved to the topical drug delivery of β_2-agonists, antimuscarinic drugs and corticosteroids for the treatment of obstructive respiratory diseases [4].

1.1.2 Challenges in Inhaled Drug Development

The lung is a highly complex organ and the transportation to the site of action can be influenced by a variety of factors [2] with dosimetry, efficacy and safety considerations being the main challenges in the development of inhaled medicine [4]. Dosing issues include contemplation of sufficient, reproducible doses and the absorbed amount of the drug by the lungs, as well as the formulation and the inhaler devices (e.g. metered dose inhaler (MDI), dry powder inhaler (DPI), nebulizer) [3]. A different inhalation technique is necessary for each inhaler devices and the

dosage at the site of action varies significantly when used incorrectly [5]. When the lungs are affected by an illness, drastic changes can occur and areas which are not ventilated, for instance because of an obstruction by excessive mucus production, can no longer be reached by the aerosol particles [2]. Once the drug has reached the lungs, safety and efficacy considerations come into play and pose critical factors in the development process [4].

When challenged with inhaled medicine, the lung activates innate defence mechanisms to cope with the particles. The bronchial network functions as a filter to hinder the particles from penetrating deeper into the lungs, proteolytic enzymes are activated leading to degradation of the molecules and physical barriers, such as the mucus layer, the alveolar lining or cells, inhibit the absorption of the drugs into the blood stream [3]. When the physiological barriers are overcome the phagocytic uptake of antigens by macrophages is the most important protection mechanism for the lungs and is decisive for the alveolar clearance [6].

If the particulate burden exceeds the lung's innate defence mechanisms, aggregation and inactivation of the drugs are the result. This acute irritation of the alveolar by the particles can further include morphological changes ranging from epithelial damage to phospholipidosis and the appearance of "foamy macrophages". The term "foamy macrophages" (FM) describes phagocytic cells with a typical vacuolated appearance when viewed in light microscopy. The formation of FM often occurs during preclinical stages of inhaled drug development and the interpretation of those foamy appearances poses a major challenge to the whole science industry. The correlation as to which extent their development can be viewed as a marker for toxicity remains unclear up to this day [4].

One of the biggest obstacle in the development process is the lack of consistent regulations throughout the whole medical industry to evaluate the toxicity or safety of a certain compound. There have been various approaches for the assessment of the toxicity of new substances, such as the monitoring of different enzymatic markers, biological markers or gene profiling. Unfortunately, the methods are all incompatible and there is no generally applicable method for

the evaluation of the FM [7]. It is indefinite whether the adaptions are a physiological response or whether pathological changes start to occur. Moreover, the nomenclature and terminology used throughout the science industry is not consistent, which makes the interpretation of the results even more challenging and inhibits the comparison between different studies [4].

In most good laboratory practice (GLP) toxicology studies of inhaled medicine the examination of stained lung tissue is suggested as primary endpoint. However, inconsistencies of how pathologies are defined still pose a big problem and the extent to which histological findings are significant in terms of the functionality of the cells remains unresolved [8].

1.2 Alveolar Macrophages and Safety Concerns in Inhaled Drug Development

1.2.1 The Function of Alveolar Macrophages

Macrophages are a type of white blood cell which can be found everywhere in the body. They do not only play an essential part in the acquired and innate immune system, but are involved in a number of different processes, ranging from host defence, proliferation of tissue, tumour growth, to phagocytosis of antigens and debris [9]. Depending on where in the body they are located, their form and morphology varies tremendously and, their differentiation being regulated by tissue derived factors, they adjust perfectly to the demands of their surrounding tissue. A perfect example of the specialization of macrophages are the alveolar macrophages in the lungs [10].

In the airways, alveolar macrophages have two major functions: inflammatory response and protective function. Firstly, alveolar macrophages are crucial for maintaining immunological homeostasis and are capable of inducing an anti- but also a pro-inflammatory response. Macrophages seem to have the ability to present and activate T-cells. The second function is the defence mechanism and the protection of the host from foreign particles, such as bacteria,

viruses or other particulate matter, like exhaust or inhaled medicine. Through phagocytosis alveolar macrophages remove antigens, debris and apoptotic cell fragments from the lungs and are therefore the terminating factor for the clearance in the lungs [7].

Due to the adaptation to their environment, there is an enormous variation in the appearance of the cells. The fact that their morphology not only varies when they are located in different parts of the body, but also within the same surrounding, seems to indicate that their appearance depends on a number of factors. Such influences are the macrophages' age and activity but also the composition and quantity of the phagocytised particles. There is a direct correlation between the quantity of inhaled particles and the outpouring of alveolar macrophages from the bone marrow, where they mainly originate from. If the particulate challenge crosses a threshold and gets too big, the provision of alveolar macrophages is exhausted and an altered appearance of the pulmonary phagocytes is the result [11].

This mechanism has to be taken into account in the development process of inhaled medicine as it is generally agreed that inhaled particles can lead to the accumulation of alveolar macrophages and further to changes in their morphology. With the aim of describing those altered cells the term "foamy macrophages" (FM) has been established [7].

The differences associated with FM are typically an enlargement of the cell compared to normal alveolar macrophages and a perforated cytoplasm when viewed in light microscopy: The number of vacuoles, as well as the percentage area of vacuoles per cell, is increased [7], [8], [12].

There are a number of reasons for the formation of a foamy morphology, ranging from an overflow of surfactant in the lungs and consequently lipid accumulation, intracellular phospholipidosis, to phagocytosis of poorly soluble particles [4].

1.2.2 Foamy Macrophages as a Safety Concern

As already discussed above, FM can frequently be detected in the development process of inhaled particles. Their occurrence still poses a major challenge to the science industry. Up to this day, the connection between the observation of foamy appearances and their significance in terms of the drug's safety continues to be unknown and can only be estimated [13]–[15].

There is reason to believe that FM can pose a threat to the patients' health, especially for patients with existing diseases of the lungs, as illnesses, such as COPD, can exacerbate [13]. But also safety concerns in healthy lungs based on the appearances of foamy phenotypes often result in the withdrawal of the drug from the development process [16].

However, not every development of a foamy appearance is necessarily a sign of toxicity. In the absence of additional histopathological changes and/or chronical inflammation and fibrosis the morphological changes are likely to portray physiological adaptions rather than an adverse event. Only when other signs of inflammation or further pathological changes occur or when the particulate load surpasses the capability of the pulmonary macrophages to clear the particulate matter, the results can be interpreted as adverse [7], [16].

The distinction between adverse and non-adverse events continues to be a big challenge, as different mechanisms of induction can result in very similar looking foamy appearances and there is no universally applicable threshold as to from which point on the pathological changes can be seen as marker of toxicity [4], [7], [12].

In addition to the visual changes, a particulate challenge on the pulmonary area can result in a functional impairment of the alveolar macrophages [17]–[20]. As the phagocytes are the cells mainly responsible for the innate host defence and the clearance of the lungs, an impaired function is likely to result in a decreased bacterial uptake, thus increasing the vulnerability to bacterial infections [21]. As a consequence, even a harmless commensal bacterium which colonizes the skin and mucosal tissue, such as *Staphylococcus aureus*, can induce serious respiratory diseases [22].

1.2.3 Amiodarone as an Inducer of Foamy Macrophages

Amiodarone is an iodinated benzofuran molecule with an inhibitory effect on the cardiac sodium, potassium and calcium channels leading to a prolonged action potential and a delay in depolarisation. As a class III antiarrhythmic agent, amiodarone is used against tachyarrhythmia [23]. Even though the remedy is highly effective, it is only used in severe cases when all other possibilities are exploited, as severe side effects can be observed in a high number of patients [24]. Due to the high lipophilicity, the drug tends to accumulate in various organs, especially in the lungs [25]. This explains the extensive pulmonary toxicity which is associated with amiodarone [26]. An orally administered daily dose of 400mg, for instance, leads to pulmonary complications in 10-17% of the patients, with a fatality of 10% [23]. Even though some factors, such as age, the sex, the duration of the therapy and the cumulative dose can increase the pulmonary risk, the toxic effects can occur at any dose and any time after the treatment has been started [24], [25].

Amiodarone can attribute directly to cytotoxic effects as it is able to induce the production of O_2 which harms the cells directly. On the other hand, an indirect immunological response can lead to the formation of FM with lamellar inclusion of phospholipids (Figure 1). The underlying mechanism is a concentration of lipids in the lysosomes of the cells [25]. Cationic amphiphilic drugs (CAD), such as amiodarone, are often associated with the induction of phospholipidosis [27]. Those drugs are able to interact with the negatively charged lipids and lead to their extensive accumulation and to the appearance of FM [28].

Due to those properties, amiodarone has pulmonary toxicity, leading to several pulmonary diseases, as one of its more serious side effects [29]. As the appearance of FM and pulmonary toxicity can be linked to amiodarone, this compound is a good candidate as positive control for non-clinical assay development of new inhaled compounds.

