Biochemical Studies on the role of *M. tuberculosis* nitrate reductase during survival in host macrophages

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By

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Dedications

This thesis is dedicated to the people who shared with me the risks and sacrifices required to complete it. The first of these is my parents for never doubting in my ability to achieve a goal and being very supportive of every endeavor I have ventured to take. The second and third are my husband, sisters, sister in-laws and brothers who has provided both inspiration and love throughout the entire doctorate program. I must acknowledge as well as all my in-laws and their families who are very supportive throughout the research period. The love and support that have given to me has been an eternal compass that kept me focused on my true north and the journey completed.

"Knowing is not enough; we must apply.
Willing is not enough; we must do."

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Sampa Sarkar

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "**Biochemical Studies on the role of *M. tuberculosis* nitrate reductase during survival in host macrophages.**" submitted by **Sampa Sarkar** was carried out under my supervision at Combichem Bioresource Center, Organic Chemistry Division, National Chemical Laboratory, Pune – 411008, Maharashtra, India. Materials obtained from other sources have been duly acknowledged in the thesis.

Dr. Dhiman Sarkar
(Research Guide)

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled "**Biochemical Studies on the role of *M. tuberculosis* nitrate reductase during survival in host macrophages.**", submitted for the Degree of ***Doctor of Philosophy*** to the University of Pune, has been carried out by me at Combichem Bioresource Center, Organic Chemistry Division, National Chemical Laboratory, Pune - 411 008, Maharashtra, India, under the supervision of Dr. Dhiman Sarkar (Research supervisor). The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University.

Sampa Sarkar

(Research Scholar)

ABSTRACT

The inevitable rise in cases of tuberculosis worldwide fuelled by the HIV epidemic highlights the need for new drugs and particularly those that can shorten the duration of treatment. After Wayne's hypothesis in 1995, we actually did not see any further advancement in the literature about the hypoxia model. This thesis describes the approaches designed for developing a better understanding of the application of hypoxia model in host system. It basically describes about the detection of *M. tuberculosis* bacilli nitrate reductase (NarGHJI) activity in host macrophages and also the direct consequence of anaerobic condition developed within the host milieu along with its implications. First approach is the development of intracellular hypoxia with subsequent lowering of intracellular ATP in association with caspase 3/7is responsible for the escape of tubercle bacilli from host macrophages. The second approach is the development of a macrophage based anti-tubercular high throughput screening system could extremely expedite the discovery programs for identifying novel inhibitors. The third approach is a series of allyl and propargyl derivatives of alkyl, aryl and heteryl substituted 1, 2, 4-triazolethiols were evaluated for their in vitro mycobactericidal activity.

Until now, collected evidences suggest that *M. tuberculosis* has evolved multiple strategies to manipulate infected host cells which include release of different interleukins as well as receiving cytokine signals to activate the apoptosis or necrosis of host macrophage cells. Although the pathogenic pathways leading to the development of disease within the host system essentially require the activation of necrotic pathway but in actual experiments in the laboratory conditions it could end up to either of the events in the host cell. In fact, until now there is no biochemical evidence regarding the characteristics of this trigger factor. So, the aspect of our study is to understand the growth of intracellular *Mycobacterium tuberculosis* (MTB) bacilli that leads to the development of hypoxia as well as dormancy phenotype which is supported from the induced expression of hypoxia biomarkers in bacterium and macrophages cells along with the use of stage specific inhibitors and development of acute hypoxia triggers mitochondrial dysfunction with subsequent lowering of intracellular ATP. The release of cytochrome C induces caspase 9 followed by caspase 3/7 of the intrinsic pathway of

apoptosis lower the level of ATP and ensures the death of infected macrophages by necrosis. So far, evidences in favor of induction of NarGHJI (NR) activity during shift down to dormancy in *Mycobacterium tuberculosis* and impaired growth of narG mutant of *M. bovis* BCG in SCID mice already indicated the potential of this enzyme as a drug target for dormant stage. More significantly, blockage of the enzyme's function by specific inhibitors led to a rapid reduction in viability of the bacilli during hypoxic stage survival in Wayne's in vitro model. Although there are other models like nutrient starvation and non-cultivable models in place, none of these are able to represent the actual dormancy state of the bacilli within the host system. Instead of using these models, evolving an infection model to identify dormant phase specific molecules would bring more convenience in developing effective drug therapy against tuberculosis. So, another aspect of our study is to find any similarity between the dormancy explained by Wayne in culture tubes with the bacilli in host macrophages under ex vivo conditions. We observed that NR activity increases with the growth of intracellular bacilli. Standard anti-tubercular agents and NR inhibitors were applied to understand its importance during this stage of bacilli residence within macrophages. So, this data clearly indicated that the increase in NR activity within host macrophages was due to the development of hypoxic environment inside the intracellular bacilli and nitrate reduction by intracellular *Mycobacterium tuberculosis* in THP1 macrophages can be used to develop an anti-tubercular screening protocol. This assay can be of great advantage as it can pick up inhibitors of both stages of the bacilli within host intracellular environment. Another major goal of the thesis was to identify the molecular target/s for three derivatives belonging to triazolethiol scaffold potentially active against non-replicating dormant phase of *Mycobacterium bovis* BCG and *M. tuberculosis*. Our study has opened up a new opportunity of optimizing potential anti-tubercular drugs by clearly establishing identification of 1, 2, 4-triazolethiols as new leads along with their novel target groEL-2 in *Mycobacterium tuberculosis*.

TABLE OF COTENTS

ABBREVIATIONS

ADAS:	Active Dormant Antitubercular Screening
ADP:	Adenosine diphosphate
AFB:	Acid Fast Bacilli
AG:	Arabinogalactan
ATCC:	American Type Culture Collection
ATP:	Adenosine triphosphate
BCG:	Bacillus Calmette Guerin
Bio-SiV:	Bioluminescent Screening In vitro
CDC:	Centre for Disease Control
CFP-10:	Culture Filtrate Protein 10
CFU:	Colony Forming Unit
DMSO:	Dimethyl sulfoxide
DOTS:	Directly observed therapy shortcourse
DISC:	Death-inducing signalling complex
E. coli:	*Escherichia coli*
EMB:	Ethambutol
ELISA:	Enzyme Linked-ImmunoSorbent Assay
ESAT-6:	Early Secretary Antigen Target-6
EPR:	Electron paramagnetic resonance
ETH:	Ethionamide
FAD:	flavin adenine dinucleotide
FDA:	Food and Drug Administration
FACS:	Fluorescence activated cell Sorting
FBS:	Fetal bovine serum
GAN:	Gene Accession Number
GS:	Glutamine Synthetase
GTH:	Glutathione
HAART:	Highly Active Antiretroviral therapy
HIV:	Human Immunodeficiency Virus
HSR:	Head Space Ratio
HTS:	High throughput screening
HMGB:	High mobility group box
IFN-γ:	Interferon-γ
INH:	Isoniazid
IUATLD:	International Union Against Tuberculosis and Lung Disease
KEGG:	Kyoto Encyclopedia of Genes and Genomes
LAM:	Lipoarabinomannan
MBC:	Minimum Bactericidal Concentration
M. bovis:	*Mycobacterium bovis*
MDR:	Multidrug resistance
MEM:	Minimum essential medium
MIC:	Minimum Inhibitory Concentration
MOI:	Multiplicity of infection
M. smegmatis:	*Mycobacterium smegmatis*
M. tuberculosis:	*Mycobacterium tuberculosis (*Mtb*)*
MTD:	Maximum tolerable dose
MW:	Molecular Weight

NAD:	Nicotinamide adenine dinucleotide
NADH:	Nicotinamide adenine dinucleotide reduced
NADP:	Nicotinamide adenine dinucleotide phosphate
NADPH:	Nicotinamide adenine dinucleotide phosphate reduced
NH_4:	Ammonia
Nir:	Nitrite Reductase
ONOO-:	Peroxynitrite
NO_3:	Nitrate
NO_2:	Nitrite
NR:	Nitrate Reductase
PAS:	Para amino salicylic acid
PCR:	Polymerase Chain Reaction
PBS:	Phosphate buffer salaine
PK/PD:	Pharmacokinetics Pharmacodynamics
PI:	Propidium Iodide
PMA:	Phorbol myristate acetate
PPD:	Purified Protein Derivative
PZA:	Pyrazinamide
RIF:	Rifampicin
RIP:	Receptor interacting proteins
SAR:	Structure Activity Relationship
SDS-PAGE:	Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis
SEM:	Scanning Electron Microscopy
STM:	Streptomycin
TB:	Tuberculosis
TEM:	Transmission Electron Microscopy
THP-1:	Human acute monocytic leukemia cell line
VEGF:	Vascular endothelial growth factor
WHO:	World Health Organization
XIAP:	X-linked inhibitor of apoptosis
XDR:	Extremely Drug resistant

Publications from thesis

1. “Depletion of intracellular ATP due to acute hypoxia play important role in the necrosis of *M.tuberculosis* infected human macrophages”, **Sampa Sarkar, Shamim Akhtar, Arshad Khan, Dhiman Sarkar**(Under review; PONE-D-11-20223).
2. "Potential use of nitrate reductase as biomarker in identifying active and dormant inhibitors of Mycobacterium tuberculosis in THP1 infection model", **Sampa Sarkar, Dhiman Sarkar.** (Under review, JBSC-11-0178)
3. “Identification of groEL 2 as potent target of 1, 2, 4-triazolethiols in killing both replicating and non-replicating *Mycobacterium tuberculosis”* **Sampa Sarkar, Arshad Khan, Shamim Akhter, Arvind M. Korwar, Preeti M. Choudhary, Sayalee R, Chavan, Anjali P. Likhite, Shailaja. P. Maybhate, Sunita. R. Deshpande, K.B. Sonawane and Dhiman Sarkar** (communicated).

Publications out of thesis

1. "Bactericidal activity of 2-nitroimidazole against the active replicating stage of Mycobacterium bovis BCG and Mycobacterium tuberculosis with intracellular efficacy in THP-1 macrophages"; **Arshad Khan, Sampa Sarkar, Dhiman Sarkar** International Journal of Antimicrobial Agents **32** (2008) 40– 45.
2. "Production, purification and characterization of taxol and 10DAB III from a new endophytic fungus Gliocladium sp. isolated from the Indian yew tree, Taxus baccata". **Sreekanth D, Syed A, Sampa Sarkar, Sarkar D, Santhakumari B, Ahmad A, Khan I.** Journal of Microbiology Biotechnology. 2009 Nov; **19**(11):1342-7.
3. "A method to extract intact and pure RNA from mycobacteria" **Shamim Akhter, Sampa Sarkar,Dhiman sarkar,** Notes & Tips / Anal. Biochem. **417** (2011) 286–288
4. "Self-Assembled Histidine Acid Phosphate Nanocapsules As Templates For Multifunctional Hollow Platinum Nanospheres In Ionic Liquid [BMIM] [BF4] For Drug Delivery" **Sarvesh K Soni, Sampa Sarkar, Dhiman Sarkar, Peter J Coloe, Suresh K Bhargava,** CHEMECA, 2011.

Patents

1. Antimycobacterial activity of propargylated 1, 2, 4-triazolethiols and 1, 2, 3-triazole derivatives. **Sampa Sarkar, D.Sarkar, A.Khan, SR.Despande, Sp.Maybhate, Ap.Likhite PM.Chaudhory, S.Chavan**, Indian patent Council of scientific & industrial Research, 0574DEL2010.
2. Antimycobacterial activity of propargylated 1, 2, 4-triazolethiols and 1, 2, 3-triazole derivatives. **Sampa Sarkar, D.Sarkar, A.Khan, SR.Despande, Sp.Maybhate, Ap.Likhite PM.Chaudhory, S.Chavan**, Indian patent Council of scientific & industrial Research, PCT/IN2011/000172.
3. Phenylcarbamoyl novel compounds anti tubercular activity in dormant stage of Mycobacterium bacilli. **Dhiman Sarkar, Sampa Sarkar, Rohit Ramesh Joshi, Vijay Murlidhar Khedkar, Raghuvir Ramakant Pissurlenkar, Evans cliffton Coutinho, Anamik Kantilal Shah.** Indian patent Council of scientific & industrial Research, 1244DEL2010.
4. Phenylcarbamoyl novel compounds anti tubercular activity in dormant stage of Mycobacterium bacilli. **Dhiman Sarkar, Sampa Sarkar, Rohit Ramesh Joshi, Vijay Murlidhar Khedkar, Raghuvir Ramakant Pissurlenkar, Evans cliffton Coutinho, Anamik Kantilal Shah.** Indian patent Council of scientific & industrial Research, PCT/IB2011/001184.
5. Identification of Mycobacterium tuberculosis bacilli dependent conversions within host macrophages and its use thereof. **Sampa Sarkar, D.sarkar,** Indian patent Council of scientific & industrial Research 3038DEL2010.
6. GroEL2 as an antitubercular target and its use thereof. **Sampa Sarkar, D.sarkar.** Indian patent Council of scientific & industrial Research 0737DEL2011.
7. Antitubercular activity of compounds from *plectranthus mollis* **S.P Joshi, D. sarkar, Sampa Sarkar, Roshan, ketaki shurpali,** Indian patent 2011-NCL-0023.
8. Useful compounds from *anisomeles* **S.P Joshi, D. sarkar, Sampa Sarkar, Roshan, ketaki shurpali,** Indian patent 2011-NCL-0024.
9. Natural selective inhibitors of *Mycobacterium tuberculosis* from *leucas stelligera* **S.P Joshi, D.sarkar, Sampa Sarkar, Roshan, ketaki shurpali.** Indian patent 2011-NCL-0026.

CHAPTER 1

Overview of *Mycobacterium Tuberculosis* and host THP-1 macrophage Interaction: Strategies for survival within macrophage

1. Introduction

1.1 Taxonomy and a global emergency of *Mycobacterium tuberculosis*

Kingdom: Bacteria

Phylum: Actinobacteria

Class: Actinobacteridae

Order: Actinomycetales

Suborder: Corynebacterineae

Family: Mycobacteriaceae

Genus: *Mycobacterium*

Species: *Mycobacterium tuberculosis*

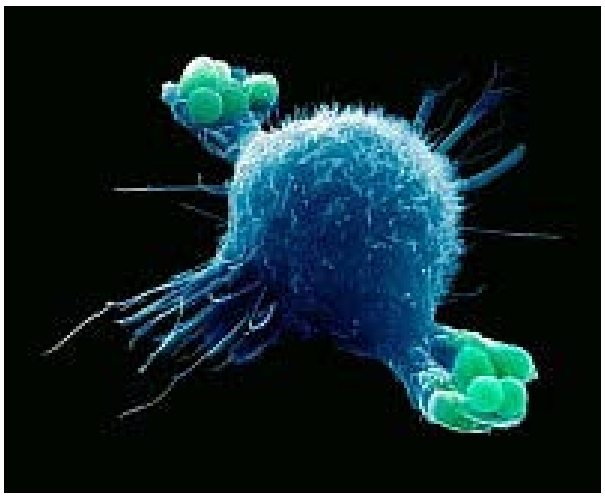

Fig. 1.1 Scaning Electron Microscopic picture of Macrophage engulfing bacteria as a part of immune system's response to infection (Adapted from http://visualsunlimited.photoshelter.com)

Mycobacterium tuberculosis (Mtb) is one of the major infectious agents of human diseases. Robert Koch identified the microbe responsible for tuberculosis (TB) in 1881 by maintaining the culture of crushed granulomas. Mtb is classified as Gram positive bacteria even though it stains with crystal violet because of its unique cell wall composition [1]. It is a slow-growing, facultative intracellular pathogen that can endure and proliferate inside macrophages (Fig: 1.1). It is an acid fast, rod-shaped, non-motile, non-spore forming, and aerobic. Its cell wall made up of mycolic acid, which gives Mtb the acid fast character and able to retain basic dyes in the presence of acid alcohol. The detailed characteristic features of the mycobacterial cell wall include the lipoarabinomannan (LAM), lipomannan, mycolylarabinogalactan, phosphatidyl-myoinositol mannoside, sulfatide, cord factor, and other acylated trehaloses, phenolic glycolipids, lipoligosaccharides, and other attenuated lipids (Figure 1.2). These components are non-covalently attached to the plasma membrane through their GPI anchors and they expand to the outer of the cell wall [1, 2]. LAM consists of a phosphatidyl-myo-inositol anchor, a D-mannan polymer attached to the inositol ring, D-arabinose chains, and capping motifs at the end of the arabinose residues [3]. LAM acts as a virulence factor of Mtb, causative to the inhibition of macrophage functions important for killing the pathogen. Inhibition of phagosomal maturation and interfering with cell signalling shift the cytokine response from pro- to anti-inflammatory [2, 4-6]. Virulent, slow-growing mycobacteria like Mtb harbour mannose-capped LAM (ManLAM) in their cell wall, while rapidly growing non-virulent species of mycobacteria such as *M. smegmatis* harbour non-capped AraLAM or phospho-myo-inositol-capped LAM (PILAM), this type of capping is important for virulence [7]. The cell wall of Mtb also contains a 19-kDa lipoprotein of unknown function which has been concerned in virulence through a role in host cell death and manipulation of bactericidal mechanisms [8]. The 19-kDa lipoprotein of Mtb, as well as LM, and AraLAM from rapidly growing mycobacteria, induce an inflammatory response in the host by binding to Toll-like receptors (TLR) on the host cell surface [9, 10]. Many of these charecteretic evidences play a major role in the virulence and pathogenesis of the bacillus. As other members of the Mycobacterium genus, the

genomic DNA of Mtb is also characterized by high guanosine plus cytosine (G + C) content. It has been sequenced and shown to be 4.4 Mb in size and contains 4006 protein-coding genes of which 52% have assigned functions .Out of these, only 376 putative proteins share no homology with known proteins and presumably are unique to Mtb [11]. The availability of its genome sequence facilitates identification of genes encoding various functions including virulence factors as well as protective antigens. It also provides so much information for identification of targets for drug and vaccine development.

