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Interaction of CPZEN-45, a novel anti-tubercular drug candidate, with immune cells *in vitro*

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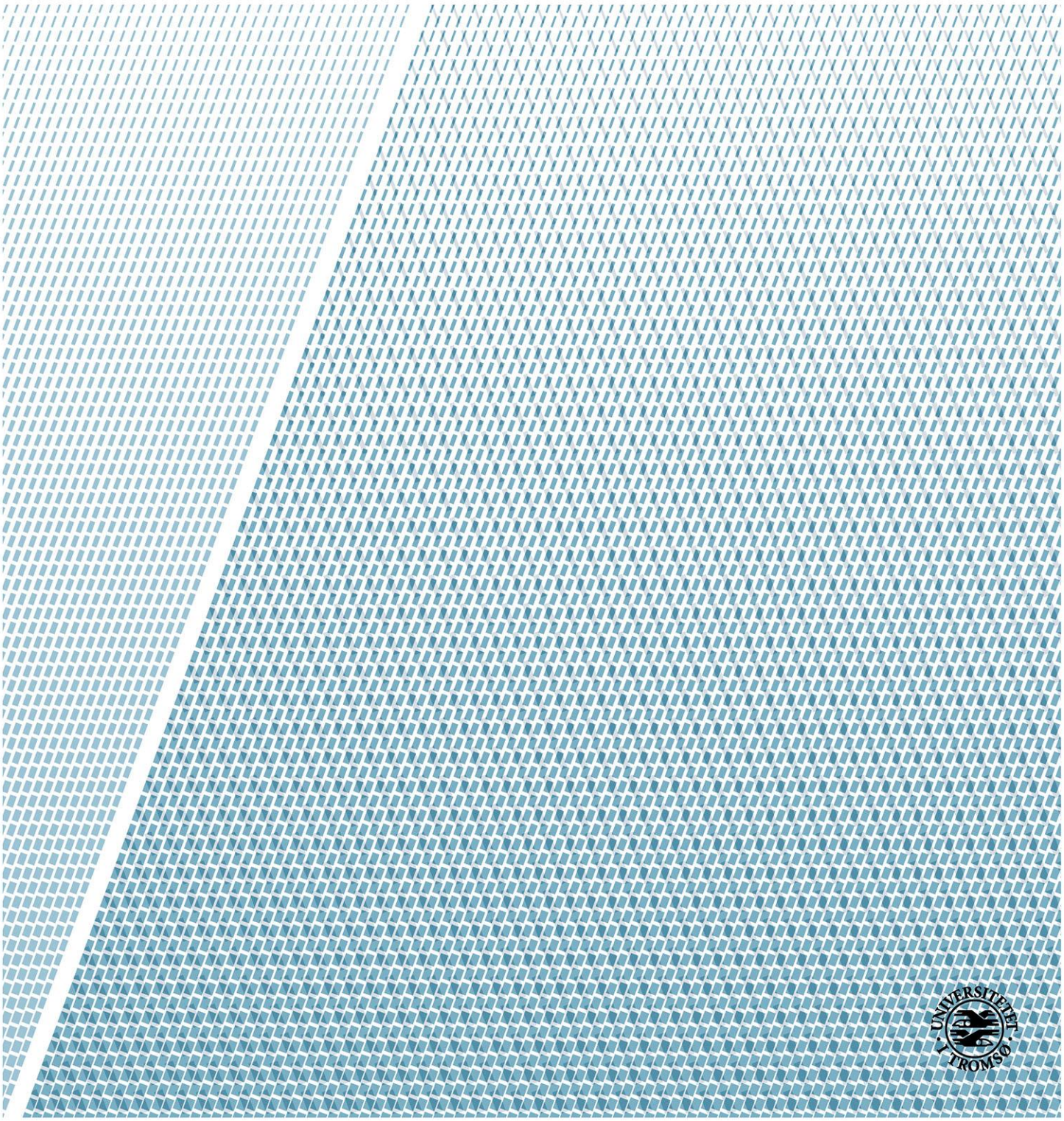
Awfa Al-shamkawy

Master thesis in Pharmacy *May 2016*



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Dublin, May 2016
Awfa Al-shamkawy

Abstract

Tuberculosis (TB) is an infectious disease that represents a serious global threat, with estimations suggesting that one third of the world's population is infected. Treating TB today requires long treatment duration and medicines must be administered orally in large doses, which leads to non-compliant patients. CPZEN-45 is a novel anti-TB drug candidate in pre-clinical development, in which pulmonary drug delivery has been proposed. As a part of the ongoing characterisation of CPZEN-45, the drug was tested for its ability to reduce the inflammation marker release *in vitro* on two types of immune cell lines; human monocytic cells (THP-1) and human mast cells (HMC-1). THP-1 cells were differentiated to a macrophage-like phenotypes and stimulated with lipopolysaccharide, and treated with CPZEN-45. A sandwich ELISA was performed to measure the tumour necrosis factor-α (TNF- α) release from THP-1 cells. HMC-1 cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, to initiate degranulation, the release of the enzyme β-hexosaminidase in particular. The cells were then compared with controls treated with only CPZEN-45, regarding their β-hexosaminidase release. Main findings showed that CPZEN-45 had an inhibitory effect on the TNF-α expression levels. THP-1 cells incubated with CPZEN-45 for 3 and 6 hours showed a decreasing trend in the TNF-α release profile with increasing CPZEN-45 concentrations. Interestingly, the findings also suggest that CPZEN-45 had an improved inhibitory effect on the TNF-α release, as the TNF-α expression level increased in the THP-1 cells. The degranulation assay showed a little increase in the release of β-hexosaminidase in cells treated with only CPZEN-45, compared to cells stimulated with PMA/ionomycin. CPZEN-45 is a promising anti-TB drug candidate, which seems to inhibit the inflammation in THP-1, while initiating a modest degranulation in HMC-1 cells. However, more repetitions are needed to ascertain the findings. Furthermore, it would be interesting to test the drug's potential in other pulmonary cell lines, i.e. lung epithelial cells and other inflammatory cytokines, i.e. histamine.

List of abbreviations

Abbreviation	Explanation
ABTS	2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt
CNRS	The national centre for scientific research
CPZEN-45	Caprezene 4-butylanilide
DOTS	Direct observed treatment short course
ELISA	Enzyme linked immunosorbent assay
EMA	European medicine agency
EMB	Ethambutol
FBS	Foetal bovine serum
FcεRI	High affinity immunoglobulin E receptor
HMC-1	Human mast cell line
IgE	Immunoglobulin E
IL	Interleukin
IMDM	Iscove's modiefied Dulbecco's medium
INH	Isoniazid
KRB	Krebs ringer buffer
LPS	Lipopolysaccharide
MCRF	Microbial chemistry research foundation
MDR-TB	Multidrug resistant tuberculosis
Mtb	Mycobacterium Tuberculosis
PAS	Paraaminosalicylic acid
PBS	Phosphate buffer saline
Penstrep	Penicillin/Streptomycin solution
PMA	Phorbol 12-myristate
PZA	Pyrazinamide
RIF	Rifampicin
TB	Tuberculosis
THP-1	Human monocytic cell line
TNF-α	Tumor necrosis factor α
WHO	World health organisation
XDR-MT	Extensively drug resistant tuberculosis

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1 Introduction

Tuberculosis (TB) is an infectious disease that represents a serious global threat, with estimations suggesting that one third of the world's population is infected (1, 2). Treatment of TB today requires long treatment duration, resistance development and large doses administered orally, leading to non-compliant patients (3). New or improved treatment strategies are thus urgently needed. In this thesis CPZEN-45, a novel anti-TB drug candidate in pre-clinical phase for pulmonary delivery, is studied with respect to its effect on release of inflammatory markers in immune cells. The following sections give an overview of the human lung, immune cells and their mechanisms of immunological responses to infection. Further, TB and current TB treatment are described, before moving on to CPZEN-45 and the current available knowledge of the drug.

1.1 The lung

The lung is big and spongy organ, providing the body with oxygen while removing carbon dioxide (4). Tubular branches, the bronchi, carry the inhaled air into the lung. The bronchi divide into smaller branches, bronchioles, and further to the alveolar sacs where the air is absorbed into the bloodstream.

Because of daily inhalation of thousands of litres of air, the lungs are exposed to countless number of pathogens (5). Yet, the lungs are lined by over 60 different cell lines (6), including several cell types involved in the innate immune defence, such as leukocytes, monocytes/macrophages, lung epithelial cells and mast cells (7). This master thesis will cover monocytes/macrophages and mast cells due to their relevance in this research.

1.1.1 Monocytes/macrophages

Monocytes are white blood cells, normally limited to the bone marrow and blood. Inside monocytes exists numerous granules which mostly contain lysosomal enzymes which come to aid when destructing pathogens (8). In the presence of inflammation, monocytes move fast to the inflamed tissue, and differentiate into macrophages. Macrophages would then attack the pathogen causing the inflammation by phagocytosis. Alveolar macrophages are crucial in the process of clearing bacteria from the alveolar surface and preventing infections (9).
When monocytes differentiate into macrophages, they are typically characterised by an increase in cell size and content. Monocytes get an extension in the plasma membrane and so the number of mitochondria, in addition to the enhanced phagocytosis capacity and inflammatory activation (10).

In order to identify pathogens, macrophages express pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), and thus trigger phagocytosis (5). TLRs recognise certain patterns on pathogens in phagosomes, which leads to activation of signalling pathways that promote phagosome maturation and inflammatory gene expression, and result in enhanced killing of infectious agents (11).

Depending on the microenvironment, macrophages can change into different phenotypes. The two most common types are pro-inflammatory (M1) and anti-inflammatory (M2) subtype,

which take part in different stages of the inflammatory response (8, 12, 13). M1 macrophages, for instance, are present in the initiating phase of the inflammatory response, and get induced by intracellular pathogens and bacterial cell wall components such as lipopolysaccharide (LPS) to secrete inflammatory cytokines such as tumour necrosis factor-α (TNF-α) (12, 14). The secretion of such cytokines leads to altering the inflammation as well as the generation of nitric oxide, which contribute in the killing of the pathogens (12). M2 macrophages are induced by numerous pathogens, apoptotic cells, and immune complexes. Once induced, the M2 macrophages deactivate the release of pro-inflammatory signalling and release pro-repair and anti-inflammatory cytokines such as prostaglandin E_2 (PGE_2) (15). In addition, M2 macrophages are more capable of phagocytosis, clearing apoptotic cells and thus secure an efficient resolution of inflammation (15).

THP-1 cells

The human monocytic cell line (THP-1) is a monocyte-like cell line derived from a one year old boy with leukaemia (16). THP-1 cells are able to differentiate to macrophages by reacting with phorbol 12-myristate 13-Acetate (PMA), thus, respond to TLR agonist (pathogens that might stimulate inflammation) (16). The classical way of stimulating macrophages into producing pro-inflammatory cytokines, is to treat them with bacterial cell wall component, such as LPS. LPS is a big component found in the outer membrane of gram-negative bacteria, and is used to stimulates the macrophages to produce inflammatory mediators, such as TNF-α (17). Therefore, the cell line has been used as an *in vitro* model for macrophages studies.

1.1.2 Mast cells

Mast cells are long-lived immune cells, derived from haematopoietic stem cells in the bone marrow (18). They are found in most tissues in the body, especially in tissues close to the external milieu such as the skin and the airway epithelium (19). Characterised by high quantities of mediator-laden granules, mast cells respond rapidly a to external triggers and pathogens and set off an inflammatory response (19). Like macrophages, mast cells are involved in airway inflammation, with cell numbers increased in pulmonary alveoli and airways. Mast cells can release potent inflammatory mediators through a process called degranulation (8). A degranulation process occurs as a response to a pathogenic insult. Prior to degranulation B lymphocytes, a type of white blood cell, produce immunoglobulin E (IgE).

