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Umashankar Rao, Komal

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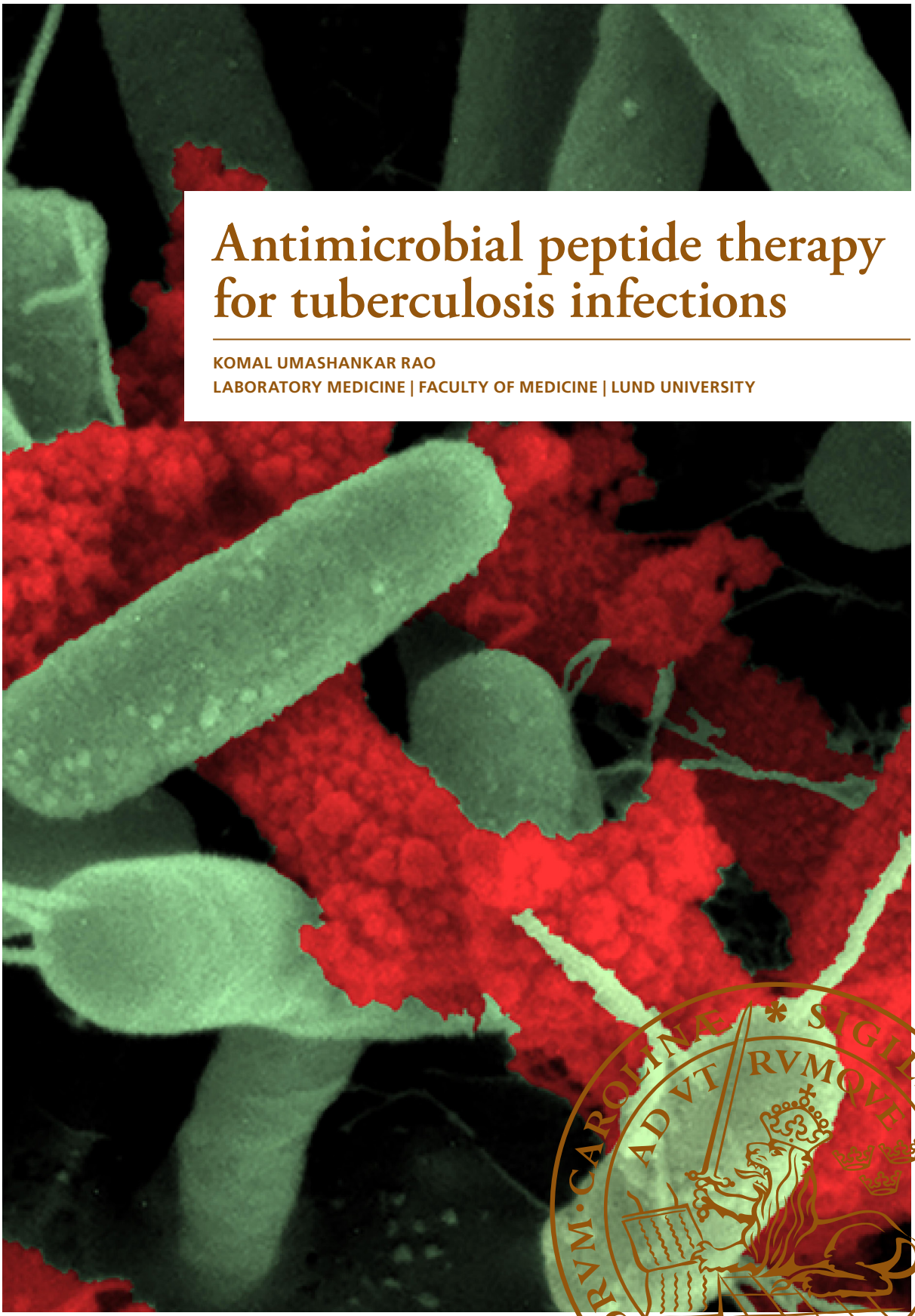

Antimicrobial peptide therapy for tuberculosis infections

KOMAL UMASHANKAR RAO
LABORATORY MEDICINE | FACULTY OF MEDICINE | LUND UNIVERSITY

KOMAL UMASHANKAR RAO, originally hailing from India, obtained her bachelor's degree in biotechnology from VTU university in Bangalore. Her academic passion has centered on microbiology and molecular biology. In 2016, she relocated to Sweden, where she pursued a master's degree in microbiology. Presently, she is actively working towards a Ph.D. in medical microbiology, specializing in tuberculosis at Lund University. She is genuinely fascinated by the profound impact these miniscule, imperceptible organisms can have on human health.

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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 6th of December at 09:00 in Segerfalksalen Hall, Biomedical Center, Lund, Sweden.

Faculty opponent
Professor Delia Goletti
Head of the Translational Research Unit at the
National Institute for Infectious Diseases L. Spallanzani, Rome, Italy

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Abstract:

Tuberculosis is a communicable disease that persists as a second leading cause for death, by an infectious agent. Several reasons contribute to this issue, of which an upsurge in antibiotic resistance is of top concern. Resistance patterns in the form of mono or multidrug resistance was reported to most clinical therapies at our disposal. This thesis aims to address this issue by studying a novel antimicrobial peptide named NZX as a potential drug candidate in the tuberculosis treatment regimen.

Mycobacterium tuberculosis, the tuberculosis pathogen is made up of a unique cell wall that renders it resistant to most compounds tested. We set out to identify the membrane interactive potential of NZX using artificial liposomes and live bacteria. The peptide appeared to interact with inner membrane of live mycobacteria by a pull and aggregate mechanism, eventually disrupting the cell integrity. A similar aggregation pattern was observed for liposomes as well as insertion into the membrane core was demonstrated. Antimicrobial peptides are known to possess multiactivity mode of mechanisms. To understand this, we investigated internal targets through a proteomics study. This led to the identification of essential protein targets such as chaperonins 60 kDa and elongation factor EF-Tu involved in bacterial growth and maintenance. Together, these findings displayed NZX's multifaceted activity against mycobacteria. The lack of mutants from resistance development studies for NZX could be asserted to their multiactivity feature.

Evidence of the therapeutic potential of NZX as an antimicrobial agent was explored. NZX displayed a wide range of activity when tested against a few clinically isolated nontuberculosis mycobacteria species and drug-resistant *Staphylococcus aureus*. The effect of drug-to-drug interactions were observed from an in vivo and in vitro standpoint and the peptide portrayed an additive effect with ethambutol, however, remained indifferent with other drug combinations. NZX retained its stability and antimicrobial property despite exposure to proteolytic molecules and human serum respectively. Directed therapy of NZX was performed by loading NZX onto nanoparticles and was found to be effectual for intracellular therapy. Moreover, nanoparticle loaded NZX exhibited better antimicrobial activity in primary macrophages.

The data presented here shows cumulative evidence on NZX as a prospective candidate against mycobacterial infections.

Key words: Tuberculosis, antimicrobial peptides, NZX, nanoparticles, novel therapy, mode of mechanism.

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Komal Umashankar Rao

LUND
UNIVERSITY

Komal Umashankar Rao
Division of Microbiology, Immunology and Glycobiology
Department of Laboratory Medicine, Lund University
komal.urao@gmail.com

Supervisor: Professor Gabriela Godaly
Department of Laboratory Medicine, Lund University
gabriela.godaly@med.lu.se
Co-supervisor: Professor Erik Sturegård
Department of Translational Medicine, Lund University

Coverphoto: Effect of peptide (NZX) on *Mycobacteria tuberculosis*
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Division of Microbiology, Immunology and Glycobiology (MIG)
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To my three pillars of life
Ratna, Asha, and Jonatan

My view on research has been well said
by Calvin, replace life with research.

Life is like topography, Hobbes.
There are summits of happiness
and success, flat stretches of boring and
valleys of frustration and failure.

- Bill Watterson (Calvin and Hobbes)

Table of Contents

Abstract

Tuberculosis is a communicable disease that persists as a second leading cause for death, by an infectious agent. Several reasons contribute to this issue to this, of which an upsurge in antibiotic resistance is of top concern. Resistance patterns in the form of mono or multidrug resistance was reported to most clinical therapies at our disposal. This thesis aims to address this issue by studying a novel antimicrobial peptide named NZX as a potential drug candidate in the tuberculosis treatment regimen.

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The data presented here shows cumulative evidence on NZX as a prospective candidate against mycobacterial infections.

Popular summary

> *Jonathan knew that I was soon going to die. I think everyone knew except me. They knew at school too, because I was away most of the time, coughing and always being ill. For the last six months, I haven't been able to go to school at all.*
>
> - A passage from Astrid Lindgren's 'The Brothers Lionheart'

Tuberculosis, a disease affecting mankind for many centuries, typically succumb to their death bed. Infected individuals suffered from symptoms that included, persistent cough, fever, night sweats, loss of weight and spitting up blood which lasted for months. The only cure recommended was complete bed rest with little to no physical movement. However, discovery of the very first antibiotic Streptomycin in 1943 radically changed the outcome of this disease. The advance in treatment seen was short-lived due to the emergence of antibiotic resistance, developed due to the use of single drug therapy. Soon the importance of multidrug therapy was understood and adapted, beginning the era of curable tuberculosis.

Globally, about 1.6 million have been reported to have died from tuberculosis in 2021. It has been noted as the second leading cause of death from an infectious agent. The spread of the causative agent, *Mycobacterium tuberculosis* (mycobacteria) is human-to-human. Classically, the disease manifestation is primarily lung infections or disseminated infections (when mycobacteria spreads to other parts of the body). The standard treatment followed for drug sensitive mycobacteria has been a combination of four drugs, rifampicin, ethambutol, isoniazid, and pyrazinamide for a course of six months. In 2022, a new conditional guideline from World Health Organization recommends use of isoniazid, rifapentine, moxifloxacin and pyrazinamide for 4 months. Even though therapeutic advances have occurred we continue to face threat from drug resistant mycobacteria. Additionally, a growing cause for concern is due to drug resistance reported to all drugs prescribed so far in tuberculosis treatment.

This thesis encompasses a detailed study on a potential novel tuberculosis drug. The compound we have been studying is a short peptide that was derived and modified from a fungus named *Pseudoplectania nigrella*. This short peptide named NZX is a type of antimicrobial peptide which is made up of 40 amino acids in length and characteristically it is negatively charged as well as hydrophobic in nature. Antimicrobial peptides are a group of short peptides that are part of the immune system of many different organisms. During a screening assay NZX was discovered to possess an antimycobacterial property. Furthermore, NZX was proven to be non-toxic to human cells and it survived immediate breakdown from proteolytic enzymes, which it may encounter when administered. These findings led to further important studies during this thesis wherein we explored the mechanistic and therapeutic facets of NZX.

As mentioned above tuberculosis treatment requires a multidrug therapy, to meet this criteria NZX was studied for its drug-drug interaction with currently used tuberculosis drugs. These drug interaction studies revealed absence of negative effects between drug combination, in fact the combined therapy of NZX and ethambutol enhanced their bactericidal property.

Next, NZX was evaluated for its therapeutic potential through some studies, to begin with stability of NZX was analyzed after dosing. Animal studies assessed the half-life of NZX, half-life can be defined as the time taken for any compound to reach half of its concentration in the blood from the time of administration. This consequently implies that the activity of the drug is decreased by 50 %. To tackle this problem and to improve cell specific targeting, NZX was loaded onto a porous silica nanoparticle. Using nanoparticle for delivering NZX not only improved bacterial death inside cells it also allowed for slow release of NZX. These findings proves that NZX could survive degradation and sustained release may reduce dosing frequency in patients. Finally, we tried to study the possibility of resistance development to NZX, as this is considered a prominent issue within tuberculosis treatment. This was investigated by attempting to produce spontaneous mutants in mycobacteria through constant exposure to NZX at conditions that might force resistance development. On the contrary, no mutants were reported from these studies, giving us some hope for an effective treatment.