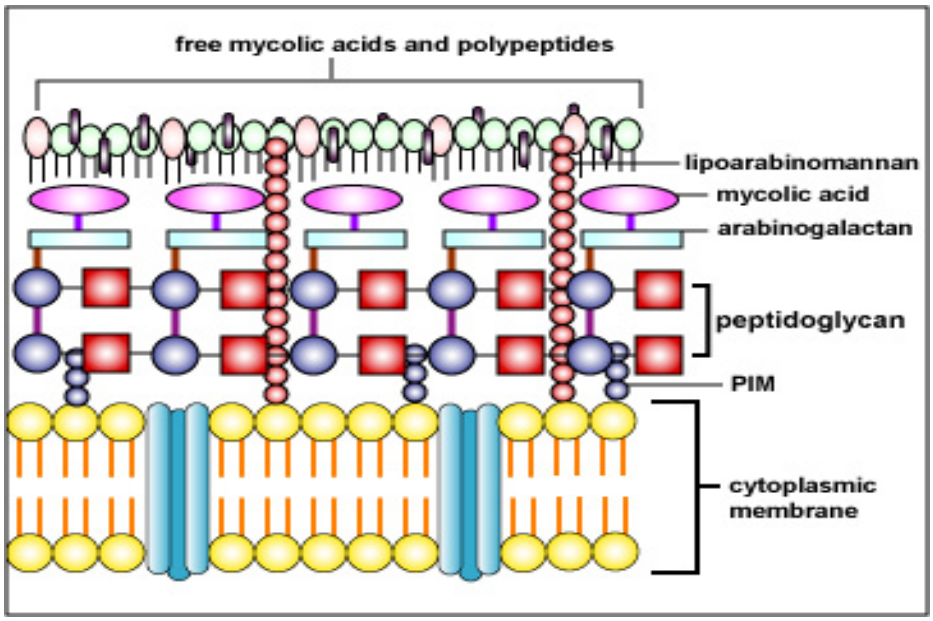

Fig. 1.2 Overview of the structure of the cell wall of *M. tuberculosis* (Adapted from Nature Reviews Microbiology **5**, 883-891).

An Mtb virulence factor that has received great attention in recent years is the 6 kDa early secreted antigenic target (ESAT-6). ESAT-6 is secreted in a 1:1 heterodimeric complex with 10 kDa culture filtrate protein (CFP-10) by a secretion system called the ESAT-6 system-1 (ESX-1) or type VII secretion system. The system is determined by the region of difference 1 (RD1) of the mycobacterial genome, and is preserved in several mycobacterial species including *M. marinum* and *M. bovis*. However, repeated passage of *M. bovis* to obtain the vaccine strain BCG led to deletion of the RD1, resulting in attenuation [12, 13]. ESAT-6, as well as the previously mentioned LAM and 19-kDa lipoprotein, express a specific immune response in the infected host, and are

therefore major antigenic determinants of Mtb [12]. Since ESAT-6 is present in Mtb but absent in BCG, a specific IFN-γ response against it is investigative of Mtb exposure and the protein is thus used in diagnostic IFN- γ release assay tests [31]. ESAT-6 is also under study for vaccine use in recombinant BCG strains [14]. ESAT-6 has multiple virulence mechanisms, but the best studied is its role in plasma membrane lysis, which play a role in the spread of Mtb from one macrophage to another [15-18]. In M. marinum-infected macrophages, it is known that ESAT-6 can lyse the phagosomal membrane, allowing escape of the bacillus into the cytoplasm of the macrophage and subsequent pore formation in the cell membrane leading to spread [19,20].

Human tuberculosis (TB) is most often caused by an Mtb strain, but *M. africanum* and *M. bovis* infection can also lead to the development of TB [1]. Mtb strains vary in phenotype and virulence, with for example the Beijing strain being particularly virulent, developing drug resistance and causing extra-pulmonary TB more often than other strains [21]. H37 is a laboratory strain that was isolated from a 19-year old pulmonary TB patient in 1905, and later dissociated into a virulent strain (H37Rv) and an avirulent strain (H37Ra), based on virulence in guinea pigs [22]. Although both strains can be cultured in suitable medium in the laboratory, only the H37Rv strain is capable of replication inside human macrophages [23]. It has recently been described how H37Rv and H37Ra differ genetically and phenotypically, and the major difference lies in a mutation in the phoP gene, which is necessary for adaptation to the intracellular environment [24-26]. PhoP forms a two-component regulatory signal transduction system together with PhoR, where PhoP acts as a transcriptional regulator. The system is important for sensing and adapting to environmental stimuli [27]. Several Studies have shown that mutations in the phoP gene lead to a defect in the secretion of ESAT-6, which can be synthesized but not released from the bacillus [28, 29]. The H37Ra, H37Rv-strains, as well as BCG and different clinical isolates, are normally used to study the pathogenesis of mycobacteria in different in vivo and in vitro models. *M. marinum* is also commonly used, as it has many of the features of Mtb and is useful to handle since it is less prone to cause disease in humans than Mtb and can be used to infect zebrafish embryos and the amoeba Dictyostelium [30-32]. It can also be used to model tuberculous lesions by infection of mouse tails [20]. However, the extrapolation of data obtained with

mycobacteria that are not pathogenic to humans should be done with great care, as many mechanisms are specific for Mtb and dependent on for example a functional ESX-1 region and PhoP/PhoR regulatory system.

Mtb can cause infection anywhere within the body, even though it mostly takes seize and resides within the lungs of infected individuals. As a result, the route of infection plays an important role in determining how signal transduction, immune activation, and the intracellular survival of Mtb occur [33]. Mycobacteria reside in structures known as phagosomes. Usually bacteria engulfed by alveolar macrophages, are destroyed within lysosomes that fuse with phagosomes. In contrast, Mtb prevents lysosomal fusion with phagosomes. The unsuccessful fusion of these two structures prevents the formation of a third structure known as the phagolysosome, which helps mycobacteria to survive within macrophage [34]. This skill avoids damage by the alveolar macrophages that restrict the immune response of the host to contineuing the infection [35]. Mtb infection begins with the inhalation of tubercle bacilli. These bacilli are ingested by alveolar macrophages, resulting in either their destruction or persistence. The tubercle bacilli that are talented of escaping destruction then multiply and interrupt the function of the alveolar macrophage. When the actions of the alveolar macrophage become disrupted, other inflammatory cells programmed to prevent the spread of infection are then engaged to the site of infection within the lungs. This inhalation of the tubercle bacilli, the mycobacteria is either destroyed, in that case no infection occurs, or the mycobacteria remain viable. In the event that the mycobacteria are not destroyed, a primary complex forms. The primary complex consists of Mtb infiltrate and a draining lymph node.

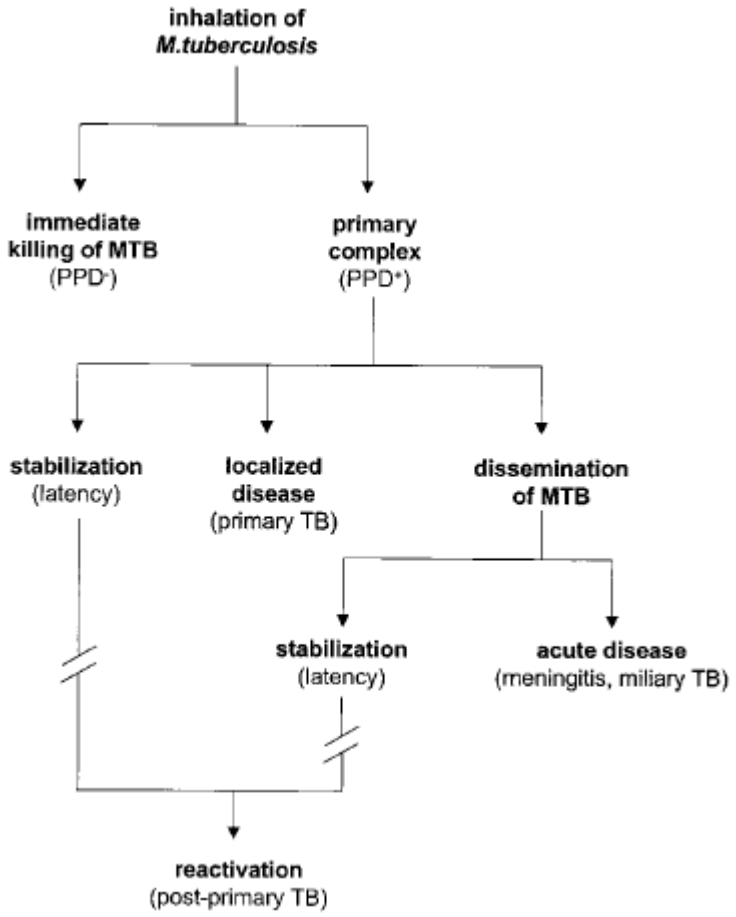

Fig. 1.3 Chronological events after inhalation of *Mycobacterium tuberculosis* (Adapted from van Crevel et al., 2002)

The primary complex gives rise to the positive skin test that marks the Mtb - specific T cell response, revealing the existence of infection. Once Mtb infection has been established, the infection is usually steady and goes into latency. In some cases however, the infection is not stabilized and active disease develops (primary tuberculosis infection). Active tuberculosis disease either remains localized; keeping infection limited to the lungs, or it disseminates and travels to other areas of the body. In the event of Mtb distribution, infection stabilization and latency are still possible however; large amounts of mycobacteria traveling throughout the body (miliary tuberculosis) and meningitis remain risks. Although establishing a state of latency in an Mtb-infected individual is one means of controlling tuberculosis infection, there always remains the risk of latent infection becoming reactivated in the event of immune surveillance failure, thereby establishing post-primary Mtb infection (Fig. 1.3) [33].

Man has been in constant fight with TB since ancient times. Mtb DNA has been discovered in Egyptian mummies from 2000 B.C. and TB was described by Hippocrates as early as 400 B.C. Historical texts identify the disease as "consumption," "wasting away," "king's evil," "lupus vulgaris," "the white plague" or "phthisis" based on its clinical manifestations [1,36]. The Industrial Revolution during the 18th and 19th centuries in Europe led to crowded living conditions in urbanized areas, which provided optimal conditions for spread of TB. This resulted in epidemic levels, with 20-30 % of all deaths being caused by the disease [36, 37]. During the second half of the 19th century, however, deaths from infectious diseases including TB drastically decreased in Europe, as housing, diet, education, and sanitation improved, and with the launch of sanatoria where patients were exposed to fresh air and a healthy diet. The decline in TB incidence in Europe occurred before the discovery of antibiotics, stressing the importance of living conditions and social factors in containing TB, and highlighting some of the difficulties that are still faced in low-income countries today [38]. With the discovery of streptomycin in 1943, TB became a medically treatable disease, and incidence continued to fall in industrialized countries throughout the 20th century. This fact, together with the hubris of the eradicationist era due to a combination of the success in eradicating smallpox, the effectiveness of DDT in eliminating malarial mosquitoes, and an immense faith in science and technology, led to infectious diseases being neglected by governments and public health agencies until the end of the 20th century. All the while, TB continued to take its toll on poor and vulnerable populations in low-income courtiers, as well as marginalized populations in high-income countries [38]. However, the HIV epidemic, the emergence of new tropical diseases, and antibiotic resistance has again made infectious disease into a recognized global threat. The evolution of extensively drug-resistant TB, the increased susceptibility of HIV-positive persons with weakened immune systems to TB, increased mobilization of people due to globalization, and the relative ineffectiveness of the Bacillus Calmette-Guérin (BCG) vaccine have put TB back on the agenda during the past twenty years [39,40]. The BCG vaccine, a live attenuated variant of *M. bovis*, has been used since the early 20th century. However, it has proven rather unsuccessful, especially in preventing adult pulmonary disease, and a more

effective vaccine is surely needed [40]. With nearly two million people dying from the disease annually, TB is truly a global emergency. Public health and financial efforts including improved access to health care, better control of transmission, improved and more available diagnostics, and increased treatment and cure rates are urgently needed. New scientific knowledge about the basic mechanisms underlying TB and how the host can overcome it is also imperative, in order to develop a new, improved vaccine and new drugs that tackle the emergence of antibiotic resistance [38, 40]. The table lists hallmark discoveries in the battle against tuberculosis (Table 1.1).

1.2 Epidemiology of *Mycobacterium tuberculosis* infection

Mtb was first well-known as the aetiological agent of TB by Robert Koch in 1882. One–third of the world's population is infected with TB [41]. Development of active disease from Primary infection is marginal (40%). The remaining 60% do not develop TB. As an alternative, they contain the infection at a low level, remain non-infectious and symptom free, it indicates that they are able of resolving infection completely, or to a level incapable of transmitting the disease. Latter on, this infection is said to be latent and capable of reactivating and of causing occurrence of disease in a future time. Persons with latent TB represent a large reservoir of Mtb. Up to 23% of these individuals are likely to develop reactivation TB during their lifetime. Out of these latently infected individuals, annually, 5-10% endures from reactivation TB due to the synergistic pathology of co-infection with human immunodeficiency virus (HIV) [42]. Joint effects of the appearance and continued spreading of multi-drug resistant Mtb strains globally [43], latent TB can cause epidemic of the disease particularly in developing countries. In 1993, the concern about the global TB situation prompted WHO to declare TB a global emergency. In recent years the annual rate of TB in the common population of developing countries has gone as high as 400 per 100,000. This combined with a tenfold increase of HIV infection has got worse of the situation [44]. In Ethiopia, the burden of TB is one of the highest in the world. Based on predictable number of cases, it ranks 7th among the 22 high burden countries globally [45]. According to WHO (2005) report from the data of the year 2003-2004, Ethiopia has an incidence of all cases of TB of 356; new

smear positive pulmonary cases (ss+) of 155 and an estimated mortality rate of 79 per 100, 000 populations. TB is a vital public health problem that still kills 136 000 people and affects 630 000 people in the Region every year. The majority (80%) of TB cases arise among the most dynamic age groups of the community (15 to 54 years) [Fig 1.4]. TB usually affects more men than women in the Region; however in some countries, particularly Afghanistan, it affects more women than men [Fig 1.5].