IgE binds to high-affinity IgE receptor (FcεRI) in the surface of the mast cell, to code the cell with antibodies against the pathogen. The cell remains inactive until it is subsequently exposed to the same pathogen. The pathogen binds to the IgE antibodies, which leads to cross linking of the IgE. Following is a series of cascade reactions within the mast cell, resulting in the degranulation of the cell as it releases all its components (18), as shown in igure 1.1.2.

Upon degranulation, the mast cell releases cytokines such as histamine and serotonin, and enzymes such as tryptase and β-hexosaminidase (8). Mediators released from activated mast cells will then spread through tissue and cause inflammation that leads to disease. In this research, the enzyme β-hexosaminidase was particularly of interest. β-Hexosaminidase, is present in the lysosome and is essential for the maintenance of cell homeostasis (20). Because this enzyme exists in large amounts in mast cells, it is used as mast cell degranulation indicator.

HMC-1 cells

The human mast cell line (HMC-1) derives from a leukaemia patient, and is proved to express several mast cell-related markers (21). HMC-1 cells undergo degranulation when activated with PMA, ionomycine. Upon activation, HMC-1 cells release the mediators contained intracellularly, such as β-hexosaminidase. The cell line has been used in this study as a mast cell model, *in vitro*.

1.2 Tuberculosis

Tuberculosis is one of the world's top fatal infectious diseases, caused by *Mycobacterium tuberculosis* (Mtb) which spreads through air and affects mostly the lungs (1, 22). Although other organs can be affected at a later stage, the lung remains the primary route of entry for Mtb (23).

Inhaled Mtb droplets are 1–5 micrometres in diameter. Due to their small size, the droplet nuclei succeed in penetrating into the terminal alveoli. Factors like exposure period and intensity of Mtb and the immune system of the individual decide whether the individual get infected or not (Figure 1.1). Once in the alveoli, phagocytic immune cells such as alveolar macrophages engulf the bacteria. After the entry of Mtb, the alveolar macrophages are stimulated to produce pro-inflammatory cytokines such as TNF-α (24). TNF-α is an inflammatory cytokine, involved in the systemic inflammation. In TB, the major roles of TNF-α is controlling acute Mtb infection in monocytes, mediation of macrophage activation, and to form granuloma and contain latent infection in granuloma in dendritic cells.

In the early phase of infection, Mtb replicates intracellularly. Thus, the bacteria laden immune cells may cross the alveolar barrier and cause systemic spread of Mtb (25, 26). These events occur prior to the development of the adaptive immune responses, and explain the remarkable ability of Mtb to establish a protected niche strong enough to keep off elimination and to keep on persisting (27, 28). 2-8 weeks after infection with Mtb, an effective cell-mediated immune response develops in order to stop further multiplication of bacteria. When activated, the immune cells, including macrophages, form granulomas (a mass of granulation tissues) that wall off the growing necrotic tissue limiting further replication and spread of the Mtb. Most of the bacteria are killed in the granuloma formation, and the disease is somehow contained. In some individuals the immune system is not strong enough resulting in uncontrolled bacteria growth. However, in most individuals the immune system is more protective and the bacterial growth is limited, but not completely erased as Mtb has developed effective survival strategies to evade the immune response (Figure 1.1) (28, 29). As the bacteria are not completely erased at this point of disease progression, the individual develops a latent TB infection, which is kept within the individual. Mtb becomes active when the immune system

of the individual drops due to serious conditions such as infection with HIV or an organ transplant.

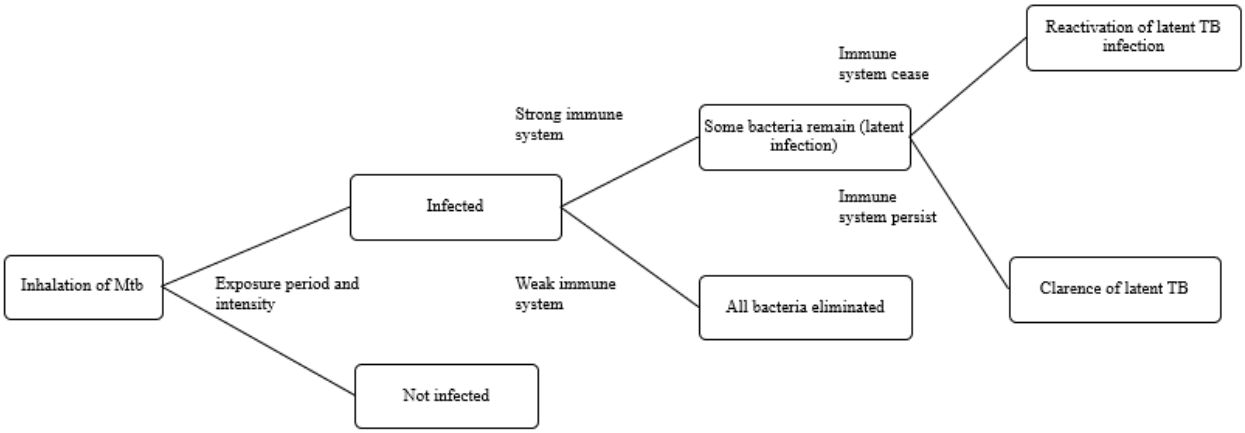

Figure 1.1 The expected course of events after infection with Mtb and outcomes in an immunocompetent patient are expected. Adapted illustration (24).

1.3 TB drug treatment

In 1944, TB treatment was first initiated with streptomycin. Two years later, paraaminosalicylic acid (PAS) was identified and combined with streptomycin. This combination was used until isoniazid and rifampicin were discovered and added to the treatment regimen in the 1950s and 1960s, respectively. Since then, many anti-TB drugs were discovered including pyrazinamide, ethambutol, and ciprofloxacin (30).

1.3.1 Current TB treatment regimen

Today, anti-TB drugs are classified in two groups; first-line anti-TB drugs and second-line anti-TB drugs. According to the therapeutic index, first-line anti-TB drugs are safer than second-line anti-TB drugs, and are therefore used as a first choice when treating TB (30). TB patients are currently treated with a four-drug regimen of the first-line anti-TB drugs, which are isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol (EMB) for two months followed by RIF and INH for four months (31). This regimen is based on a strategy called *directly observed treatment short course* (DOTS). DOTS is internationally recommended by *World Health Organisation* (WHO), and aims at limiting the drug resistance Mtb strains. The strategy is effective even in patients resistant to one of the four anti-tuberculosis drugs.

Multi-drug resistant Mtb (MDR-Mtb) is registered when a patient develops resistance to at least INH and RIF. Today, there are 600,000 cases of multidrug-resistant tuberculosis registered worldwide (32). The treatment gets more complicated, is more toxic and the treatment duration is prolonged from 6 months to approximately 2 years (33). MDR-Mtb is treated with second- line anti-TB drugs. Also, HIV patients infected with TB is treated with second- line anti-TB drugs. In spite of the good effect against Mtb, second-line anti-TB have more severe side effects than first-line anti-TB drugs, they are administered parenterally, and requires characterisation of the Mtb strains in order to offer more personalised treatment. Furthermore, studies have reported cases in which patients are resistant to both first-line anti-TB drugs and second-line anti-Mtb drug, a condition called extensively drug-resistant TB (XDR-TB) (34).

Current anti-TB treatment has some obstacles to overcome. The treatment is administered orally, in large doses to ensure that the drug reaches to the site of action. As a result, patients often suffer from side effects. Furthermore, MDR-TB and XDR-TB has long treatment periods, which results in high treatment costs and leads to patients being non-compliant. These factors make TB-treatment more complex what it already is. Taking these factors into account, new and efficient anti-TB drugs and treatment strategies are urgently needed.

1.3.2 Pulmonary drug delivery

In the case of infection by Mtb, the lungs are the most important route of administration (35), due to the fact that Mtb establishes infections mainly in the alveolar macrophages. Pulmonary drug delivery offers an attractive alternative to oral administration with quite a few advantages. The extended absorption surface of lung mucosa, the thin alveolar epithelium allows a faster absorption through the air-blood barrier (36). It also makes it possible to avoid the hepatic first pass metabolism upon orally given medication. The selectivity of this route of administration allows targeted drug delivery and thereby, reduces possible side effects (37). Moreover, unlike the parenteral route, pulmonary route is non-invasive, and can easily be self-administered. Moreover, pulmonary drug administration allows better bioavailability in the targeted region, compared to other organs, because of the limited drug-metabolising enzyme activity (38).

So far, the treatment of TB is done by oral administration. This route of administration has caused undesirable side effects, in addition to the necessity of monitoring the liver and kidney functions. As a pulmonary drug candidate, CPZEN-45 is a promising candidate that will eliminate these concerns.

1.3.3 Current approaches in TB drug development

A wide range of new anti-TB drug candidates is currently in research and in clinical trials. Below are listed the drugs in preclinical development and phase II trials. In December 2012 diarylquinoline, the derivative bedaquiline completed phase II trials and was approved by FDA as a part of a combination therapy for the treatment of MDR-TB in adults, when other alternatives are not available (39). In April 2014, delamanid completed phase II trials and was conditionally approved by the European Medicine Agency (EMA) for MDR-TB treatment in

adults and children (39). However, the use of these drugs are limited, due to toxicity issues, and further studies in phase III and IV trials are necessary to fully understand the effect of these drugs.

Table 1.1 Drugs in pre-clinical phase.

Drug	Mode of action/ targets	Anti-bacterial activity
PNU-100480 (Oxazolidinone)	Inhibition of protein synthesis by binding to 23S RNA	3.2 times more active than linezolid
BDM31343	Decrease of *INH-A* by interfering with INH-R's DNA binding function of ethionamideR	Synergist of ethionamide (analogue of INH)
SQ609	Unknown	Better anti-mycobacterial activity than it is in TB standard care
SQ641	Inhibition of peptidoglycan synthesis by interfering with activity of the enzyme TL1	Intracellular mycobactericidal activity
CPZEN-45 (Caprazene nucleoside)	Unknown	Active against replicating and non-replicating *M. tuberculosis.* Active against MDR/XDR-TB.
RBx 8700 (Oxazolidinone)	Inhibition of protein synthesis	Mycobactericidal activity against both drug-susceptible and drug-resistant TB. Active against intracellular mycobacteria
DC-159a (Fluoroquinolone)	Inhibition of DNA synthesis by interfering with activity of gyrases	More potent *in vitro* compared to available fluoroquinolones Active against both drug-susceptible and drug-resistant TB
BTZ043	Inhibition of cell wall synthesis by interfering with activity of DprE1	Mycobactericidal activity

Table 1.2 Drugs in phase II of the clinical trials.