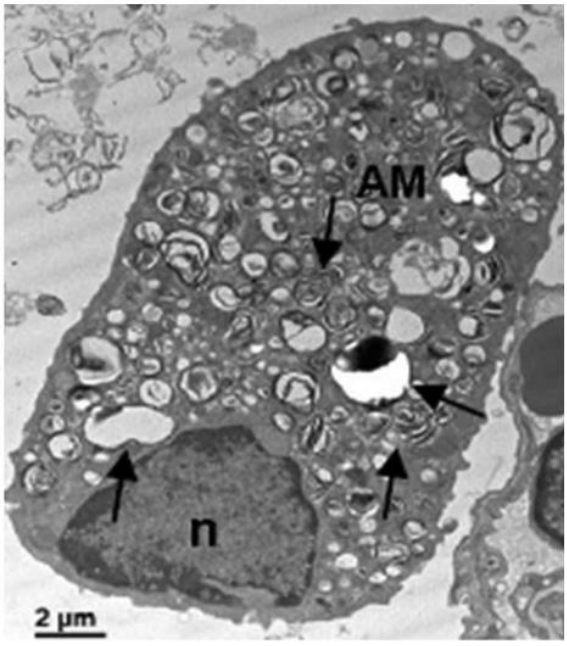


Figure 1 – Foamy Macrophage
T.E.M of a rat's lung with lamellated inclusions in the alveolar macrophages induced by amiodarone. (Image obtained from Mesens, N. et al. [28])

1.3 Models for Studying Foamy Macrophages

1.3.1 *In-vitro* Models

Macrophage cell lines are a commonly used model for toxicology studies of inhaled medicine. Even though a broad range of cell lines is accessible, there are some that are used especially often: RAW 264.7(a murine leukemic monocyte derived macrophage cell line) [30], J774A.1 (a murine sarcoma-derived macrophage cell line) [31], U937 (a human lymphoma derived cell line) [32] and THP-1 (a human leukemic monocyte cell line) [33].

The reoccurring use of those *in-vitro* models is due to the fact that there is already a lot of data available which enables the direct comparison between different studies and simplifies the meta-analysis of the outcomes. Other than cell lines, primary cells and triple- or tetra-co-cultures, containing epithelial and endothelial cells, can be used with the advantage that they closer resemble the *in-vivo* situation [7].

The *in-vitro* models are chosen according to the needs of the desired information and provide an easy to handle, convenient test system for the evaluation of toxicity markers, such as cytotoxicity, cell proliferation, gene expression, apoptosis or phagocytosis [12]. However, all *in-vitro* testing systems underlie certain limitations as the *in-vivo* situation is much more complex. The results are thus not always predictive of the physiological toxicity in the human body, which is why animal testing is needed as well [7].

1.3.2 *In-vivo* Models

Animal testing is necessary before proceeding to first-in-men studies [4]. By performing *in-vivo* tests, predictions about the deposition as well as the pharmacokinetics, pharmacodynamics and the safety of a new inhaled drug can be made [34].

The species mainly used as *in-vivo* models are small rodents like rats, mice or guinea pigs but also larger mammals such as dogs or non-human-primates are required as model organisms [34]. As neither rodents nor mammals provide the perfect preconditions, the testing takes usually place on both, rodent and non-rodent. Moreover, both sexes should be included in the experiment [16].

Even though animal models are a crucial stage of the drug development process, their disparity with the human anatomy displays limitations. Due to the variation between different species, predictions based on *in-vivo* models remain to be a major challenge. Therefore, differences concerning metabolic pathway, the physiology or the anatomy from the physiological situation in humans must be taken into account. Rodents, on the one hand, are solely able to breathe through the nose, and their respiratory anatomy differs tremendously from human's physiological features of the lungs. On the other hand, mammals, with the exception of pigs and non-human primates, are able to breathe through the mouth and the nose. But while they resemble the physiological situation in terms of the breathing pattern, their nasal anatomy varies [34].

As lung tolerance is one of the most critical aspects, *in-vivo* models remain irreplaceable for the drug development. Despite the fact that ethical reservations favour the use of rodents rather than larger species, mammals are nevertheless necessary in addition to the small rodents for the toxicological assessment due to their closer resemblance to the human biochemistry and immunology. Even though *in-vitro* testing cannot replace animal studies, there is the need for more decisive *in-vitro* models, which could then perform preparatory work for the *in-vivo* tests and hence reduce the use of animals [34].

1.3.3 Phagocytosis Models

High particulate burden on the lungs increasingly becomes associated with pulmonary diseases. As a result, infections, especially pneumonia, are on the rise and contribute significantly to an increase in morbidity due to lung diseases [21].

Macrophages play an important role in the host's defence against intruders, hence a decreased phagocytic function is likely to result in a greater susceptibility to bacterial infections [19]. Consequently, challenging the macrophages with bacteria is a convenient technique to assess the antimicrobial activity of the phagocytes [17], [18]. The most commonly used bacteria are *Streptococcus pneumonia* [21] and *Streptococcus aureus* [19], last of which were used in this experiment.

Staphylococcus aureus (S. aureus) is physiologically present on the healthy mucosal tissue and skin as commensal bacteria. If, however, the immunological defence mechanisms are impaired, the bacteria can induce severe respiratory infections [22]. The bacterium's capability to evade innate defence mechanisms and its growing resistance to antibiotics contributes to the serious threat of *S. aureus* infections [35]. *Methicillin-resistant S. aureus (MRSA)* infections no longer remain limited to hospitalized patients as also community-acquired MRSA are on the rise [36].

1.4 Techniques to Evaluate the Toxicity and Phagocytic Function

1.4.1 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT)-Assay

The MTT-Assay is the most commonly used parameter in cytotoxic testing for cell viability [37]. It is a high throughput method for studying the cell viability and proliferation. The assay's endpoint is the effect of the treatment on the mitochondrial function and is based on the capacity of the mitochondrial enzyme succinate-dehydrogenase in viable cells to cleave the tetrazolium rings of the yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide, a yellow tetrazole). The reduction reaction results in the formation of purple formazan crystals which accumulate in healthy cells. The scheme of the reduction of MTT into formazan can be seen in Figure 2. The crystals can be solubilised in detergent (SDS) with the concentration of formazan being directly proportional to the number of active mitochondria in viable cells. The absorbance of this coloured solution can be quantified by a spectrophotometer at a certain wavelength (570 and 650 nm).

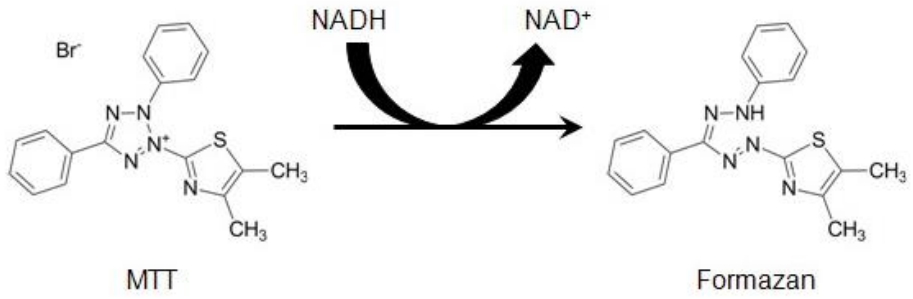


Figure 2 – Reduction of MTT to Formazan
(Image obtained from Riss, L. et al. [37])

With the aim of excluding an effect of the inducer on the bacterial growth, a growth curve was prepared in the presence of various concentration of amiodarone.

As soon as any possible influences of amiodarone on the bacteria were ruled out, the antimicrobial activity assay was established as described above.

1.4.2 Antimicrobial Activity Assay

The second experiment involved a coculture of macrophages with *S. aureus* to reveal the antimicrobial activity of the macrophages. The phagocytic uptake of bacteria was measured after the cells had been exposed to different concentrations of amiodarone. The phagocytic capability of the cells served as a marker for the physiological function dependent on various concentrations amiodarone.

Minimal inhibitory concentration (MIC)

Prior to the experiment itself, possible effects of amiodarone on the bacteria had to be confirmed. Therefore, the minimal inhibitory concentration (MIC) of amiodarone on the *S. aureus* strain was determined. The MIC is the lowest concentration which prevents visible growth of microorganisms. Typically, the method is used for antimicrobial susceptibility experiments of antimicrobial substances, e.g. antibiotics [38]. In this study, the MIC of amiodarone on *S. aureus* was determined before performing the actual experiment, namely determining the antimicrobial activity of the macrophages, in order to make sure that the concentration of the drug used in the experiment was below the MIC. Otherwise, observed results in the phagocytosis-experiment could possibly come from the effect of amiodarone on the bacteria and not from the loss of activity of the macrophages.

In order to be able to detect a colorimetric change when a loss of cell viability occurred, the indicator of oxidation-reduction resazurin was added to the wells [39]. Resazurin, a widely used indicator in toxicology assays, was used here to establish the MIC, as it is an easy, quick and cheap way to get information about the metabolic activity of the tested cells [40]. Living cells in an active state are able to reduce the blue substance to resorufin, thus changing the colour to pink (Figure 3). A further reduction results in the formation of colourless hydroresorufin. Furthermore, due to the additions of resazurin the cell viability is quantifiable [40].