Table 1.1 Discoveries in the field of Tuberculosis

When	Who	What
1865	Villemin	Infectious nature of tuberculosis. Inoculation of a rabbit with purulent liquid from a tuberculous cavity resulted in extensive TB.
1882	Koch	Discovery of tubercle bacillus (Koch-Henle postulates)
1883	Ziehl & Neelsen	Development of Ziehl-Neelsen (ZN) stain based on the acid-fastness of the tubercle bacilli
1890	Koch	Developed tuberculin, a substance from tubercle bacilli, to cure TB. Proved not to be successful as a cure; "Koch reaction"
1907	Von Pirquet	Developed tuberculin skin test (TST); intracutaneous injection of tuberculin for diagnosis of TB
1908	Mantoux	Cannulated needle and syringe to inject tuberculin intracutaneously
	Seibert	Developed purified protein derivative (PPD), used for the TST
1909	Von Pirquet	Introduced the term latent tuberculosis; positive tuberculin
1921	Calmette & Guérin	Developed a vaccine Bacille Calmette-Guérin (BCG) through attenuating M. bovis
1943	Lehmann	First therapeutic agent: para-amino salicylic acid (PAS)
1943	Waksman & Schatz	First mycobactericidal agent: streptomycin
1952		First oral mycobactericidal drug: isoniazid (INH)

1957		Rifampycins
1998	Cole et al.[1]	Deciphering of the Genome sequence of M. tuberculosis
2000	Andersen et al.[2]	Development of in vitro M. tuberculosis-specific immunodiagnostic assays
2007		Several new TB vaccines candidates in phase one and two clinical trials.

1. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D et al. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 1998; 393(6685):537-44.

2. Andersen P, Munk ME, Pollock JM, Doherty TM. Specific immune-based diagnosis of tuberculosis. Lancet.2000; 356(9235):1099-104.

Age distribution of TB cases in EMRO (2000)

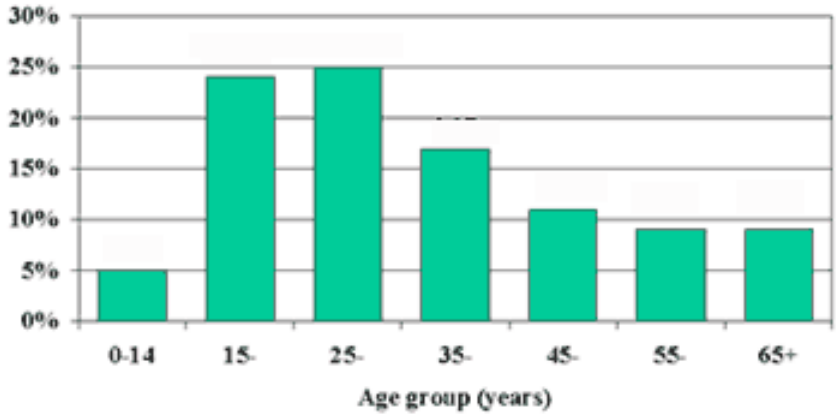

Fig 1.4 Age distribution of TB cases in EMRO. (Adapted from http://www.emro.who.int/stb/images)

Male-female ratio among TB cases

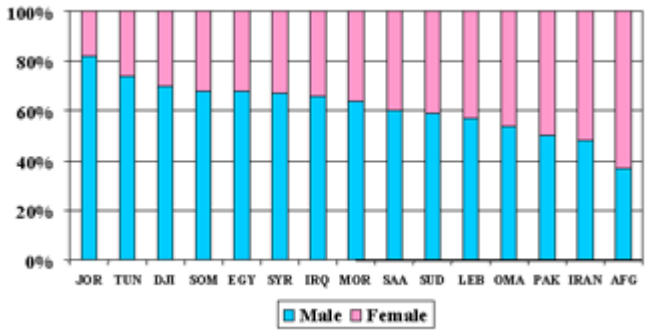

Fig 1.5 Male-Female ratio among TB cases (Adapted from http://www.emro.who.int/stb/images)

In short, TB is an important problem in social and economic development. The incidence of TB varies greatly within the Region, from more than 300 per 100 000 population to less than 10 per 100 000 [Fig 1.6].

TB epidemiology map

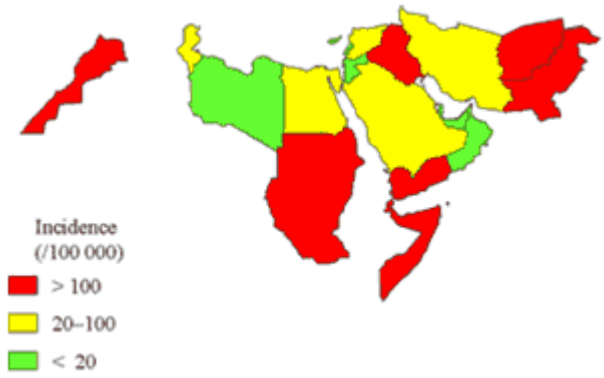

Fig 1.6 TB epidemiology map (Adapted from http://www.emro.who.int/stb/images)

There are 9 countries with high TB burden which account for 94% of the regional TB burden [Fig 1.7]. Unless all countries achieve the global targets of 85% treatment success rate and 70% case detection rate the total number of TB cases in the Region is projected to increase, reaching 672 000 in 2005.

9 high burden countries (2000) based on estimated TB cases by country

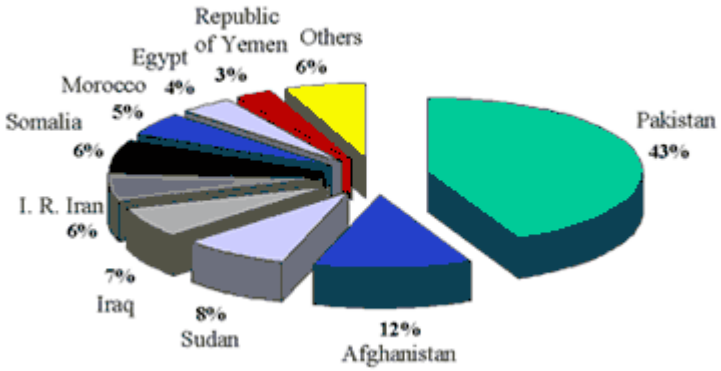

Fig 1.7 9 high burden countries (2000) based on estimated TB cases by country (Adapted from http://www.emro.who.int/stb/images)

1.3 Pathology of and immunity to TB in humans

The TB infection starts mostly by inhalation of aerosol containing tubercle bacillus. Mtb infection is characterized by three organized stages. In the first stage, a few air-borne droplets of Mtb by inhalation are ingested and reside in resident phagocytic cells mainly the alveolar macrophages. After the engulfment of the macrophages, it initiates host immune responses. However, the tubercle bacilli have the extraordinary ability to persist and even to replicate in this extremely hostile environment, where most other pathogens die. Thus, macrophages do not eliminate the bacilli completely and some bacilli remain persistent within the tissue in a non-replicating state [46]. This is regulated by specific genes and their proteins. In the second stage, a cell-mediated immune response causes condensed granuloma formation due to the infiltration of CD4+, CD8+ T cells, B cells, activated macrophages, and other host cells [47] to the sites of bacterial implantation and multiplication (Fig 1.8). At this stage, infection depends upon the immune status of the host. The host will either establish an acute infection characterized by bacillary proliferation or resolve the initial infection. The third stage of infection is characterized by reactivation of latent bacilli /Non-replicating persistent (NRP) TB to cause subsequent acute secondary infection. Mtb at this stage exhibits uncontrolled bacterial growth, secondary transmission occurs to susceptible hosts. Cell mediated immunity is a complex phenomenon and TB is mainly dependent on this process. It involves CD4+ and CD8+ T cells, as well as alternative T cells such as γδ T cells and CD1-restricted

CD4- CD8- or CD4+/CD8+ single positive αβ T cell subsets, and NK cells are involved but generally CD4+ T cells play a central role in defense [48,49]. Acquired immunity to Mtb is dominated by CD4+ and CD8+ T cells with the Th1 cytokines, such as IFN-γ and IL-12 [49]. IFN-γ is a key cytokine involved in the immune response against Mtb. It plays a very important role in the activation of macrophages and stimulation of Th1 dominated immune responses [51]. As well as processing and presenting antigens to T-cells, activated macrophages act as effector cells. Th1- CD4+ cells get stimulated by releasing IL-12 and IL-18 which induced the release IFN-γ [52]. Some of the CD8+ T cells, and CD1 restricted T cells secrete perforin and granulysin in human which apparently directly kills mycobacteria within macrophages [53]. CD1 presents the T-cells with specificity for mycobacterial glycolipid (CD1a, b, and c) molecules which play a unique role in human tuberculosis. CD1 molecules are in large quantities expressed on dendritic cells (DCs) and that is why CD1-glycolipid specific T cells produce IFN-γ and express cytolytic activity [54] In recent years, as we have riched our knowledge and understanding of NRP stage of TB, but complete understanding of latent infection of TB from the host immunity and the bacterial perspective is still faraway.

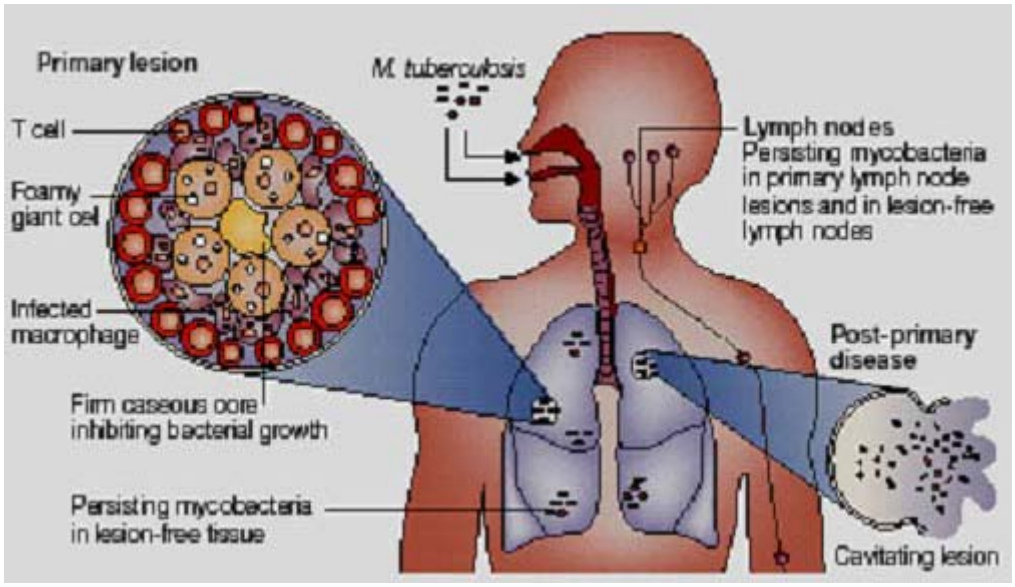

Fig 1.8. Composition of mature granuloma. The granulomatous lesions in Mtb infections are complex structural entities, which are organized aggregates of immune cells surrounding Mtb–infected tissues, mainly necrotic tissue and macrophages fused to form

multinucleated giant cells or epitheliod-like cells (the center), activated macrophages and CD4+ and CD8+ T cells (periphery). The barricade-like structure of the granuloma acts to wall off Mtb and prevent further bacillary multiplication and spread to other infection sites (Adapted from: Stewart et al., 2003).

1.4 Latency (NRP) in *Mycobacterium tuberculosis* infection

In recent years, dormancy of Mtb has received attention very well. Dormancy can describe both the disease and metabolic state of the tubercle bacilli. This term is also similar with Mtb that bacilli has grown under limited oxygen tension in vitro [46] to consider the fact that bacilli could equally survive in a closed, necrotic lesion during clinical latency. On the other hand, the word persistence has been used to describe the capacity of the bacteria to survive a wide variety of ex vivo condition including desiccation, nutrient deprivation, and osmotic shocks. It also shows the ability of the bacteria to reside within cells such as macrophages, its ability to avoid elimination from the host immune system by its immune elusive mechanisms. The present concept seems to accept the use of both terms “latency or NRP to describe the sum total picture of inactive state and physiology of the bacilli as well as TB disease.

Latent stage of TB is a clinical syndrome that occurs after an individual has been exposed to Mtb, after establishment of infection, an immune response has been generated to control the pathogen and force them to enter into a dormant state [42]. Sometimes clinical symptoms, sputum of acid-fast bacilli, and the progression of X- ray dense lung lesions will not show any positive result although the patient is carrying the latent TB [55]. In this case, 90% of bacilli remain latent and do not transform into active clinical disease. Approximately, 10% of infected individuals subsequently develop clinical disease during the first two years of infection [56]. In contrast to the cases with active TB, individuals with latent TB do not transmit the disease. The pathogen persists within the functioning immune system and survives within professional phagocytes in granulomatous lesions. Latent TB can reactivate after years or even decades of subclinical persistence, leading to progressive disease. Health Organization, based on Tuberculin Skin Testing (TST) estimates that nearly 2 billion people (one third) of the world’s population are infected latently with Mtb and this could provide as the largest reservoir of infection [44]. The chance of the latently infected individuals developing clinical tuberculosis is approximately 2-23%

within their life time, with the risk rising to about 5-10% per year among HIV-infected individuals (Fig 1.9). [42].

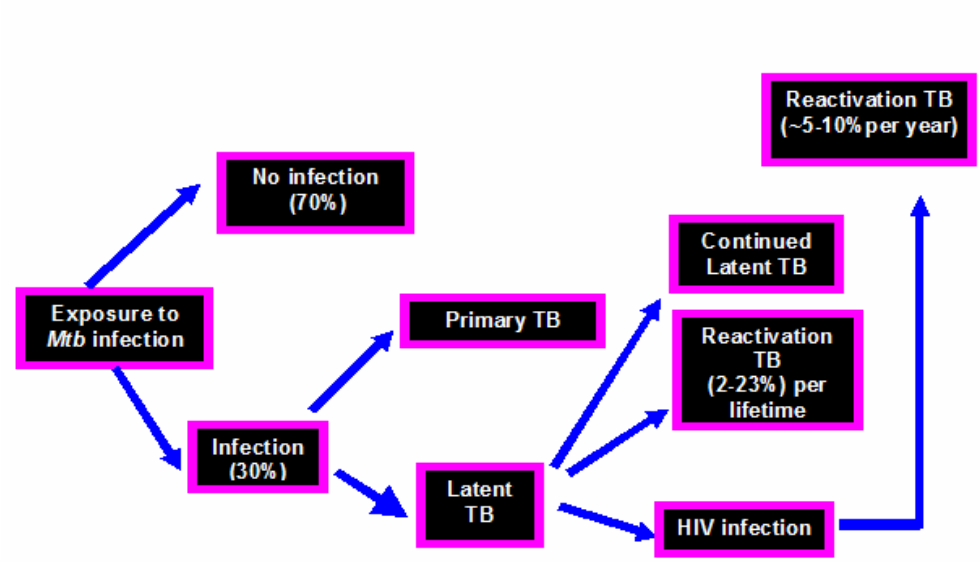

Fig 1.9 Outcome associated with exposure to Mycobacterium tuberculosis (Adapted from Parrish et al., 1998)

In the later life Post-primary TB develops by exposed individuals due to endogenous reactivation of the NRP Mtb from the early infection or due to exogenous reinfection with the same or different Mtb strain. Both reactivation and reinfection contribute significantly to the total TB burden which considers the local route of the epidemic and risk factors. The mechanisms of Mtb latency from a actively growing bacilli and reactivation from its NRP state are still unknown. In 2000, nearly half of all TB deaths in Africa were HIV associated and many of these cases were jointly due to reactivation of infections acquired in childhood or puberty. From recent studies, a few bacterial metabolites briefly described below have been concerned in induction of latency and /or reactivation [56].

1.5 Concerned Genes for Latent/NRP and reactivation TB

There are so many evidences that *Mtb* can regulate differentially the expression of some of its genes, when shifted from exponentially growing to stationary/latency phase. There are so many *in vitro* models are available based upon oxygen and nutrient starvation [58]. Ex vivo as well as in vivo models are

well known to study the NRP state of the bacteria within the host. Such studies have identified multiple gene loci that allow the mycobacteria to metabolically adapt to the environmental changes during latent infection. The *acr* gene (Rv2031c), encoding the 16-kDa α-crystallin like protein induced during mycobaterial stationary phase is a prototype in this respect. It is necessary for survival of the bacteria in macrophages and stabilization of cell structure by assisting protein refolding [59].