Drug	Mode of action/ targets	Anti-bacterial activity
TMC207 (Diarylquinoline)	Inhibition of the proton pump of ATP synthase	Similar activity to that of INH and superior to that of RIF *in vitro*. Mycobactericidal activity 10-fold superior to that of INH and RIF in murine models. Active against numerous monodrug-resistant TB strains (INH, RIF, EMB, PZA, moxifloxacin and streptomycin). Excellent activity against non-TB mycobacteria including *M. ulcerans*
SQ109 (Diethylamine)	Disruption of cell wall assembly – MmpL3	Similar activity to that of INH and superior to that of EMB. Excellent against drug-susceptible and drug-resistant mycobacteria
PA-824 (Nitroimidazole)	Pro-drug activated by a nitroreductase Inhibition of protein synthesis and cell wall lipid synthesis	Strong activity against latent TB infection by encouraging nitrogen oxide production Mycobactericidal and sterilizing activity similar to that of INH and RIF
Delamanid or OPC-67683 (Nitroimodazole)	Inhibition of mycolic acid synthesis	Better anti-mycobacterial activity than that of PA-824
Linezolid (Oxazolidinone)	Inhibition of protein synthesis by binding to 23S RNA	Low early mycobactericidal activity. The compound is the least favourable of all compounds presented in this list Used on patients failing MDR- and XDR-TB treatment

1.3.4 CPZEN-45

Caprazamycins are newly discovered Anti-TB drug compounds, isolated from the culture if an actinomycete strain. The drug compounds are a group of novel liponucleoside antibiotics effective against gram positive bacteria and mycobacteria (32). One of these compounds, caprezene 4-butylanilide (CPZEN-45), has demonstrated anti-TB activity against Mtb H37Rv (40), which is one of the most used models in TB genotyping (41).

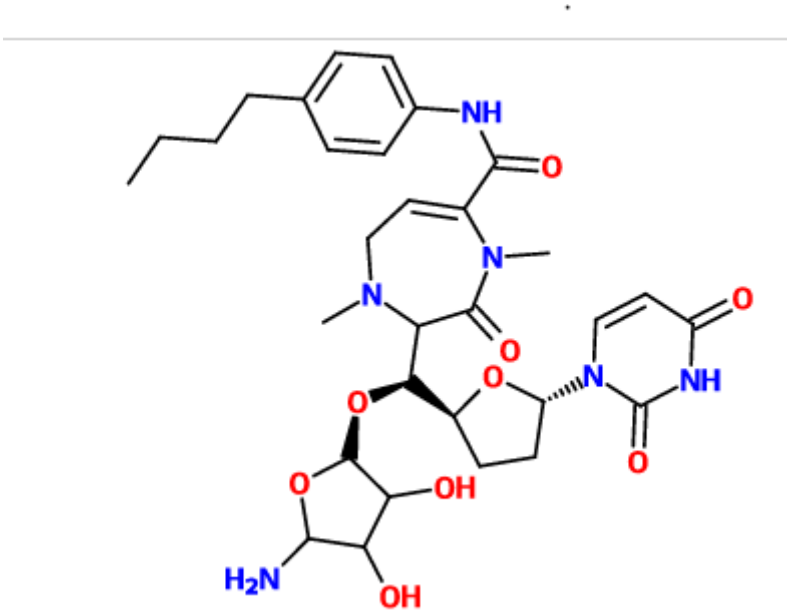

Figure 1.2 The chemical structure of CPZEN-45. Adapted illustration (40).

CPZEN-45 is meant to be administered by inhalation; the compound was first reported in 2003 by researchers at the Microbial Chemistry Research Foundation (MCRF) and Meiji Seika Kaisa, Japan. Although the mechanism of action is still poorly understood, CPZEN-45 was found to be active against both replicating and non-replicating Mtb *in vitro*. It has also shown effect against MDR-TB and XDR-TB in an animal study performed on a mouse model of acute TB, in which CPZEN-45 was administered by subcutaneous injection (32). When administered with other anti-TB drugs, CPZEN-45 showed improved efficacy against MDR-MTB. Furthermore, CPZEN-45 had a narrower antibacterial spectrum and better water solubility, compared with other caprazamycin derivatives (32).

A study carried out to determine the *in vitro* cytotoxicity and permeability of CPZEN-45 showed no acute cytotoxicity of CPZEN-45 up to 3 mg/ml (42). In transport studies, the Calu-3 cell line was used, a human airway epithelial cell line, to investigate the permeability of CPZEN-45 through the cell monolayers. Permeability of CPZEN-45 across the Calu-3 cell monolayers in absorptive and secretive directions was identical, with no significant net directionality, suggesting that the compound is transported through the cell monolayer by concentration dependent passive diffusion. Spite the promising findings, CPZEN-45 is still in the preclinical phase and more experiments are required to understand further aspects of the drug candidate.

2 Aims and objectives

This research aims at determining the effect of CPZEN-45, an anti-TB drug candidate, on immune cells with respect to its ability to modify the inflammation marker release in two chosen immune cell lines.

1. The THP-1 cells are monocyte-like cells, which are differentiated to macrophages, and an ELISA was performed to measure the TNF-α release the cells when treated with CPZEN-45.
2. The HMC-1 cells are mast cells and will be tested for whether CPZEN-45 modulates the degranulation processes. A β-hexosaminidase assay was performed to measure the β-hexosaminidase release.

3 Materials and methods

3.1 Materials

Table 3.1 Used chemicals in the ELISA.

Chemicals/ proteins	Function	Supplier
Bicarbonated Krebs-Ringer Buffer (KRB)*	Diluent in sample procedure	-
CPZEN-45 HCl	Testing of THP-1 cells	The Infectious Disease Research Institute (WA, USA).
Deionised water	Dilute most solutions	Trinity Biomedical Science Institute, pharmacy bio lab (Dublin, Ireland)
TNF-α ELISA Kit**	Sandwich ELISA	PeproTech (London, UK)
Foetal Bovine Serum (FBS)	Culturing the THP-1 cells	Sigma-Aldrich (Wicklow, Ireland)
L- glutamine solution	Culturing THP-1 cells	Sigma-Aldrich (Wicklow, Ireland)
Lipopolysaccharides (LPS)	Stimulates macrophages to release TNF-α	Sigma-Aldrich (Wicklow, Ireland)
Phorbol 12-myristate 13-acetate (PMA)	Differentiate THP-1 cells to macrophages	Sigma-Aldrich (Wicklow, Ireland)
RPMI 1640 medium	Culturing THP-1 cells	Thermo Fisher Scientific (Dublin, Ireland)

*1.8 mM $CaCl_2$, 0.81 mM $MgSO_4$, 116.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH_2PO_4, 25 mM $NaHCO_3$, 5.55 mM glucose, 15 mM HEPES.

** Capture antibody, detection antibody, human TNF-α standards, avidin-HRP, phosphate buffered saline (PBS), diluent, blocking buffer, wash buffer, 2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS).

Table 3.2 Used chemicals in the β-hexosaminidase assay.

Chemicals/ proteins	Function	Supplier
4-nitrophenyl-N-acetyl-β-D-Glucosaminide	Isolation of β-hexosaminidase	Sigma-Aldrich (Wicklow, Ireland)
CPZEN-45 HCl	Testing the HMC-1 cells	The Infectious Disease Research Institute (WA, USA).
Glycine	Stop the enzymatic reaction	Sigma-Aldrich (Wicklow, Ireland)
Ionomycin	Activating the HMC-1 cells	Sigma-Aldrich (Wicklow, Ireland)
Iron-supplemented calf serum 10%	Culturing the HMC-1 cells	Sigma-Aldrich (Wicklow, Ireland)
Iscove's modified Dulbecco's medium	Culturing the HMC-1 cells	Thermo Fisher Scientific (Dublin, Ireland)
Monothioglycerol	Culturing the HMC-1 cells	Sigma-Aldrich (Wicklow, Ireland)
Pencillin/Streptomycin solution, (PenStrep),	Culturing the HMC-1 cells	Source BioScience (Nottingham, UK)
PMA	Activating the HMC-1 cells	Sigma-Aldrich (Wicklow, Ireland)
Sodium citrate	Dilute 4-nitrophenyl-N-acetyl-β-D-glucosaminide	Sigma-Aldrich (Wicklow, Ireland)

Table 3.3 Instruments used.

Instrument / machine	Manufacturer
Autoclave	Austell (Dartford, UK)
Axiovert 200M microscope	Zeiss (Cambridge, UK)
Centrifuge 5415 D	Eppendorf (New York, USA)
Centrifuge 5415 R	Eppendorf (New York, USA)
Fluostar OPTIMA plate reader	BMG Labtech (Aylesbury, UK)
Magnetic stirrer	VWR (Radnor, USA)
Magnetic stirring hotplate	Heidolph (Essex, UK)
PH meter	Mettler Toledo (Leicester, UK)
Vortex Genius 3	IKA (Straufen, Germany)
Incubator	Thermo Fisher Scientific (Dublin, Ireland)

3.2 Cell culture

3.2.1 Human monocytic cell line (THP-1)

THP-1 cells were maintained in RPMI 1640 medium, 10% of FBS, and 1% L-glutamine solution at 37°C in 5% CO_2. The weekly routine of managing the THP-1 cells was to passage them on Monday, and feed them on Wednesday and Friday. The passaging of the cells was done according to the following protocol: The cell suspension was transferred from the T75 flask to a 50 ml centrifuge tube and centrifugation at 900 rpm for 5 min. The pellet was checked. The medium was removed carefully using the vacuum pump, and the pellet re-suspended in 10 ml of new medium. Nine millilitres of fresh medium were added to a new 75 ml flask. One millilitre of the cell suspension was then added to the flask and incubated at 37°C.

3.2.2 Human leukaemic mast cell line-1 (HMC-1)

HMC-1 cells were kindly provided by Professor Michel Arock (The National Center for Scientific Research (CNRS), France), and grown in Iscove's modified Dulbecco's medium (IMDM) with 10% iron-supplemented calf serum, 1.2 mM monothioglycerol and 1% of PenStrep solution at 37°C in 5% CO_2. The cells were passaged twice a week due to their ability to grow fast, and were split in a ratio of about 0.3.

3.3 The TNF-α sandwich ELISA

To study the effect of CPZEN-45 on the TNF-α release in THP-1 cells, a sandwich ELISA (Enzyme linked immunosorbent assay) was performed. Briefly, the monocyte-like THP-1 cells were differentiated to macrophages by the addition of PMA, and later stimulated with LPS to produce TNF-α. The cells were then treated with different concentrations of CPZEN-45 to investigate any anti-inflammatory effect caused by the drug. To quantify the TNF-α released from the cells, a sandwich ELISA was performed (Figure 3.1). The effect of time of incubation with LPS and different LPS concentrations were also studied. THP-1 cells were stimulated with LPS for 2, 3, 6, 24 and 48 h, and concentrations of 0.125 µg/ml and 0.01 µg/ml were used.

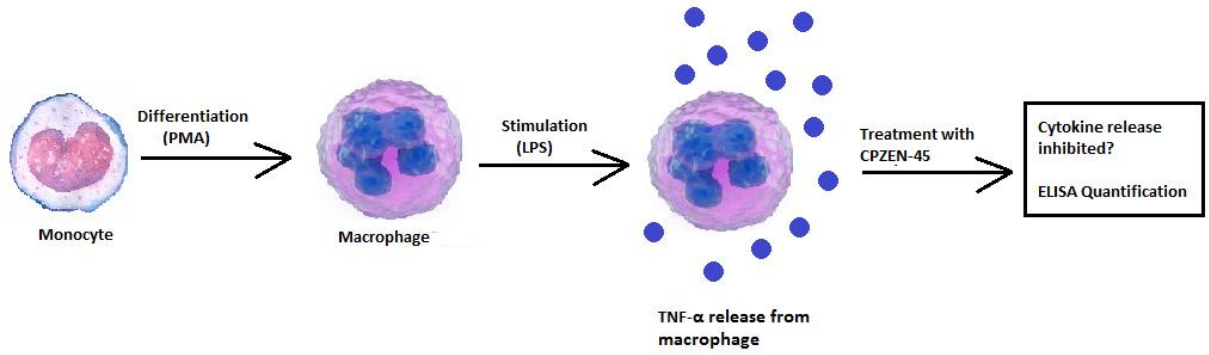

Figure 3.1 Overview of the steps in the testing of the THP-1 cells.