Interestingly, antimicrobial peptides possess broad range applicability, which means that they can target different types of bacteria and exhibit their antimicrobial property. NZX displayed this characteristic feature against several mycobacterial species including some drug resistant Gram-positive bacteria. This broad range effect is particularly important because NZX could perhaps be used against other bacterial disease, like penicillin.

To understand the biological mechanism with which NZX targets *Mycobacterium tuberculosis*, a set of biophysical and molecular biology techniques were used. The importance of knowing NZX's mechanism of action is that it helps to identify potential toxic effects in mycobacteria and gain confidence in the molecule's ability to perform, hence improving the likelihood of a successful drug development. We discovered that NZX has a strong initial attraction towards the mycobacterial cell surface and this interaction led to bacterial cell death. The peptide was also found to target some essential pathways by binding to specific proteins present inside the cell. The cell death observed could be speculated to ensue from either one or both these processes.

Through these studies we gathered compelling data to support NZX as a potential novel antimicrobial peptide therapy against tuberculosis disease.

List of Publications

Papers included in this thesis

I. **Rao KU**, Li P, Welinder C, Tenland E, Gourdon P, Sturegård E, Ho JC, Godaly G. Mechanisms of a Mycobacterium tuberculosis Active Peptide. Pharmaceutics. 2023 Feb 6;15(2):540.

II. **Rao KU**, Henderson DI, Krishnan N, Puthia M, Glegola-Madejska I, Brive L, Bjarnemark F, Millqvist Fureby A, Hjort K, Andersson DI, Tenland E. A broad spectrum anti-bacterial peptide with an adjunct potential for tuberculosis chemotherapy. Scientific Reports. 2021 Feb 18;11(1):4201.

III. Tenland E, Pochert A, Krishnan N, **Umashankar Rao K**, Kalsum S, Braun K, Glegola-Madejska I, Lerm M, Robertson BD, Lindén M, Godaly G. Effective delivery of the anti-mycobacterial peptide NZX in mesoporous silica nanoparticles. PLoS One. 2019 Feb 26;14(2):e0212858.

Peer-reviewed papers outside this thesis

I. Grønberg C, Hu Q, Mahato DR, Longhin E, Salustros N, Duelli A, Lyu P, Bågenholm V, Eriksson J, **Rao KU**, Henderson DI. Structure and ion-release mechanism of PIB-4-type ATPases. Elife. 2021 Dec 24;10:e73124.

Book chapter

I. **Rao KU**, Godaly G. Isolation and Purification of Mycobacterial Extracellular Vesicles (EVs). InBacterial Pathogenesis: Methods and Protocols 2023 Jun 1 (pp. 55-60). New York, NY: Springer US.

Abbreviations

AcpM	Acyl carrier protein
AMs	Alveolar macrophages
AMK	Amikacin
AMPs	Antimicrobial peptides
BCG	Bacillus Calmette–Guérin
BPaLM	Bedaquiline, pretomanid, linezolid and moxifloxacin
CLRs	C-type lectins
CL	Cardiolipin
Cpn60	Chaperonins 60 proteins
COPD	Chronic obstructive pulmonary disease
CFU	Colony forming units
CR	Complement receptors
CF	Cystic fibrosis
DRAMP	Data repository of antimicrobial peptides
DCs	Dendritic cells
DOTS	Directly observed Therapy Shortcourse
DLS	Dynamic light scattering
ELISA	Enzyme linked immunosorbent assay
EMB	Ethambutol
NMV	Gram-negative vesicles
PMV	Gram-positive vesicles
H&E	Hematoxylin and eosin
HNP	Human neutrophil peptide
IFN- γ	Interferon gamma
IGRA	Interferon gamma release assay
INH	Isoniazid
LTBI	Latent tuberculosis infection
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
MHC	Major histocompatibility complex
MSPs	Mesoporous silica nanoparticles
MSRA	Methicillin-resistant *Staphylococcus aureus*
MICs	Minimum inhibitory concentrations
MBCs	Minimum bactericidal activity
MGIT	Mycobacterial growth Indicator tube
MABCS	*Mycobacterium abscessus* complex
MAC	*Mycobacterium avium* complex

Mtb	*Mycobacterium tuberculosis*
NPs	Nanoparticles
NTM	Nontuberculosis mycobacteria
NTM-PD	Nontuberculous mycobacterial pulmonary infections
PCR	Polymerase chain reaction
POPE	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
POPG	1-palmitoyl-2-oleoyl-sn-glycero-3[phospho-rac-(1-glycerol)
PS	Phosphatidylserine
PG	Phosphatidylglycerol
PBS	Phosphate buffered saline
PPD	Purified protein derivative
RGM	Rapid growing mycobacteria
REMA	Resazurin microtiter assay
RD1	Region of difference 1
SEM	Scanning electron microscopy
SGM	Slow growing mycobacteria
TLRs	Toll-like receptors
TEM	Transmission electron microscopy
TB	Tuberculosis
TST	Tuberculin skin test
TNF	Tumor necrosis factor
WHO	World Health Organization
WGS	Whole genome sequencing

Background and clinical aspects of Mycobacterial infections

Brief history on tuberculosis

This ancient disease is presumed to be as old as 150 million years, around the time of Gondwanaland, marking the end of the Jurassic period (1) . Records of evidence of tuberculosis (TB) from ancient times have been reported throughout history. Documentation as writings on papyri tombs from ancient Egypt to sculpture inscription from the Babylonian era, and description of a consumptive disease in the Vedic Indian literature are a few examples (2-6). The contagious nature of TB was first described by Girolamo Fracastoro in 1546 as 'invisible yet living and transmissible' disease. This laid a foundation for public health protection by isolating and treating the infected individuals in special infirmaries (7). French military surgeon Jean Antione Villemin was the first one to demonstrate the transmission of TB through a study involving blood samples from sick rabbits to infect other animals. The monumental discovery led him to propose that this disease is caused by a specific organism that must be present in the air (8).

> *– The phthisic soldier is to his roomates what a glandered horse is to its stablemates*
> - Jean Antione Villemin (9).

In 1882, a historic moment for research in TB; Robert Koch a German scientist visualized a tubercule with a special staining technique. He presented this in a lecture for the first time at the Physiological Society at the Charite Hospital in Berlin on the 24th of March 1882. He received the Nobel Prize in Medicine or Physiology in 1905 for his discovery of TB bacillus (10). Today, March 24th is remembered as World Tuberculosis Day. Prior to the discovery of antibiotics only treatment followed was isolation and complete bed rest of infected individuals. This concept was reinvented by Hermann Brehmer who suggested isolating the patient up in the mountains with lots of fresh air, sun, rest, and adequate nourishment. As a result, the rise of sanatoriums began. Some patients recovered miraculously for short terms, however, in the long term about 60 % of these patients died within 6 years, probably due to recurrence (9, 11).

Figure 1: TB patients being treated in open air by the river Thames, London UK.
A widely accepted treatment for TB before chemotherapy included resting in bed, good nutrition along with daily exposure to sunlight and fresh air. Reprinted from The Guardian, published August 2019. Reference (12).

This was followed by the era of chemotherapy that revitalized TB treatment with the discovery of Streptomycin. Soon it became evident that monotherapy led to failure in treatment and after many trials a triple therapy was studied (13). The emergence of resistance to these drugs led to the search for newer and better therapeutics which still seems to be a theme in the TB battle.

Mycobacterium tuberculosis

Aetiology

TB is a communicable disease spread through an airborne pathogen named *Mycobacterium tuberculosis* (Mtb). TB is present in two forms, active or latent state (LTBI). TB primarily causes pulmonary infections and about 15% are extrapulmonary infections (14). Mtb is related to a group of mycobacterial species called the *Mycobacterium tuberculosis* complex and the other members of this complex are *M bovis*, *M. africanum, M. canetti, M. mungi and M. microti*. They are known to be 99% related, indicating they belong to the same species but differ in pathogenicity (15-17). Formerly it was hypothesized that Mtb had branched out from *M. bovis,* a cattle infecting bacterium. However, this was disregarded later through single-nucleotide polymorphism studies suggesting both evolved at the

same time from *M. canetti* (16, 18). *M. bovis* infections in humans occurred occasionally through unpasteurized milk. *M. bovis* infections were commonly seen among people who suffered from comorbidities and were immunocompromised (19). Mycobacteria are described as rod shape, aerobic, catalase positive, non-motile and non-spore forming (20). Typically, they are known as acid-fast bacteria due to their unique cell wall composition composed of mycolic acids which renders them impermeable to most antibiotics (21, 22). Characteristically, mycobacteria have distinctive colony morphology (cloudy rough colonies) and slow doubling time (20). Generation time is estimated to be around 24 hours taking them up to 3-4 weeks to grow on an agar plate (23, 24).

Morphology of cell wall

The cell wall of mycobacteria is composed of four components: the inner membrane (IM), the peptidoglycan and arabinogalactan layer, the mycomembrane (MM) and the capsule /outer membrane (OM) (Figure 2). IM is mostly composed of glycerophospholipids such as phosphatidylethanolamine (PE) and cardiolipin (CL), phosphatidylinositol (PI), lipoarabinomannen (LAM), and phosphatidyl-myo-inositol mannosides (PIM) (25, 26). The peptidoglycan layer is similar to Gram-positive and Gram-negative bacteria and is made up of highly cross-linked repetitive disaccharide units which are interlinked covalently with arabinogalactan (27). The peptidoglycan layer is followed by MM, the characteristic mycolic acid layer. However, MM is a key component that identifies mycobacteria as this layer is important for identification via acid-fast staining, but also limits permeability to nutrients and antibiotics, and plays a role in virulence. It is further interlinked with free lipids that are important for survival (27, 28). The OM is composed of trehalose-based glycolipids, phenolic glycolipids (PGLs) phthiocerol dimycocerosate (PDIMs), lipomannan (LM), lipoarabinomannan (LAM) and sulfolipids (25, 28). PIMs, LM, LAM, PDIMs are recognized by specific receptors responsible for eliciting an immune response in the host cell (29).

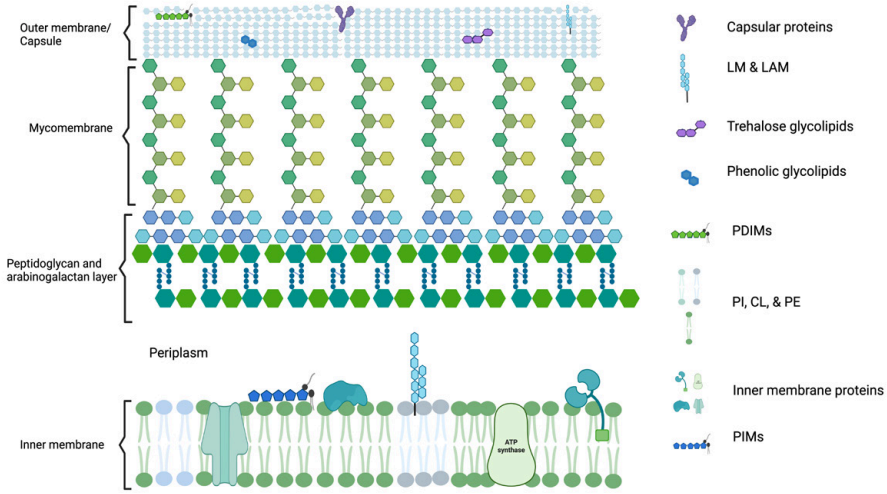

Figure 2: A brief schematic representation of the mycobacterial cell wall and its components. The mycobacterial cell wall is a complex wall composed of several lipids giving it the thick waxy coating. The different layers are (bottom to top), inner membrane, peptidoglycan & arabinogalactan layer, mycomembrane, and the capsule layer. Within these layers are some free or non-covalently linked lipids along with essential proteins. Figure was created using Biorender and adapted from references (30, 31). Created with BioRender.com

Epidemiology

The End TB Strategy, an initiative by WHO has targeted an ambitious deadline to End TB by 2020-2035. This target was well under way until Covid-19 pandemic emerged and derailed the progress made so far. WHO reported 10.6 million people fell ill from TB in 2021 an increase by 4.1 % from 2020 (14) (Figure 3). It is estimated that over 1/3rd of the world's population is infected and exist in LTBI form of which 5-10% of this population can develop TB during their lifetime, with the probability decreasing after the first two years after infection (32). The countries Bangladesh, India, China, Pakistan, Democratic Republic of Congo, Nigeria, and Philippines counts by WHO to be high burden and makes up to 87% of all TB cases. The mortality from TB infection rose by 0.1 million from 2019 to 2021 to 1.3 million deaths among HIV-negative individuals and 214 000 deaths among HIV positive (14). Region wise epidemiology of TB varies vastly. Incidence and mortality rates are high in the African subcontinent because of the high number of HIV infections. Correspondingly, the incidence rates are high in Southeast Asia due to factors such as poverty and undernourishment (33). The prevalence of drug resistance in TB adds to the burden of cases reported yearly. Drug resistance reported were estimated to be 450,000 cases in 2021 which was up by 3.1 % since 2020. This was probably due to the decline in treatment of drug-resistant TB during the COVID-19 pandemic (34).