Other important examples of latency-related genes of Mtb described so far consist of the four *narGHJI* genes (Rv1161-4) that encode a functional nitrate reductase enzyme *in vivo* which plays a role in adapting the bacteria to the oxygen-limiting microenvironment (alternative pathway to utilize nitrate) [60]. Isocitrate lyase gene (Rv0467), icl, which allows the bacteria to have alternative pathway to utilize fatty acids as carbon and energy source during latent infection within lipid rich caseous granuloma debris [61,62]. The pcaA gene (Rv0470c), which encodes a cyclopropane synthase, an enzyme required for cord factor formation and mycolic-acid cyclopropane-ring biosynthesis in the cell wall, which may be used to alter antigen presentation by the host's immune system [63]. Up regulation of genes involved in stress responses such as PGRS and other sigma factors including the homologues of Mtb PE-PGRS genes (Rv3812/Rv1651c) [64] and the sigma factors, sigB (Rv2710) and sigF (Rv3286c) which may also contribute to mycobacterial survival in vivo [65]. sigH (Rv3223c) is required in vivo by Mtb for progressive pulmonary disease during latent infection [66]. These genes may give important clues about the physiology of the pathogen in the host, which may lead to the finding of important vaccine and drug targets against NRP TB.

In earliar literature so many models has been established .The aim of the development of these modeles, from which people can understand the complex environment encountered by Mtb during infection and which could provide advantages in future studies. One group suggested that survival in sealed liquid cultures held at 37C^{o} for as long as 12 years; variations were seen in the pH of these cultures, but only those between pH 6.1 and 7.6 yielded viable bacilli. In this, the study described the apparent linear arithmetic growth of tubercle bacilli. They noted that bacilli suddenly stopped growing at a critical turbidity and autolysis occurred as the glycerol, which stimulates oxygen consumption by Mtb,

caused abrupt catastrophic depletion of oxygen and death of the bacilli. In subsequent studies of so-called linear growth, the bacilli adapted to hypoxic conditions on settling through the gradient and became tolerant to anaerobiosis, on the basis of these observations, Wayne demonstrated the shiftdown to hypoxic NRP to have an orderly, regulated process [67]. In sealed tubes with slow stirring condition tubercle bacilli cease replicating, when oxygen concentrations decrease to the microaerobic level (1% oxygen saturation) and they enter a nonreplicating persistence state (NRP-1). With continued incubation the oxygen levels decrease further to anaerobic levels (0.06% oxygen saturation) and the second state, NRP-2 follows. This model has been used extensively as a simple model of dormancy for Mtb. While taking about the TB bacilli are surrounded by layers of immune cells and a fibrotic layer in the granuloma structure, Wayne hypothesized a microaerobic environment for *M. tuberculosis* in vivo. Oxygen limited in the granuloma is supported by gas concentration measurements of cavities from the lungs of living tuberculosis patients, where the overall pressure is negative, is enriched for carbon dioxide, 10.5% on average versus 3.5% for open cavities, partially depleted for oxygen, 6.3% on average versus 17.8% in open cavities. In the aspect of in vivo models, the Cornell model was the first animal model for dormant bacilli. This model involves partial clearance of Mtb infection by incomplete chemotherapy to induce the latent state [68]. Thus this model mimics the natural latency in humans where the bacilli may remain dormant for many years before being reactivated. As these models still have their limitations about explaining metabolic state of persistent mycobacteria and host immunity. A significant body of evidence accumulated over the last century suggests a link between hypoxic microenvironments within the infected host and the latent phase of tuberculosis. Since the study against Mtb grown in microbiological culture can differ from the bacteria present in human body. The use of an ex vivo cell-culture model, to assess the study on Mtb in an environment resembling that encountered during infection (objective of chapter 2) is very essential.

1.6 Other survival strategies

Apart from the strategies used by Mtb which can assist to survive inside the macrophages and defend from microbicidal functions described above, there

are several others, including the inhibition of apoptosis, which will be discussed in detail below. Another mechanism is the inhibition of the macrophage response to pro-inflammatory cytokines including IFN-γ by interfering with signalling events downstream of the IFN-γ receptor through the 19-kDa lipoprotein [66]. Furthermore, Mtb can suppress the expression of MHC class II on macrophages to prevent antigen presentation to CD4+ T cells by blocking transport and processing of the molecules, thereby avoiding an IFN-γ response [70]. Another potential virulence mechanism is the disorder of actin, as actin is essential for the scaffolding of endosomes during phagosome-endosome interactions and a correlation between the disruption of actin by Mtb and a delay in phagosomal maturation has been observed [70-72]. Thus, Mtb has evolved an array of mechanisms to allow survival within the macrophage, and it is not completely understood how the human macrophages can be stimulated to produce the factors necessary for killing the pathogen, nor whether this is possible at all *in vitro* or *in vivo*.

1.7 Controlling intracellular Mtb growth

It is normally very difficult to stimulate a macrophage to kill Mtb *in vitro,* even though an organized infection can sometimes be achieved [69]. In one study people have shown the killing of Mtb inside human macrophages has been attained using stimulation with bacterial lipoproteins (TLR ligands) [73]. In another study, IFN-γ or TNF-α activation of human macrophages containing avirulent mycobacteria led to more effective phagosomal maturation [74]. Previous data gives conflicting views as to the effect of the pro-inflammatory cytokine IFN-γ on intramacrophage replication of Mtb in primary human cells, as it was shown that IFN-γ could either inhibit [75] or promote [76] growth. Vitamin D has also been reported to be involved in the killing of intra-macrophage Mtb in human cells through the induction of cathelicidin in human cells. This was achieved upon TLR stimulation to upregulate the vitamin D receptor and the vitamin D-1–hydroxylase genes, or through simultaneous stimulation with pro-inflammatory cytokines [77-79, 80]. It is known that TNF-α as well as IL-1β are crucial for control of human TB, as medications that include antagonists of these can lead to reactivation of latent TB [81,82]. IFN-γ is also crucial as mutations in

the IFN-γ receptor gene leading to defective signalling result in higher susceptibility to Mtb infection [49]. However, it is still not known exactly what component of the phagolysosome kills Mtb in human infection and how the functionality of the macrophage can be enhanced and it is difficult to make out how the phagolysosome can be induced to kill the bacilli without knowing how or whether the cell can be stimulated to achieve killing.

1.8 Cell death during Mtb infection

Cell death is important for many processes in the body, development and immune regulation. Cell death can be programmed, where the cell decides to die in a tightly regulated manner, or more accidental in emergency situations such as tissue damage. Different types of cell death are defined based on morphologic and biochemical features, enzymologic criteria such as dependence on caspases, functional aspects such as whether death is induced to kill a pathogen or occurs accidentally, and immunological characteristics including whether death is accompanied by release of pro-inflammatory cytokines or anti-inflammatory manner [83]. The major known cell death pathways that macrophages can enter are shown in a diagram (Fig 1.10)

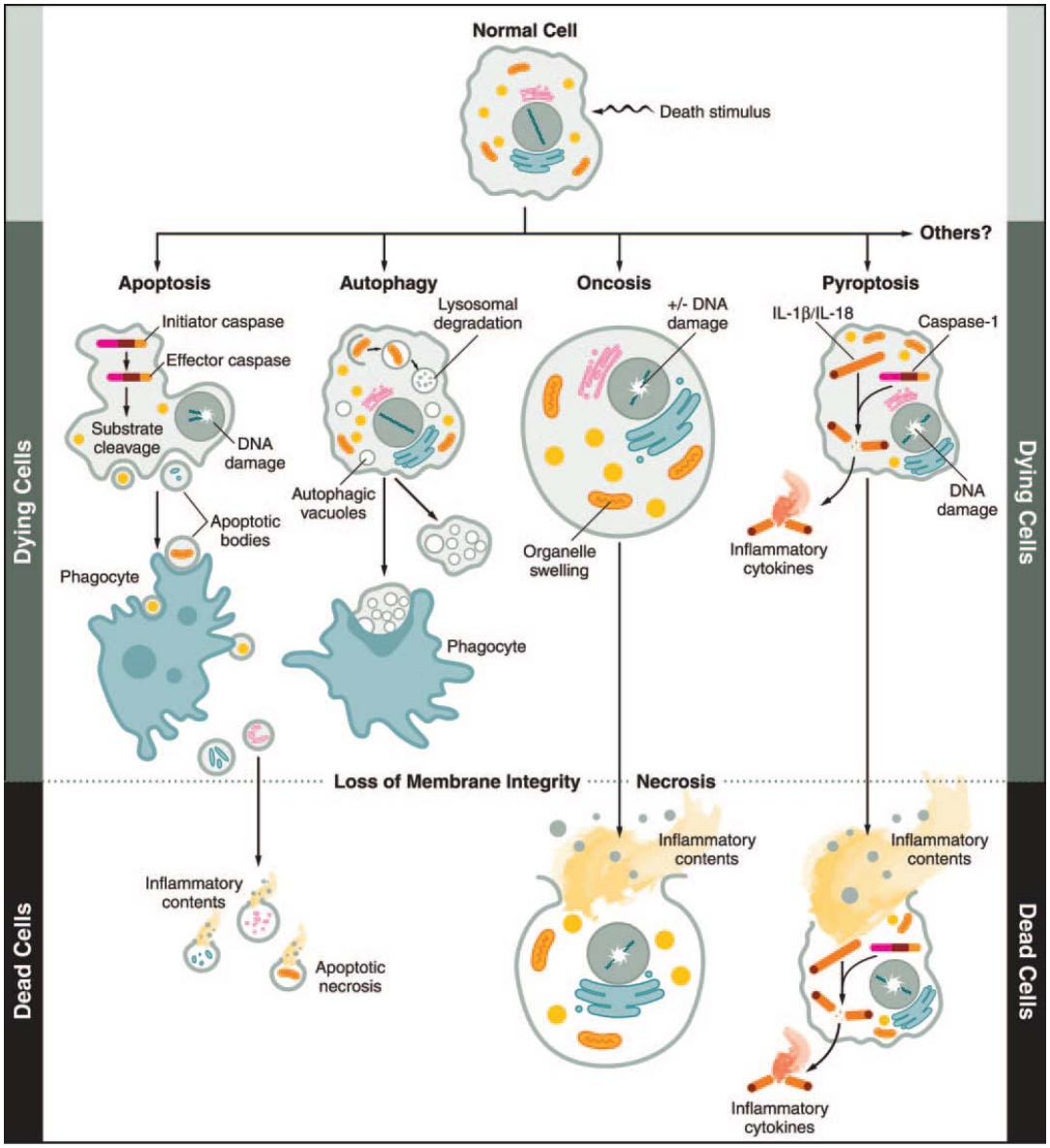

Fig 1.10 Pathways leading to cell death. (Adapted from Susan L. Fink1 et al., 2005)

In response to infection of a cell, inflammatory pathways are induced and the immune system attempts to clear the invading pathogen through activation of complement, antimicrobial mechanisms and cytokine release leading to immune cell recruitment. However, if the infection remains unresolved, the infected cell can undergo programmed cell death in order to deprive the microbe of its replication niche and expose it to extracellular immune defence components. This is a typical response to viral infections, but cells can also undergo apoptosis in response to bacterial infection, and many viruses and bacteria have evolved strategies to evade this response [84]. Mtb is one of the pathogens that is able to

manipulate the host macrophage cell death response to its own advantage. For a long time, there was a difference in the results obtained concerning host macrophage death upon Mtb infection, as some studies have shown Mtb to be pro-apoptotic [85-88], while others have shown the bacterium to be anti-apoptotic in macrophages [89,90]. However, it is becoming clear that virulent Mtb is able to both inhibit apoptosis initially upon infection in order to retain its replication niche, but also induce necrosis-like cell death in order to escape from its host cell enabling infection of new macrophages [91]. It is still not completely clear, however, what type of cell death is induced by Mtb and how this affects the immune response.

1.8.1 Apoptosis

Apoptosis is the classical programmed cell death type, with an important role in embryonic development. Apoptosis is non-inflammatory as the cell contents are kept inside the plasma membrane and the cell is rapidly taken up by neighbouring phagocytes by a process named efferocytosis, and apoptosis is thus mainly considered to have an anti-inflammatory and immunoregulatory role [92]. However, recent work points to a proinflammatory function of apoptotic neutrophils upon phagocytosis by macrophages [93]. Morphological features of apoptosis include plasma membrane blebbing, cell shrinkage, nuclear condensation and fragmentation, and formation of apoptotic bodies, but the plasma membrane remains intact. Biochemically, apoptosis is characterized by retention of high mobility group box 1 protein (HMGB1) in the nucleus and permeabilization of mitochondrial membranes which leads to a decrease in mitochondrial membrane potential. The processes are dependent on apoptotic caspases, and these are grouped into initiator caspases (caspase-2, -8, -9, and -10) and executioner caspases (caspase-3, -6, and -7). Apoptosis can be induced through the extrinsic or intrinsic pathway. In a resting healthy cell, the pro-apoptotic Bcl-2 family proteins Bax and Bak are kept inactivated through the anti-apoptotic Bcl-2 proteins, and the mitochondria remain intact. In the intrinsic pathway, activated by some microbial species, DNA-damaging agents, irradiation, growth-factor depletion, and some chemotherapeutics, Bax and Bak are activated through inhibition of anti-apoptotic Bcl-2 by Bcl-2-homology 3

(BH3)-only proteins. Thus, the outer mitochondrial membrane becomes compromised, and apoptogenic proteins, including cytochrome c, are released from the mitochondrial innermembrane space through a channel formed by the oligomerization of Bax and Bak [81, 82, 89, 92]. Recent work revel to a direct effect of BH3 on Bax and Bak through a conformational change leading to oligomerization and activation, as well as the indirect effect through inhibition of anti-apoptotic Bcl-2 [95]. Upon mitochondrial membrane permeabilization, cytochrome c forms the apoptosome complex together with apoptosis protease activating factor-1 (Apaf-1), and this mediates the activation of caspase-9. In the extrinsic pathway, binding of ligands (TNF, Fas ligand, and TRAIL) to death receptors leads to the assembly of the death-inducing signalling complex (DISC), and activation of caspase-8 and -10. Furthermore, the extrinsic pathway can be amplified by initiation of the intrinsic pathway as well through caspase-8-mediated cleavage of Bid, which then acts on the mitochondrion. The initiator caspases proteolytically activate the executioner caspases, and the executioner caspases are liberated from their inhibitor X-linked inhibitor of apoptosis (XIAP) by the mitochondrial death proteins SMAC/Diablo and HtrA2/Omi. The mature caspases are then able to cleave several different substrates giving different effects, including the inactivation of a DNA-repair enzyme and a DNase inhibitor, resulting in the mentioned morphological features such as DNA fragmentation. Additionally, apoptosis can be induced by granzyme B released from NK cells and cytotoxic T cells, and by a lysosomal pathway following lysosomal membrane permeabilization and release of cathepsins, which in turn act on mitochondria [84, 83, 91, 92]. Apoptosis can lead to killing of pathogens through removal of the replication niche of obligate intracellular microbes, exposure to immune surveillance outside the cell, and through recognition and uptake of apoptotic bodies leading to more efficient microbicidal mechanisms in the phagocytosing macrophage as well as more effective antigen presentation [84]. Apoptosis has been assigned a role in the immune response against Mtb, as the bacterium has been found to inhibit host cell apoptosis. This is achieved in part by the product of the gene*nuoG*, as deletion of this gene from mycobacteria results in elevated levels of apoptosis [89, 90]. Furthermore, avirulent strains of Mtb have been found to be more potent inducers of TNF-α-dependent apoptosis than their virulent counterparts [95], and apoptosis in response to avirulent strains can be

enhanced by addition of TNF-α while that in response to virulent Mtb cannot [97]. Apoptosis exerts an antimicrobial effect on intracellular Mtb, as evidenced by reduced bacterial viability upon infection of macrophages and subsequent chemical induction of apoptosis [96]. Uptake of apoptotic macrophages containing Mtb by fresh cells also results in reduced bacterial viability [86, 98]. Thus, prevention of macrophage apoptosis by virulent Mtb appears to be a mechanism of virulence, and apoptosis is used by the host as a defence mechanism and is detrimental to the bacilli. In neutrophils, on the other hand, Mtb is a very potent inducer of apoptosis [93, 99].