3.3.1 Preparing the samples for the TNF-α sandwich ELISA

THP-1 cells were differentiated to macrophages by treatment with PMA (cell density was $1x10^6$ cells/ml) and then transferred to a 96-well plate. The cells were then incubated at 37°C for 48 h. Meanwhile the cells were incubating, the solutions needed to perform the ELISA were freshly prepared every week. The solutions; PBS, diluent, blocking buffer and washing buffer, were all diluted in deionised water to desired concentrations at stored at 2-8°C. The different ELISA kit proteins; capture antibody, detection antibody, human TNF-α standards and avidin-HRP conjugate, were also reconstituted in deionised water according to the provided protocol from PeproTech, and stored at -20°C.

After 48 h incubation, the cells were washed twice with KRB (200 µl/well). Hundred and sixty microliters of LPS (0.125 mg/ml) were added to each well, but four wells, to stimulate

the cells to release TNF-α. Two hundred microliters of the negative control, KRB was added to the remaining 4 wells, to serve as a negative control, shown in Figure 3.2. The plate is incubated for 2 h at 37°C. The LPS concentration used here was later lowered to 10 ng/ml, and the incubation time increased to 3, 6, 24, and 48 h.

During the incubation of THP-1 cells with LPS, the CPZEN-45 HCl is weighed and the required concentrations/dilutions were prepared. The drug was first dissolved in water and the concentrations made by diluting with KRB. The prepared concentrations (mg/ml) were: 0.5, 1, 5, 10, 50, 100, 250, 500, 750, 1000, and 2000.

Forty microliters of the different CPZEN-45 concentrations were added to the well plate in quadruplicates, shown in Figure 3.2. Forty microliters of KRB was added to the positive control; and the well plate was incubated for another 2 h at 37°C. The samples were then stored at -20°C to the next day.

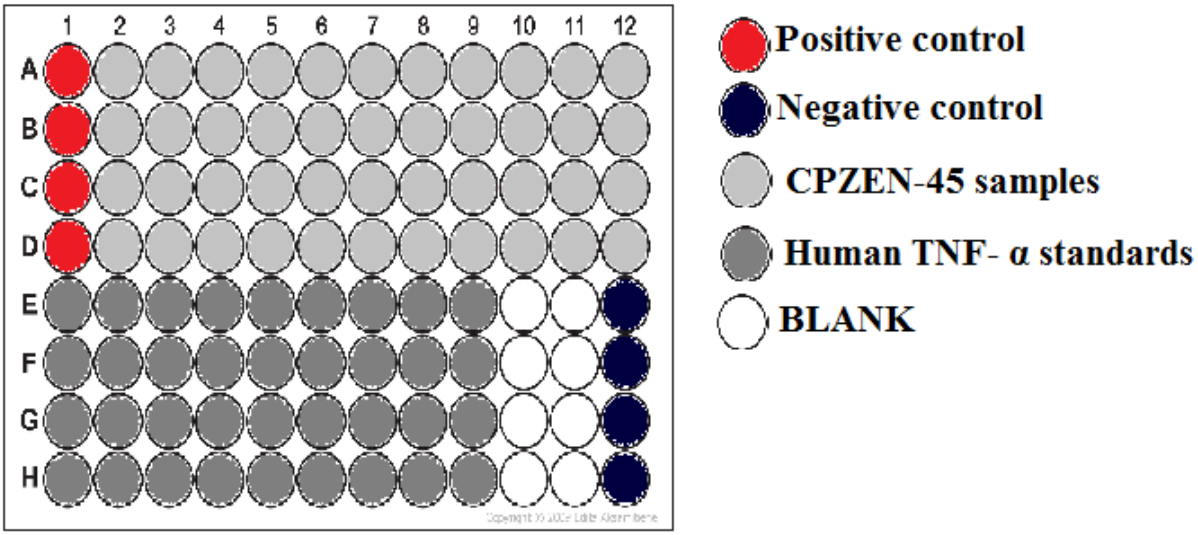

Figure 3.2 The layout of 96-well plate used in the ELISA. Samples added in quadruplicates.

3.3.2 ELISA phases I-VI

The sandwich ELISA is shown in Figure 3.3. Briefly, an adhesive 96-well plate was coated with capture antibody for 2-4 h at 37°C (phase I). The plate was washed with wash buffer, and any nonspecific binding sites were blocked by adding the blocking buffer (phase II). The plate was then incubated for 1 h at 25°C. The CPZEN-45 samples and the human TNF-α standards were added to the well plate and captured as shown in Figure 3.2. The human TNF-α concentrations were decreased by half for each column in the well plate, starting with 2

ng/ml (1X) and ending with 0.00781 ng/ml (1/256X). The plate was incubated for 2 h at 25°C (phase III). Detection antibody was added to the plate to bind to the antigen and form a "sandwich", and the plate is incubated again for 2 h at 25°C (phase IV). The avidin-HRP (a converting enzyme) was added, and the plate incubated for another 30 min at 25°C (phase V). ABTS was added in the end for colour development (phase VI). The plate was then monitored in the plate reader with 5 min intervals for 20 min at 405nm.

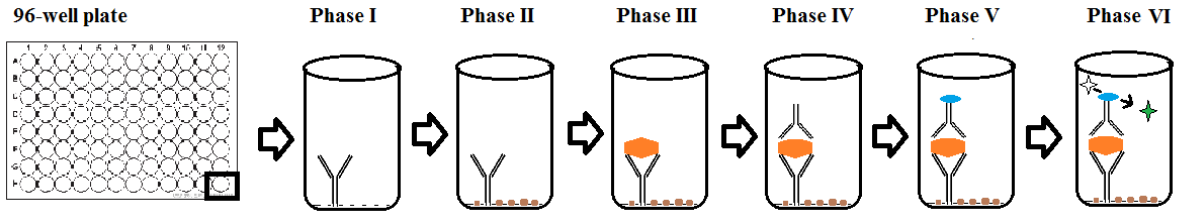

Figure 3.3 The steps of the sandwich ELISA. Plate is coated with capture antibody (phase I), and the remaining sites are blocked with a block buffer (phase II). The antigen/ CPZEN-45 samples are added and binds to the capture antibody (phase III). Detection antibody is then added and binds to the antigen to form a sandwich ELISA (phase IV). Avidin HRP is added (phase V), followed by ABTS for colour development (phase VI).

3.4 β-hexosaminidase assay in HMC-1 cells.

3.4.1 Sample preparation

HMC-1 cells were transferred to a 50 ml centrifuge tube and a small sample was taken for cell count. The cells were centrifuged at 900 rpm for 5 min, and the pellet checked. Carefully, the old medium was removed. The pellet was then re suspended in 10 ml of freshly made KRB. The cell suspension was added (depending on the cell count) to a new 50 ml centrifuge tube and filled up to 10 ml with fresh made KRB. The cells were seeded in a density of 4 x 10^5 cells/ml. One hundred microliters of the cell suspension were added to each well of a 96-well plate. The cells are rested for 2-3 hours before making the samples.

The 96-well plate was seeded with different samples, in quadruplicates (Figure 3.2). Briefly, the positive control was fifty microliters of 0.1 µg/ml PMA and 1 µg/ml ionomycin added to four wells. The negative control was cells treated with fifty microliters of freshly made KRB. Total cell lysates were obtained by the addition of fifty microliters of 0.1% Triton -X100 to four wells, as these wells served as the 100% value of β-hexosaminidase release from HMC-1 cells. Higher concentrations of Triton-X100 were also used at a later stage of the research (0.5% and 1%). Fifty microliters of CPZEN-45 were then added to not treated cells, to

investigate whether CPZEN-45 initiates any inflammatory response in HMC-1 cells. Following concentrations were used: (0.5, 1, 5, 10, 50, 100, 250, 500, 750, 1000, 2000) µg/ml.

At a later stage of the research, some cells were also treated 0.1 µg/ml PMA and 1 µg/ml ionomycin, in addition to CPZEN-45 to investigate any inhibitory effect of CPZEN-45 on the inflammation processes in HMC-1 cells. The well plate was then incubated for 30 min at 37°C. No reproducible results were obtained.

3.4.2 Isolation and measurement of β-hexosaminidase

Fifty microliters samples were transferred to a new 96-well plate. The samples were incubated with hundred microliters of 1.3 mg/ml p-nitrophenyl-N-acetyl-β-D-glucosaminide in 0.1 M sodium citrate (pH 4.5) plate at 37°C for 1 h. The enzymatic reaction was stopped by addition of fifty microliters of 0.4 M glycine (pH 10.7) to each well. Enzymatic activity was then measured using a plate reader at 405 nm (absorbance reading). The release of β-hexosaminidase in medium was expressed in percentages of the total release from cells lysed.

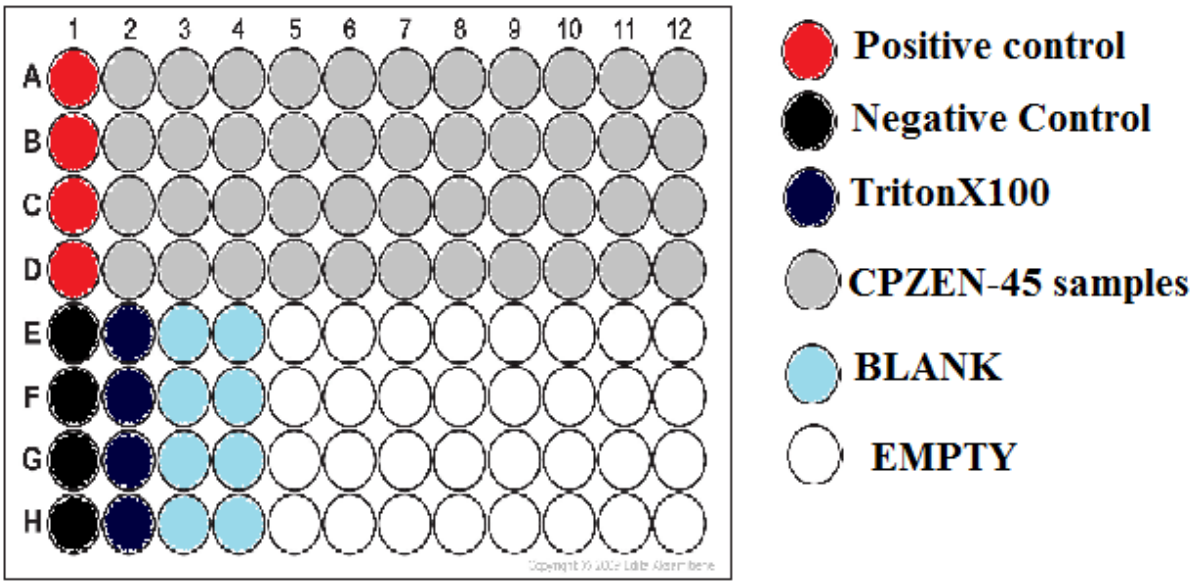

Figure 3.4 Layout of the 96-well plate used in the β-hexosaminidase assay. Samples added in quadruplicates.

3.5 Statistical analysis

The softwares used in this research are GraphPad PRISM 5 and Microsoft Excel 2010.
All experiments were performed in quadruplicates. Absorbance values were first organised in an Excel sheet then transferred to GraphPad PRISM 5 to be presented in a statistical analysis. Statistical analysis was performed by one-way ANOVA test with Borferroni data comparisons. *P*-values of < 0.05 were considered statistically significant.