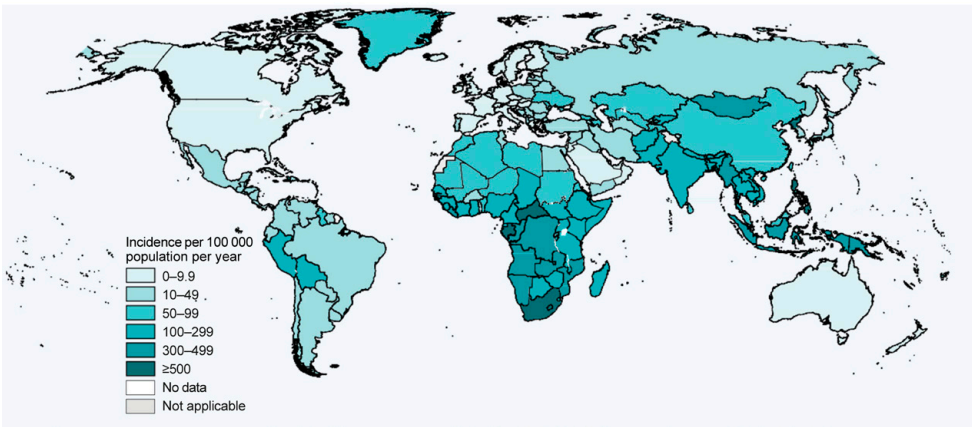

Figure 3: Estimated TB incidence rate from 2021.
Reprinted from Global tuberculosis report from 2022. Geneva: World Health Organization; 2022. Licence: CC BY-NC-SA 3.0 IGO. Reference (34).

Establishing an infection

Transmission

Over 90% of TB cases are transmitted through the inhalation of aerosol droplets and in rare cases via oral or placental routes (35, 36). Aerosols are formed through coughing, singing, sneezing, or talking (37). Transmission from sick individuals are enhanced by certain factors namely, smear-positive culture (high bioburden), proximity, poor UV levels and ventilation (38). Finally, host factors for example compromised immunity by HIV or malnutrition etc, culminate to increase the probability of spread (38, 39).

Mycobacterial uptake via aerosol

TB infections are acquired from breathing in aerosols produced by infected individuals (40). Close contact with sick patients advances to infect only a few subsets of individuals and this is influenced by a combination of bacterial load, bacterial lineages and host factors (41). Once inhaled, the pathogen enters alveolar space and encounters alveolar epithelial type II pneumocytes followed by alveolar macrophages (AMs) (42). Even though macrophages were believed to be the first cells that encounter the bacillus, studies have shown that mycobacterial interacts with epithelial cells, provokes uptake, replication and suppression of pro-inflammatory cytokines (43). Nevertheless, Mtb is an intracellular pathogen, and it resides in AMs, a hallmark of TB infection. Other phagocytic cells that take up Mtb are dendritic cells (DCs) and neutrophils. The predominance of Mtb within these cells changes over time, dependent on the stage of infection (44). Macrophages and neutrophils are further known to initiate innate immune responses by producing antimicrobial peptides that inhibit extracellular bacterial growth (45).

Macrophage and neutrophil intervention

Recognition and uptake of Mtb by AMs are mediated through a variety of pattern recognition receptors namely C-type lectins (CLRs), toll-like receptors (TLRs), Fc receptors, complement receptor (CR), scavenger receptor, surfactant protein and glycophosphatidylinositol- anchored membrane receptor (46-48). Upon uptake, bacteria are sequestered into phagosomes followed by fusion with lysosomes to form phagolysosomes. In the phagolysosome the bacteria confronts a harsh environment mediated by low pH and formation of reactive oxygen species (49). However, as a defence mechanism mycobacteria block phagosome formation (50, 51). AMs infected with Mtb replicate and progress to either apoptosis or necrosis during which macrophages mediate through cytokines namely, tumour necrosis factor (TNF) and interleukin (IL-12), resulting in recruitment of other immune cells (52).

The role of neutrophils in TB infections can be contradictory. Recognition of Mtb by neutrophils occurs through TLR2 and TLR4. Neutrophils contribute to bacterial killing through several processes. This includes phagocytosis, the release of human neutrophil peptides (HNP), chemotaxis, generation of reactive oxygen species, and neutrophil extracellular traps (53, 54). Neutrophils also cooperate with macrophages and DCs during TB infections and help by recruiting more immune cells via cytokine productions and promote antigen presentation respectively (54). On the contrary, the surplus in neutrophils has been associated with improved disease outcomes which can be due to the accumulation of toxic compounds from necrotic neutrophils (55).

Antigen presentation and T cell priming

Dendritic cells (DCs) are antigen presenting cells that present Mtb antigen to T cells via major histocompatibility complex (MHC) class I and class II molecules. They form a link between the innate and adaptive immune system. DCs are normally present in an immature state in tissues and the process of maturation is activated upon interaction with a foreign antigen (56). Interacting with Mtb antigen matures DCs cell to migrate to the draining lymph node and effectively activate T cells (57). In addition, AMs along with sheltering Mtb inside them serve as antigen presenting cells (58).

Delayed onset of T cell response

The onset of adaptive immunity is dependent on antigen presentation by dendritic cells in the lymph node (or AMs presentation by MHCs) and this is characteristically delayed in TB infection, a known virulence mechanism (59). The onset of adaptive immunity development has been estimated with the help of TB diagnostic analysis, tuberculin skin test (TST) which essentially measures CD4+ T lymphocyte reaction. Interference of antigen presentation has been reported through specific examples such as 19-kDa lipoprotein and lipid trehalose 6,6′-dimycolate present in the cell

membrane of Mtb. This has been reported to block antigen presentation (60, 61). If a good TB immune response is present, activated CD4+ T-cells differentiate into Th1 cells, Th17 and cytotoxic T effector cells. They relocate to the lungs where they encounter bacteria infested neutrophils, macrophages and DCs and participate in granuloma formation (62).

Consequences of Granuloma formation and maturation

The granuloma is an organized structure that is produced as a consequence of the immune response during TB infections. It encompasses engulfed Mtb surrounded by different immune cells. The formation of granuloma is a characteristic feature of intracellular pathogens orchestrated by the expression of pro- and anti-inflammatory cytokine. At the core, macrophages infected with bacteria are found and surrounded by recruited monocyte-macrophages that undergo epithelioid cell differentiation. The tight network of aggregated epithelioid cells is characteristic to TB granuloma. These epithelioid cells can be further surrounded by multinucleated giant cells. Macrophages also transform into foamy cells that contain lipid droplets. The cascade effect of pro-inflammatory cytokines leads to recruitment of other cells that contribute to the structure of granuloma including neutrophils, DCs, natural killer cells, T-cells and B-cells, and fibroblasts (63-66). Classically, granulomas formed during TB infections are either necrotic (active) with a typical caseous cheese-like centre containing high bioburden of bacteria or a calcified granuloma (inactive), representing containment of bacteria (63). A third structure has also been mentioned with respect to TB granulomas, called the solid granuloma, that lacked necrosis (67) (Figure 4). Heterogeneity in granulomas is found within the same lung tissue which unfortunately leads to problems during clinical treatment (63).

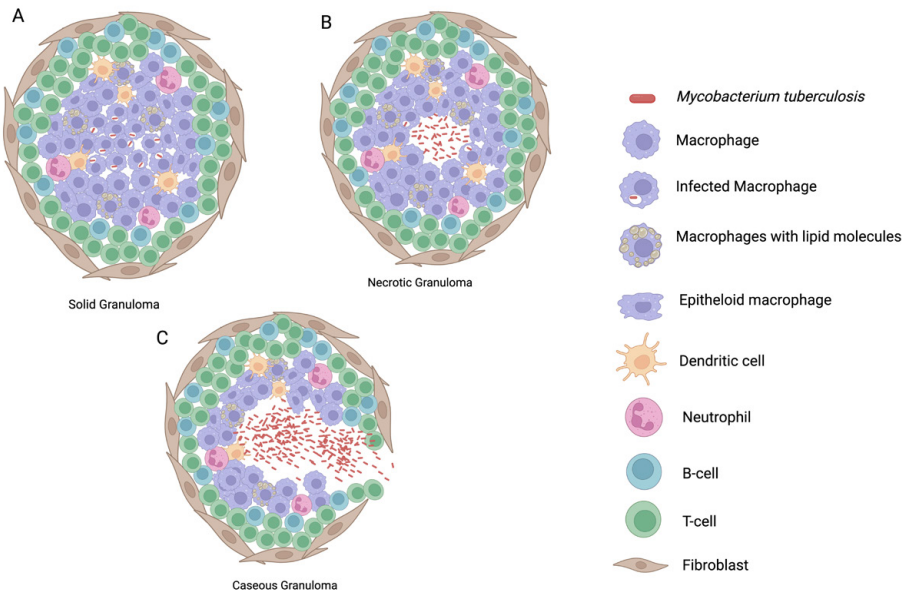

Figure 4: Stages of granuloma formation and progression in tuberculosis.
The figure represents the three distinctive stages in granuloma formation seen during TB infection. A. Solid granuloma: this the first stage wherein the bacteria is still contained within the macrophages. B. Necrotic granuloma: in this stage some of the macrophages undergo necrosis and bacteria are now extracellular in the central core, but they are contained in by other immune cells within the granuloma structure. C. Caseous granuloma: this stage is the active infection stage when the bacteria have multiplied exponentially, and the granuloma structure has rupture to spread the bacillary load. Figure created using Biorender and adapted from references (65, 68). Created with BioRender.com

Diagnosis

Standard immunological diagnostic techniques

Tuberculin skin test (TST) or Mantoux skin test is based on purified protein derivative (PPD) administered intradermally at 2 tuberculin units to test for type IV delayed hypersensitivity (69, 70). The test result should be read between 48-72 hours post administration (70). The test is considered positive if the induration is 5 mm or larger and gives a false positive result in vaccinated individuals (69). The main disadvantage of the PPD test is that the proteins are not specific to Mtb. For example, vaccinated individuals or exposure to NTM will elicit a positive reaction (71, 72).

Interferon-gamma (IFN-γ) *release assay* (IGRA) is an *ex vivo* analysis of IFN-γ levels released in response to Mtb antigens by T-cells in whole blood (69). QuantiFERON-TB Gold IN-Tube assay and the T-SPOT.TB assays are two commonly used assays for TB diagnostics. The analysis is based on enzyme-linked immunosorbent assay

(ELISA) which uses region of difference 1 (RD1) antigens to sensitize T-cells. The advantage of this assay over TST is its specificity to Mtb with no chance of a false positive result with respect to strain ambiguity seen in TST (73, 74). However, one drawback of IGRA testing lies in the low mitogen response among certain high risk TB populations with an indeterminate IGRA between 2% - 11% (75).