1.8.2 Necrosis

Necrosis is a different type of cell death, which is usually not planned, and which occurs in response to excessive stress, microbial invasion or tissue damage. Necrosis elicits inflammation as the cell membrane is permeabilized and the cell contents are spilled into the surrounding milieu. Morphological features of necrosis include organelle, nuclear and cell swelling, and DNA fragmentation in a disorganized fashion due to hydrolysis, but no chromatin condensation. Necrosis occurs independently of caspase activation, and was long thought to always be completely accidental and uncontrolled. However, it is becoming evident that necrosis can also be the result of the failure of death receptor ligation to induce caspase activation and thus apoptosis. This can lead to activation of the serine/threonine kinases receptor interacting proteins (RIP) 1 and 3 and subsequent ROS production, calpain activation, destabilization of lysosomes, and release of cathepsins. In large quantities, these mediators act as stress signals which can induce necrosis [83, 84]. Necrosis can thus be considered an emergency cell death pathway which is detrimental to the host as it results in excessive inflammation on the one hand, and a cry for help that attracts the attention of surrounding immune cells in response to infection on the other hand. A large body of evidence is accumulating that virulent Mtb induces necrosis of the infected macrophages upon infection and replication to a certain threshold in bacterial load. Different reports give different views of the features of this necrotic cell death [91]. One study showed high-MOI infection (MOI≥ 25) to induce propidium iodide-positivity, indicating plasma membrane

permeabilization, of infected cells within 6 h of infection, as well as externalization of phosphatidyl serine and nuclear condensation, in a manner independent of caspases but dependent of cathepsins [86]. Another study found H37Rv to induce caspase independent plasma membrane permeabilization within 24 h after infection of macrophages at MOI 10-20 and this was dependent on serine proteases [87]. A third study showed that infection with H37Rv for 72 h at MOI 10 led to lysis of the host macrophages in a manner dependent on mitochondrial membrane destabilization [85]. Furthermore, recent work shows that Mtb is capable of inhibiting the production of the eicosanoid lipid mediator PGE2, which plays a role in preventing mitochondrial damage as well as repairing the plasma membrane. This leads to necrosis instead rather than apoptosis, a situation which is favoured by the bacterium [100, 101]. Additionally, H37Rv can prevent completion of the apoptotic envelope by removing a domain from annexin-1, and this is also thought to play a role in inducing necrosis rather than the more hostile apoptosis, allowing dissemination in the lung [102]. A mouse study using *M. marinum* also found the bacilli to be cytotoxic, and that this was a prerequisite for bacterial spread to neighbouring cells and throughout the body [38]. Thus, induction of necrosis is an important step in the pathogenesis of TB, allowing rupture of the host cell and spread to other cells once the bacterial load is sufficiently high.

1.8.3 Other types of cell death

Apart from the types of cell death described above, macrophages can also lose viability through autophagic cell death, which can occur upon autophagy of vesicles containing the microbe and subsequent delivery to mature phagosomes and killing of the microbe, in cases where engulfment of the cell's own cellular material is excessive. This type of cell death is generally beneficial for the host as it allows removal of the cell in a quiescent manner, simultaneously to promoting the antimicrobial functions of the cell [103], and autophagy has been shown to play a role in the effective macrophage response to Mtb infection [104, 105, 106]. It is also likely that future cell biology studies will lead to the discovery of yet more modes of cell death (Fig1.10).

1.9 Diagnosis of tuberculosis

TB can be a difficult disease to diagnose, mainly due to the difficulty in culturing this slow-growing organism in the laboratory (4–12 weeks for blood culture). A complete medical evaluation for TB must include a medical history, a chest X-ray, and culture based examination.

1.9.1 Medical symptom

The medical history includes obtaining the symptoms of pulmonary TB: productive, prolonged cough of three or more weeks, chest pain [107]. Systemic symptoms include fever, chills, night sweats, appetite loss, weight loss, and easy fatigability. Other parts of the medical history include prior TB exposure, infection or disease; past TB treatment; demographic risk factors for TB; and medical conditions that increase risk for TB disease such as HIV infection. Tuberculosis should be suspected when a persistent respiratory illness in an otherwise healthy individual does not respond to regular antibiotics.

1.9.2 Chest X-ray

Tuberculosis creates cavities visible in X-rays like this one in the patient's right upper lobe (Fig. 1.11). In active pulmonary TB, infiltrates or consolidations and cavities are often seen in the upper lungs with or without mediastinal or hilar lymphadenopathy or pleural effusions (tuberculous pleurisy) [107]. However, lesions may appear anywhere in the lungs. In disseminated TB a pattern of many tiny nodules throughout the lung fields is common - the so called milliary TB. In HIV and other immunosuppressed persons, any abnormality may indicate TB or the chest X-ray may even appear entirely normal. Abnormalities on chest radiographs may be suggestive of, but are never diagnostic of, TB. However, chest radiographs may be used to rule out the possibility of pulmonary TB in a person who has a positive reaction to the tuberculin skin test and no symptoms of disease.

1.9.3 Acid Fast Staining

Ehrlich-1882 discovered the AF nature and Franz Ziehl 1882 and F. Neelsen 1883 modified it: Ziehl-Neelsen stain. Acid-fastness is a physical property of some

bacteria referring to their resistance to decolorization by acids during staining procedures. The high mycolic acid content of certain bacterial cell walls, like those of Mycobacteria, is responsible for the staining pattern of poor absorption followed by high retention. The most common staining technique used to identify acid-fast bacteria is the Ziehl-Neelsen stain, in which the acid fast bacilli are stained bright red and stand out clearly against a blue background (Fig 1.12).

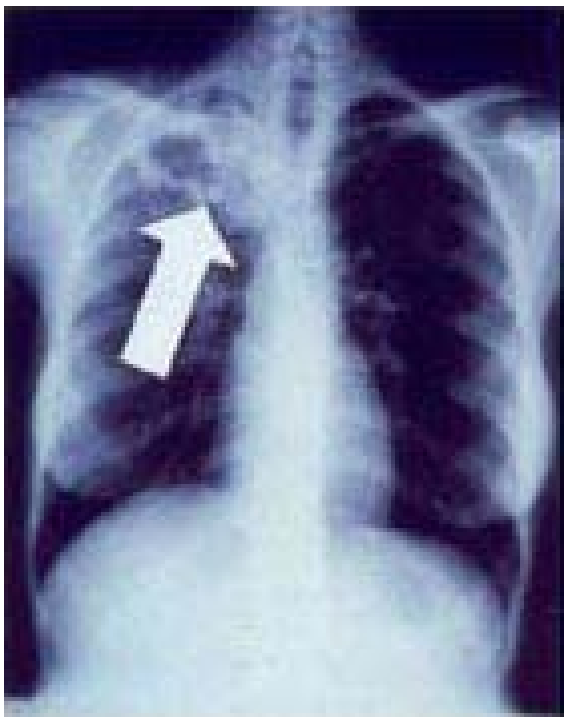

Fig. 1.11 Chest X-ray of a patient suffering from TB.

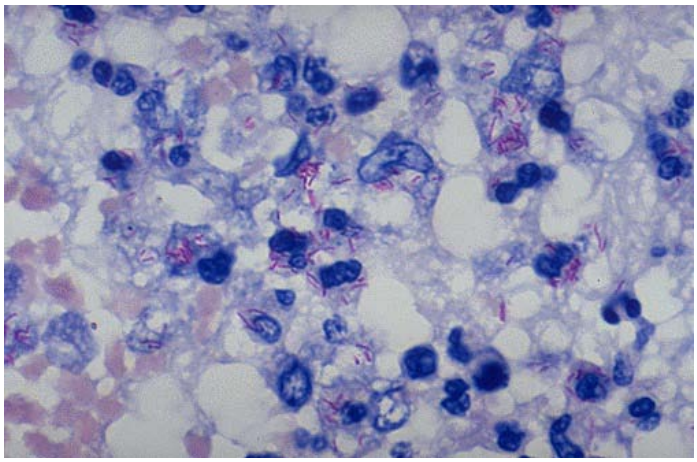

Fig. 1.12. Acid fast stain of sputum containing *M. tuberculosis*. The bacteria are visible as small pink stained rods

The gold standard of TB diagnostics is confirmation with its growth in selective media [108]. This culturing is 1000 times more sensitive than microscopy, allows precise species identification, can be applied to drug susceptibility testing and may be useful to identify epidemiological links between patients or to detect laboratory cross contamination. In general, the sensitivity and specificity of culture method is 80-85% and 98% respectively [109]. However, their outcome is delayed by extremely low growth rate of mycobacteria. Contrary to a number of environmental mycobacteria that are rapid growers, yielding colonies in 7 days or less, Mtb exhibits a slow growth rate, requiring 14-21 days to generate visible colonies and does not produce any pigment (Fig. 1.13). With the advancement in culture system in 1980s BACTEC and biphasic culture methods were developed for faster recovery than traditional culture system [110].

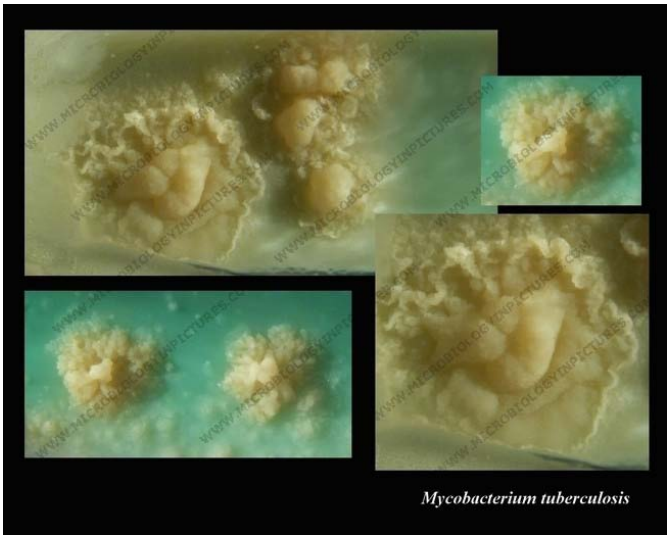

Fig. 1.13 Colonies of *M. tuberculosis* as appeared on selective Dubos medium.

The introduction of nucleic acid amplification assays using polymerase chain reaction (PCR) in 1985 brought the most progress in TB diagnostics [111]. In 1989, PCR was first applied to clinical samples, sputum, gastric aspirates, abscess aspirates and biopsy samples to detect a mycobacterial gene which allows the differential diagnosis of Mtb from non-tuberculous mycobacteria. A number of candidate genes have been tested for usage as diagnostic targets. Among them, IS6110 repeat sequence has been used to detect Mtb directly in clinical samples

[112]. However, some other factors such as an endogenous amplification inhibition factor of Mtb or unreliable quality control can influence susceptibility to both false positives and negatives and have hampered clinical uses of this assay [113]. However, the severity of problems began to reduce after development of automated, robust, commercial tests which made clinical risk assessment possible [114].

Another old diagnostic method is the tuberculin skin test developed by Dr. Koch [115]. The test is for the identification of the host infected with Mtb. Tuberculin skin tests involve the intracutaneous injection of five tuberculin units of Purified Protein Derivative (PPD) prepared by the Montoux technique (Fig. 1.14). The skin reaction of Mtb infection may discriminate from *M. avium* or other non-tuberculous infections [116]. However, the cross reactivity of PPD with *M. bovis* BCG vaccinated individuals represents a major limitation in applying this method to TB patients [117]. Therefore, the interpretation of the skin test needs to be made in the clinical context and with evaluation of other risk factors for infection.

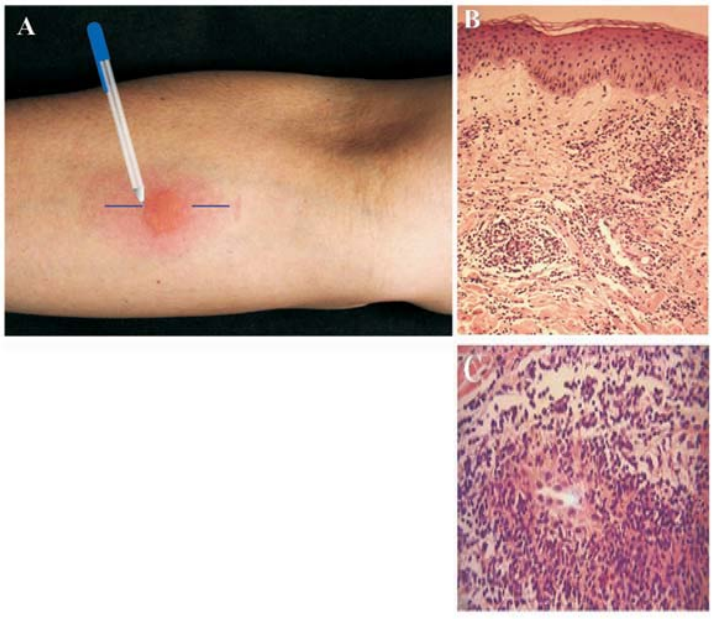

Fig. 1.14 Tuberculin test performed with 2 units of purified protein derivative, yielding 92-mm indurations after 72 hours.

The identification of regions of the Mtb genome those are not present in *M. bovis* BCG and non-tuberculous mycobacteria provide a unique opportunity to develop new specific diagnostic reagents. Fortunately, genomic studies have shown that

the region of differentiation (RD-1) is shared only by Mtb, *M. szulgai, M. marinum* and *M. kansasii*. The RD-1 region contains the early secretary antigen target -6 (ESAT-6) and the culture filtrate protein-10 (CFP-10) that are potential targets of the specific immune response against Mtb [118]. Interferon (IFN)-γ secreted by TB patient's memory and effecter T-cells by the response of these proteins or overlapping peptides is a novel diagnostic marker of TB infection and could be discriminated from BCG vaccination or non-tuberculous mycobacteria infection [119]. In vitro blood test measuring of IFN-γ is very useful to identify contacts of TB cases and show remarkable concordance with the tuberculin skin test [120]. Accordingly, the blood test has shown a greater sensitivity than the tuberculin skin test in active TB of HIV co-infected subjects.

The recent progress in TB serology is also represented by the multi-antigen test using purified antigens. The recent studies of an Enzyme Linked-ImmunoSorbent Assay (ELISA) test using the 38Kda antigen, lipoarabinomannan (a mycobacterial lipoglycan, LAM), MPT-64, and glutamine synthase could achieve 93% sensitivity and 76% specificity with combination of AFB microscopy, suggesting that in the appropriate epidemiological and laboratory context this test could be used to improve the performances of the AFB smear test alone [121]. However, serological tests alone do not appear to help diagnosis of sputum positive pulmonary TB [122].

1.10 Treatment of Tuberculosis

About one third of the world's population has latent tuberculosis, caused by Mtb infection [123]. From this pool, roughly 9 million cases of active tuberculosis emerge annually, resulting in 2-3 million deaths. Most new cases occur in the most populated nations, India and China but the highest rates of disease are seen in sub-Saharan Africa, the Indonesian and Philippine archipelagos, Afghanistan, Bolivia, and Peru. In these regions, case rates typically exceed 300 cases per 100 000 per year [123,124]. Although the incidence of tuberculosis declined in North America and Western Europe throughout most of the latter half of the 20th century, case rates have increased over the past 10 years mainly because of immigration, HIV/AIDS, and the neglect of tuberculosis control programmes [125,126]. One vital factor in curbing the increase of tuberculosis is the

instigation of proper treatment that not only encompasses an effective regimen but also ensures compliance with and response to treatment. This review highlights current treatment recommendations for tuberculosis.