4 Results

4.1 CPZEN-45's effect on the THP-1 cells

The ELISA gave reproducible results at 3, 6, and 48 h of incubation of THP-1 cells with LPS. TNF-α release from THP-1 cells incubated with 125 µg/ml LPS for 3 h showed a decreasing trend as the CPZEN-45 concentrations were increased (Figure 4.1). Still, the decrease in TNF-α release was not pronounced enough to be considered statistically significant. A One Way ANOVA test showed no significance between the positive control (cells treated with only LPS) and cells treated with LPS and CPZEN-45, or between the positive control and the negative control (cells treated with only KRB).

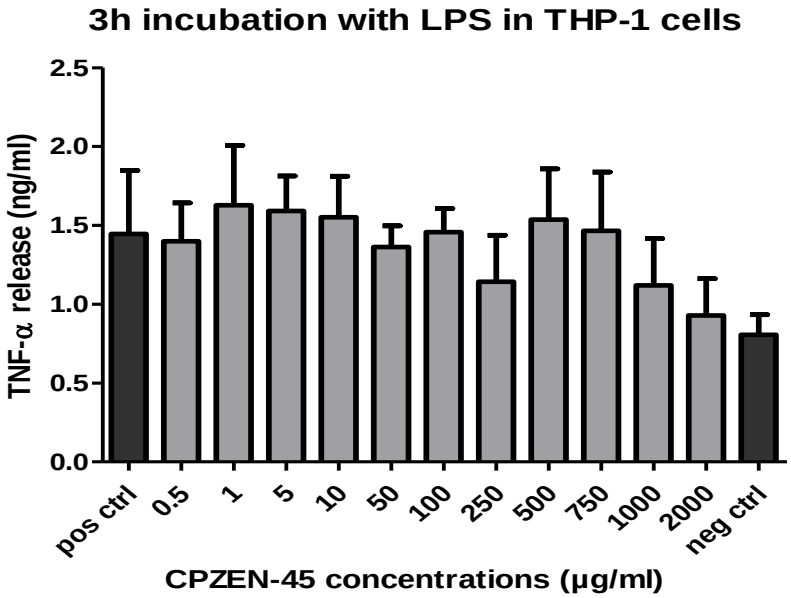

Figure 4.1 THP-1 cells incubated with LPS (125 µg/ml) for 3 h, and treated with CPZEN-45 for 2 h. The TNFα release is decreased as the CPZEN-45 concentration increase. No statistical significance was proven. Data represent means ±SEM; n=4.

TNF-α release after 6 h incubation of the cells with LPS (125 µg/ml) showed a TNF-α release profile which was even more clearly decreased with increasing concentrations of CPZEN-45 than what was observed for 3 h incubation. One Way ANOVA test confirmed the significant TNF-α decrease in cells treated with higher CPZEN-45 concentrations (50 µg/ml and above) compared to the positive control.

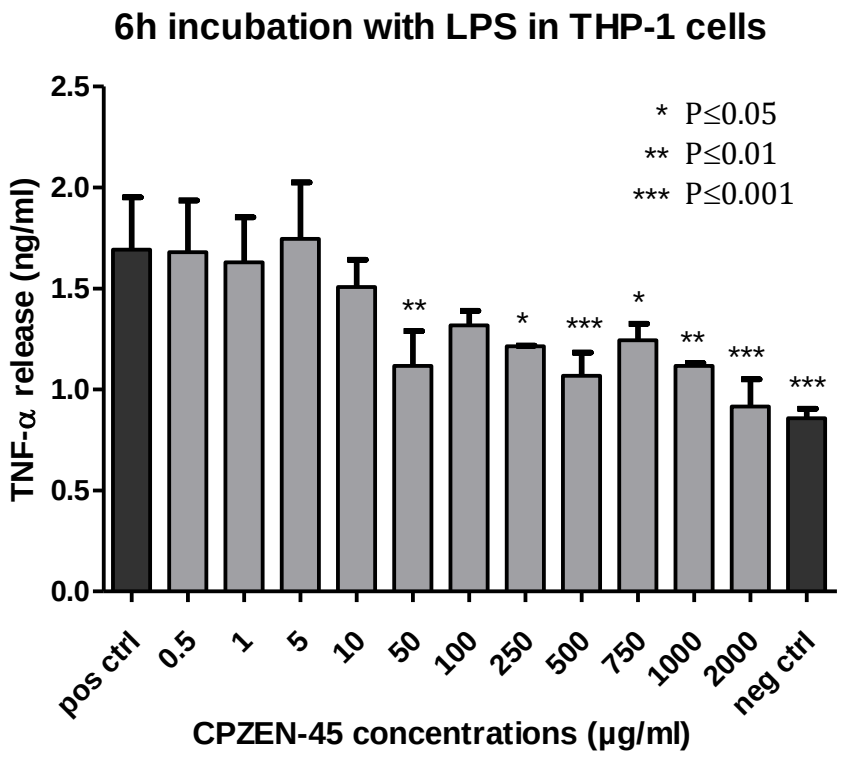

Figure 4.2 6 h incubation. THP-1 cells incubated with LPS (125 µg/ml) for 6 h, and treated with CPZEN-45 for 2 h. The profile of TNFα release has a pattern of more decreased release as the CPZEN-45 concentration increase, compared to the trend seen in Figure 4.1. Statistical significance is shown in the following CPZEN-45 concentrations when compared with the positive control (µg/ml); 50 (P≤0.01), 250 (P≤0.05), 500(P≤0.001), 750 (P≤0.05), 1000 (P≤0.01), and 2000 (P≤0.01) as well as in the negative control (P≤0.001). Data represent means ±SEM; n=4.

TNF-α release measured after 48 h incubation of THP-1 cells treated with LPS (10 ng/ml), showed a similar release profile, with significant decrease in the 4 highest CPZEN-45 concentrations (Figure 4.3).

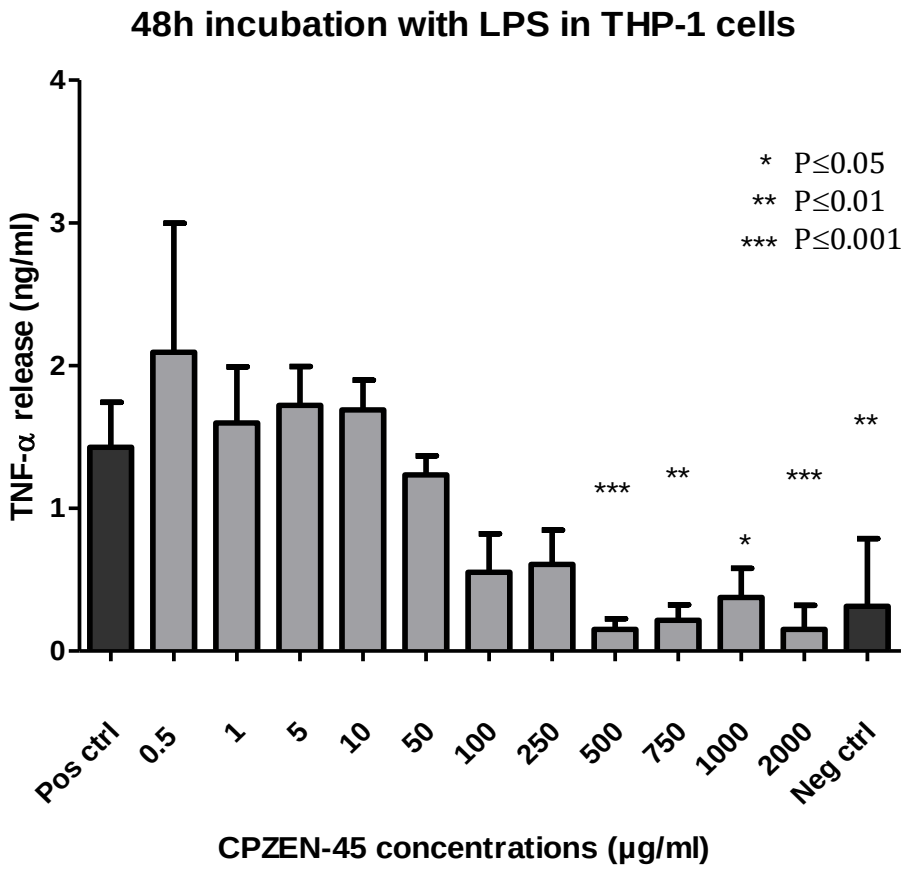

Figure 4.3 THP-1 cells incubated with LPS (10ng/ml) for 48 h, and treated with CPZEN-45 for 2 h. Statistical significance is shown in the following concentrations when compared with the positive control(µg/ml); 500 ((P≤0.001)), 750 (P≤0.01), 1000 (P≤0.05), 2000 (P≤0.001), as well as in the negative control (P≤0.01). Data represent means ±SEM; n=4

The TNF-α release from THP-1 cells was decreasing as the CPZEN-45 concentrations were increased. Similar trend in the three repetitions was shown. The third repetition was done with much lower LPS concentration (0.10 µg/ml) compared to earlier repetitions (125 µg/ml), while the incubation time was much longer (48 h). Therefore, it was more reasonable to compare the two first repetitions to each other (Figure 4.4).

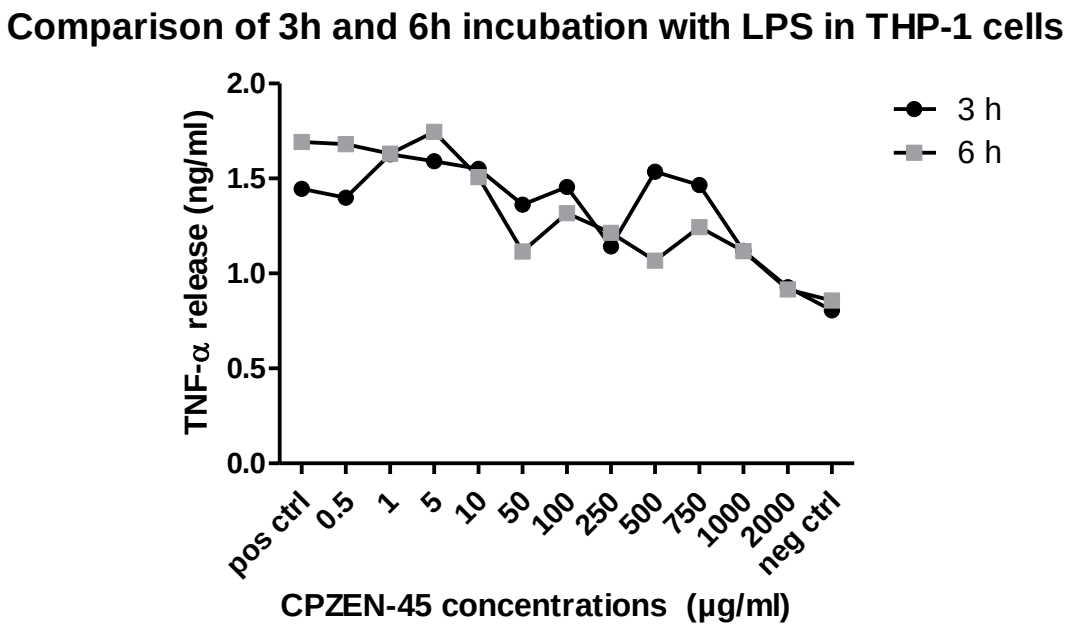

Figure 4.4 Comparison of the TNF-α release profile after 3 h and 6 h incubation of THP-1 cells with LPS (125 µg/ml); n=4.