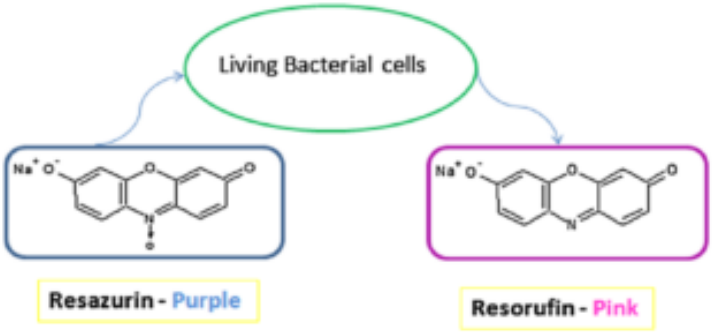


Figure 3 – Resazurin Reduction
Vital cells reduce the blue resazurin to pink resorufin. (Image obtained from Elshikh, M. et al. [40])

Bacterial Growth Curve

After having guaranteed that the metabolic activity of the macrophages is not reduced by amiodarone, any effects of the drug on the growth of the bacteria had to be excluded. It had to be secured that not only the cell viability, but also the bacterial replication was unimpaired in order to receive reliable results concerning the antimicrobial activity in the final experiment. Therefore, a series of bacterial growth curves in the presence of various concentrations of amiodarone were prepared.

A growth curve is typically performed to examine the cell division process of a microbial population. The growth of microorganisms in a batch culture is characterized by different stages of growth (Figure 4). The lag phase, where no immediate growth is visible, is followed by the exponential (log) phase which is characterized by maximal growth of the cells, and then resulting in the stationary phase the growth rate stagnates. A lack of nutrients and accumulation of toxins finally leads to a loss of cell viability, becoming apparent in the death phase [41].

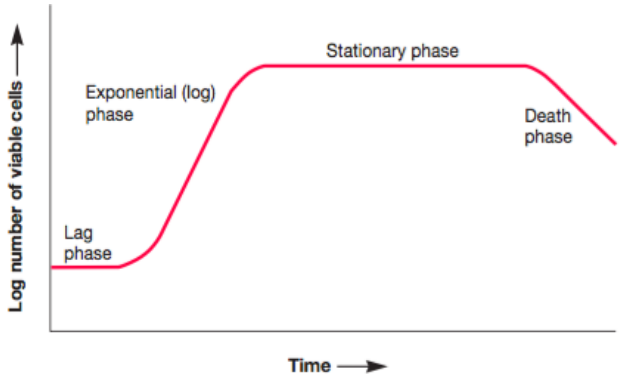


Figure 4 – Microbial Growth
Different stages of growth in a closed system. (Image obtained from Koch, A. [41])

As soon as any possible influences of amiodarone on the bacteria were ruled out, the antimicrobial activity assay was established as described above. The experiment was performed with concentrations lower than the MIC and at a concentration where the growth of the bacteria was not impaired.

1.5 The Aim of the Study

Safety studies are designed to meet certain 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 [4]. Hoffman, E. et al. developed a hierarchical *in-vitro* screening strategy to answer this problem [12]. They focused on the differentiation and the characterization of different mechanisms of drug-induced FM tested on a murine macrophage cell line, J774A.1. The objective of their work was to discover unsuitable drug candidates in early stages of drug development. To differentiate alveolar macrophage responses to inhaled pharmaceuticals, the high throughput *in-vitro* screening strategy was divided into four parts: 1) model set-up, 2) morphometric and vacuolation, 3) cellular status and 4) functional activity.

The aim of this study was to propose a complementary assay to the functional activity, focusing on the response to pathogens after having challenged them with the drug. The assay will be implemented on one cell line, J774A.1, the same which was used by Hoffman, E. et al. and amiodarone, a well-known FM-inducer will be used to establish the assay.

The first part of the study is focused on the evaluation of the toxicity of the tested compound, whereas the second part investigates the phagocytosis function.

2. Materials and Methods

2.1 Materials

100 U penicillin/0,1mg/ml streptomycin	Sigma-Aldrich Company LTD., Gillingham, UK
25 cm^2 cell culture flasks	Greiner bio-one, Frickenhausen, Germany
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma-Aldrich Company LTD., Gillingham, UK
75 cm^2 cell culture flasks	Greiner bio-one, Frickenhausen, Germany
96-well cell culture plates	Greiner bio-one, Frickenhausen, Germany
AH1178 = Staphylococcus aureus strain Newman	Univerity of Iowa Health Care, Iowa City, USA
Amiodarone-Hydrochloride	Sigma-Aldrich Company LTD., Gillingham, UK
ATCC29213 = Staphylococcus aureus reference strain	American Type Culture Collection, USA
Chloramphenicol	Sigma-Aldrich Company LTD., Gillingham, UK
Dimethyl-sulfoxid (DMSO)	Sigma-Aldrich Company LTD., Gillingham, UK
Dulbecco's modified Eagle's medium (DMEM)	Sigma-Aldrich Company LTD., Gillingham, UK
Dulbecco's modified Eagle's medium (DMEM) phenol red free	Life Technologies, Oregon, USA
Fetal bovine serum (FBS)	Sigma-Aldrich Company LTD., Gillingham, UK

Gentamycin	Santa Cruz Biotechnology Inc., Texas, USA
HEPES (1M)	Sigma-Aldrich Company LTD., Gillingham, UK
J774A.1 cell line	American Type Tissue Culture, Rockville, USA
L-glutamine 200mM	Sigma-Aldrich Company LTD., Gillingham, UK
Müller Hinton Agar	Oxoid LTD, Basingstoke, Hampshire, UK
Müller Hinton Broth	Oxoid LTD, Basingstoke, Hampshire, UK
Phosphate buffered saline (PBS)	Sigma-Aldrich Company LTD., Gillingham, UK
Phosphate Buffered Saline System (PBS)	Oxid LTD, Basingstoke, Hampshire, UK
Resazurin	Sigma-Aldrich Company LTD., Gillingham, UK
Sodium Chloride	Thermo Fisher Scientific, Massachusetts, USA
Sodium dodecyl sulfate (SDS)	VWR Life Science, Leighton Buzzard, UK
Sodium pyruvate (100mM)	Sigma-Aldrich Company LTD., Gillingham, UK
Triton® X-100	Sigma-Aldrich Company LTD., Gillingham, UK
Trypan Blue	Sigma-Aldrich Company LTD., Gillingham, UK

2.2 Methods

2.2.1 Cell Culture Conditions

Cell Maintenance

J774A.1, a monocyte-macrophage cell line, originating from a mouse [31], was used to perform the experiments. The cells were grown in 75 cm^2 cell culture flasks at 37°C in 95% air, 5% CO^2. The cells were cultured in High Glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% v/v foetal bovine serum, 1 mM sodium pyruvate and 1 µg/mL penicillin and streptomycin (P/S). The medium was changed every 2 to 3 days. All processes were carried out using appropriate precautions and techniques for cell culture, using a Class II Flow cabinet.

Subculture

When the confluency reached approximately 80-90%, the cells were detached by scrapping using a cell-scraper. To avoid a selecting for subpopulation, 100% of the cells were detached at each passaging procedure. The cells were collected by centrifugation (at 300g for 5 min), the supernatant was discarded and the pellet was resuspended in medium. A sample of 100µL was taken out and homogenized with the equal amount of trypan blue and the cells were counted using a haemocytometer. The quantity of cells was calculated and the required cell concentration for subculturing was prepared.

To prepare a cell suspension for the cell viability assay, the cells were re-suspended in 6 mL of DMEM without phenol red supplemented with 2% FBS, 1 mM sodium pyruvate and 1 µg/mL penicillin and streptomycin (P/S). A homogeneous suspension was achieved by pipetting the suspension up and down at least 5 times. 100 µL of this cell suspension was transferred to an Eppendorf microtube and diluted with 100 µL of trypan blue to stain the cells which were counted on the haemocytometer. Dead cells, which were stained blue, were not counted. The

cell suspension was diluted with DMEM without phenol red to prepare a cell suspension of 0.5 x 10^5 cells/ml.

2.2.2 Test Solution – Amiodarone

The tested compound, amiodarone, was diluted in dimethylsulfoxid (DMSO) at 10 mM and was reconstituted with DMEM for the preparation of all further dilutions of amiodarone used in the experiments. The final concentration ranged up to 31.6 µM in solutions containing a final maximum concentration of 1% v/v DMSO. All experiments were performed at 37°C.

2.2.3 Cell Viability Assay Using MTT

The effect of eight different concentrations of amiodarone (31.6 µM, 25.0 µM, 20.0 µM, 15.0 µM, 10.0 µM and 3.16 µM, 1.0 µM, 0.32 µM)) was measured as a reduction in metabolic activity using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay. A final cell suspension of $0.5x10^7$ cells/ml was required, which equals $1x10^4$ cells/well, when 200 mL are put into each well. The cells were seeded in a 96-well plate, and after having incubated them for 24h, they were exposed to 200 µL of varying concentration of the compound suspended in 2% FBS in DMEM. After the exposure period of 24h, the medium was aspirated and replaced by fresh 2% DMEM. MTT (50 µL; 5 mg/mL) was added to the wells and the plate was incubated for further 4h. Due to the photosensibility of MTT, the solution had to be wrapped with aluminium foil and all steps involving MTT had to be performed in the dark. After 4h, the medium was removed and the resulting intracellular formazan crystals were dissolved over 24h in 100 µL of 10% SDS prepared in 1:1 water:DMF; after which the absorbance from the solubilized formazan was measured spectrophotometrically at the absorbance wavelength of 570 nm and 650 nm.