1.10.1 Principles of chemotherapy and rationale for multidrug treatment

Treatment using more than one drug is based on two principles: preventing acquired drug resistance and enhancing efficacy. Tubercle bacilli undergo random chromosomal mutations that have made them resistant to every drug used to treat tuberculosis. Fortunately, these mutations are infrequent because they are unlinked (in terms of chromosomal location or function) and specific to a drug or drug class, spontaneous generation of an organism with multi-drug resistance is extremely doubtful [127]. Acquired drug resistance for tuberculosis is almost caused by insufficient treatment. This can include failure of the patient to take the prescribed drugs, failure of the physician to prescribe appropriately, failure of the health- care system to ensure that drugs are available, or rarely malabsorption of the drug(s) due to dysfunction of the digestive system or substandard bio-availability of the preparation. Treatment that uses a combination of drugs has been shown to accelerate the response of the disease to treatment and to shorten the length of treatment required to cure (table 1.2). Rifampicin and isoniazid are the main drugs used today, rifampicin being the more important agent in terms of reducing the duration of treatment and assuring favourable outcomes [128]. Nine month regimens using rifampicin and isoniazid, together with an introductory phase of streptomycin or ethambutol, or both, have been predicted to cure 95% or more patients [129]. Studies from the UK's Medical Research Council showed that, if pyrazinamide is included in the treatment for the first two months, the length of treatment could be reduced to six months and still retain cure rates of 95% or more [130]. A regimen of rifampicin, isoniazid, and pyrazinamide given to patients who have strains of the bacilli resistant to isoniazid—the most common type of resistance—is thought to result in treatment failure and acquired resistance to rifampicin. Therefore, the American Thoracic Society and US Centers for Disease Control and Prevention recommended in 1994 that a fourth

drug, ethambutol, should initially be included in the treatment for patients in whom the bacilli might be susceptible to resistance [131]. Such individuals may be immigrants from regions known to have a high prevalence of resistance, people from urban areas, or individuals with a medical history that might predispose them to resistance; arbitrarily, the fourth drug was to be included in areas in which the level of resistance was known to be 4% or more. In 1998, the British Thoracic Society embraced regimens that use four drugs for the initial phase of treatment as standard practice. It is recommended that all patients with tuberculosis undergo a test for HIV. Supplements of pyridoxine (vitamin B6) not to exceed a daily dose of 50 mg—are suggested for patients taking isoniazid to prevent peripheral neuritis. Particular attention should be given to patients at risk of neuropathy, including patients who are malnourished or pregnant. Baseline liver function tests and periodic and regular monitoring are advocated in view of the potential hepatotoxicity of isoniazid, rifampicin, and pyrazinamide. The risk of major liver damage is less than 1%, but mild asymptomatic increases in transaminase blood concentrations are seen in up to 20% of patients. Doses of ethambutol should be carefully adjusted in patients with renal impairment. In addition, patients taking ethambutol should have their visual acuity checked initially and monitored monthly (Snellen acuity and Ishihara colour). They should be instructed to report promptly any perceived disturbances in their vision. Hospital admission is not routinely indicated for patients with tuberculosis unless the clinical illness merits such care, justifying psychosocial circumstances exist, or patients have prognostic factors associated with poor short term outcome (respiratory failure or death) such as lymphopenia, advanced age, or alcoholism [132]. To prevent nosocomial transmission, patients with tuberculosis (and suspected cases) should be placed in rooms of negative pressure and frequent air changes and, ideally, the means to filter out or lethally irradiate the tubercle bacilli with ultraviolet radiation. The first line agents and their common drug toxicity are listed in table1. 2. A low and unavoidable risk of relapse is present after treatment. For the regimens described in table 1.2, the probability of relapse is less than 5%. Most recurrences occur within six months and the disease usually has the same drug susceptibility profile as before treatment [133]. Current guidelines do not state the need for surveillance after treatment, especially when drugs have been given under supervision. Rather, patients should be instructed to

return to their clinic or physician after treatment when their clinical status changes; in these instances, suitable tests, including examination of sputum samples and chest radiographs, should be carried out.

Table 1.2 Dosages of first line antituberculosis drugs and major adverse effects (Data adapted from BMJ VOLUME 325 30 NOVEMBER 2002)

Drug	Dosage		Adverse effects
	Daily	Twice or thrice weekly	
Isoniazid	5 mg/kg oral (maximum 300 mg)	900 mg twice weekly 600 mg thrice week	Hepatitis, peripheral neuritis, drug induced lupus, seizures, and hypersensitivity with rash and fever. Drug interactions with dilantin and disulfiram
Rifampicin	10 mg/kg oral (maximum 600 mg)	10 mg/kg 600 mg twice weekly 600 mg thrice weekly	Orange body secretions, flu-like syndrome, hepatitis, thrombocytopenia, nausea, anorexia, diarrhoea, renal failure, and multiple drug interactions
Pyrazinamide	25-30 mg/kg oral	30-35 mg/kg	Hyperuricemia, hepatitis, rash, nausea, and anorexia
Ethambutol	25 mg/kg initial 2 months, then 15 mg/kg oral	50 mg/kg twice weekly 30 mg/kg thrice weekly	Optic neuritis and gastrointestinal discomfort
Streptomycin	15 mg/kg intravenously or intramuscularly (maximum 1.0 g) 5 days a week	15 mg/kg (maximum 1.5 g) twice weekly or thrice weekly	Ototoxicity, vestibular dysfunction, nephrotoxicity, rash, and hypersensitivity reactions

1.10.2 The mode of action of current drugs for tuberculosis

According to their mode of action TB drugs can be grouped as inhibitors of cell wall biosynthesis (D-cycloserine, isoniazid, ethionamide and ethambutol), inhibitros of protein syntheis (streptomycin, kanamycin and capreomycin),

inhibitors of nucleic acid synthesis (rifampin, quinolones) and inhibitors of membrane energy metabolism (pyrazinamide) (Table 1.3) [134]. D-Cycloserine targets peptidoglycan biosynthesis in various bacteria, including mycobacteria [134]. It is a structural analogue of D-alanine and competitively inhibits the action of D- alanine racemase and D-alanyl-D-alanine synthetase. Isoniazid is a prodrug which targets mycolic acid biosynthesis. This prodrug is activated by catalase-peroxidase encoded by *kat G* (Rv1908c) [136]. This was shown when *KatG* from Mtb was cloned and transformed into *M. smegmatis* which is a naturally more resistant to isoniazid. KatG is thought to be able to oxidize isoniazid into an electrophilic species, which is presumed activated form of isoniazid. The molecular target for isoniazid has been debated for some time. Jacobs and colleagues proposed the target of isoniazid is an enoyl reductase-NADH binary complex [137]. The enoyl reductase is encoded by *inhA.* Ethionamide is now thought to act in a similar manner as isoniazid including the activation step [138]. The proposed target of ethambutol was provided by Takayama and Kilburn who showed that it is likely inhibitor of arabinan biosynthesis of arabinogalactan (AG) and LAM [139]. They demonstrated that the incorporation of [^{14}C] from [^{14}C] glucose into AG arabinan was immediately inhibited upon exposure to ethambutol. This implicated that AG specific arabionosyl transferases were the target for ethambutol. Three putative arabinosyltransferases have been identified, *embC, embB* and *embC* [140,141]. Ethambutol resistance was observed upon the over expression of the latter two genes and a third termed *embR*, which has been postulated to act as a regulator. Recent studies have shown that *embB* is the most mutated gene in ethambutol -resistant strains of mycobacteria and that amino acid 306 of *embB* is the most mutated position in Mtb. From this, it is clear that arabinosyltransferases are the site of action of ethambutol [142].

Rifampicin is responsible for the reduction of the duration of therapy. Its mechanism of action is based on the inhibition of bacterial DNA dependent RNA polymerase, which is crucial for bacterial transcription. Despite the importance of rifampicin in TB treatment, the emergence of different rifampicin-resistant bacteria, increase the problems to global TB control. This resistance occurs during therapy against active TB and normally arises from mutations in the β-subunit of the ribosomal polymerase gene (*rpoB*) [143]. Quninolone derivatives possess

potent antibacterial activities with a broad spectrum organism including Mtb. The inhibition of bacterial multiplication caused by quinolone derivatives is in general due to the inhibition of two bacterial enzymes: DNA gyrase (topoisomerase II) and topoisomerase IV enzymes. DNA gyrase is an essential protein involved in the replication, transcription and reparation of bacterial DNA. Topoisomerase IV is responsible for decatenation that is removing the interlinking of daughter chromosomes thereby allowing segregation into two daughter cells at the end of the replication round [144].

Pyrazinamid is an important sterilizing drug. However, the mode of action is poorly understood. It is a prodrug and activated by Mtb pyrazinamidase which is only active at acidic pH. Pyrazinamidase converts Pyrazinamid to the active form, pyrazinoic acid. Pyrazinoic acid and Pyrazinamid de-energize the membrane by collpsing the membrane potential and affect the membrane transport function at acid pH [145].

Table 1.3 Classes of AntiTB drugs and their mode of action (Data extracted from *Respiratory Research* 2006 **7**:118.)

Agent	**Mechanism of Action**	**Activity Against *M. tuberculosis***
First-line agents		
Rifampicin (RIF)	Inhibits bacterial RNA synthesis by binding to the β subunit of bacterial DNA-dependent RNA-polymerase (DDRP) Inhibition of DDRP leads to blocking of the initiation chain formation in RNA synthesis. One of the most effective antituberculosis agents available and is bactericidal for intra- and extra-cellular bacteria.	RIF inhibits susceptible organisms at concentrations of less than 1μg/ml.
Isoniazid (INH)	Most active drug for the treatment of TB caused by susceptibe strains. Is a pro-drug activated by katG, which exerts its lethal effect through inhibition of synthesis of mycolic acids, an essential component of mycobacterial cell walls, through formation of a covalent complex with an acyl carrier protein (AcpM) and KasA, a beta-ktoacyl carrier protein synthetase.	INH inhibits tubercle bacilli at a concentration of 0.2μg/ml.

Pyrazinamide (PZA)	Converted to the active pyrazanoic acid (encoded by pncA) by pyrazinamidase in susceptible organisms. Pyrazanoic acid lowers pH in the immediate surroundings of *M. tuberculosis* – organism is unable to grow. May also function as an antimetabolite of nicotinamide and interfere with the synthesis of NAD, inhibiting the synthesis of short-chain, fatty-acid precursors.	Inhibits *M. tuberculosis* and other mycobacteria at concentrations of 20 μg/ml.
Ethambutol (ETB)	Inhibits mycobacterial arabinosyl transferases (encoded by the embCAB operon) involved in the polymerization of D-arabinofuranose to arabinoglycan, an essential cell wall component.	Ethambutol is generally bacteriostatic, but at high doses (25mg/kg) can be bactericidal. Inhibits susceptible strains of *M. tuberculosis* at concentrations of 1–5μg/ml.
Streptomycin, kanamycin, amikacin, capreomycin	The aminoglycosides are irreversible inhibitors of protein synthesis through binding to specific 30S-subunit ribosomal proteins.	Bactericidal. *In vitro* and *in vivo* clinical data support use.
Ciprofloxacin, ofloxacin, levofloxacin, moxifloxacin, gatifloxacin, sparfloxacin	Inhibit bacterial DNA synthesis through inhibition of bacterial topoisomerase II (DNA gyrase) and topoisomerase IV, which are responsible for the relaxation of supercoiled DNA and the separation of replicated chromosomal DNA, respectively.	Bactericidal, broad spectrum antibacterials. In-vitro and in-vivo clinical data support use. Ciprofloxacin and levofloxacin inhibit strains of *M. tuberculosis* at concentrations of less than 2μg/ml. Newer agents (moxifloxacin, gatifloxacin, sparfloxacin) have lower minimum inhibitory concentrations.
Ethionamide	Chemically related to INH, converted via oxidation to ethionamide sulfoxide, blocks the synthesis of mycolic acids.	Inhibits most tubercle bacilli at concentrations of 2.5μg/mL or less.
Cycloserine	Structural analogue of D-alanine, inhibits incorporation of D-alanine into peptidoglycan pentapeptide through inhibition of alanine racemase.	Inhibits strains of *M. tuberculosis* at concentratins of 15–20μg/ml.
P-aminosalicylic	Anti-metabolite interfering with incorporation of	Inhibits tubercle bacilli at concentrations

acid (PASA)	para-aminobenzoic acid into folic acid – folate synthesis antagonist. of 1–5µg/ml.

1.10.3 Non-adherence to treatment and directly observed therapy (DOT)

Some patients with tuberculosis, as in virtually all chronic disorders, will fail to take their drugs [146]. Unique public health philosophies and practices have evolved, however, which have helped to tackle this problem. For example, the people in industrialised nations has come to expect the air to be free of tuberculosis, in the same way that the water is free of such potentially lethal pathogens as typhoid and cholera. This has led to mandated treatment, quarantine, or even short term incarceration of patients in the United States or other countries [147]. DOT programmes use a nurse or surrogate to deliver and observe patients taking all the doses of their drugs, rather than relying on patients to take them on their own. To facilitate DOT, intermittent (less frequently than daily) regimens have been used. Two popular intermittent regimens are represented in table (1.2); these six month regimens have been shown to be comparable in efficacy to daily treatment of equal length. The patients may either come to a health facility (clinic based DOT) or be seen elsewhere— for example, at home, work, or shelter (community based DOT) [146,147] (fig 1.15). Preparations using drug combinations— for example, isoniazid and rifampicin (Rifamate; Hoechst Marion Roussel) and isoniazid, rifampicin, and pyrazinamide (Rifater; Hoechst Marion Roussel) — are also available, and may improve adherence.Whether such drug combinations prove to be more beneficial, given their increased cost and the reduced ability to discriminate which drug is responsible for toxicity or intolerance, has yet to be shown. DOT is highly effective at promoting successful treatment. A comparison of self treatment versus various forms of DOT has shown that completion of treatment is significantly higher when the treatment is supervised [150]. Some observers have argued that DOT is an infringement of individual libertybut well designed DOT programmes can be regarded as enhancing the health service and as a manifestation of community care [151]. Successful DOT programmes provide a variety of incentives and enablers (practices that facilitate the treatment programme) to make them “consumer

friendly." Incentives may include rewards for making oneself available for treatment—for example, provision of social services, food stamps, assistance with housing or, in some cases, cash payments for the inconvenience. Some enablers facilitate treatment by being open during convenient hours, being in accessible locations, and providing help with transport, child care in clinics, or comprehensive services at a single site—for example, radiology, blood drawing, and sputum induction services. Concern exists that governments cannot afford to provide DOT, but recent analyses show that, by assuring prompt cure, preventing relapses, and lessening acquired drug resistance, DOT programmes result in net savings to the community [152,153].The impact of DOT

Fig 1.15 Directly observed therapy in Rangoon, Burma. The two Buddhist nuns are receiving antituberculosis drugs at a public health facility that is also a poultry market

programmes may be seen in the reduction in the number of cases of TB in the 1990s in the United States.All together an increase in the proportion of patients, receiving DOT in 1990 from 4% to over 70% by 2000 [154]. From 1995 to 2000, the rate of tuberculosis in the United States fell by an average of 7.8% per year. Although the broad implementation of DOT was not the only intervention during this period (improved measures to limit nosocomial transmission were also introduced), we believe it was the major fac- tor driving these improved rates.

1.10.4 Treatment in developing countries

In theory, the diagnosis and treatment of tuberculosis is the same in developing countries and industrialised countries, but economic limitations mean

that significant differences exist in practice. As advocated by the World Health Organization's DOT short course policy, microscopy of sputum is the primary and often sole means of diagnosis in nations that have limited resources. It has notable limitations: firstly, diagnosis by microscopy of unconcentrated sputum is far less sensitive than that of concentrated sputum smears (better) or sputum culture (best); secondly, culture of the tubercle bacilli is required for the early detection of drug resistance, which may compromise the response of the bacteria to standard treatment. Historically, many of the poorer nations have used a highly economical drug regimen consisting of isoniazid and thiacetazone given for 15 to 18 months, typically costing a total of only US$10-15 per person. Although the regimen is attractive in terms of cost, it is undesirable because the treatment takes longer, has marginal efficacy, and is ineffective in the presence of resistance to isoniazid. Regimens containing thiacetazone also have a greater risk of causing potentially lethal cutaneous drug reactions in people with AIDS [155]. Most nations have now developed the standard WHO six month regimens, which includes isoniazid, rifampicin, pyrazinamide, and ethambutol. Because of the profoundly deleterious effects of resistance to rifampicin, strong emphasis is placed on DOT when rifampicin is used.