Currently, these are the only analyses that can be used to test for LTBI. New modifications to the IGRA test have shown potential in identifying incipient TB (71, 74).

Conventional and new microbiological diagnostic techniques

Sputum smear test performed with Ziehl-Nielsen stain remains one of the cornerstones of TB diagnostic methods. This low-cost method is important in low income countries because of its quick and simple (76). The specificity of the stains is due to acidic mycolic chain in the cell membrane which persists even when bacteria is dead. Sensitivity to the experiment is considered moderate since the samples can be affected by the Mtb concentration of the sputum. Fluorescence microscopy with fluorochrome dyes was introduced to improve the smear test analysis with better staining dyes that improve on time and sensitivity. Sensitivity was 10% higher than conventional methods (76, 77).

Culturing mycobacterium has been the gold standard in TB diagnosis. The two main advantages it provides are high sensitivity and drug susceptibility testing. The disadvantage of this analysis lies in the time taken to get results is between 4-6 weeks which is a challenge in clinical settings (69, 78). However, the development of Mycobacteria Growth Indicator Tube (MGIT) has provided an alternative to bacterial growth on plates. These automated liquid culture tubes are used to obtain rapid results for the initial diagnosis of pulmonary and extrapulmonary TB (79).

Gene Xpert MTB/RIF is a molecular method developed for TB diagnostics. It utilizes real time polymerase chain reaction (PCR) technology that uses single-use cartridges that are pe-prepared to process and identify Mtb and resistant Mtb. The gene of interest that is amplified is the *rpoB* gene which detects both Mtb strain and rifampicin (RIF) resistant strain (80). After detecting a few false-positive results from some studies for RIF resistance, the WHO used to recommend combining conventional antibiotic resistance testing along with gene amplification (81).

The latest method in molecular techniques that has been incorporated as a diagnostic tool is whole genome sequencing (WGS). WGS provides an advantage over many of the older methods due to its specificity and the ability to catch mutations for different drugs as well as mutations occurring outside of target regions (82, 83).

Imaging diagnostic techniques

It is one of the tools that has been used since the discovery of X-ray in 1895 by physicians to examine organs (84). Today developments in imaging have improved

TB diagnosis, the methods used are magnetic resonance imaging, positron emission tomography, computed tomography, and radiography (85, 86). These techniques can be used in diagnosis of both pulmonary and extrapulmonary TB (86).

Resistance

Drug resistance is defined as the capability of bacteria to survive in the presence of a drug that ordinarily could kill it. Resistance in mycobacteria is acquired through chromosomal mutations (87). However, studies have begun to also recognize its intrinsic resistance to drugs observed due to its unique cell wall and additionally efflux mechanisms (88). Some of the common resistance patterns reported for first-line TB drugs are, RIF, which targets β-subunit of RNA polymerase is affected by mutation in *rpoB* gene. This reduces the drug's affinity by inducing conformational changes (88). Resistance to isoniazid (INH) is seen as mutations generated in different genes but the most prominent ones reported are *inhA* and *katG*. INH usually acts by interrupting mycolic acid production (88). Pyrazinamide (PZA), a pro-drug is responsible for disrupting membrane transport. Activation of the drug is by an enzyme named pyrazinamidase produced by Mtb and resistance is acquired by mutation to the *pncA* gene which produces this enzyme (88). Ethambutol (EMB) interferes with the transfer of arabinogalactan, a component of the cell wall by causing mutation in *embB* therefore leading to morphological changes (88). Similar mutation-based resistance is acquired for most TB drugs used in practice (89).

Reasons for resistance development involve two facets, bacterial properties and human interventions (87). Bacterial properties such as diverse sensitivity within the same sputum sample from a study revealed the existence of heteroresistance, indicative of different subpopulations, probably from selective pressure (90). The rate of spontaneous mutation is linked to the lineage of the strain and higher risk of drug resistance in those infected with species displaying higher mutation rate (91). Mutations in *rpoB* or *inhA* have been associated with cross-resistance i.e., resistance acquired for one drug can influences resistance development for another (92). Human interventions that increase the risk for mutations are related to errors by physicians made during the prescription of drug regimens and poor adherence to treatment by patients (93). Resistance within TB disease is classified into six categories as coined by WHO (Table 1).

Table 1: Classification of TB drug resistance (94, 95).

Types	Description
Mono-resistance	Resistance to any one of the first-line drugs
Multidrug resistance (MDR)	Resistance to INH and RIF
Poly-resistance	MDR and any other first-line drug
Pre-extensively drug-resistant TB (pre-XDR TB)	Resistance to RIF maybe INH and one fluoroquinolone (levofloxacin/ moxifloxacin)
Extensive drug resistance (XDR)	Resistance to fluoroquinolones and one of second-line injectable drugs
Rifampicin resistance (RR)	Resistance to RIF and/or any, first /second-line drugs

Treatment

The course of treatment in case of drug susceptible TB is standard four drug therapy consisting of RIF, EMB, INH and PZA for the first two months, the induction phase. The consolidation phase is the following four months with rifampicin and isoniazid to eliminate any persister Mtb strains (96). This treatment is lengthy and evidence of hepatotoxicity, gastrointestinal and neurological disorders and allergies has been reported (97). Regardless this treatment regime is necessary to prevent acquired drug resistance and improve efficacy (98). Globally the success rate for treatment in 2020 was reported as 86 % (34). This progress is also owed to Directly Observed Therapy Shortcourse (DOTS), an initiative established by WHO to improve treatment outcomes by monitoring the administration of drugs by a healthcare personnel at a clinic. (99, 100).

Drug resistance in TB treatment is challenging due to the difficulty of using which a standard treatment regimen. WHO classifies TB resistance profiles into five categories (Table 1) (101). To effectively treat various resistance profiles, several medications need to be administered with the right balance between combinations and durations. An update from WHO guidelines from 2020 recommendation suggests the use of an all-oral bedaquiline, pretomanid, moxifloxacin and linezolid (BPaLM) regimen for six months for MDR/RR-TB. BPaLM only for pre-XDR for six months and an individualized 18-month program if failed. The data used in consolidating this report comes from 55 different studies collected across 38 countries (101, 102). The success rate for RR-TB treatment is reported to be reaching 60% globally, while XDR-TB and pre-XDR-TB is regrettably low (34).

Prevention

Bacille Calmette-Guérin (BCG) vaccine is the only vaccine that is used in TB prevention and was developed over 100 years ago. In 1900, Albert Calmette and Camille Guérin started their research in finding a vaccine towards TB infection.

They created an attenuated strain from the virulent bovine strain by subculturing 230 times, this was over a period of 13 years. The BCG vaccine was administered to guinea pigs, rabbits, cattle, and horses and did not elicit an active disease (103). By 1921, they started testing this attenuated strain on humans. The first patient to receive this vaccine was an infant who was administered orally(103). The effectiveness of the BCG vaccine in protection from pulmonary TB is contradictory. It has been documented to help prevent severe forms of TB in children, but it is not effective against adult/adolescent TB (104). Studies have shown the effectiveness ranges between 0-80% (104). The reason behind this heterogeneity is suggested to depend on for example sensitization of surrounding mycobacteria and the time of immunization. A recent systematic review from 2022 analyzed 26 cohort studies from high burden countries and concluded that the BCG vaccine is good in protecting children below five years of age from any form of TB (104). To reach the goal of ending TB by WHO, prevention is one of the strongest strategies and to attain this goal efforts in discovering new vaccines is imperative. As of September 2022, 16 candidates are in various stages of clinical trials (34).

Nontuberculous mycobacteria

Brief background

Non-tuberculosis mycobacteria (NTM) or atypical mycobacteria are a group of saprophytes that cause pulmonary and other disseminated diseases in humans (105). Historically, NTM infections drew attention only in the 1940s. The susceptibility to these infections among healthy individuals is low. Conversely, predominant in individuals with low immunity from lung diseases or immunosuppression (106). Some known comorbidities that are associated with NTM infections include cystic fibrosis (CF), bronchiectasis, previous pulmonary tuberculosis, lung cancer, chronic obstructive pulmonary disease (COPD) and pulmonary immune deficiency syndromes (107). NTM infections clinically manifest in five forms, nontuberculous mycobacterial pulmonary infections (NTM-PD), lymph node infections (lymphadenitis), skin and soft tissue infections, bone and joint infections and finally disseminated infections (108).

NTM are omnipresent opportunistic bacteria found in soil and water with about 175 species identified so far (109). They are categorized into two main groups based on their growth rate, slow-sgrowing mycobacteria (SGM) and rapid growing mycobacteria (RGM). They are commonly found in water distribution systems at homes such as showers and hot tubs. Naturally they form biofilms, this feature serves as a vantage point by protecting them from disinfectants and antibiotics (110). Not all NTM species are known to cause infections in humans, but the ones reported are *M. avium* complex (MAC), *M. abscessus* complex (MABCS), *M. kansasii, M. chelonae, M. xenopi, M. malmoense, M. lentiflavum, M. marinum, M. ulcerans, M. haemophilum, M. sulgai,* and *M. fortuitum* (16, 105, 106, 108, 111).

They can grow both on liquid and solid media with varied growth rate which is dependent on the species (111).

NTM Infection epidemiology

In contrast to TB epidemiology, NTM infection rates are poorly reported mainly because of two reasons: lack of awareness among health care professionals and inadequate diagnostic tools. In some situations, these infections are overlooked as drug-resistant TB (112, 113). Additionally, NTM infections are not required to be reported in many countries. However, in the past decade the rise in incidence rate is attributed to improvements in the shortcomings in diagnosis. Ascribing to their ubiquitous nature isolation from a patient is common but corresponding this to clinical infection is still hard to conclude (111, 114). A study from India highlights precisely this issue where about 83 % of the patients from the sample pool were misdiagnosed (115). NTM infections are famously known to be an underlying disease in association with other respiratory diseases, such as CF. A study from the US in CF patients had co-infection with 48.2% by MAC and 25.5% by *M. abscessus* (116). Even though MAC is predominant in most countries, species specific prevalence varies across countries. This variance is also observed in ethnic subgroups (117, 118). Similarly, susceptibility among men and women varies, but bias towards one gender is prevalent in specific regions, probably due to the presence of region-specific species (118, 119). In a systematic review of global trend of NTM infections, the main findings were the increased infection rates. This was suspected to originate from better laboratory techniques such as DNA-based identification and larger prevalence of chronic lung infections. Another finding reported a decrease in TB burden in some areas have contributed to rise in NTM infections (114). A study from 2014 reported data from five European countries (UK, France, Spain, Italy and Germany) and concluded that 75% of patients were male with pulmonary infections mostly associated with smoking (120). Among them 33% of them were diagnosed with COPD and MAC being the causative agent (120). NTM-PD was more prevalent among male above the age of 60 as reported in a study from France with data collected over 8 years (2010-2017). Several comorbidities such as cystic fibrosis, history of TB, pneumonia, malnutrition and HIV were the few with higher incidence rates compared to controls that increased the risk of morbidity in these patients (121) Overall, the epidemiological reports from global settings fluctuate and no one trend seems to apply to all countries or regions. Timely and accurate identification of NTM infections can help in reducing the infection rates. Being aware of risk groups and proper reporting will aid in monitoring the current situation and may help in tackling these infections.