The relative cell viability (% viability) was calculated using Eq. 1 as follows:

Equation 1:

$$Viability\ (\%) = \frac{A*S}{CM-S} * 100$$

Where A is the absorbance obtained for each concentration of the tested compound, S is the absorbance obtained for the positive control (1% v/v Triton) and CM is the absorbance for untreated cells (negative control, DMEM alone). The latter reading was defined as 100% cell viability.

2.2.4 Preparation of the *S. aureus* Suspension

Staphylococcus aureus strain Newman (AH1178) were revived from -80°C storage in 80% glycerol by streaking onto Mueller Hinton Agar (MHA) and incubation at 37°C overnight. The Müller Hinton Agar which was poured into the petri dishes consisted of dehydrated beef infusion (300 g/L), casein hydrolysate (17.5 g/L), starch (1.5 g/L) and also contained 17 g/L of agar.

For further experiments a liquid culture was required, hence one single colony forming unit (CFU) from the MHA plate was inoculated into 30 ml of Mueller Hinton Broth (MHB) and incubated at 37°C while shaking at 110 rpm overnight. The Mueller Hinton Broth contained dehydrated beef infusion (300 g/L), casein hydrolysate (17.5 g/L) and starch (1.5 g/L).

The resulting suspension was centrifuged and the pellet was resuspended in medium. The necessary concentration of bacteria was prepared by measuring the optic density at a wavelength of 600nm (OD_{600}) with a spectrophotometer and diluting until the final concentration of colony forming units/mL (CFU/mL) was reached.

The correlation between CFU/mL and the OD_{600} was established by preparing different dilutions of the bacteria overnight suspension, plating them on petri dishes, incubating those at 37°C overnight and counting the colonies the next day.

2.2.5 Bacterial Growth Curve

S. aureus strain Newman growth curve experiments were performed with a series of amiodarone concentrations and as a comparison the growth curve was performed without the drug. The growth rates in presence of the different concentrations of amiodarone were compared to the replication rate of the growth curve without the drug.

Quarter-logarithmic dilutions (100.0 µM, 56.2 µM, 31.6 µM, 17.7 µM, 10.0 µM) of amiodarone were prepared in MHB.

Using 96-well plate 190µL of amiodarone solution and 10µL of *S. aureus* overnight culture set to an OD_{600}=1.0 were combined in each well. The plates were incubated at 37°C and samples for CFU/mL identification taken periodically for 12 hours (after 0h, 1h, 2h, 3h, 3.5h, 4h, 4.5h, 5h, 5.5h, 6h, 6.5h, 7h, 7.5h, 8h, 8.5h, 9h, 10h, 11h, 12h). To capture the stationary phase of the growth curve, a last sample was taken after 24h. These samples were then further diluted in saline, plated onto MHA and incubated at 37°C overnight. Colonies grown on the agar were counted manually and the colony forming units per mL (CFU/mL) calculated by taking the dilution factor into account. The growth curves were finally plotted as log of viable bacteria (CFU/mL) over growth time.

2.2.6 Minimal Inhibitory Concentration (MIC)

The minimal inhibitory concentration of amiodarone was determined on *S. aureus strain Newman* in order to exclude any effects of the inducer on the bacteria. A quarter logarithmic dilution series of 10 different concentrations, from 52.6 µM to 0,56 µM, of amiodarone in DMEM (without P/S) were freshly prepared and 50 µM pipetted into a 96-well plate. 50 µL of *S. aureus* overnight culture with a concentration of $1x10^6$ CFU/mL was added, resulting in a final bacteria concentration of $5x10^5$ bacteria/well. The plate was incubated at 37°C without shaking overnight, after which resazurin (0.2 g/L) was added to each well. After a further

incubation time of two hours, the outcome was primarily assessed visually as after two hours, an occurring colour change from pink to blue indicated a decline in metabolic activity of the bacteria and marked the value of the MIC read by eye. The plates were read at a wavelength of 600 nm. The received value was subtracted from the blanc value of 570 nm to get rid of the background noise, and the MIC was calculated.

Since no MIC guidelines and cut-off values for amiodarone were available, the *S. aureus reference strain ATCC29213* was tested alongside against chloramphenicol and the results interpreted according to the BSAC guidelines [42]. This additional assay was performed to ensure that the ATCC29213 and the test organism *S. aureus strain Newman* gave trustworthy results also against amiodarone.

2.2.7 Antimicrobial Activity of Macrophages

The J774A.1 cells were seeded at a density of $1x10^5$ cells/well into a 24-well plate and incubated for 24 hours at 37°C, 5% CO_2. After 24h of incubation, the cells were incubated with four different concentrations of amiodarone in DMEM. Starting from 31.6 µM, quarter-logarithmic dilutions were used (31.6 µM, 17.8 µM, 10.0 µM and 5.6 µM). The plate was incubated for 24h at 37°C and 5%CO_2 overnight.

After the incubation period, the medium was removed from the wells and the cells washed with phosphate buffered saline (PBS) for three times. The macrophages were challenged with bacteria at a multiplicity of infection (MOI) of 0.01. Overnight cultures of *S. aureus strain Newman* were centrifuged at 5000 xg for 30 minutes, the supernatant discarded and the remaining pellet re-suspended DMEM (without P/S). The optical density of this suspension was identified at the wavelength of 600 nm and the bacterial working suspension of $1x10^3$ CFU/mL prepared. One mL of this suspension was added to each well. By centrifuging the plates at 1300 xg for ten minutes, interactions between the bacteria and the cells were encouraged. The plates were then incubated for 2 hours at 37°C and washed with PBS for three times afterwards. Each

well was inoculated with 1 mL of 50 µg/mL gentamicin and incubated for 10 minutes with the aim of killing all the bacteria that were not phagocytised by the macrophages during the incubation period. The gentamycin was removed by washing with PBS for three times and in order to lyse the macrophages, 1 mL of sterile, cold H_2O was added into each well and the plates were left at room temperature for 15 minutes. The lysed macrophages with floating bacteria were fully scraped off the surface of the wells and the content of the well transferred to a 1 mL eppendorf tube which was then centrifuged for 5 minutes. The supernatant was discarded, the pellet resuspended in saline and the suspensions plated on MHA. To identify the real CFU/mL of the bacterial solution used in this phagocytosis assay, the bacterial solution was diluted in PBS, plated on MHA and the colonies enumerated the next day.

2.2.8 Data and Statistical Analysis

The cell viability was used as a marker to assess the toxicity of the inducer amiodarone. The cytotoxicity of the compounds was visible as a loss in cell viability and was expressed dependent on the concentrations of the compound. As marker for the antimicrobial activity the phagocytic uptake of bacteria was used. The numbers of the up taken bacteria was compared between the different concentrations of the inducer. All experiments were carried out in triplets and a blank was included as a negative control. The data analysis was performed using GraphPad Prism version 7.

3. Results

3.1 Cell Viability Assay

To determine experimental conditions and to assess the biocompatibility for further experiments, the cell viability assay was evaluated using different concentrations of amiodarone. J774A.1 cells were cultured for 24h, then exposed for 24h to varying concentrations of amiodarone (0.32 – 31.6 µM)

Amiodarone concentrations below 3.2 µM did not affect the cell viability, the cell health remained at around 100%. A decrease in cell viability was observed in amiodarone concentrations above 10.0 µM. After the exposure to 10.0 µM amiodarone, the cell viability decreased by 20.87%. A decrease in cell viability by 85.95% was observed after incubation with 15.0 µM amiodarone compared to the control (untreated cells). Only 1% of the cells remained alive, when 20.0 µM, 25.0 µM or 31.6 µM of amiodarone were tested. Amiodarone showed a dose dependent toxicity profile (Figure 5). A rapid decline in cell health for concentrations >3.2 µM can be observed. From those results an EC_{50} of 11.87 µM was calculated.

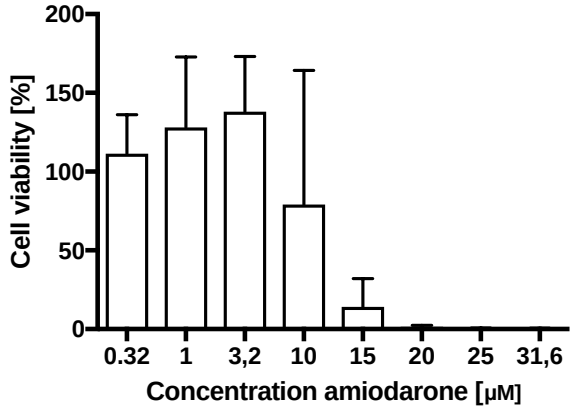


Figure 5 – MTT-Experiment
The cell viability plotted against different logarithmic concentrations of amiodarone.