Comparison of 3 h and 6 h incubation of THP-1 cells showed higher TNF-α release from the positive control after 6 h, as shown in Figure 4.5. Furthermore, the TNF-α release in cells treated with concentrations of CPZEN-45 up to 5 µg/ml was higher after 6 h. Yet, after 6 h the TNF-α release was lower in cells treated with higher concentrations of CPZEN-45. The decrease in TNF-α release is particularly detectable at a CPZEN-45 concentration of 50 µg/ml and above. The higher CPZEN-45 concentration used the more inhibition of TNF-α release was detected.

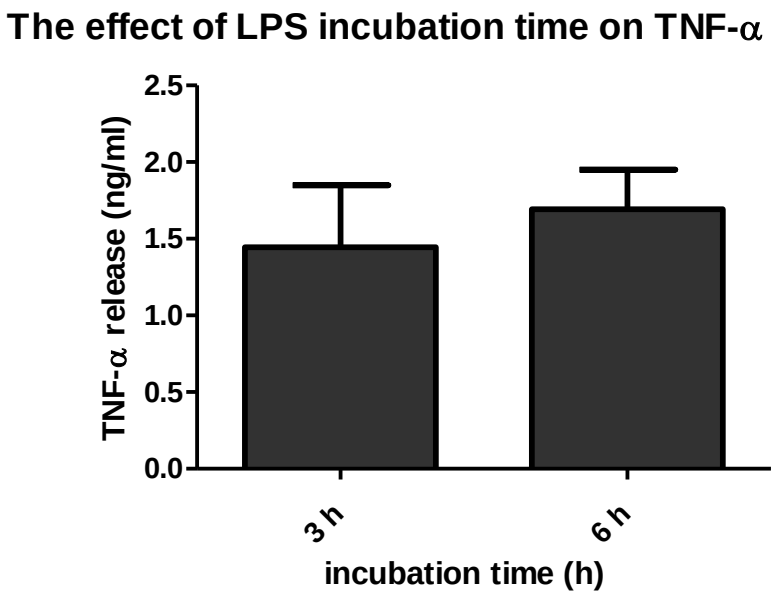

Figure 4.5 Incubation of THP-1 cells with LPS. The effect of LPS on the release of TNF-α from THP-1 cells after 3 and 6 hours. Data represent means ±SEM; n=4.

Incubation time of THP-1 cells with LPS, and LPS concentrations used to stimulate the cells, as well as the CPZEN-45 concentrations are the main parameters that effect the TNF-α release profile. These parameters will be discussed further in the next chapter.

4.2 The effect of CPZEN-45 on HMC-1 cells

β-hexosaminidase assay suggested slight inflammatory effect caused by CPZEN-45 on the HMC-1 cells, compared to the positive control, shown in Figure 4.6. Interestingly, the enzyme release was higher in cells treated with lower concentrations of CPZEN-45, compared to higher concentrations. Significant decrease in the enzyme release were registered in concentrations above 10 µg/ml ($p \leq 0.05$).

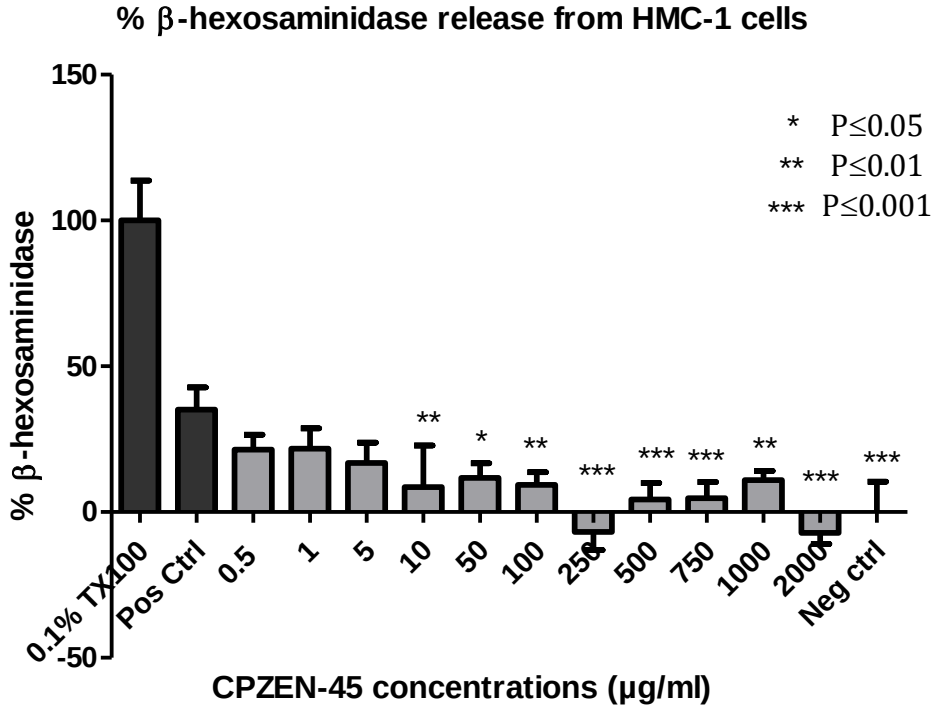

Figure 4.6 Percentage of β-hexosaminidase release from HMC-1 cells. Cells treated with different concentrations of CPZEN-45, compared to lysed cells (0.1% Triton-X 100) and activated cells (Pos ctrl). Significantly low release was registered at concentrations above 5 µg/ml ($p \leq 0.05$). Data represent means ±SEM; n=4.

5 Discussion

5.1 Main findings on CPZEN-45

The anti-inflammatory effect of CPZEN-45 on THP-1 cells was investigated by performing a sandwich ELISA. Reproducible results were obtained at 3, 6 and 48 h of incubation with LPS. The main findings of the assay suggest that

- CPZEN-45 inhibits the TNF-α released from THP-1 cells at concentrations above 50 µg/ml.
- The higher CPZEN-45 concentration on cells, the more decrease in TNF-α expression levels.
- The higher expression levels of TNF-α, the more visible inhibition effect of CPZEN-45.

The inflammatory effect of CPZEN-45 on HMC-1 cells was investigated by performing a β-hexosaminidase assay. Main findings suggest that

- CPZEN-45 activates the degranulation of HMC-1 cells to some extent, compared to the combination of PMA and ionomycin.
- The higher CPZEN-45 concentrations on cells, more decrease in β-hexosaminidase levels.

5.2 Parameters affecting the TNF-α expression levels from THP-1 cells

CPZEN-45 concentrations, incubation time of THP-1 cells with LPS, and LPS concentrations used to stimulate the THP-1 cells are major parameters affecting the TNF-α expression level. The ELISA was thus optimised for incubation time of THP-1 cells with LPS, and LPS concentrations used to stimulate the THP-1 cells.

5.2.1 CPZEN-45 concentrations

The pattern of which the TNF-α release is decreased is more pronounced as the CPZEN-45 concentrations are increased. The decreased TNF-α release curve suggests that higher CPZEN-45 concentrations are more effective in lowering the TNF-α release. More specifically, concentrations of 50 µg/ml and above have shown the ability to reduce the TNF-α release in THP-1 cells. Clinically, the findings suggest that CPZEN-45 might have better potential, with concentrations above 50 µg/ml, in patients with high inflammation caused by Mtb. Still, the decrease is not as steady as desirable. This might have been due to the uneven amount of cells in each well. A steadier decrease of the cytokine could be obtained by performing a protein normalisation.

Protein normalisation

To obtain more accurate cytokine release from THP-1 cells, a protein normalisation assay may be a good tool. A protein normalisation assay helps to determine the cytokine release either by normalisation of cell number (cell count after harvesting the supernatant), or by normalisation for total cytokine content in cells (lysing cells). By adjusting for either the cell number or total cytokine content, the cytokine quantification will be more accurate, which may give a steadier cytokine release curve, and hence more pronounced effect of CPZEN-45. This normalisation was not performed.

Toxicity testing

The CPZEN-45 concentrations used in the ELISA are in the range of 0.0005 mg/ml to 2 mg/ml. After initiating the CPZEN-45 project with the mentioned concentrations, a toxicity test (Alamar blue) was run by a fellow student, to investigate the cell viability of CPZEN-45 treated THP-1 cells. The results showed no toxicity of CPZEN-45 on THP-1 when treated with the CPZEN-45 concentrations, as the viability of cells was around 100% in most drug

concentrations, as shown in Figure 5.1. Furthermore, earlier toxicity studies support the chosen concentrations as it was reported no toxicity of CPZEN-45 up to 3 mg/ml in bronchial epithelial cell lines (40).

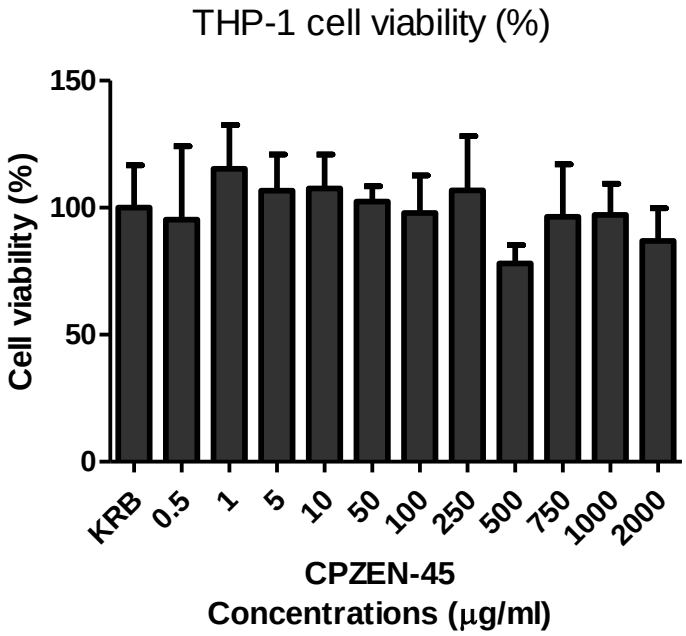

Figure 5.1 THP-1 cell viability. THP-1 cells treated with CPZEN-45 in the concentrations 0.5, 1, 5, 10, 50, 100, 250, 500, 750, 1000, and 2000µg/ml. No clear toxic effect was reported. Data represent means ±SEM; n=5. Figure reproduced from the interim report of the CPZEN-45 project.

5.2.2 Incubation time with LPS

The incubation time with LPS is reckoned to be an important parameter in the part of investigating the effect of CPZEN-45 on the release of TNF-α from THP-1 cells. LPS is known to be a classic stimulus of pro-inflammatory cytokines (17, 43). As long as the THP-1 cells are in contact with LPS, the cells will continue releasing TNF-α. It would thus be expected to have more TNF-α release with longer incubation time. The ELISA showed higher TNF-α release in cells treated with LPS only (positive control) after 6 h incubation with LPS, compared to the 3 h incubation, shown in Figure 4.5. Based on earlier publications (17, 44) the THP-1 cells were incubated with LPS for longer time, 24 h (no reproducible results obtained) and 48 h. Yet, it was not possible to compare the cytokine release from 3 h and 6 h incubation with 48 h incubation, due to different LPS concentrations used. Therefore, more data is needed on longer incubation time (i.e. 24 h, 48 h) with the same LPS concentrations to

gain better understanding of incubation time in relation to TNF-α release, and further the effect of CPZEN-45 on the cytokine release.