NTM treatment

Several guidelines exist for treating NTM infections. The treatment plan is dependent on the type of strain causing infections, where the infection is located and NTM susceptibility to antibiotics (122). NTM-PD, the most common reported

infection, has an initial phase of a 3-4 antibiotic treatment regimen up to a duration of 12 months where the continuity is based on sputum data. The drugs commonly used in treating NTM-PD involve INH, RIF, EMB, macrolides (clarithromycin or azithromycin), moxifloxacin and amikacin that are administered either orally, intravenously, or nebulized (123). Macrolides are the most widely used antibiotics in most NTM infections. Other forms of NTM manifestations are addressed individually with respect to duration, choice of antibiotic and the treatment action is based on the severity of symptoms (123). Alternatively, treatments using non-pharmaceutical therapy is also applied in NTM treatment along with surgical interventions in extreme cases (18).

Antimicrobial peptides

Background

Antimicrobial peptides (AMPs) are naturally existing small protein molecules with a typical size ranging between 5-50 amino acids (124). They are mostly composed of amino acids with cationic and hydrophobic properties. Briefly, they are crucial in the innate immune system of any organism and are known to possess antimicrobial properties against bacteria, viruses, fungi, and parasites. Additionally, some AMPs exhibit anti-carcinogenic, angiogenetic, and wound healing properties making them very versatile (124-129). Although the actual discovery and understanding of AMPs was made late in the 20th century, the first mention of antimicrobial-like serum was reported by Nutell in 1888 (130, 131). In another case, the presence of AMPs was mentioned by Alexander Fleming in 1922, wherein he observed tissues and body fluids from a sick individual showed antimicrobial properties. Later he named this compound ¨lysozyme¨ (132). The discoveries continued and the first commercially produced AMP was Gramicidin S, which was used for wound healing during World War II (133). With these findings, the field of AMPs research began to flourish and today around 22499 entries have been recorded in the database 'Data repository of antimicrobial peptides´ (DRAMP, updated from the database (134). Concisely, they can be classified under four main groups based on one, sources: mammalian, insect, amphibian, and microorganism derived AMPs. Second by composition of amino acid, they are grouped into four: proline rich, tryptophan and arginine, histidine rich and lastly glycine rich. Third, structurally they mostly form α-helical, β-sheet, linear extension structure, and both α-helix and β-sheet structure. Finally, activity-based categorization is formed from their choice of targets which include bacterial, antiviral, antiparasitic, antifungal and anti-carcinogenic. (126).

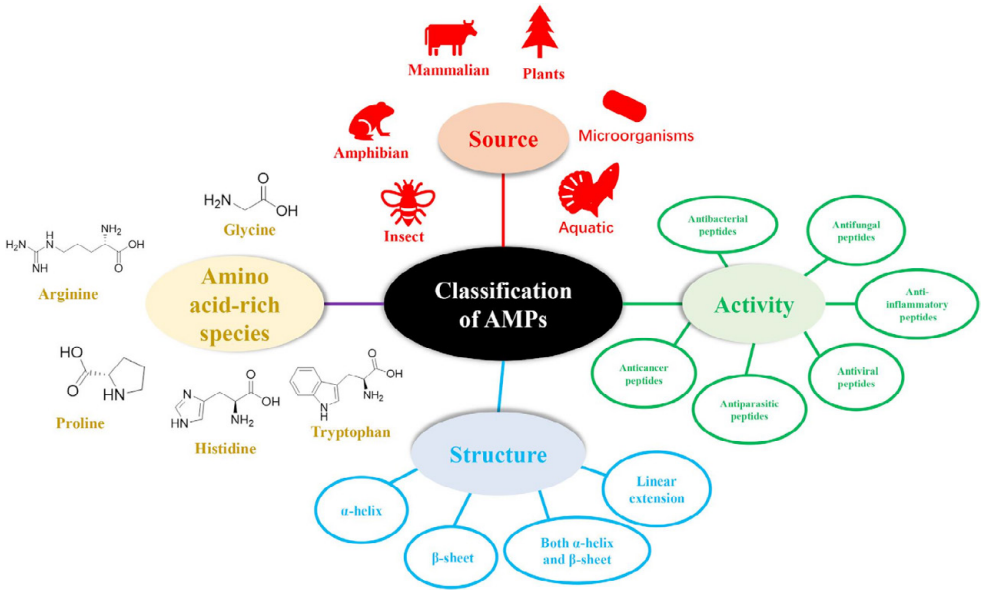

Figure 6: Antimicrobial peptides classified based on different features of peptides.
Reprinted from the article ¨ Antimicrobial Peptides: Classification, Design, Application and Research Progress in Multiple fields¨. License: CC BY. Reference (126).

Mode of action with respect to bactericidal activity

AMPs act by either disrupting the cell membrane (direct killing) or targeting specific molecules internally (indirect killing) (129).

Membrane target

Cationic peptides initial contact with bacterial membranes is mediated by electrostatic interactions due the presence of specific anionic lipids in bacterial membranes. Principally, bacterial membranes are composed of anionic phospholipids, as phosphatidylserine (PS), PG and CL, giving them the net negative charge required for AMP interaction. Membrane-peptide interactions can be categorized into four types of models (129). The first interaction is called barrel stave model where the peptide initially interacts by lying parallel to the membrane. When a substantial number of peptide molecules aggregates, it causes conformational shift by inserting into the membrane and forming a central lumen leading to membrane thinning (129, 135, 136). However, this mechanism has been reported by only a few peptides; probable as this interaction possibly requires a unique peptide sequence (135, 137, 138). In the toroidal pore model, the peptide inserts itself into the lipid bilayer vertically and binds to the phospholipid group to form a membrane curvature. The peptide orientation is such that the hydrophobic region is inside with the hydrophobic core while the hydrophilic region faces

aqueous phase. Famously known peptides displaying this type of interactions are melititin, auerin 2.2, lacticin Q and maganin 2 (128, 129, 136). The third interaction is the carpet model, in which the peptide molecules cover the membrane by orienting themselves parallel to the membrane. The peptide does not create pores or cause membrane curvature in this case but requires optimal high concentration to disorient the membrane and form micelles in a detergent-like manner. Some examples of peptides that follow the carpet model are cecropin and auerin 1.2 (128, 129, 136, 139, 140). The fourth interaction model, i.e., aggregation model, is characterized by peptide-lipid complexes turning into micelles, leading to the leakage of internal contents. This formation contrasts with the carpet model where only partial membrane lysis is observed (128, 129, 136). The aggregate model requires the formation of lipid membrane domains formed by concentrated anionic lipids. This occurs in regions of the membrane that can be induced in bacteria with cationic substances. The interaction is described as membrane perturbation by formation of a complex between lipids and AMP (141-144). An example of this model is reported by Powers *et al*. where a crab peptide named polyhemusin-I that was shown to translocate into the cytoplasmic area without causing extensive damage to the membrane (143).

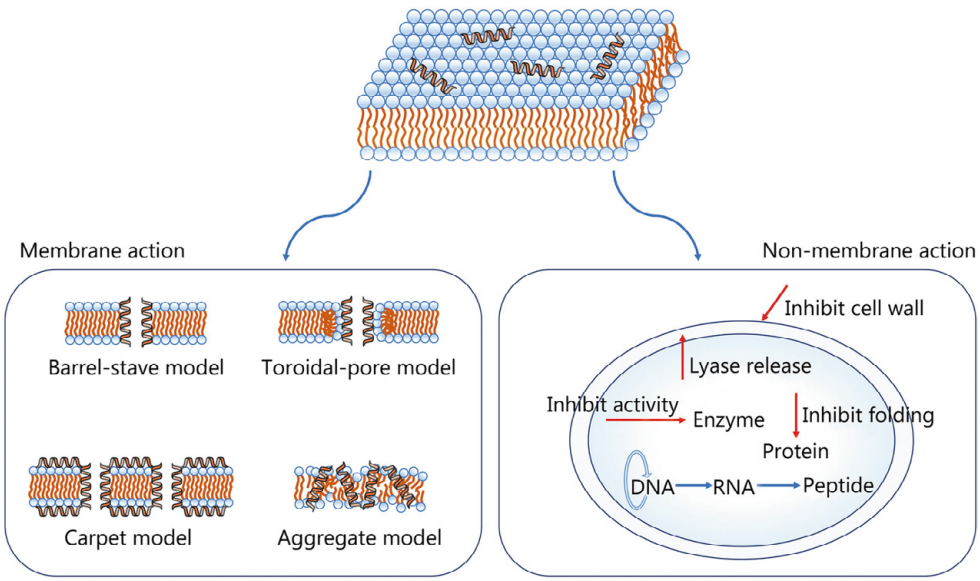

Figure 7: Different types of mechanism of action for AMPs.
AMPs mode of action is broadly classified into two paths which includes membrane action (membrane target) or non-membrane action (intracellular target). Membrane target model consists of four types, and they are barrel-stave, toroidal-pore, carpet, and aggregate model. Some peptides have intracellular target that interact with different targets. Reprinted from the article ¨ Antimicrobial peptides: mechanism of action, activity, and clinical potential¨. License: CC BY 4.0, reference (129).

Intracellular target

AMPs multifaceted nature is a well acknowledged concept (145). After translocation into the cytoplasm AMPs can target specific intracellular targets such as nucleic acids, proteins, enzymes or lipids involved in biological processes of bacterial survival (146). The target interests of AMPs intracellularly can be categorized into few groups based on their actions. Peptides that target nucleic acid usually hinder bacterial survival and here are a few examples. Buforin II, an AMP derived from gastrointestinal tissue of Asian toads demonstrated affinity towards nucleic acid binding from a 2D gel-based analysis and lipid vesicle models, potentially interrupting DNA/RNA activity (147). Likewise, affinity to DNA sequence by a 13 amino acid short AMP named indolicidin from bovine neutrophils was reported. Indolicidin not only displayed affinity towards DNA it interrupted DNA relaxation, a process of unwinding DNA during transcription (148, 149). Binding of these AMPs to DNA and blocks processess that are essential to bacterial repair and survival. Protein synthesis inhibiting AMPs has been reported previously as a proline rich AMPs (150). PR-39 originating from the pig intestine kills Gram-negative bacteria through a process that inhibits protein synthesis and targets the metabolically active bacterial population (151). A cathelicidin AMP, Bac7 is another proline-rich peptide that inhibits protein synthesis by binding to specific subset of the ribosome, which was observed both *in vitro* and *in vivo* (152). AMPs can also target other enzymes such as a heat shock protein involved in protein folding. Studies with analogues of proline rich pyrrhocoricin, drosocin and apidaecin were found to be stereospecifically bound to DnaK of *E. coli*, both in solution and solid phase (153). These are just a few examples of the various targets of AMPs from different studies. AMPs can target several other processes too, such as cell wall biosynthesis, cell division, protease inhibitors, and biofilm inhibition (154). However, the ultimate advantage with AMPs is their ability to target multiple pathways when dealing with any microorganisms.

AMPs explored as TB Treatment

The multifunctional properties of AMPs make them a perfect choice to test as novel treatment for TB disease. A brief list on different AMP explored as potential Mtb treatment have been summarized in Table 2. The sources covered in the table are from mammalian, insects, and plants. AMP from mammalian source is broadly categorized as cathelicidins, these peptides produced as a pre- and pro-peptide sequence. The N-terminal of the peptide is conserved, and the C-terminal is the variable domain responsible for antimicrobial property (155). The second group is named defensins, they are a large group of small peptides characteristically affiliated with β-sheet structure and six cysteine residues stabilized with disulphide bonds. They get their name from their association to host immunity (156). Mammalian defensins are subdivided into three classes based on their structure: α-defensins, β-defensins and θ-defensins (157).