3.2 Exploratory Studies for the Antimicrobial Activity Experiment

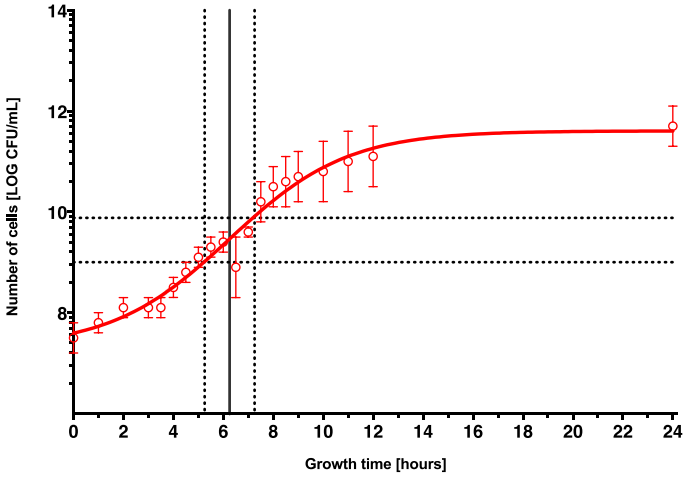
3.2.1 Bacterial Growth Curve

Before proceeding to the main experiment of testing the effect of amiodarone on the antimicrobial activity of the macrophages, a potential effect of the inducer on the *S. aureus strain Newman* growth behaviour had to be excluded. A series of bacterial growth curves were performed without and with various concentrations of amiodarone to make an assessment about the influence of amiodarone on the growth of *S. aureus strain Newman*.

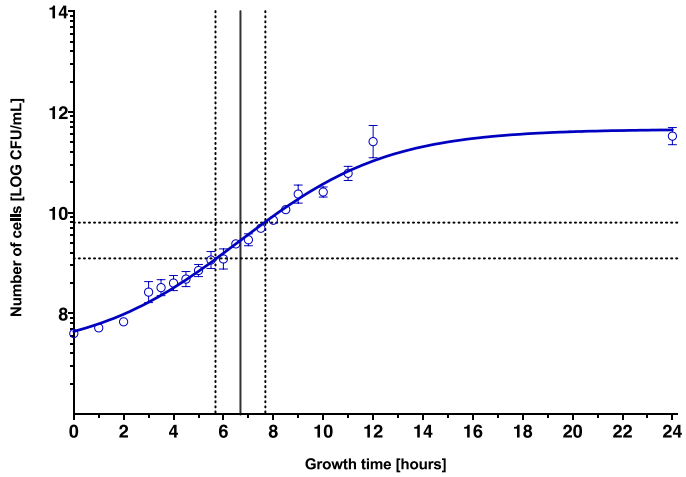
The data (Figure 6) shows, with minor non-significant deviations, that at lower concentrations of amiodarone (10.0 µM, 17.7 µM and 31.6 µM) the bacterial growth after 24h was in the same range as when no amiodarone was present. With the concentration of 56.2 µM amiodarone the maximum bacterial growth clearly ended at a lower value compared to lower concentrations of amiodarone. The growth curve with a concentration of 100 µM amiodarone showed a significantly greater decline in the maximal growth of the bacteria after 24 hours.

The timepoint at which the bacteria entered the log phase was also dependent on the concentration. While amiodarone-concentrations <17.7 µM (Figure 6A, 6B, 6C) entered the log phase between 6.3 to 7.4 hours after the start of the experiment, it took between 7.7 to 8.2 hours for amiodarone concentrations >32.6 µM (Figure 6D, 6E, 6F) to enter the exponential growth phase.

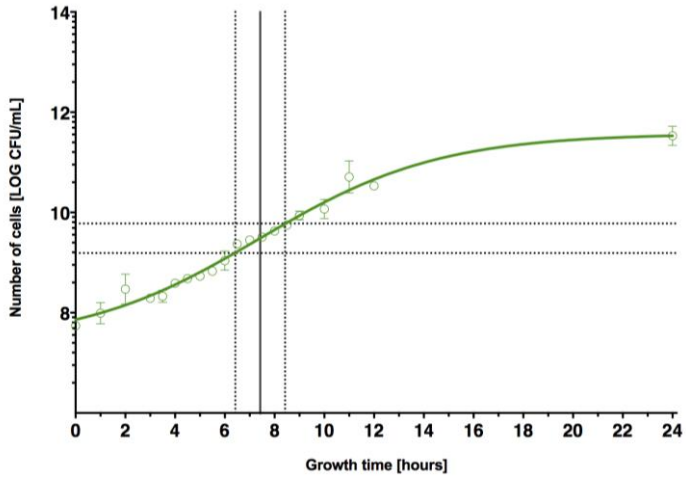
A)


Number of cells [LOG CFU/mL]
Growth time [hours]

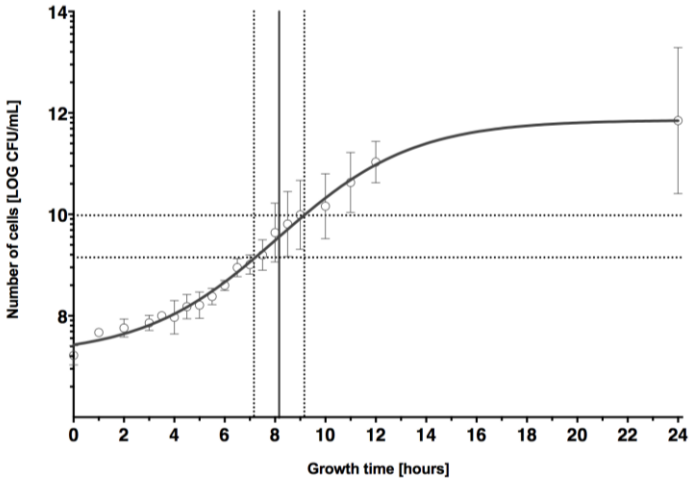
B)


Number of cells [LOG CFU/mL]
Growth time [hours]

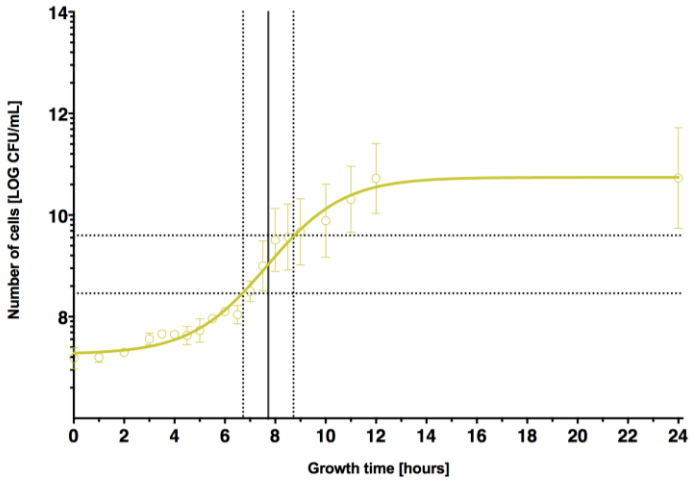
C)


Number of cells [LOG CFU/mL]
Growth time [hours]

D)



E)



F)

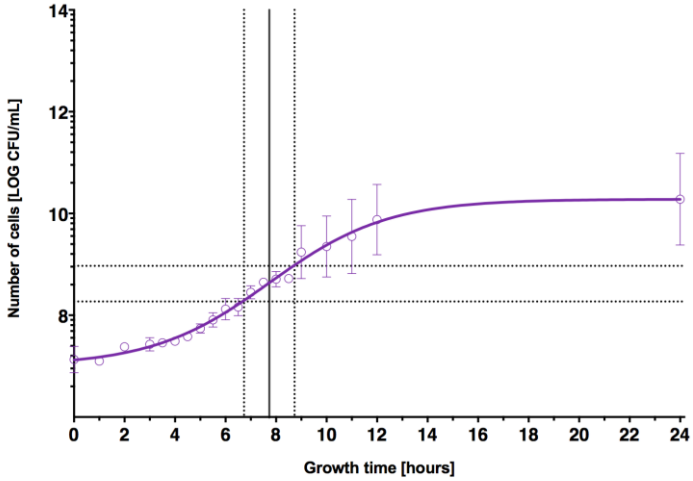


Figure 6 – *S. aureus* Growth Curves with Amiodarone
The graphs show the growth behaviour of *S. aureus strain Newman* when treated with the following concentrations of amiodarone: A) 0.0 µM, B) 10.0 µM, C) 17.7 µM, D) 31.6 µM, E) 56.2 µM, F) 100.0 µM. The timepoint of the beginning of the log phase is marked in the figures.

When the *S. aureus* growth behaviour with different concentrations of the inducer is compared (Figure 7), it becomes evident that amiodarone concentrations <31.6 µM have shown similar growth behaviour to the negative control, hence bacterial growth was not influenced by the inducer at that particular concentrations. Concentrations >56.2µM lead to a significant decline in CFU/mL after 24h and enter the log phase at a later timepoint than concentrations below, suggesting that amiodarone has an effect on the *S. aureus* growth when higher concentrations are used. Consequently, for concentrations >56.2µM the data suggests a dose-dependent effect of amiodarone on the growth behaviour of *S. aureus*.