Furthermore, the study suggests an improved effect of CPZEN-45 on the inflammation of THP-1 cells in concentrations above 50 µg/ml. Clinically, this would mean that the alveolar macrophages would try to defeat the Mtb as long as it is in the lung, by sending inflammation signals by releasing TNF-α. If CPZEN-45 is given at concentration above 50 µg/ml, in a prolonged manner, it will have a better effect on defeating the bacteria.

5.2.3 LPS concentrations and incubation time

Working with LPS-induced macrophages, the researchers became aware of the balance between the incubation time and the concentrations of LPS on the cytokine release. A growing number of publications report an LPS incubation range of 2-72 hours, and an LPS concentration of 10 ng/ml - 1µg/ml (43, 45-47). Therefore, as longer incubation time of cells with LPS was tested, the LPS concentration was considerably lowered, from 125 µg/ml to 0.01 µg/ml. This was based on earlier publications, reporting that LPS increased the TNF-α release in THP-1 cells in concentrations down to 10 ng/ml (17). Lowering the LPS concentration while increasing the incubation time seemed to give the same TNF-α release trend as in the higher LPS concentration and lower incubation time.

5.2.4 Other parameters affecting the ELISA findings

Cell density

The cell density used in the ELISA was 10^6 cells/ml. Earlier published studies use different cell densities, up to 10^6 cells/ml (48-50). Thus, the cell density used in the ELISA is acceptable. Still, examining the seeded cells under microscope, a density of10^6 cells/ml seems too high, shown in Figure 5.2. Cells of high density would function as one unit and stop the LPS from reaching to all cells, and hence a total cell stimulation will not be achieved. This would affect the cytokine release largely, and result in non-representative cytokine quantification.

Figure 5.2 Micrograph of THP-1 cells seeded in a density of 106 cells/ml.

LPS first or CPZEN-45 first?

The THP-1 samples were prepared with LPS incubation prior to treatment with CPZEN-45. The incubation time and concentration of LPS allows the THP-1 cells to release most of the TNF-α contained in the cell. The cells are then treated with CPZEN-45 for only 2 hours. One would then assume then that the drug will not have much effect on the cells. This is explained by the CPZEN-45 mechanism of action (which is still poorly understood) aimed at the defeating the pathogen, not the mechanism of which TNF-α is produced. By inhibiting LPS, the macrophages will not be induced to release TNF-α, and hence the inflammation is contained, shown in Figure 5.3.

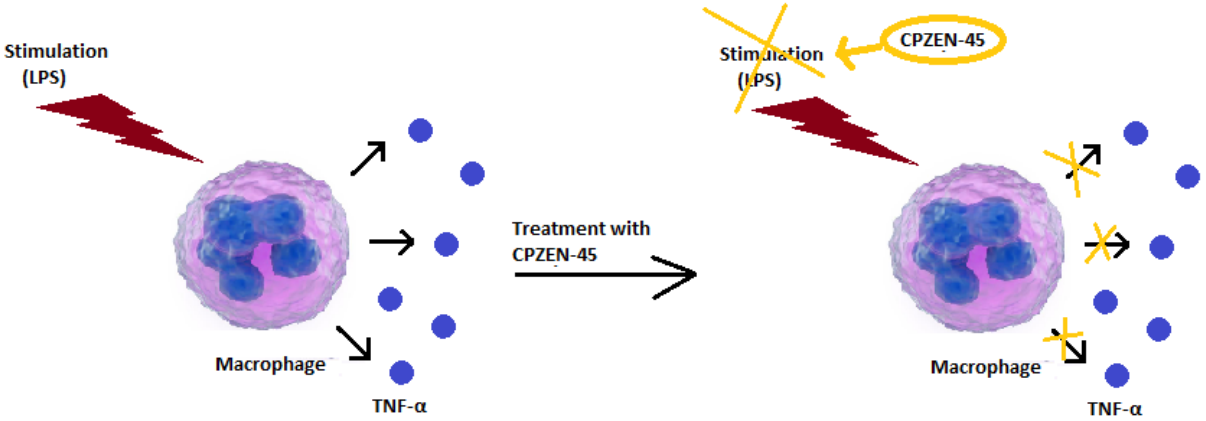

Figure 5.3 LPS induced TNF-α from macrophages. When treated with CPZEN-45 inhibits the LPS, which will cease the TNF-α release.

However, preparing the samples the other way around by treatment with CPZEN-45 prior the induction of LPS was performed first by fellow students. It was reported that CPZEN-45 does not cause release of TNF-α from THP-1 cells *in vitro*. The compound seemed to have an anti-inflammatory effect as LPS-mediated TNF-α release was inhibited by increasing concentrations of CPZEN-45. The pattern of which the TNF-α is decreased is the same in the findings of this study, shown in Figure 5.4. This suggests no difference in in the chronology of which is added first, CPZEN-45 or LPS. Yet, the addition of CPZEN-45 to the cells first is not clinically correct in terms of treating a person prior to infection with Mtb. Taking this into account, the samples in our study were incubated with LPS first to trigger the inflammation, and then treated with CZPEN-45.

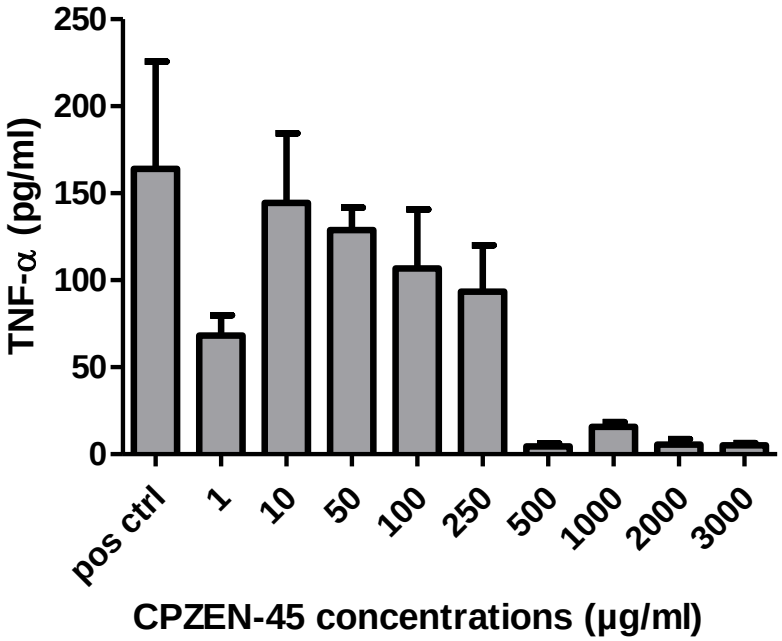

Figure 5.4 Release of TNF-α by THP-1 cells in response to exposure with increasing concentrations of CPZEN-45 for 2 h, followed by incubation with LPS for 4 h. Data represent means ± SEM; n=3. Figure adopted from the interim report of the CPZEN-45 project.

5.2.5 TNF-α sandwich ELISA

ELISAs are widely known and used in clinical practice, as a method to quantify cytokines (51). In this study, the ELISA was repeated many times in order to quantify the TNF-α from THP-1 cells. The difficulties faced in this assay were regarding low absorbance readings and poor standard curve. Some modifications were done on the assay to get reproducible results.

Trouble-shooting

The manufacturer (Peprotech) of the ELISA kit that were used in this study provided a trouble-shooting manual. For weak/ colour development, the manufacturer listed few error sources and solutions, including "plate washed inadequate", suggesting to decrease the number of washing cycles between steps. Therefore, the washing frequency was reduced from 4 to 3 washing cycles, which helped get remarkable higher O.D. readings. The O.D. readings for the positive control, for instance, increased from an average O.D. value of 0.2 up to 0.9.

Also for unsatisfactory standard curve, the manual listed some error sources and solutions, including pipetting errors, dilutions made too early, storage of components, and insufficient washing. All these sources were taken into account. The standard curve got more linear after some repetitions, probably due to improved lab techniques such as pipetting skills.

In order to get reliable TNF-α values, the R^2 value of the standard curve should ideally be 0.99, or above. This linearity was difficult to obtain. However, R^2 values over 0.95 were permitted to proceed with the calculations of the TNF-α values. Not obtaining the right linearity, raised questions like; what is the range of the ELISA kit? The kit had a range of 31-2000 pg/ml. Taking this information into account, the two lowest concentrations of the standard curve were excluded. This gave standard curve with better r^2 values.

Alternative assays

In general, ELISAs are known to have high test sensitivity and specificity (52). It was earlier known that ELISAs are designed to measure one cytokine. However, new ELISA strategies have been established, and different manufacturers provide multi-analyte ELISA arrays. Up to 15 cytokines can be tested at the same time. For the future, more cytokines could be tested to further investigate the anti-inflammatory effect of CPZEN-45. The family of the pro-inflammatory cytokine interleukin (IL) is widely used to determine inflammation in macrophages. Multi-analyte arrays could thus be a good tool in this case.

5.3 β-hexosaminidase release from HMC-1 cells

The β-hexosaminidase assay showed that CPZEN-45 activates the degranulation of HMC-1 cells in a certain degree. The release in the positive control (PMA and ionomycin) was still higher, as PMA and ionomycin are classical mast cell activators (53), and the high enzyme release from these samples was expected. The CPZEN-45 on the other hand, has not been previously tested with respect to its effect on mast cells. Yet, having an anti-inflammatory effect on macrophages, the drug was expected to have the same effect on mast cells. Furthermore, the increase in β-hexosaminidase was more pronounced in the lower set of CPZEN-45 concentrations. This phenomenon was not expected as the degranulation should be more pronounced with higher concentrations of CPZEN-45.

5.3.1 How does CPZEN-45 effect the degranulation in HMC-1?

The mechanism of action of CPZEN-45 is still unknown. Studying the β-hexosaminidase release in the CPZEN-45 treated cells, higher enzyme expression level was detected. When compared to the negative control, the enzyme expression level in the CPZEN-45 treated cells cannot be reported as spontaneous. The cells were not pre-treated with any stimulator, like PMA, ionomycin, or both. The degranulation is therefore mainly assumed to be caused by CPZEN-45. Having said that, it is only logical to further assume that higher concentrations of the drug would result in even higher enzyme expression level. Nevertheless, the results give an opposite enzyme release pattern. In fact, the degranulation is higher in cells treated with CPZEN-45 concentration in the range of 0.5 – 100 µg/ml, compared to cells treated with higher drug concentration. This phenomenon could be explained by the fact that the cells have already been stimulated before the addition of CPZEN-45. Such assumption gives another interpretation of the results. Having another source of degranulation stimuli gives CPZEN-45 an anti-inflammatory effect on the HMC-1 cells, which is more pronounced in higher drug concentrations. This statement is preliminarily counteracted due to low values of degranulation in the negative control. However, more studies have to be performed in order to ascertain the findings.

5.3.2 Other parameters affecting β-hexosaminidase release from HMC-1 cells

Although the role of β-hexosaminidase remains partly understood, the enzyme is commonly used as a biomarker in mast cell degranulation (20). The β-hexosaminidase assay is an

inexpensive and straight forward method. Still, establishing the right assay for the CPZEN-45 experiment was not as straight forward as anticipated.

The difficulties faced in this assay were related to no colour change in the samples. Because this is a colorimetric assay, colour change is essential. The colour change occurs due to the β-hexosaminidase level in the supernatant. Higher levels of the enzyme result in sharper colour. Therefore, parameters like cell density, cell viability, Triton-X100 concentration and the use of wrong substrate were questioned. After few repetitions, the error was discovered in the pH adjustment of glycine. Being a colorimetric assay, the right pH is essential for colour change.