Table 2: List of antimicrobial peptides explored for Tuberculosis treatment

AMP group name	Source	Name	Study summary	MIC & % dead	Reference
Mammalian origin: Cathelicidins	Porcine leucocytes	PR-39	It was tested against drug susceptible and drug resistant clinical Mtb isolates. Bacterial killing was better with drug susceptible strain.	50 µg/ml (80%)	(158)
	Bovine	Indolicidin	Tested against Mtb and *M. avium*	32-64 µg/ml & 128 µg/ml (99%)	(159)
	Human immune cells	LL-37	L and D-LL37 forms were investigated against H37Rv and a clinical resistant strain. Only D-enantiomer LL37 showed activity against only H37Rv.	200 µg/ml (99%)	(160)
Mammalian origin: Defensins	Human neutrophil peptide	HNP-1	It was tested investigated against H37Rv	2.5 µg/ml (99%)	(161)
	Human beta defensins (HBD)	HBD2 & HBD3-M-HBD2	HBD2 was tested against H37Rv and HBD3-M-HBD2 against MDR yielded good results	1.5 µM & 2.7 µM (99%)	(162)
	Human Colostrum	Lactoferrin	Tested against Mtb and *M. avium*	1 mg/ml	(163)
	Hepatocytes	Hepcidin	Tested against Mtb	200 µg/ml (50%)	(164)
Insect origin	Wasp venom	Mastoparan	Tested against Mtb	32-64 µg/ml (99%)	(159)
	Bee venom	Melittin	Tested against Mtb	32-64 µg/ml (99%)	(159)
Bacterial origin	*Lactococcus lactis*	Nisin	Tested against Mtb, *M. avium* subsp and *M. kansasii*	7.5 µg/ml, 60 µg/ml & 15 µg/ml (90%)	(165)
	Lactococcus lactis subsp. *lactis* DP C3147	Lacticin 3147	Tested against Mtb, *M. avium* subsp and *M. kansasii*	>60 µg/ml , 60 µg/ml & >60 µg/ml (90%)	(165)
	Lentzea kentuckyensis *sp.*	Lassomycin	Tested against H37Rv, *M. avium, M. smegmatis,* and several clinical MDR and XDR isolates	0.71-3.1 µg/ml (99%)	(166)
Plant origin	Capsicum annum fruit	F2 fraction	Tested against H37Rv and clinical Mtb M299 strain	100ug/ml (60% & 30%)	(167)
	Capsicum annum fruit	F3 fraction	Tested against H37Rv and clinical Mtb M299 strain	100ug/ml (50 % & 30%)	(167)
Fungal origin	*Pseudoplectania nigrella*	NZX	Tested against H37Rv and MDR clinical isolates	3.2 µM (99%)	(168)

Clinical application of AMP

So far FDA has approved formulations for topical application and they are gramicidin and polymyxins for ocular infections, daptomycin for skin infections, nisin A in food preservation, and melittin for its anti-inflammatory property (169). Despite AMPs being a great resource, their potential has not been exploited due to some pitfalls. Production of AMPs bare high costs and the choice of method in manufacturing needs to address issues like complexity of chemical synthesis and efficiency in genetic engineering. To achieve scalability researchers have explored biological hosts for recombinant expression, AMPs with cyclic structures are more easily produced versus peptides with disulfide bonds (170). Some AMPs might be susceptible to proteolytic cleavages in biological fluids that render them inactive (155). Additionally, AMP have a short half-life. To address this problem studies are exploring modifications to AMPs sequence to protect them from proteolysis and focus on site directed delivery using nanoparticles as carriers. (171, 172). Overall research in AMP has progressed to overcome these complications by peptidomimetics, a strategy that modifies the chemical structure of peptides for example through glycosylation, PEGylation, lipidation etc to improve therapeutic potential in clinical setting (173).

NZX

Plectasin is an AMP derived from the fungus *Pseudoplectania nigrella*. The fungus is primarily found in European pine forests. Plectasin, was identified through sequence similarity search programs like BLASTX and SEARCHq and a 55% match was found with defensins from invertebrates. The cDNA was transformed into *Aspergillus oryzae* to produce the defensin of 40 amino acids, purity was checked using mass spectrometry (174). The peptide was found to have great effect *in vitro* against several Gram-positive bacteria, especially *Streptococcus pneumoniae. E. coli* was also analysed but found to be resistant. Minimal inhibitory concentrations (MICs) and Minimal bactericidal concentrations (MBCs) had similar values for most isolates. This suggested that the mechanism of plectasin was bactericidal (33). Two mouse models for systemic pneumococcal infection were used to determine the effect of plectasin *in vivo*. Both models yielded good bacterial clearance when comparing treatment between plectasin and other standard antibiotics (33).

Plectasin was subjected to a high-throughput screening in 2005 aimed at improving antimicrobial properties by amino acids substitution (175). In our lab we work with NZX, a third-generation variant derived from plectasin. The sequence differs in amino acid composition to plectasin at three sites. In our previous paper the peptide was tested for its *in vitro* and *in vivo* effect and was found to be active against susceptible and drug resistant Mtb. The level of bactericidal effect was like conventional TB drugs. Toxicity was studied on primary cells using up to a 100μM concentration which did not show any toxicity. We also demonstrated that NZX is resistant to degradation from proteases.

EM experiments could verify intracellular activity, and gold labelled NZX was found to co-localize with Mtb inside an activated macrophage (168). In this thesis, NZX is investigated for its mode of mechanism in **Paper I** and therapeutic potential as combination therapy in **Paper II**.

Nanoparticles in AMP-delivery

Delivering potent agents to the target site is beneficial in many aspects. It helps to minimize nonspecific binding, lowers toxicity by using required dosage, and ability to traverse tissue (98). Nanoparticles (NPs) are colloidal particles with a size range of 1-1000 nm. During drug delivery an individual NP is coated/loaded with respective drug molecules and materials used to prepare NPs are either from natural or synthetic sources. Some NPs can intrinsically display antimicrobial properties, enhancing the activity of respective drugs (99,100). As mentioned above, many AMPs are easily degradable which lowers their bioavailability in a therapeutic setting. One approach to protect them from proteolysis is to encapsulate them in a porous material that target infectious sites. This way the peptide is protected, allows for sustained release, improves treatment, and reduces frequent dosing, subsequently reducing the risk of toxicity (105). A summary of current research on different NPs for AMP in TB treatment is listed below in a table.

Table 3: Summary of NPs explored as carriers of AMPs in TB treatment.

Particle	AMP	Size (nm)	Activity	Reference
Gold NP	Pep H (motif of HNP-1)	20	Antimicrobial activity against H37Rv was tested for intracellular activity with monocyte derived macrophages	(176)
Poly (lactic-co-glycolic acid) PGLA NP	B5	206.6 ± 26.6	Antimicrobial activity against *M. bovis* was lower for loaded B5 compared to B5 was tested in a in vitro setting	(177)
Biogenic Silver NP	NK-2 LLKKK-18	NP1 (50) NP2 (100)	Antimicrobial activity against *M. marinum* and *M. smegmatis* was tested in a in vitro setting	(178)
Chitosan NP	Pep H (motif of HNP-1)	244	Antimicrobial activity against was tested for intracellular activity with monocyte derived macrophages	(176)
Dendritic Mesoporous silica particles	NapFab	163 ± 7	Antimicrobial activity against H37Rv was tested for intracellular activity with monocyte derived macrophages	(179)
Phosphatidylcholine-cardiolipin liposome	Bcn1-5	Average size was <150	Antimicrobial activity against H37Rv inside peritoneal mouse macrophages was observed	(180)

In this thesis we investigated the therapeutic potential of NPs made from mesoporous silica nanoparticles (MSPs) to deliver NZX in **Paper III**.

Aims

The work in this thesis presents a novel therapeutic (NZX) in TB treatment. It tries to address the various aspects of treatment challenges that is encountered during design and evaluation of novel therapeutics. To summarize, my main research questions were,

1. What are the bactericidal mechanisms of NZX against Mtb?
2. Can NZX be incorporated in combination with other standard TB drugs?
3. How can bioavailability of NZX be improved to survive within host environments?

Paper I

Mechanisms of a *Mycobacterium tuberculosis* Active Peptide

Background

AMPs are naturally occurring short peptides recognized as a part of the host immune response. They are produced by every single and multicellular organism (181). In our previous work, NZX emerged as a strong candidate among other AMPs tested (168). This peptide is derived from plectasin, a fungal defensin (174). The peptide displays bactericidal effect on clinical isolates of *M. tuberculosis* and was found inside alveolar macrophage *in vivo* where it interacted with residing Mtb (168). In this paper we wanted to understand the mechanism by which the peptide interacts with mycobacteria and responsible for its bactericidal activity.

Aim

To understand the mode of action of NZX of *M. tuberculosis* bactericidal effect.

Results

Bacterial approach

The influence of NZX on Mtb clinical isolates was studied by growth kinetics. A concentration dependent decline in bacterial growth was seen for both isolates. Bacterial elimination was recorded for Mtb 1 at 12,5 ug/ml whereas a higher concentration was needed with Mtb 2, a MDR strain (Figure 1A).

Scanning electron microscopy (SEM) imaging of H37Rv and the clinical Mtb isolate was observed with gold labelled NZX. Both samples were treated with 6.3 μM of NZX for up to 24 hours. The addition of peptide prompted morphological changes to the bacteria seen as membrane protrusions followed by formation of bubble-like structures. These structures progressed with time and in the end the whole cell appeared disintegrated, and the debris resembled massive membrane vesicles formation (Figure 1B).

Transmission electron microscopy (TEM) was performed to visualize the interaction between H37Rv after treatment with gold labelled NZX. At first NZX seems to be associated with the outer membrane, later it appears to be traversing into the bacterial cell membrane with no visible damage to the outer membrane. As the peptide was found near the plasma membrane, it seems to disrupt the layer by a ¨pulling¨ mechanism, where the plasma membrane winds up into tubular-like structures. Surrounding small vesicle-like bodies were also observed. Complete cell disruption was observed resembling a ghost cell, characterized by a less electron dense cell. These assays exemplify NZX's affinity towards bacterial cell wall and its bactericidal properties (Figure 2B).

Biophysical approach

Understanding the interactive nature of NZX with Mtb membrane gave us an insight of possible mode of action, which seemed to be plasma membrane dependent. To further elucidate this theory a model membrane mimicking standard plasma membrane lipids from mycobacteria and gram-positive species were prepared. The model membrane setup comprised of 100 nm lipid vesicles prepared using 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3[phospho-rac-(1-glycerol) (POPG) in two formulations, POPE/POPG (85/15, mol/mol, termed G-negative vesicles (NMV) and POPE/POPG (30/70, mol/mol, termed Gram-positive vesicles (PMV).

To examine NZX interaction with lipid vesicles, intrinsic fluorescence of cyclic amino acids- tryptophan and tyrosine present in the peptide was exploited. Spectrophotometer reading records slight shifts in emission spectra caused by reduction in polarity of the environment surrounding tryptophan (λex = 295 nm) or the tryptophan/tyrosine residues (λex = 280 nm) resulting in increased quantum yield (182). Changes in emission spectra of tryptophan and tyrosine/tryptophan were observed when NZX was added in presence of PMV causing a shift from 360-364 to 346-350 with an increased (>10 nm) fluorescence intensity. This shift in energy is due to a change in hydrophobic environment encountered by these aromatic amino acids, probably at W8, likely stemming from anchoring of the peptide at the lipid vesicle's interracial region. In contrast, NMV and NZX interaction did not induce any changes to the spectra indicating the membrane anchoring property of NZX is dependent on charge (Figure 2C).

As observed in TEM, NZX's pulling mechanism intrigued us to understand this process. To study this, membrane permeabilization of lipid vesicles was measured through quantification of fluorescence intensity release by calcein, encapsulated within the vesicles. Addition of peptide in varying concentrations to vesicles resulted in decrease in fluorescent intensity as opposed to an expected increase or no change. The decrease was observed to be concentration dependent. The addition of triton resulted in release of encapsulated calcein, meaning the membrane wall was intact (Figure 3). To understand the dip in fluorescence intensity a dynamic

light scattering (DLS) experiment was performed. As we speculated that the decrease could be associated with changes in vesicle size, kinetic size measurement of PMV and NMV was recorded for 30 min in presence of the peptide. We observed a concentration dependent increase in Z-average hydrodynamic diameter and polydispersity index. However, a concentration of 25-33 μM culminated in random fluctuation in PMV. Even though the effects were scaled down for NMV a similar fate as PMV was observed at higher concentrations, but no change was observed at lower concentration (Figure 4).