1.10.5 Treatment of multidrug resistant tuberculosis

Multidrug resistant tuberculosis—which occurs when tuberculosis strains are resistant to at least isoniazid and rifampicin—is important clinically because it substantially increases the risks of treatment failure, further acquired resistance, and death. Its prevalence varies widely and generally reflects poorly organised treatment practices [156]. People who are particularly at risk include those with histories of treatment for tuberculosis, those from high risk areas, and patients or healthcare workers from institutions (hospitals, clinics, prisons, or nursing homes) in which there has been epidemic transmission of resistant strains. Initial therapy for patients with suspected multi-drug resistant tuberculosis might reasonably use extended empirical regimens, especially if patients have extensive lung disease or perilous extrapulmonary forms of tuberculosis such as miliary or meningeal disease. For patients with proved disease, it is important to give at least four drugs to which the mycobacteria are susceptible—usually three oral drugs and one

injectable drug. Generally, an injectable drug such as an aminoglycoside is given for three to six months after the initial date of conversion of sputum cultures from testing positive for Mtb to testing negative, and the patient continues to take oral antimycobacterial drugs for 15-18 months after the last positive sputum culture.
Treatment of latent infection for people exposed to multidrug resistant bacilli is problematic because the only drugs widely deemed appropriate are isoniazid and rifampicin. A Delphi survey of a panel of experts on tuberculosis failed to reach a defined consensus on the most appropriate regimen for people exposed to multi-drug resistant tuberculosis, although a combination of pyrazinamide and ciprofloxacin was considered some-what appropriate [157]. Experimentally, the combination of pyrazinamide and ofloxacin has been shown to have a favourable intramacrophage antimycobacterial effect [158]. In light of the recent cases of severe hepatotoxicity associated with preventive treatment comprising either pyrazinamide and rifampicin or pyrazinamide and fluoroquinolone however, fluoroquinolone monotherapy without pyrazinamide may be considered for people whose tuberculin skin test recently converted who have been exposed to multidrug resistant TB, with the caveat that long termefficacy data on these treatments are lacking [159-162].

1.10.6 Potential chemotherapeutics

The Darwinian principle of natural selection predicts that drug resistant strains of TB will continue to develop. Research into new forms of treatment is therefore important. Fluoroquinolones are the most promising new agents for treatment of tuberculosis [163]. Additional potential therapeutics include other classes of pharmaceuticals such as oxaolidinones (eg linezolid), treatments that affect the immune system such as improvement of BCG or *Mycobacterium vaccae* vaccines with or without cytokine augmentation treatment and sterilisation of the semidormant population by targeting the citrate lyase pathway [164-165].

1.11 Control of tuberculosis

Over a century ago Robert Koch already declaimed: “This is the time to control TB” [166]. However, after a strong decline in the incidence of tuberculosis in the developed world, the incidence decreased only little in the past

decades mostly due to reactivation of latent TB in immigrant from TB endemic regions. In the developing world the incidence had only slightly decreased, when the emerging HIV pandemic resulted in a significant increase in incidence again. Thus, 100 years later, it became clear that control and eradication of TB were not imminent. In 1993, TB was declared "a global emergency" by the World Health Organisation (WHO) and "control of TB" became part of the United Nations Millennium Development Goals. International TB control programs focused almost entirely on early detection of active tuberculosis and effective treatment. Direct observed therapy (DOTS) is being used to improve compliance to therapy and cure rate. There are two important factors that preclude the success of this TB control strategy [167]. First, in many areas of the world where TB is highly endemic, there is also a high prevalence of HIV-infection. In HIV and *M. tuberculosis* co-infected individuals, the risk of progression to active TB is significantly higher, up to 8-10% per year, which greatly increases the number of active TB cases [168,169]. Secondly, effective TB treatment is complicated by the increasing presence of multidrug-resistant TB and the emerging of extensively drug-resistant TB. Thus, in order to be able to effectively control or even eradicate TB in the future, other strategies, focussing on latent M. tuberculosis infection are needed.

1.11.1 Control of latent tuberculosis infection

New cases of active TB are in part attributed to ongoing community transmission, including reinfections, but a substantial number of new cases will arise from the enormous reservoir of individuals with a latent Mtb infection, which is estimated to consist of 2 billion persons. Therefore, efforts to control and finally eradicate TB should also be directed towards the prevention of progression from latent infection to active disease. This could be achieved through two different strategies: 1. adequate/accurate diagnosis and treatment of individuals with latent infection and 2. an immunotherapeutic/ post-exposure vaccine which prevents reactivation from latent infection. With regard to the first, the currently available methods to detect persons with a latent *M. Tuberculosis* infection and select those with an increased risk to develop TB disease are insufficient. Contact tracing and treatment of latent infection is only achievable in a setting where most

persons are tuberculin skin test negative, this being only the case in industrialized countries (without standard BCG vaccination) where TB incidence is already low. Even in this setting, the effectiveness of the currently available regimens used for the treatment of latent Mtb infection is limited, due to problems with low compliance to treatment, drug toxicity mainly in the elderly, and prevalence of antibiotic resistant strains (9,4% in the Netherlands in 2005; multi-drug resistant strains: 0,8%). Further, in vitro studies demonstrated that dormant Mtb are moderately to highly resistant to commonly used drugs such as rifampin and isoniazid (INH) that are bactericidal to replicating bacilli [170,171]. With regard to vaccination, the only currently available vaccine against TB is *M. Bovis* bacillus Calmette-Guérin (BCG) that affords limited and highly variable protection against pulmonary TB in adults, which is mostly due to reactivation of latent tuberculosis infection [172,173].

1.12 Thesis objectives

The general goal of this thesis was to understand the mechanisms and role of nitrate reductase in the survival and multiplication of Mtb inside the human host macrophages. Given the observation of the presence of narGHJI operon in Mtb genome, which codes for a respiratory type of nitrate NarGHJI, we investigated the hypothesis that nitrate respiration through NarGHJI could provide energy during latent stage survival of the pathogen. If the latent stage is assumed to be anaerobic, reduction of nitrate into nitrite may generate necessary ATP to keep the pathogen alive in absence of oxygen as terminal electron acceptor. Therefore, NarGHJI may play a major role in maintaining viability of the pathogen and could be an attractive drug target to kill the latent tubercle bacilli.

For long time, macrophages are known as host of Mtb to support their pathogenesis and growth in humans. Our initial objective was first to examine the the production of nitrite inside Thp-1 macrophage model that increases with time as the intracellular bacilli growth occurs. If the growth and nitrate reduction follow a similar pattern when Mtb bacilli are infected to Thp-1 macrophages and incubated in culture to monitor both the parameters then it could be indicated that NarGHJI activity increases as a consequence of growth of bacilli with simultaneous development of hypoxia within intracellular environment Following

the confirmation of the relationship between the increased bacilli burden with and the induction of NarGHJI activity as a result of gradual entry of the culture to anaerobic stage, next objective would be to confirm the gradual decrease in relative oxygen concentration within intracellular environment with time. As an extension of this hypothesis, the importance of NarGHJI as well as its activity could be validated during this stage of bacilli residence within macrophages with the help of standard anti-tubercular agents like rifampicin, isoniazid, streptomycin, ethambutol, pyrazinamid. If NarGHJI activity represents the extent of hypoxia generated within the macrophage, then we could carry out identical experiment on human and mouse cell lines as host. The role of hypoxia within infected host cells could be evaluated in the context of elucidating mechanism of tubercular pathogenesis (Chapter 2).

Once the results of development of hypoxia within host system based on bacterial NarGHJI activity from infected macrophages as model system were confirmed, screening as well as basic research could be carried out in a convenient way. In fact, this screening protocol could be useful in identifying both active and dormant stage inhibitors of Mtb (Chapter 3).

Another major goal of the thesis was to identify novel inhibitors and take them to the lead stage. Successful evaluation of novel inhibitors needs the complete characterization of its mode of action particularly when the inhibitors were identified from a whole cell based screening. If the inhibition is found to be specific in nature, in most probable reason, the target should be protein in nature. Therefore, another major objective of this thesis was to identify the molecular target/s for these leads that will create an opportunity for starting lead optimization program (Chapter 4).

1.13 References

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CHAPTER 2

Depletion of Intracellular ATP due to Acute Hypoxia Triggers Necrosis of *Mycobacterium tuberculosis* Infected Human Macrophages

2.1. Introduction

The survival of persistent tubercle bacilli within granulomatous lesions of human beings for lifelong period raise major obstacle to the eradication of tuberculosis, bacilli having the ability to persist asymptomatic infection for a long time which is known as latent tuberculosis, a essential part of the disease [1]. All the standard drugs are available in the market preferentially effective against replicating bacteria. Durable cure of tuberculosis requires eradication of both replicating and non-replicating or persistent Mtb [2]. How the pathogen develops latency within the host as well as reactivates suddenly is still a mystery, the solution of which is linked to eradication of the disease. It is well recognized that non-pulmonary tissue oxygen concentrations within the human body are far below the oxygen concentration in ambient room air and the oxygen tension is playing a major role for production of nitric oxide (NO) within the macrophage [3, 4]. Furthermore, people have already shown that the oxygen concentration in the phagosome of activated macrophages was shown to be lower than the extracellular oxygen concentration by a new electron paramagnetic resonance (EPR) method [5]. Wayne's hypoxia and nutrient starvation induced dormancy models were earlier developed to explain certain features seen in persistent tubercular bacilli obtained from hosts. Various mycobacterial factors have also recently been observed which may be involved in the persistence or dormancy and resuscitation of dormant organisms and host immune system is also very critical in controlling latent TB infection from reactivation [6]. Thus, a shift from aerobic to anaerobic respiration during adaptation to dormant state, where nitrate rather than O_2 would serve as the terminal electron acceptor, is signaled by the induction of narX, which encodes nitrate reductase, narK2, which specify a nitrate/nitrite function. Recent reports suggest that NO generated inside host macrophages, leads to the development of either dormancy of the bacilli or able to kill the pathogen by forming peroxynitrite ($ONOO^-$) in association with superoxide (O_2^-) in the host [7]. Antioxidants like vitamins A, C and D3 are shown to induce dormancy of Mtb inside macrophages and these models can be very attractive for the study on persistent bacilli. Recently a study showed that vitamin D along can induced autophagosome formation to kill internalized Mtb. Adaptation to hypoxia and exposure to NO is implemented through the

DevRS/DosT two-component system which induces the dormancy regulon. Vitamin C can trigger the bacterial growth arrest and dormancy phenotype development of intracellular bacilli [8, 9]. The mechanism by which virulent Mtb inhibits apoptosis in macrophages is still unclear, People have already shown that host macrophages undergo apoptosis by two different pathways, extrinsic pathway by activation of caspase 8 activity and intrinsic pathway by activation of caspase3/7 followed by caspase 9 activity. Different findings in the literature regarding inducing or inhibiting apoptosis may be depends to the many factors. It has recently been demonstrated that virulent Mtb induces LXA4 and inhibits PGE2 production [10]. Under such conditions, it is suggested that mitochondrial inner membrane damage leads to macrophage necrosis. Thus, virulent Mtb subverts eicosanoid regulation of cell death to foil innate defense mechanisms of the macrophage. In earlier reports, development of hypoxic condition inside infected macrophages in necrotic animal tissues has been evidenced. It has been observed that break down of the lymphoid component of this complex result in hematogenous dissemination of Mtb to other regions of the lung, in which caseous necrotic granulomas form containing hypoxic regions. Mtb persisting within hypoxic region of caseous necrotic granulomas where most of the frontline drugs are ineffective in killing these bacilli to cure the patients. Mtb infected human alveolar macrophages are most likely enclosed in a hypoxic environment within the granulomas where the pathogen becomes dormant. It was recently established that tuberculous granuloma in guinea pigs, rabbits and non-human primates were hypoxic. It has been observed that granuloma is preventing the spread of infection throughout the organs. Both CD4 and CD8 T contribute to the maintenance and function of the granuloma in wild type mice. CD4 T cells are important in the initial formation of granuloma and also for the elimination of bacilli within granuloma, so CD4 T cells are required for resistance to tuberculosis. During dormant stage, pathogen contained within granuloma which is made up of infected macrophages surrounded by another set of foamy lipid loaded macrophages and persists for decades until the host immune system is weakened and then reactivates to cause active disease. The pathogen is likely to go into dormant state within macrophages that are in the hypoxic environment of the granuloma. Occurrence of necrosis within infected tissues has been suggested as one of the critical events that create hypoxia as well as dormancy of bacilli

within host system which does not explain the reason of developing tissue necrosis; drug induced dormancy as well as non-culturability of the persistent bacilli. It has already been demonstrated that growing bacilli develop multi-drug tolerance after they infect the macrophages. It has also been shown that these dormant bacilli undergo ovoid shape which is important for elucidation of the molecular mechanisms that underline non-replicating persistence of mycobacteria [13, 14**,** 15]. Mtb bacilli within lipid-loaded macrophages accumulate lipid droplets containing triacylglycerol, lose acid-fast staining and become phenotypically resistant to the two frontline anti-mycobacterial drugs, rifampicin (Rif) and isoniazid (INH) as well as induce gene transcripts involved in dormancy and lipid metabolism within the pathogen but the nature of the host environment that causes Mtb to develop latent state is still poorly understood. It is also established that under multiple stress conditions in vitro Mtb bacilli acquire all of the major characteristics of in vivo dormancy [16-20]. None of the reports so far, has clearly established the triggering factor as well as sequence of events that take place within macrophage leading to the escape of the pathogen.

This study demonstrates that increased bacilli burden is responsible for developing acute hypoxic condition inside infected Thp-1 macrophages under in vitro culture condition which leads to mitochondrial dysfunction followed by drastic lowering of intracellular ATP and finally culminates into necrosis. Briefly, it demonstrates first, the identification of mycobacterium dependent conversions of nitrate to nitrite during intracellular residence and its correlation with gradual depletion of oxygen, second, approach is to identify the role of anaerobic condition generated within the host due to increased intracellular bacilli burden in deciding the pathway of apoptosis and necrosis of the host cell.

2.2. Results

2.2.1 Effect of NR inhibitors on aerobic and dormant culture of *M. tuberculosis*

It is established that narK2 (a nitrate/nitrite antiporter), narG (nitrate reductase) and genes of dos regulon are induced during hypoxic shift from an aerobically active stage of the bacilli under in vitro conditions [21-24]. Nitrate reductase (NR) is also an established biomarker in mycobacterium bacilli whose activity is induced during gradual shift of culture takes place to hypoxic stage to support the bacteria survive without oxygen [24,25]. Pentachlorophenol, a well-known inhibitor of prokaryotic NR, was earlier used to specifically inhibit respiratory NR of *M. smegmatis* [26].

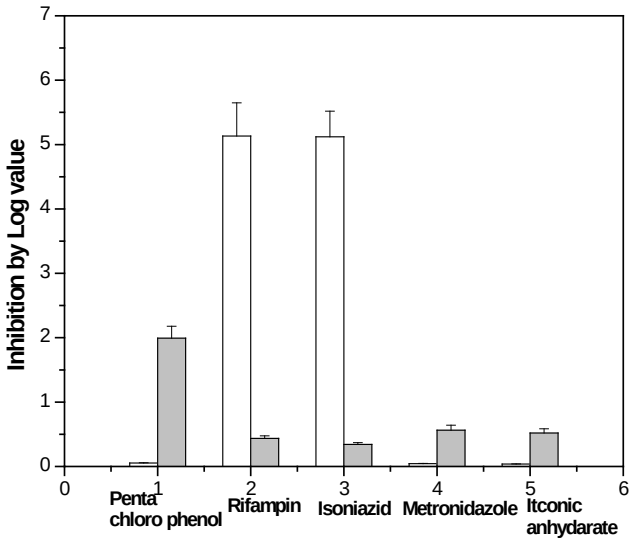


Fig.1.1. **Effect of different inhibitors on the viability of aerobic and anaerobic dormant *M. tuberculosis*.** Rifampicin (0.121µM), Isoniazid (0.729µM), Metronidazole (1mM), Pentachlorophenol (1.08µM) and Itaconic anhydride (2mM) were added just after inoculation in aerobic culture **(light bars)**, after 8 days of inoculation in Wayne culture **(dark bars)** and the CFU was determined after 4 days. Further details of the experiment are described in "Materials and Methods". Results are mean ± SD of three identical experiments.