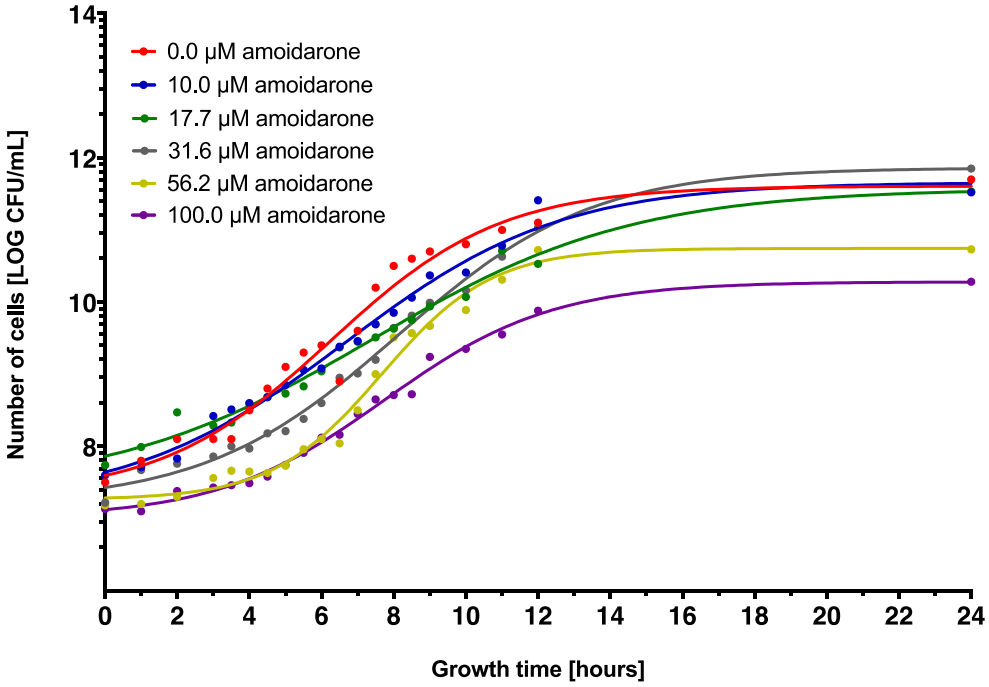


Figure 7 – Comparison of Growth Curves
Direct comparison of the *S. aureus* growth curves with different concentrations of amiodarone.

When the growth rates and the doubling times of the growth curves with different amiodarone concentrations are compared, no correlation becomes apparent, suggesting the conclusion that amiodarone does not influence the growth rate (k) and the doubling time (g) but that the deviations exist because of the natural variety in biological systems (Table 1).

For the analysis of the data, besides the comparison of the different growth curves, the exponential growth phase was characterized by the growth rate (k) and the mean generation (doubling) time (g). The calculations were based on manual readings. The growth rates (k) were similar for all concentrations of amiodarone and lay within a range from 1.0 to 1.9 generations/hours, for 17.0 µM of amiodarone having the lowest growth rate of k = 1.0 generations/hrs) and 56.2 µM of amiodarone having the highest of k = 1.9 generations/hrs. All growth curves showed a doubling time between 31.7 to 61.2 minutes per generation, which equals 0.5 to 1.0 hours per generation. The findings have shown that the growth curves of *S. aureus strain Newman* with 17.0 µM amiodarone and with 52.6 µM amiodarone mark the extreme values with 17.0 µM having the longest doubling time of 61.2 min/generation and 52.6 µM having the shortest of 31.7 min/generation.

Table 1 – Growth Rate and Doubling Time of *S. aureus* Growth Curve

Amiodarone conc.	growth rate (k) gen/h	doubling time (g) min/gen
0.0µM	1.5	41.0
10.0µM	1.2	50.9
17.7µM	1.0	61.2
31.6µM	1.4	43.5
56.2µM	1.9	31.7
100.0µM	1.2	51.6

All provided evidence leads to the conclusion that amiodarone concentrations >32.6 µM had an effect on the start of the log phase and on the CFU/mL after 24h.

In order not to influence the further experiments, 36.2 µM was set as the highest concentration used for performing the antimicrobial activity assay. Moreover, the timepoint of the addition of the macrophages in the final experiment takes place after 2h, when the *S. aureus strain Newman* have not yet entered the log phase (Figure 8). As the bacteria still remain in the lag phase, there is no difference in growth behaviour at that timepoint. Therefore, it can be stated with confidence that amiodarone has no influence on the bacteria in the antimicrobial activity experiment.

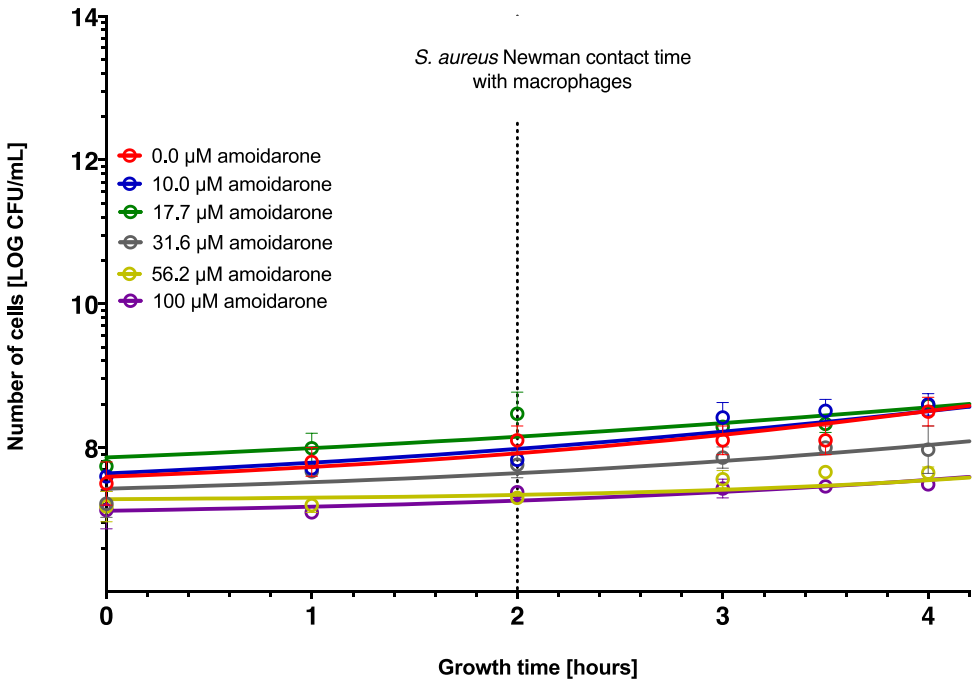


Figure 8 – Lag-Phase of the Growth Curve
Growth curves at the timepoint when the cells are added. The figure shows that the bacterial growth is not in the exponential phase during the 2h incubation period at 37°C of the cell culture experiment.

3.2.2 Minimal Inhibitory Concentration (MIC)

The minimal inhibitory concentration (MIC) of amiodarone was determined prior to the actual experiment, to establish the amiodarone concentrations that could later be used without affecting the *S. aureus strain Newman* in the antimicrobial activity experiment.

The MIC of amiodarone on *S. aureus* was identified with >56,2 µM, which was the highest concentration of amiodarone which was tested in this MIC experiment (Table 2). The plate reading and the calculation lead to the same result as the visual assessment.

Table 2 - Plate Layout of the MIC
Minimal inhibitory concentration (MIC) of amiodarone on S. aureus strain Newman.
The results are shown as 3 independent repeats.

	MIC	MIC	MIC	mean
Repeat 1	>56.2	>56.2	>56.2	>56.2
Repeat 2	>56.2	>56.2	>56.2	
Repeat 3	>56.2	>56.2	>56.2	

Besides amiodarone, chloramphenicol was tested on the *reference strain ATCC29213* as an internal control. The procedure was the same as with amiodarone. To receive reliable results, the MIC of chloramphenicol had to lie in a certain range specified by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). For chloramphenicol the MIC by eye was 13 mg/L and the calculated MIC was 7,5 mg/L. The received values were compared to the EUCAST table [43]. Both results were within the EUCAST range meaning that the MIC values we received for amiodarone are reliable (Figure 9).

The outcomes showed no change of colour for the cells that were treated with amiodarone, which means that the macrophages are alive and that the MIC of amiodarone is >56.2 µM.

As the highest concentration used for the testing of the antimicrobial activity was 31.6 µM and therefore lower than the MIC, the potential risk of an interaction between the bacterial strain

and amiodarone was excluded. To summarize, with the performance of the MIC assay any possible effects of amiodarone on the cell viability of *S. aureus* was ruled out for the concentrations we used in the experiment.

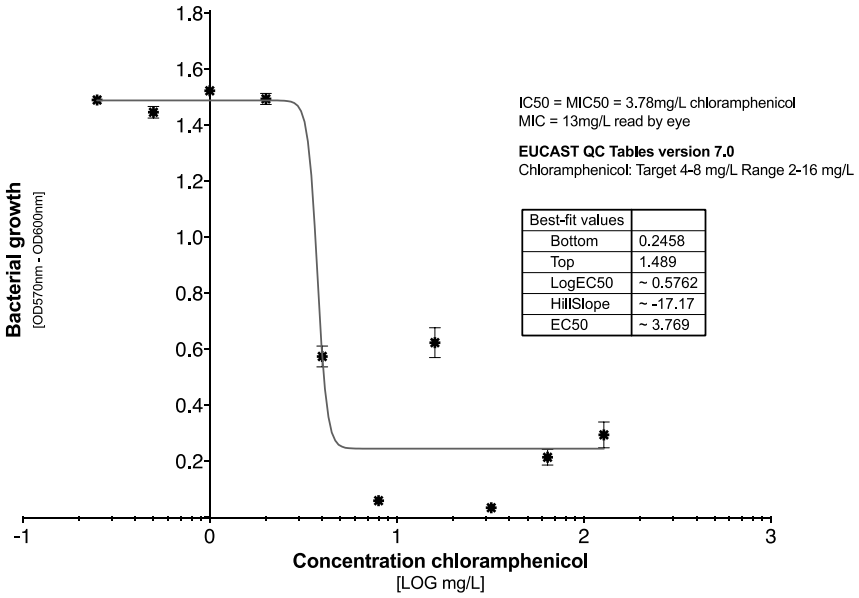


Figure 9 – Chloramphenicol Dose vs. *S. aureus ATCC291213* Response
Minimal inhibitory concentration of chloramphenicol on the *S. aureus reference strain ATCC291213* as an internal control.