Upon interacting with lipid bilayers AMPs are reported to change their structures which aid in their antimicrobial properties. Using far-UV circular dichroism, change of peptide's secondary structure was measured. The measurements were fed into an algorithm K2D3 to estimate changes in secondary structures. The control peptide was largely composed of helical fold with the minima measure laying around 208 and 222 nm. CD-spectra of PMV in presence of NZX estimated a reduction of about 30% in alpha helical content and formed 16 % beta strand. No change was observed to CD-spectra of NMV as the recorded spectra was similar to that of NZX control (Figure 2D).

These results suggest that NZX has affinity to lipids present in PMV, that the peptide changes its secondary structure upon anchoring into its interfacial membrane, but that it does not rupture the membrane in lipid vesicles. However, these results suggest that interactions with lipid vesicles or plasma membranes in bacteria are guided by other factors other than charge as they cause aggregation or ¨pulling¨ instead.

Proteomics approach

To understand and identify possible peptide-protein interactions with bacterial proteins we performed co-immunoprecipitation and liquid chromatography with tandem mass spectrometry (LC-MS/MS). Histidine and Streptadin-tagged fractions NZX were incubated with whole cell lysate of BCG for an hour followed by elution and bound proteins that were identified using a mass spectrometer. The proteins detected were identical irrespective of the NZX tag. The identified proteins were Cpn1, Cpn2, EF-Tu and acyl carrier protein (AcpM) found to be essential in mycobacterial survival. Another unidentified protein Rv3269 was also targeted by the NZX. These results align with the theory that the NZX possibly has other properties in conjugation with the membrane associated mode of mechanism.

Paper II

A broad spectrum anti-bacterial peptide with an adjunct potential for tuberculosis chemotherapy

Background

Antibiotics are widely used in many aspects of life ranging from agriculture, medicinal purpose, and livestock farming. The goal being to prevent or treat microbial infections that could be detrimental. However, overuse of antibiotics has led to adverse effects with the emergence of antibiotic resistance. It was in 1940 when Abraham and Chain reported first antimicrobial resistance in *E. coli* to penicillin (183). With growing antibiotic resistance patterns in many bacterial infections including TB, it is imperative to explore other antimicrobial molecules (184). AMPs have been a promising path as new therapeutics in microbial and cancer treatments (185). Currently, research in AMPs shows discovery of different classes of peptides from several sources (185). To attain a good drug regime to complement the existing TB drugs it is necessary to evaluate them in combination. In this article we set out to evaluate pharmacokinetics and pharmacodynamics aspects of NZX. Along with resistance potential as well as broad spectrum application.

Aim

The aim of this study was to further understand the potential of NZX as TB treatment.

Results

Diverse antimicrobial targets of NZX

In our previous work, Tenland *et al*. reported bactericidal activity of NZX towards pathogenic Mtb and MDR strains at concentrations comparable to first line TB drugs (4). In this article NZX was tested against several clinical isolates of ubiquitous NTM species, Gram-positive bacteria such as *S. aureus*, MRSA and *S. pneumoniae* and the Gram-negative *E. coli*. NZX displayed antimicrobial activity against all

screened species, although we observed different MIC, except for *E. coli*, where the antibacterial property was absent even at a high concentration (>100 ug/ml). Some biological replicates of NTMs demonstrated high MIC at 25 μg/ml, while others ranged between 0,4-12 μg/ml (Figure 1).

Pharmacodynamics of NZX

Drug interaction studies were evaluated with both *in vitro* and *in vivo* settings. Drugs used for combination therapy were RIF, INH, EMB and AMK. Checkerboard assay between NZX and each drug combination individually had a positive outcome, meaning none were antagonistic. Interaction scores followed either additive (EMB and INH) or indifferent interaction (AMK and RIF). Using a murine model, combinations showing an additive effect in the checkerboard assay were evaluated. Bacterial load of H37Rv from lungs were measured pre and post treatment. Reduction in colony forming units (CFU) for treated groups was observed. Comparing INH with untreated mice 92% clearance was seen; similarly, 79% and 75 % clearance was observed for EMB and NZX respectively. However, the p-value between independent treatments was not significant, implicating similar clearance of bacteria. Among the drug combination therapy i.e., NZX alone, EMB/INH and EMB/NZX combinations significantly ($p = 0.0317$) reduced the CFU with respect to EMB treatment alone. While the other combinations had lower CFU counts from the untreated control, they were not significant (Figure 2A). Tissue damage was checked using hematoxylin and eosin (H & E) staining from the lungs of mice by rating three parameters: perivascular, peribronchial and alveolar infiltration. Blinded lung inflammatory score showed INH maintained better alveolar structure while EMB treatment destroyed the lungs sooner. Interestingly, in combination therapy EMB+NZX scored like NZX while INH+NZX was similar to INH (Figure 2B &C).

Pharmacokinetics of NZX

Serum half-life of NZX was calculated by administering 33 mg/kg in mice from a single intravenous dose. Samples were collected from three mice at 5-, 20-, 60- and 120-min time points. LC/MS analysis quantified mean NZX concentration to be 61 mg/l after 5 minutes and the estimated terminal half-life was 42 minutes. Post 240 mins NZX was completely cleared from the circulation. Human serum stability was investigated by measuring MIC_{99} after NZX incubation for up to three hours. MIC values were established using resazurin microtiter assay (REMA) and values were found to be stable up to the maximal time (Figure 3).

Susceptibility to resistance

Spontaneous mutants of *M. smegmatis* were evaluated for development of resistance to NZX. Bacteria were grown with and without NZX treatment on agar plates/liquid media with varying NZX concentrations. No resistant mutants were isolated irrespective of NZX concentration or growth condition.

Paper III

Effective delivery of the anti-mycobacterial peptide NZX in mesoporous silica nanoparticles

Background

Mtb is an intracellular pathogen primarily residing within alveolar macrophages. Most antibiotics are effective on external bacteria, but lose their effect on intracellular pathogens (186). As AMPs are gaining in popularity as a new antimicrobial drug against resistant bacteria, they need to be protected from degradations during administration until they reach the site of infection (187). In this study we evaluated mesoporous silica nanoparticles (MSPs) designed to protect and increase bioavailability of NZX by controlled release.

Aim

Increase bioavailability of NZX using MSPs to eliminate intracellular Mtb.

Results

Characterization of mesoporous silica particles (MSPs)

MSPs used in this experiment had a mean pore size of 200 nm to increase the efficacy to enter macrophages. The size and shape of the vesicles were evaluated visually with transmission electron microscopy. The particles look dense in the core area, depicting a loaded state (Figure 1A).

NZX and MSPs interaction

Adsorption studies between NZX and MSPs was studied in phosphate buffered saline (PBS) buffer. The affinity between them was found to be strong and the particle concentration was 5 mg/ml having an NZX-loading of 17% weight (Figure B). Release kinetics of NZX from MSPs was evaluated in PBS and simulated lung fluid. NZX desorption rate in PBS was comparatively lower to SLF, with 14.1 μM concentration recorded after 48 hours whereas NZX concentration from SLF was

about 16.2 μM at the same time (Figure 1C & 1D). After release, antimicrobial function of released NZX was tested for MIC using a REMA assay. The MIC was found to be the same (3.2 μM) as the free NZX antimicrobial value.

Uptake of NZX loaded MSP

Particle uptake into cells was analyzed with THP-1 monocytic cell line and primary macrophages. The particles were conjugated with a fluorochrome having an excitation wavelength at 488 nm. The percentage of phagocytosed particles was measured using a flow cytometer. Particles successfully internalized into both types of cells, with uptake being more efficient in primary macrophages than THP-1 monocytes. Higher concentration (300ug/ml) was internalized completely (100%) by half an hour in primary macrophage and about 80 % in THP-1 monocytes. At lower concentration, 25 μg/ml, the uptake was 80% and 40% in macrophages and THP-1 monocytes respectively (Figure 2B & 2C). TEM analysis was performed to study the uptake and internalization of loaded MSPs in macrophages. Primary macrophages were incubated with 300 μg/ml and the images were collected from 2-72 hours. Particle uptake can be seen as quick as 2 hours and particles appear disintegrated at 72 hours. Interestingly, loaded MSPs were observed near vacuoles of macrophage early on (Figure 3A).

Cytotoxicity of NZX loaded MSP

Cytotoxicity was performed for MSPs at different concentrations via MTT and ATPlite toxicity assays (Figure 4A & 4B). A dose dependent toxicity was observed with both methods. Toxicity was also measured *in vitro* from an experiment with macrophages infected with Mtb H37Rv. A similar pattern of dose related toxicity was observed.

Bactericidal effect of NZX loaded MSP

Intracellular antimicrobial activity was tested for loaded MSPs by *in vivo* and *in vitro* analysis (Figure 4C, 4D & 5). Infected primary macrophages with H37Rv revealed a better bactericidal activity from loaded MSPs compared to free NZX. In the murine TB model, bactericidal activity was observed for both loaded and free peptide with no significant difference among the groups.

Discussion

Multi-faceted mechanisms of NZX

In a previous study a screening analysis identified that NZX, a fungal defensin-like peptide was a promising antimycobacterial agent (168). Antimicrobial nature of NZX was demonstrated with the H37Rv strain and two clinical isolates, one of which was a drug resistant strain (168). To understand this antimicrobial mechanism, we explored two aspects: direct and indirect mechanisms of NZX against mycobacteria.

Indirect targeting of mycobacteria

Affinity of NZX towards mycobacterial membrane was demonstrated through TEM analysis, wherein the gold-labelled peptide is seen interacting with the outer membrane in a non-pore forming manner. This initial interaction is driven by electrostatic forces but also due to the hydrophobic nature of NZX. Net charge of AMPs is mostly cationic and this feature makes them good candidates in targeting anionic bacterial/carcinogenic cell membranes (139). Following initial interaction with the bacterial capsule, the peptide appears to be traversing into the cell to interact with plasma membrane by remodelling the mycomembrane. However, the exact process through which NZX is capable of traversing through complex layers of tightly packed impermeable mycomembrane is yet to be characterized (188). To further understand if the peptide interacts with plasma membrane and drives cytoplasmic leak after passing through the mycomembrane we prepared calcein incorporated unilamellar liposomes. These experiments revealed NZX does not release calcein upon interaction, but the peptide still interacts with the vesicles. Perhaps the mode of interaction with lipids in the inner membrane is not charge motivated but specificity to certain lipids (128). A study by Epand *et al*. reported formation of lipid domains between anionic and zwitterionic/ neutral lipids in the presence of cationic peptides (189). This possible domain inducing feature is detrimental to cell survival, as seen in TEM images.

Interaction with the liposome membrane induced a change of the peptide's secondary structure. This was observed during a CD analysis, where the secondary structure of the peptide was monitored in presence and absence of liposomes. This change in secondary structure is attributed to changes in the hydrophobic

environment which NZX encounters upon embedding itself into the membrane. Even though liposomes were structurally intact, embedding of NZX is bound to bring upon changes and compromise membrane fluidity (190). Proteins with tryptophan (Trp) residues are preferred in membrane interactions because of their shape that facilitates the interactions. Thus, by including tryptophan to the N-terminal of a cationic AMP can enhance the antimicrobial properties of a peptide (191, 192). NZX intrinsically contains a Trp residue in the W8 position that contributes to its interaction both in liposomes and bacterial plasma membranes.