Further, an explanation of the high β-hexosaminidase release in CPZEN-45 samples might be due to cells being removed with supernatant prior to the addition of substrate (52). This would give higher absorbance reading compared to the actual enzyme level released from the cells. Such problem could be avoided by letting the seeded cells rest for few hours before initiating the experiment, also showing caution when working with the well plate afterwards, so no cells would be transferred to the next well plate, only the supernatant.

5.3.3 What are the consequences of the mast cell activation?

With findings suggesting that CPZEN-45 induces an activation of mast cells, possible consequences of degranulation must be looked into. Mast cells express TLRs such as TLR-9 and TLR-3. These receptors get activated by pathogens and result in the generation of several inflammatory mediators, i.e. TNF-α (54). When activated, mast cells have damaging effects on their surroundings in the vessel wall, which leads to matrix degradation and natural cell death, in addition to the enhancement in inflammatory cells (55). This will make the inflammation greater, and harder to control.

TB patients have already an inflammation caused by Mtb. Treating patients with a drug that induces more inflammation does not appear appropriate. On the other hand, the magnitude of this effect CPZEN-45 has on mast cells is not determined. Furthermore, findings suggest that CPZEN-45 has an anti-inflammatory effect in macrophages. Therefore, further studies are required to gain more knowledge about of the drug *in vivo*.

5.3.4 Other mediators to measure the level of degranulation in HMC-1 cell?

Besides β-hexosaminidase, pro-inflammatory cytokines like histamine are widely used as a degranulation mediator (56). Histamine is known as the predominant mediator of acute reactions in mast cells (57). Quantifying histamine release in mast cells requires an ELISA method, which is usually far more expensive than a simple β-hexosaminidase assay. After completing the β-hexosaminidase assay, a histamine ELISA might however be run to confirm the results.

5.4 *In vitro* systems

The THP-1 and HMC-1 are immortalised cell lines used as *in vitro* models to investigate the effect of CPZEN-45 on inflammatory markers. In general, *in vitro* cell modelling is well acknowledged as it offers a higher and quicker throughput possibility compared to *in vivo* testing (58).

THP-1 and HMC-1 cells were chosen to in this study to investigate CPZEN-45 effect in human immune cells. These cell lines were chosen due to their ability to mimic properties in human cell lines, and give a closer approach of the drug's effect.

THP-1 cells

When compared to other human bone marrow cell lines, the behaviour of differentiated THP-1 cells are the closest to the indigenous monocyte-derived macrophages (59). In fact, the THP-1 cell line is the most used model in monocytes/macrophages studies (60). THP-1 cells are therefore useful in studying mechanisms involving macrophage differentiation.

HMC-1 cells

HMC-1 cells are the only established cell line to provide a similar phenotype to the one in human mast cells, compared to other models for mast cells, i.e. the LAD2 cell line (21). HMC-1 expresses several biomarkers related to mast cells such as histamine and β-hexosaminidase. A study carried out to compare the HMC-1 to mature skin mast cells with respect to the biomarker release, showed that HMC-1 represent immature malignantly transformed mast cells (61).

Immortalised cell lines for in vitro studies

It is well established that *in vitro* systems are unable to imitate complex disease conditions. Experiencing inflammation, for instance, the inflamed tissues under steady state shelter several signals – lipids, proteins, lipo-proteins, etc. Inflammation is a dynamic process of sequentially-released mediators secreted by diverse amount of cells, and can be hard to predict (62). Having said that, immortalised cell lines provide an extremely powerful tool in *in vitro* experimenting. In general, such cell models are far more accessible in research than primary human cells. However, the function and cellular differentiation of immortalised cells

are significantly influenced by the cell culture conditions, and thus, it is essential to show caution when changing any cell culture parameters (63).

5.5 CPZEN-45 project, what's next?

The CPZEN-45 is a promising anti-TB drug candidate, suggesting a pulmonary route of administration. CPZEN-45 is still in the pre-clinical phase, current research is in progress around the world to characterise and drive the drug candidate to the next stage. In fact, researchers at MCRF and Meiji Seika Kaisa, Ltd of Japan, are working on the animal studies of CPZEN-45, which are performed on TB infected mice to investigate the potential of the drug *in vivo* (42). In the United States, researchers at Research Triangle Institute International are currently working on the development of the right pulmonary drug formulation for CPZEN-45. No papers have yet been published on this research, as it is still ongoing.

As mentioned earlier, further studies must be carried out to ascertain the findings on the CPZEN-45 on the selected immune cells, THP-1 and HMC-1. More ELISAs are needed, with longer incubation time and constant concentration of LPS, to determine the effect of the drug on macrophages. More β-hexosaminidase assays are needed to gain further knowledge of the role of CPZEN-45 in modulating degranulation processes. Moreover, it could be more advantageous to quantify other inflammation mediators in these cells, i.e. histamine.

5.6 What does this study add to the field of TB treatment?

CPZEN-45 is a promising novel anti-TB drug candidate, only a few studies have been performed on the compound, and only two papers, covering main characterisation of the drug, published. This kind of study has not been performed earlier, and aimed at investigating the effect of CPZEN-45 on immune cells. This study contributes in the characterisation of CPZEN-45, so the compound could pass the pre-clinical phase.

6 Conclusions

The effect of CPZEN-45 has been investigated with respect to its ability to modify the inflammation marker release in THP-1 and HMC-1 cells. The ELISA suggested that CPZEN-45 does not cause release of TNF-α from macrophages *in vitro*. In fact, the drug seemed to have an anti-inflammatory effect as LPS-mediated TNF-α release was inhibited by increasing concentrations of CPZEN-45. On the other hand, a β-hexosaminidase assay suggested that CPZEN-45 activated mast cells to a degranulation process. Yet, more studies are required to confirm the role of CPZEN-45 in modifying inflammation processes. However, CPZEN-45 is a promising anti-TB drug candidate suggesting a pulmonary drug administration.

7 TB treatment and future perspectives

Although less common in Europe and North America, TB is still considered public health concern responsible for huge economic burden and high prevalence in Africa, Asia and South America (64). Efforts have been established to defeat TB, and the search for new drug candidates is ongoing. The discoveries has been excruciatingly slow, as the most recent anti-TB drugs back to 50 years (35). Although vastly researches, the pulmonary drug delivery face difficulties that are hard to overcome, and no inhalation treatment for TB has made it to the marked yet.

Nevertheless, for the first time in decades, a list of new promising drug candidates for the treatment of TB is in development, of which inhalation therapeutics (65). Moreover, increased knowledge around TB and Mtb gives the hope of finding new drug targets and strategies.

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9 Appendix

Appendix 1: Cytotoxicity test

Alamar Blue - ASSAY

Materials needed:

- Alamar blue solution
- Working solution alamar blue in KRB (1:10 dilution)
- KRB
- Triton-X100 and PQ
- Pipette tips and micro-pipette (yellow=10-200µL)
- Multi-step pipette and tips
- Pasteur pipette and vacuum pump
- Tissue/gloves etc.

1. Pre-warm incubator/water bath to 37°C.
2. Prepare KRB.
3. Prepare serial dilution of controls and treatments, pre-warm to 37°C.
4. Aliquot KRB in 50 ml tubes and place in water bath (37°C) or on ice.
5. Carefully aspirate the medium (without disturbing the monolayer) using a Pasteur pipette and vacuum pump.
6. Wash cells twice with 200µl pre-warmed KRB/well. Use Multi-step pipette and shake gently for a few seconds. Aspirate KRB as described above.
7. Add 200uL of controls and treatments (according to scheme)

(+) control: Triton-X100.

(-) control: KRB.

Background: KRB only (do not add alamar Blue).

Table 9.1 CPZEN-45 dilutions

CPZEN-45 Concentrations (µg/ml)	Volume of SS. (µl)	Volume of KRB (µl)
0.5	0.25	999.75
1	0.5	999.5
5	2.5	997.5
10	5	995
50	25	975
100	50	950
250	125	875
500	250	750
750	375	625
1000	500	500
2000	1000	0

96 well plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.5	0.5	0.5	0.5	0.5	750	750	750	750	750		
B	1	1	1	1	1	1000	1000	1000	1000	1000		
C	5	5	5	5	5	2000	2000	2000	2000	2000		
D	10	10	10	10	10	Background						
E	50	50	50	50	50	KRB						
F	100	100	100	100	100							
G	250	250	250	250	250	Triton-X100						
H	500	500	500	500	500							

8. Incubate for 4 h on 37°C.
9. Aspirate the serial dilutions, add 100 µl of alamar blue working solution in KRB.
10. Incubate for 3 h on 37°C and protected from light (for cell densities below 10^5 cells/ml incubate for 3-4 h).

11. Read fluorescence using a fluorescence excitation wavelength of 540–570 nm (peak excitation is 570 nm). Read fluorescence emission at 580–610 nm (peak emission is 585 nm).

Appendix 2: Sterility testing

When possible contamination is suspected:

1. Add 2,06 g of LB Broth to 100 ml of deionised water in a glass bottle and with the blue cap loosely on place in autoclave.
2. Add 100 ml of deionized water to another glass bottle with blue cap. This will serve as the negative control.
3. Autoclave samples for 20 min.
4. Remove 1 ml of samples tested and add to 9 ml of sterilised LB Broth.
5. Incubate overnight.
6. Investigate the samples under the microscope for possible contamination.

Appendix 3: Thawing cells

Thawing/ defrosting cells

Note

- Thawing cells must be done rapidly.
- Dilute thawed lysate slowly using pre warmed medium.
- Plate thawed lysate at high density to optimise recovery.
- Always use proper aseptic techniques, and work in a laminar fume hood.
- Some freezing media contain DMSO, which is known to facilitate the entry of organic molecules into tissue.
- Wear PPE.

General protocol

1. Prepare new medium
2. Remove the cryovial containing the frozen cells from the liquid nitrogen storage and immediately place it into 37°C water bath.
3. Quickly thaw cells (around 2 min) by quickly swirling the vial in the water bath until there is a small bit of ice left.
4. Transfer the vial into the fume hood and clean.
5. Transfer the content to a 15 ml tube.
6. Add 1 ml of pre warmed medium dropwise to the tube; dilution 1:1.
7. Add further 3 ml of medium (total 5 ml).
8. Centrifuge a 900rpm for 5 min.
9. After centrifugation, check the clarity of supernatant and visibility of a complete pellet.
10. Aseptically remove the supernatant without disturbing the cell pellet.
11. Gently, re suspend the cells in 10 ml of medium and homogenize
12. Add 5 ml of medium to culture vessel.
13. Transfer 10 ml of cell suspension to culture vessel, and incubate overnight.

Appendix 4: Freezing cells

1. Count the cells.
2. Centrifuge the cells for 5 min at 1.000 rpm.
3. Carefully remove the supernatant by aspirating.
4. Re-suspend the cell pellet in freezing medium. Note: add first ml of medium very carefully, drop by drop. Amount of added medium depends on your cell count and on how much cells you want to have per vial, e.g. you have 15 Mio. cells and want to have 5 Mio. cell per vial, then you have to add 4.5 ml freezing medium (1.5 ml/vial).
5. Aliquot the cell suspension to cryovials (1.5 ml/vial).
6. Put the vials to -20°C until frozen (2-3 h).
7. Transfer cell vials to -80°C overnight.
8. Put the cell vials into liquid nitrogen tank.

Composition of the freezing medium:

"normal" medium for the relevant cell line, but with 20% FBS, 10 % DMSO (sterile filtered) and preferably without antibiotics.