3.3 Antimicrobial Activity of Macrophages

Four different quarter-logarithmic dilutions of amiodarone (31.6 µM, 17.7 µM, 10.0 µM, 5.6 µM) were tested on the cells. The concentrations were chosen according to the outcomes of the pre-experiments described above. The aim was to make sure that the *S. aureus strain Newman* were not influenced by the inducer amiodarone and that the results of the main experiment were due to an impaired function of the macrophages.

In this experiment, the antimicrobial activity of the untreated macrophages was compared to the antimicrobial effect of those that were challenged with amiodarone. The percentage of

phagocytised *S. aureus strain Newman* by amiodarone-treated macrophages was measured in correlation to the bacterial uptake of the untreated cells.

The final bacterial suspension with a concentration of $1x10^3$ CFU/mL (measured via the optical density) was added into each well.

The number of phagocytised bacteria by the healthy macrophages (0 µM amiodarone) was determined. Untreated macrophages phagocytised on average 605.83 CFU/ml (=20.75%) of the *S. aureus strain Newman* (with an average concentration of 2920 CFU/ml) which were present in the wells. This means that 20.75% of the bacteria were taken up when the macrophage function was unimpaired. With increasing concentrations of the inducer amiodarone, the number of phagocytised *S. aureus strain Newman* declined significantly, as can be seen in Figure 10.

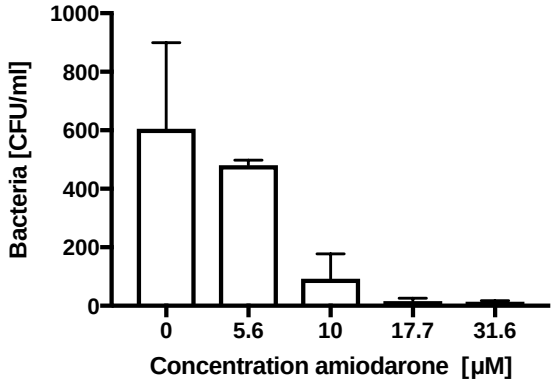


Figure 10 – Bacterial Uptake of the Macrophages
Count of bacteria which were phagocytised by macrophages when treated with different concentrations of amiodarone.

In Figure 11 the number of phagocytised *S. aureus strain Newman* by the untreated macrophages is the marker for the antimicrobial activity. As the physiological function of the untreated cells is unimpaired when no amiodarone is present, the antimicrobial activity for the untreated macrophages is at its maximum. The bacterial uptake (=marker for the antimicrobial

activity) of the untreated macrophages marked the full physiological function and was therefore set to 100%. For all tested concentrations of amiodarone, a loss in bacterial uptake becomes evident, meaning that the antimicrobial activity of the macrophages is impaired. A loss of antimicrobial activity becomes evident by plotting the percentages of the bacterial uptake relative to the phagocytic uptake of unimpaired macrophages (=100%). All tested concentrations of amiodarone result in a significant decrease in antimicrobial activity and concentrations >10.0 µM reduce to physiological activity by greater than 50%. The data clearly suggests that the antimicrobial activity decreases with ascending concentrations of amiodarone.

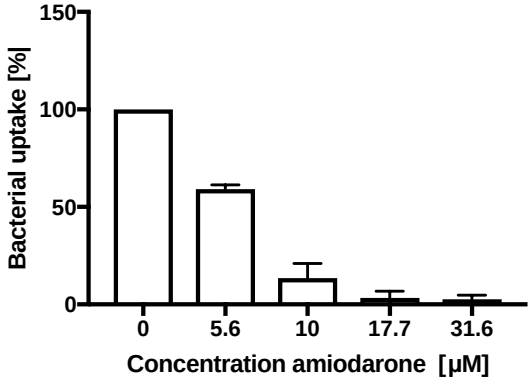


Figure 11 – Antimicrobial Activity of the Macrophages
Percentage by which the antimicrobial activity of the macrophages declined dependent on different concentrations of amiodarone. The bacterial uptake in percent served as a marker for the antimicrobial activity.

The toxicity of amiodarone becomes obvious by a significant functional decrease of the phagocytes with all tested concentrations of the drug (Figure 12). Already a concentration of 5.6 µM, which was the lowest tested concentration of the inducer, shows a significant decrease in function of 20.50%. With an amiodarone concentration of 10.0 µM amounting to a loss of function of 84.59%, 17.7 µM amounting to 97.35% and a drug concentration of 31.6 µM amounting to a functional decrease of 97.87%.

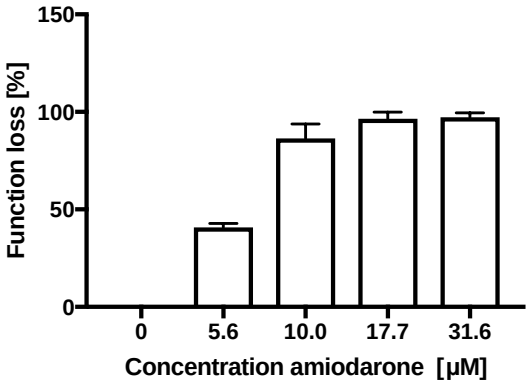


Figure 12 –Function Loss of the Macrophages
Antimicrobial activity of amiodarone treated macrophages, plotted against the function of the untreated cells.

4. Discussion

4.1 Experiment Setup

The experimental design was based on prior research of Hoffman, E. et al. [12], where an *in-vitro* screening strategy for the differentiation of foamy macrophages was established. In that publication, three inducers of foamy macrophages (amiodarone, staurosporine, PVAcNP) were tested concerning a variety of parameters, including cytotoxicity, cell morphology, gene expression and phagocytosis, while in our study the focus lay solely on amiodarone and its effect on the cell viability as well as the antibacterial capacity of the cells.

The test substance, amiodarone, is a widely prescribed antiarrhythmic drug and a known inducer of foamy macrophages [15], [26]. Just like chloroquine, amiodarone is a cationic amphiphilic drug (CADs) and leads to phospholipidosis due to the complexion of phospholipids in the cytoplasm of the macrophages. Consequently, the digestion and metabolism of the macrophages is impaired and the phospholipids accumulate inside of them, which gives an explanation for the lung toxicity of amiodarone [7], [12]. Adverse pulmonary effects can be observed in 5% of the patients [24] and usually occur as subacute or acute pneumonitis, pleural disease or migratory infiltrates [25].

The cell line which was used for the study was J774.A1, which is a murine macrophage cell line derived from a mouse reticulum cell sarcoma. The cell line was mainly used because of its quick growth and easy handling [44]. Moreover, J774.A1 is a widely used cell line in scientific research and therefore allows the comparison of the results of different studies as there is a lot of data already available [45]–[47]. The primary aim was to work with a well-established cell line which allowed to optimise an assay that can later be transformed and tested for other cell lines and primary human macrophages, which better comply with the physiological situation in humans.

4.2 Cell Viability Assay

Our findings clearly suggest a dose-dependent toxicity of amiodarone on the macrophages. These findings are in accordance with the study of Hoffman, E et al. where also a dose-dependent loss of metabolic activity was observed. However, in their paper the toxicity was observed for amiodarone-concentrations >5.0 µM while we did not test that specific concentration and found a decrease in cell viability for concentrations > 3.2 µM. In our experiment, for concentrations of 10.0 µM and above a rapid decline in cell viability becomes apparent, with 20.0 µM already leading to a loss of cell viability of more than 99%. Those findings contradict earlier results, where a concentration of 20.0 µM amiodarone only lead to a decline of less than 50%.

Our findings are in accordance with the thesis of Tillman, N. [48] who based her experimental work on the research of Hoffman, E. et al as well. In her MTT-experiment a dose-dependent toxicity of amiodarone for concentrations of 10.0 µM and above was found and the EC_{50} for amiodarone was 10.3 µM, which is in accordance with our outcome and an EC_{50} of 11.9 µM. Minor differences in the results can be attributed to the variation of biological systems.

4.3 Antimicrobial Activity

After any possible effects of amiodarone on the cells have been ruled out prior to the experiment, it can be stated with confidence that all the results only come from the cells themselves and are not influenced by any interactions between the bacteria and amiodarone.

The choice for the range of concentration was based on the outcome of the previous test. As the cell viability assay showed toxicity for amiodarone-concentrations of 10.0 µM and above, 10.0 µM marked the midpoint of the quarter-logarithmic dilution range in this experiment. Two concentrations that were higher, thus toxic to the cells, as well as lower concentrations, which did not influence the cell viability in the MTT-experiment, were included.