Whether the mode of mechanism involved in NZX, and mycobacterial membrane interaction follows one of the classical AMP-membrane interactions is hard to conclude. But the fact remains, that the type of the observed interaction bears some resemblance to the toroidal-like model as suggested in many mechanistic studies of AMPs (193). The toroidal-like model behaviour was seen in liposomes where the aggregation of vesicles was observed in DLS experiments as the concentration of the peptide was increased. Additionally, aggregation formation was stochastic, emphasizing on toroidal-like models (193). However, the lack of pore formation in liposomes made us question the toroidal model. Though, a pore-like formation was observed in bacterial membrane in TEM analysis, suggesting that the peptide appears to be pulling the inner membrane while some peptide molecules enter the cytoplasmic region. The rationale behind this varied outcome in liposomes and bacterial membranes can be explained with the fact that interactions between them are not solely dependent on phospholipid bilayer, but also depends on the presence of anionic lipids, making it resemble the aggregated mechanism model (190). Interestingly, images from SEM analysis reveal sudden bubbling-like morphology on the surface of bacteria likely caused by an increase of peptide concentration. These bubble-like forms resemble membrane vesicles released by bacteria under stress induced conditions (194, 195). Similar structures can be observed around the dislocated bacterial membrane in TEM images. Studies have indicated that AMPs can release vesicles like structures from bacterial membranes by binding to lipid domains (196). To summarize, it is not possible to assume that one mechanism is responsible for NZX-membrane interactions contributing to the bactericidal effect.

Direct targeting of mycobacteria

AMPs are known to possess multiple properties that make them a valid choice to use as antibacterial drugs. Whether membrane interaction is the sole reason for mycobacterial death is difficult to say, we predict there is more than one mechanism involved. NZX was found colocalized next to a condensed chromosome inside the cytoplasmic space. Experiments that can predict DNA-peptide interactions were not performed; however, we have a reason to believe this may be happening due to recent publication by Zhang *et al*. revealing that two NZX variants have an effect with bacterial chromosomal DNA (197).

Proteomic results of proteins bound to NZX through co-immunoprecipitation were interpreted. Two separate experiments using different affinity tag chromatography yielded five common proteins of interests that have affinity to NZX. The proteins were the 60kDa chaperonin 1, elongation factor Tu, 60kDa chaperonin 2, acyl carrier protein and Rv3269 from the BCG protein database. Chaperonins 60 proteins (Cpn60) are a family of heat shock proteins present across various species which are expressed upon encountering stress conditions. Mycobacteria has two analogues of Cpn60: Cpn60.1 for Hsp 60 and Cpn60.2 (GroEL) for Hsp65 and both are important in bacterial survival and pathogenesis (198, 199). NZX seems to bind to both these Cpn60 proteins which interferes with mycobacterial cell survival, affecting important folding of housekeeping genes or effecting survival in hypoxic environments (199-202). Binding of AMPs to proteins is highly influenced by amino acid sequence. A recent study discusses how small proline-rich AMPs have affinity to GroEL both in solid and liquid medium (150). Presence of proline in NZX perhaps promotes binding to GroEL protein, nevertheless its impact on bacterial growth cannot be confirmed without further studies (153). Mycobacteria has been reported to manipulate host defence by recruiting Hsp60 and Hsp65 to interact with TLRs to manipulate the pathogenesis (203-205). Mtb acyl carrier protein (AcpM) is an important carrier protein and a part of the fatty acid synthase II system, responsible for mycolic acid synthesis. Binding of NZX to this protein can be its prime target with a severe effect on mycobacterial survival. AcpM has also been identified to interfere in macrophage apoptosis pathway (206, 207). Elongation factor (EF-Tu) primarily plays a role in transferring aminoacyl-tRNA to ribosomes in the cytosol; however, it also has been associated with canonical activities in the cell (208). By binding to this protein NZX potentially can inhibit several processes in the cell and in the protein synthesis (208, 209). Finally, the last protein hit was Rv3269 whose function is uncharacterized but has homology to mycobacterial chaperonins (210).

These multifaceted potential targets of NZX support the therapeutic potential in antimycobacterial therapy. Furthermore, having a multi-target mechanism is beneficial in the context of antimicrobial resistance. Binding of these proteins to NZX is a promising discovery especially considering they were generated from two independent affinity chromatography techniques. However, it is premature to confirm that these findings verify bactericidal effects. Proof of concept studies are needed to claim with certainty which among these proteins bound to NZX are responsible for its antimycobacterial activity.

Therapeutic potential of NZX

Broad-range antimicrobial property of NZX

Antimicrobial resistance problem is seen in every infectious disease where the treatment is using antibiotics. To solve this rising problem, one of WHO's sustainable development goals (SDGs) is to find sustainable alternative molecules to combat resistant infections. NZX, an AMP, is among the molecules that have a broad range activity (129). We analyzed NZX's antimicrobial activity against a wide range of clinically isolated NTM's and Gram-positive strains. Among the Gram-positives screened, important strains belonging to the problematic group of Methicillin-resistant *Staphylococcus aureus* (MSRA) and *S. pneumonia* were analyzed (211). Gram-positive strains were highly susceptible to NZX at low concentrations, with the MRSA strains showing similar sensitivity. The major membrane lipids present in both these species are PG and CL, mostly anionic lipids, a preference seen by NZX during membrane interactions (212, 213). NZX's broad range was also tested against a clinical Gram-negative strain, *E. coli*, where the bacterial growth was not affected. Perhaps the interaction between NZX and Gram-negative bacterial membrane is not compatible owing to lipid composition and complex outer membrane (214).

Within the NTMs, several strains were analyzed of which some significant ones are MAC and MABCS. NZX displayed antimicrobial activity against 15 species tested. The MIC values were not uniform across species, with a trend towards growth rate (MIC > rapidly growing NTM). This variance in MIC among NTMs was expected and has been as reported in other studies (215, 216). Most NTM infections are pulmonary, and the most common causative agents are MAC, MABCS, *M. xenopi* and *M. kansii* (18, 110, 217). The two severe issues in NTM-PD management is treatment failure and antibiotic resistance to macrolides (218). Recently, there has been a sudden increase in incidence rates of NTM infections perhaps because of improved diagnosis and general awareness (219). To the best of our knowledge NZX is the first reported AMP capable of inhibiting bacterial growth for a wide range NTM species *in vitro*. Most AMP publications are targeting specific groups or species of NTM infections (88, 220, 221).

Pharmacodynamics and pharmacokinetics of NZX

In a previous study, we demonstrated bactericidal activity of NZX *in vitro* against clinically relevant strains and the peptide was shown to clear infection in line with current TB drug, rifampicin, in a murine study (168). To continue with these findings, we evaluated combinations of drug therapy using NZX and other Mtb first line drugs. NZX interactions with ethambutol and isoniazid were additive, meaning

the MIC values are decreased by one-fold for drugs tested. To evaluate these results *in vivo*, a chronic murine study was performed for the same drug combinations. Bacterial clearance had a similar additive effect for combination treatment between NZX and ethambutol. Likewise, tissue samples from murine lungs showed good preservation of lung tissue in comparison to other TB drugs tested. Ethambutol as a stand-alone treatment displayed poor tissue structure retainment. Toxicity from ethambutol has been reported in many studies in the form of hepatotoxicity, renal toxicity, and ocular neuropathy (88, 222, 223). Monotherapy with NZX exhibited good tissue preservation and for ethambutol in combination with NZX.

Preliminary pharmacokinetics studies demonstrated NZX stability in human serum and serum-half life in murine study. Previously, we have reported that NZX was not susceptible to enzymatic degradation (168). One of the challenges in using AMPs as therapeutics is that they are easily susceptible to degradation from enzymes subsequently problematic in therapeutic application (131). Substitution with different types or forms of amino acids in AMPs can help improve peptide stability and antimicrobial activity (224, 225). However, NZX is intrinsically stable, which was observed in human serum studies with retained mycobacterial inhibitory concentration after serum incubation for three hours. Serum half-life of the peptide in circulation in murine studies was found to be 25 minutes. AMPs and their short peptide sequence is also an advantage for stability since shorter peptides have been reported to fare better (226-229).

Another hindrance encountered with AMPs is the resistance bacteria may acquire to it. Even though mycobacteria have slow mutation rates mainly involving point mutations, while horizontal gene transfer remains to be debated. Nevertheless, resistant strains have been recorded to every drug against TB that has been used in treatment (91, 227, 230). To screen for potential mutants against NZX, liquid and agar based spontaneous forced mutants were isolated. No resistant colonies were recovered, irrespective of drug concentration. In a similar study with short peptides, resistant mutants emerged at a frequency of 10^7, suggesting that the lack of mutants in NZX can be ascribed to a higher threshold (231).

Overall, these studies address some of the potential challenges seen in pre-clinical stage of evaluating novel therapeutics. Nevertheless, NZX has a great potential as a candidate for TB therapeutics.

Improving bioavailability of NZX

Drug delivery is an important aspect that defines the bioavailability of drugs. Nano-particle based drug delivery has gained popularity as a choice of drug delivery vehicle (232). In our research we explored MSPs as potential drug delivery system for NZX. MSPs are non-toxic, spheric or rod shaped and highly porous. Properties

that make them desirable are the pore shape and the ability to coat surfaces to be increasingly target specific (233).

The MSPs size used in our study was 200 nm in diameter and they displayed strong adsorption to NZX. The desorption studies were performed in PBS and SLF, and activity of NZX was retained upon disassociation. The sustained release observed for NZX in SLF highlights the therapeutic potential of using these nanoparticles in TB treatment. A study demonstrating peptide encapsulated within MSPs has proved to be beneficial in improving the solubility and the antimicrobial activity by delivering the peptide to the target site (234). Additionally, as AMPs manufacturing is costly, loading AMPs into nanoparticles is cost effective by minimizing the loss of free peptide (234).

Mycobacteria primarily reside in macrophages after initial infection, they continue to survive and replicate and later only to get disseminated to establish an infection. In latent TB these bacteria reside in macrophages within granulomas. Targeting drugs to penetrate both macrophage layer and granuloma is key in resolving TB infection (235). In our study we demonstrated internalization of MSPs through THP-1 cells and primary macrophages. In the presence of macrophages, MSPs uptake was as early as two hours. We observed uptake of MSPs into vacuoles followed by degradation over three days. Macrophages displayed higher affinity to take up vesicles compared to THP-1 cells. Efficient uptake of nanoparticles and clearance has been previously described in macrophages during *in vitro* analysis (236). In our study, we showed that the sustained release of peptide from MSPs did not interfere with NZX bacterial killing capacity in a murine model and did not affect the intracellular killing in macrophages.

Finally, we evaluated for potential toxicity elicited by MSPs by two methods. A dose dependent toxicity was noticed for macrophages. Toxicity from MSPs has been recorded previously but they were dependent on surface functional groups and size (237, 238).

In summary, nanoparticle-based delivery of NZX is an interesting option since bacterial elimination was successfully achieved. This study has given insights into better targeted therapy of AMP for TB treatment, potentially improving their bioavailability to an intracellular pathogen.

Conclusions

- NZX possibly has two mechanisms involved in its antimycobacterial activity against Mtb. First, our findings suggest NZX has a strong affinity towards mycobacterial membrane and this interaction leads to membrane remodelling and consequently promotes cell deformity. Second, proteomic analysis revealed explicit binding to specific intracellular proteins which seem to be necessary in bacterial survival.
- Characteristically, AMPs are well known to target different pathogens. NZX seems to have this feature, it is active against not only Mtb but also several NTM species and MRSA.
- NZX shows potential as an adjunct for antituberculosis therapy since monotherapy in TB management gives rise to resistant strains. It also preserved tissue morphology in the lungs compared to EMB, a standard first-line drug used in TB treatment.
- Bioavailability of NZX could be improved by loading it on mesoporous silica nanoparticles for intracellular therapy. Additionally, antimycobacterial property of NZX was not altered after desorption.

How inscrutable and incomprehensible are the hidden works of Nature!

-Antonie van Leeuwenhoek

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