To have a margin of security, the highest concentration tested in this experiment was set one quarter-logarithmic step lower than the highest concentration tested in the MIC-experiment, which was 56.2 µM. Furthermore, the growth curves showed lower numbers of CFU/mL after 24h for concentrations of 56.2 µM and above, which was another reason why 31.6 µM was the highest concentration used in this experiment. That choice allowed absolute certainty about the exclusion of any interference between amiodarone and the bacteria.

In contrast to the phagocytosis experiment recently performed by Hoffman, E. et al. [12], where the phagocytic activity was tested using polystyrene beads, in this study the antimicrobial activity of the alveolar macrophages was evaluated with *S. aureus*. The usage of living bacteria allowed to make a statement not only about the phagocytic but also about the antimicrobial activity. Bacterial challenge of macrophages is a commonly used method to evaluate the physiological function [17], [18] and is often carried out using *S. aureus* [19], [20]. *S. aureus* is a commensal bacterium and is physiologically present on the skin and mucosal tissue. When the natural barrier is overcome, the bacteria can spread and induce infections mainly of the skin or the soft tissue. With an increasing number of strains acquiring resistance against drugs, nowadays methicillin resistant S. aureus (MRSA) amount to over 50% of S. aureus. [22] This bacteria was chosen in our experiment as it increasingly becomes associated with community-acquired infections and can induce severe respiratory disease. Moreover, the bacteria have the ability to evade the phagocytic uptake of the immune cells and their resistance to antibiotics is on the rise [22], [35].

The ratio between the infectious agent and the target, in this case the macrophage cell, is described as the multiplicity of infection (MOI). A commonly used ratio between the bacteria and the cells, which was also used in this experiment, is the MOI of 0.01 [17], [20].

As mentioned above, a growth curve-experiment was performed before, to exclude any possible interactions with the bacteria. The same approach was chosen in the study of Braydich-Stolle, L.K. et al. [20].

4.4 Cell Viability vs. Antimicrobial Activity

The intention of the experiment was to establish a correlation between the cell viability and the antimicrobial activity. Therefore, the results of the two experiments were compared. After the MTT-experiment was performed, the phagocytic capacity of the macrophages was on the one hand tested with concentrations of amiodarone that were already toxic in the MTT, and on the other hand with lower concentrations where no toxicity became apparent. The outcome should allow a statement about the antimicrobial activity of the cells and an assessment whether the cellular function is already impaired with amiodarone concentrations which did not affect the cell viability in the previous test.

It was found that with increasing amiodarone concentrations, both, the macrophage viability and the macrophage functionality decreases significantly. Interestingly, the impairment of the physiological function already becomes apparent at concentrations that have no toxic effect on the cells. While the EC_{50} for the cell viability is between 10.0-15.0 µM amiodarone, the EC_{50} for the phagocytic function of the macrophages is significantly lower, between 5.6-10.0 µM. Those findings suggest that amiodarone can promote the pathogenicity of *S. aureus* even before a detectable loss in cell viability occurs.

5. Conclusion

Up to this point, the distinction of foamy macrophages and the evaluation of new inhaled medicine still poses a major challenge to the science industry [4], [7], [12], which is why the aim of our experiment was to enable the *in-vitro* assessment of the influences of new inhaled drugs on alveolar macrophages. In order to make a qualified statement about the safety and the toxicity of a new inhaled medicine, the establishment of consistent methods throughout the science industry is crucial. Ideally, the used methods should be cheap, simple and easy to handle and, above all, universally implementable.

Besides, uniform biomarkers are needed and pharmacodynamic endpoints should be established and used whenever it is possible. The pulmonary function is a good example of a pharmacological endpoint for the evaluation of safety and could also be used in early stages [4].

In this experiment, amiodarone, a substance with known toxicity on the cells and an inducer of foamy macrophages was used [25]. We aimed to establish a method to make a loss in macrophage function detectable and correlate the loss of phagocytic activity with the loss of cell viability.

The method could be used at an early stage of drug development to rule out candidates which induce to pathological responses in the lungs and thus save cost and time that could be invested in promising substances.

However, before the method is applicable in drug development, much more research needs to be carried out. As all the experiments were only performed on one cell line (J774A.1) and with one compound (amiodarone), in the future, this assay should be applied to different cell lines and more drugs should be tested. Preferably, the experiments should be performed on human cell lines, like THP-1, before progressing to applying the assay to primary cells, which closer resemble the *in-vivo* environment in the human body.

Besides the comparison of the cell viability and the antimicrobial activity, there is yet another unclear correlation between the visual appearance and the function of the macrophages. Does a change in morphology automatically imply a loss of functionality or is the physiological function of the cells even already impaired before the changes in appearance occur [12]?

A potent imaging technique for the examination of the physiology of foamy macrophages is the transmission electron micrograph (TEM). The downside is that it is not applicable for usage on a big scale due to the high expenses and because it is tremendously time consuming. In a study by Hoffman, E. et al. [12], very similar results to TEM could be achieved by tissue staining, followed by the analyzation of cell morphometry, vacuolation and lipid accumulation with the IncellAnalyzer. In the future, this method could be used in addition to the assay we established. Furthermore, a data set of normal physiological responses as well as of pathological responses should be established. It is crucial to receive as much information as possible as early as possible in order to make a statement about the toxicity and the safety of a new drug and to be able to save resources, lower expenses and cut down on animal studies [4].

ZUSAMMENFASSUNG

Die Inhalation von Arzneistoffen bietet eine gute Möglichkeit zur gezielten Therapie von respiratorischen Erkrankungen. Da bei der pulmonalen Applikation der First-Pass-Effekt vermindert ist, eignet sich diese besonders für die lokale Verabreichung von Arzneistoffen, die oral eine geringe Bioverfügbarkeit aufweisen. Außerdem kann, um den selben Effekt zu erzielen, die Dosierung im Vergleich zur peroralen Applikation verringert werden, wodurch Nebenwirkungen beträchtlich vermindert werden können. Die Lunge bietet aufgrund ihrer großen spezifischen Oberfläche, der hohen Perfusionsrate und des dünnen Epithels auch ideale Voraussetzungen um systemische Blutspiegel zu erhalten. Bis zum heutigen Zeitpunkt ist die Anwendung von Inhalanda jedoch fast ausschließlich auf die lokale Anwendung von β_2-Agonisten, Anticholinergikern und Glukokorticoiden zur Therapie von respiratorischen Erkrankungen beschränkt. Grund dafür ist vor allem die Einschätzung der Arzneimittelsicherheit bei der Entwicklung von neuen Wirkstoffen. Trotz der verwendeten *in-vitro* Modelle ist es nicht möglich genaue Vorhersagen über die physiologischen *in-vivo* Wirkungen zu treffen, da die Situation in den Lungen viel zu komplex ist um diese in Zellkulturen zu simulieren. Neben physiologischen Barrieren (Zellen, Mukus) und proteolytischen Enzymen, sind alveolare Makrophagen maßgeblich für den Schutz der Lunge verantwortlich. Übersteigt die Anzahl der inhalierten Partikel die Kapazität dieser pulmonalen Fesszellen, kann dies im Auftreten von „foamy macrophages“ (FM) resultieren. Die Beurteilung der entstandenen perforierten Makrophagen stellt eine große Herausforderung dar, kann deren Entstehung doch sowohl eine unbedenkliche physiologische Anpassung als auch eine toxische Nebenwirkung bedeuten.

Das Ziel dieser Studie ist daher die Etablierung eines Tests um die Sicherheit neuer inhalativer Arzneistoffe einschätzen zu können. Es soll eine Korrelation zwischen der Zytotoxizität einer Substanz, welche zur Entstehung von FM führt, und deren Auswirkung auf die physiologische

Phagozytosefunktion etabliert werden. Getested wurde die FM-induzierende Substanz Amiodarone, ein bekanntes Antiarrhythmikum, mit dem kommensalen Bakterium *Staphylococcus aureus (S. aureus)* auf der murinen Zelllinie J774A.1.

Methoden: Die Zellgesundheit wurde mit unterschiedlichen Konzentrationen von Amiodaron auf der Zelllinie J774A.1 untersucht. Zuvor wurden unterschiedliche Konzentrationen von Amiodaron auf *S. aureus* getested, um einen Effekt der Substanz auf die Bakterien auszuschließen. Dazu wurde die MIC (minimal inhibitory concentration) und der Effekt von Amiodaron auf das bakterielle Wachstum untersucht. Konzentrationen ohne Effekt auf *S. aureus* wurden auf eine Beeinträchtigung der phagozytären Funktion untersucht.

Ergebnis: Amiodaron zeigt eine dosisabhängige Toxizität auf die Zellen und der EC_{50} wurde mit 11,87 µM errechnet. Die Anlaufphase (lag -Phase) der Wachstumskurve blieb unbeeinflusst und die MIC war größer als die höchste getestete Konzentration von 56,2 µM. Die Phagozytosefunktion verminderte sich mit steigenden Konzentrationen von Amiodaron.

Conclusio: Die erhaltenen Resultate stimmen mit zuvor erhaltenen Ergebnissen überein.

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