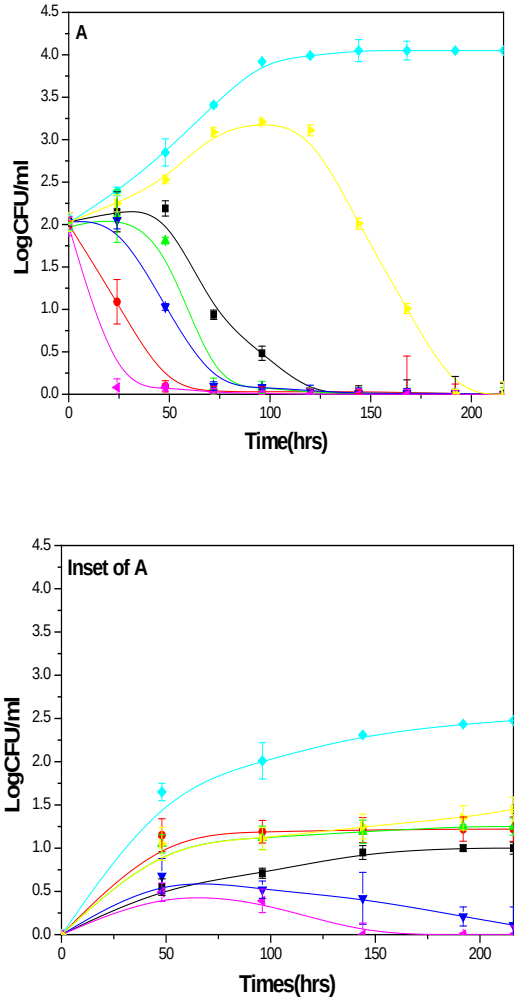
Rifampicin and isoniazid were used as standard aerobic whereas metronidazole and itaconic anhydride used as dormant stage inhibitors of the bacilli at respective

minimum inhibitory concentrations (MIC) [25, 27]. Amongst them, only rifampicin and isoniazid were significantly inhibiting the growth of the bacilli under aerobic condition (Fig. 1.1). The effect of various inhibitors on the viability of NRP stage of Wayne's culture was determined from CFU which indicated that Pentachlorophenol (1.08μM) reduced the viability of Mtb bacilli by1.99logs whereas, rifampicin(0.121μM) and isoniazid (0.729μM) marginally reduced it by 0.43 and 0.34 log respectively. Metronidazole (1mM) and itaconic anhydride (2mM) reduced the CFU by 0.4 and 0.35 log of these dormant bacilli. Initially, the dose response effect of Pentachlorophenol was seen on aerobic and anaerobic bacilli to identify the MIC which clearly indicated that the inhibitor is specifically effective on hypoxic bacilli. Pentachlorophenol inhibited NR activity in membrane associated and pure enzyme preparations by 94 % and 97 % respectively. These results suggested the dependence of the survival of bacilli on the alternate respiratory chain using narGHJI took place under hypoxic condition and be used as marker for development of hypoxic condition[29].

2.2.2 Effect of NR inhibitors on growth of the bacilli in human macrophage

Infection profile of Mtb in presence of different inhibitors at their respective MIC could provide an insight about the environment prevailing inside infected macrophages (Fig 1.2). Pentachlorophenol did not affect the viability of Mtb in Thp-1 macrophages till the growth reached 3.01log value after ~100hrs of incubation and then the CFU reduced by 0.34 log compared to the control (Fig 1.2A). At this concentration, pentachlorophenol did not show any toxic effect on Thp-1 macrophage cells (data not shown). Thus, the reduction in cell count was due to specific killing of intracellular Mtb bacilli by pentachlorophenol. Earlier studies using narG mutant of Mtb had shown that the bacilli was not able to survive within the host macrophages [21]. The effect of rifampicin, on viability of intracellular bacilli could be seen immediately after its addition in the medium and complete killing was achieved within 72hrs. This observation indicated that a microaerophilic environment might have been created due to the overburden of Mtb during the late growth in Thp-1 macrophages. Under such conditions, the demand for oxygen probably exceeds the supply for continuing aerobic

respiration. During hypoxia, use of alternate respiratory process is mainly decided by the presence of appropriate electron acceptor and in presence of nitrate, narG becomes essential. Nitrate could be available from the rearrangement of peroxynitrite produced in infected macrophages [30]. This could be one of the possible explanations why the bacilli have to depend on NarGHJI for survival inside the host macrophage.



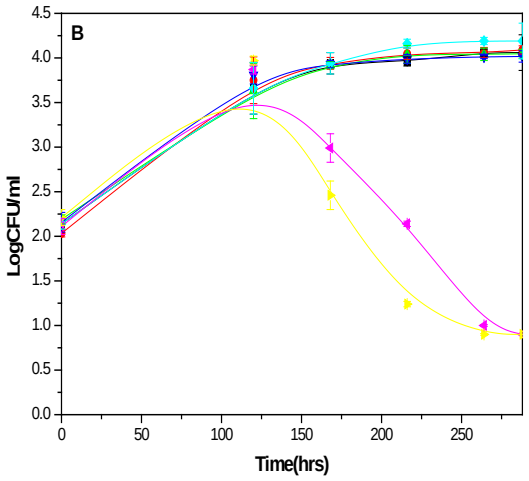


Fig. 1.2. **Effect of stage specific inhibitors on viability of intracellular *M.tuberculosis*.** Infected THP-1 macrophages were treated with Rifampicin (0.2µg/ml) (◀), Pentachlorophenol (0.29µg/ml) (▶), Streptomycin (0.15µg/ml) (■), Ionized (0.06µg/ml) (▲), Ethambutol (2µg/ml) (▼), Pyrazinamid (10µg/ml) (●) and Control (♦) were added just after the infection (A), after 5days of infection (B) to obtain viable counts (CFU) at different periods of incubation. Effect on extracellular bacilli counts due to addition of same inhibitors just after the infection were shown (inset of A). The CFU values are shown as mean ± SD of three identical experiments.

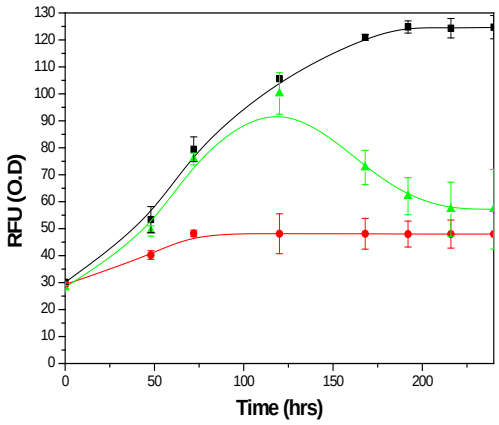
Ineffectiveness of anti-tubercular agents specific against growing bacilli inside infected macrophages indicated that the bacterium was residing in dormant stage when these inhibitors were added after 5days of infection (Fig 1.2B). Interestingly, the significant killing effect of pentachlorophenol on the intracellular bacilli also supported the presence of dormant mycobacterium within macrophage. These results also suggested that indigenously produced nitrate within activated macrophages is used by Mtb as alternate electron acceptor for respiration [29]. The dependence of the bacilli on NR activity at that stage suggested the development of a possible hypoxia within the niche where the bacilli are residing. The effect of inhibitors on intracellular bacilli was also supported from the viable count of extracellular bacilli which clearly indicated that the release of the bacteria into extracellular medium did not occur in presence of either pentachlorophenol or rifampicin (Fig 1.2A inset). CFU data of the extracellular bacilli also indicated that apart from Rifampicin and Isoniazid,

Pentachlorophenol is a potent killer of intracellular bacilli. The finding also pointed towards the potential of nitrogen metabolism as target for identifying novel inhibitors against TB. Subsequently, Rifampicin as well as Pentachlorophenol was used as aerobic and hypoxia stage specific inhibitors to probe the stage of the bacillus residing inside infected macrophages during further investigations.

2.2.3. Relative level of oxygen inside macrophages infected with *M. tuberculosis*

In order to assess the level of intracellular hypoxia and the role of bacilli burden in it, fluorescence emitted from polystyrene coated beads carrying oxygen sensitive Ru-complex [(bpy) 2Ru (bpy-pyr)] Cl_2inside the infected Thp-1 macrophages was monitored (Fig 1.3). In presence of oxygen, the fluorescence of the Ru complex will remain quenched and the fluorescence intensity will increase because of the increased hypoxia to finally attain saturation [31].

A



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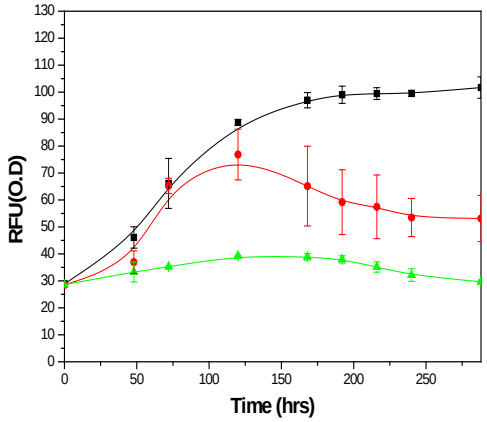


Fig. 1.3. R**elative level of oxygen within *M .tuberculosis* infected macrophages.** Relative level of oxygen was measured within (A) Thp-1 and (B) J774 infected macrophages already treated with vehicle (■), Rifampicin (●), and Pentachlorophenol (▲) at their respective MIC. The details of the experiments are provided in "Materials and Methods" section. Results are mean ± SD of three identical experiments

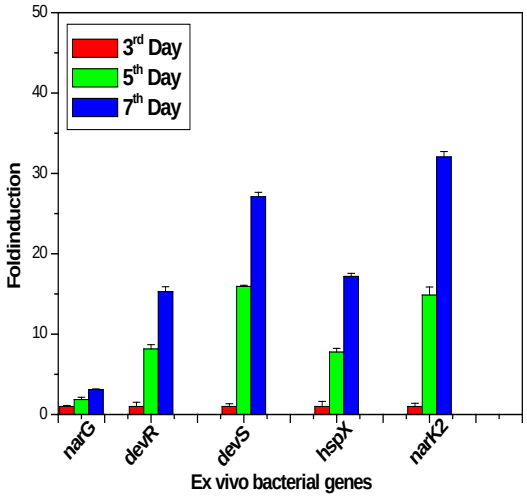
This result indicated that the level of hypoxia attained the peak level on the 5th day of infection in control macrophage cells under the experimental conditions mentioned. In presence of Rifampicin, there was no change in the level of intracellular O_2 which could be attributed as a result of the killing of intracellular bacilli whereas in presence of Pentachlorophenol, the development of hypoxia was found to build up till 4th day after infection and after that returned to almost normal level within 8th day which indicated the demand for oxygen was proportionately decreased as the viable count of intracellular bacilli reduced within the infected macrophages. In order to verified the same thing by using J774 murrain macrophages and observed that the rate of generation of anaerobic condition was more prominent in human than murrain macrophages.

2.2.4. Hypoxia induced expression of dormancy related genes within infected macrophage

To further investigate the development of hypoxia inside infected macrophages, the expression of biomarkers genes (devR, devS, hspX and narK2) of the bacillus and genes (IL-8 and VEGF) of the infected Thp-1 macrophage were monitored at

different periods during growth of intracellular bacilli (Fig 1.4) [9, 32, 33]. Quantitative real-time PCR was carried out using samples at 3, 5, 7 days of post infection and found a gradual increase in expression of these genes with time and reached the plateau on the 7th day. Bacterial 16S RNA as well as macrophage actin was used as internal controls. The result clearly indicated that all the genes under consideration, was at basal level on the 3rd day of infection and then increased to the peak level of expression between 5th and 7th days post-infection. Amongst the bacterial genes, nark2 and devS was found to express 32.33 and 27.09 fold respectively on the 7th day after infection greater than the internal control. Interestingly, the expression of IL-8 and VEGF genes of the host macrophage was also found to increase at the same time by 20.11 and 20.82 fold respectively with respect to the internal control. There was no significant level of expression of these genes found to occur within uninfected macrophage cells.

A



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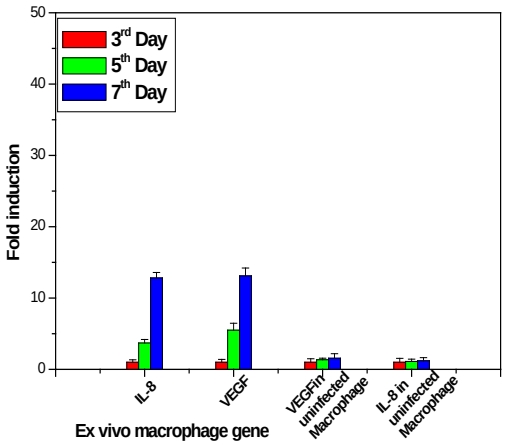

Fig.1.4. **Real-time PCR analysis of dormancy and hypoxia markers during growth of *M.tuberculosis* within infected macrophage.** Kinetics of expression of devR, devS, hspX, narG and narK2 genes of MTB was monitored with RNA isolated from infected Thp-1 macrophage cells (A) vs. IL 8, VEGF genes of macrophage from infected and uninfected control macrophage cells (B). MTB and macrophage fold induction of individual gene transcription is a ratio of 16S rRNA and Actin -normalized transcript number respectively. Results are expressed as mean ±SD of pools of 3 independent infections, each pool analyzed in triplicate.

The real-time quantitative assay confirms that hypoxia is gradually developed within host macrophages and reaches the peak on 7^{th}day after infection under the condition mentioned when narK2 plays major role in protecting the bacilli within the host. The expression of host genes was also monitored to confirm the hypoxia induced within infected host cells. The increase in expression of host genes was also maximum during the period between 5^{th} to 7^{th} days of infection. The induced expression of hypoxia related genes in both bacilli and host suggested that acute hypoxia was gradually achieved between 5^{th} to 7^{th} days after infection and probably its consequences were also seen.

2.2.5. Effect of stage specific inhibitors on the host macrophages infected with *M. tuberculosis*

Although host cell necrosis is the major outcome of mycobacterial pathogenesis under physiological conditions, during infections of isolated cells under ex vivo culture conditions, apoptosis was found to occur in the earlier studies [9, 34, 35]**.** As attenuated Mtb strain H37Ra was used in our study to infect Thp-1 cell line,

we wanted to check the fate of host macrophages as well as effect of these stage specific inhibitors on it (Fig 1.5). The microscopic

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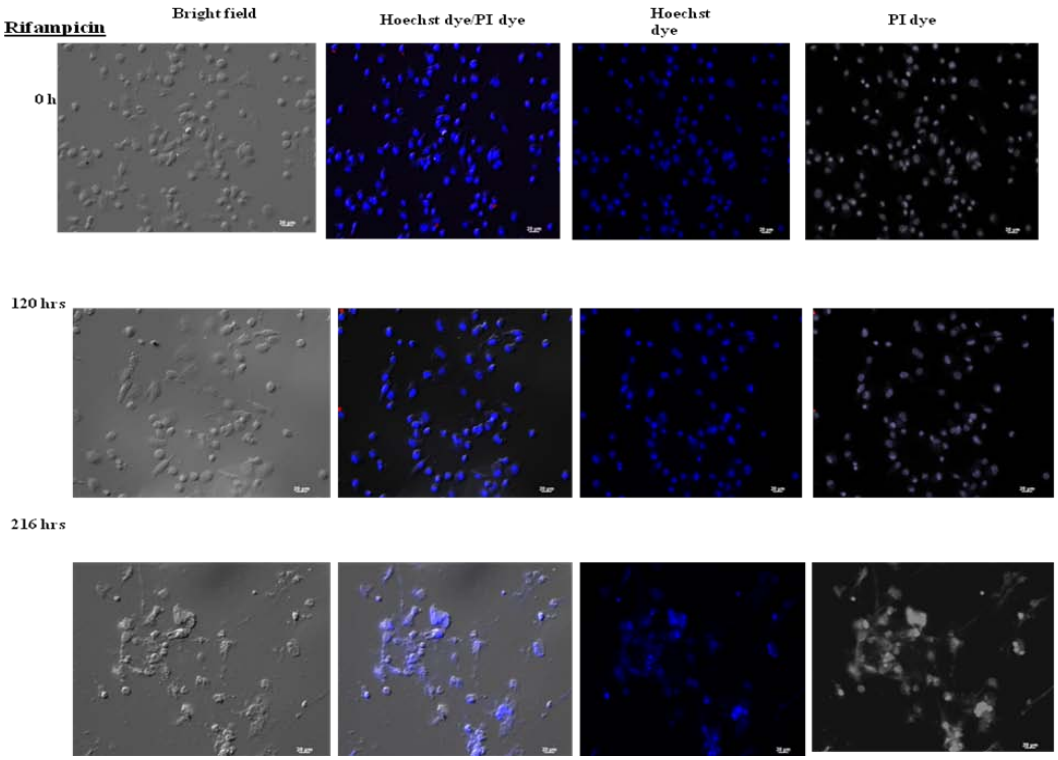

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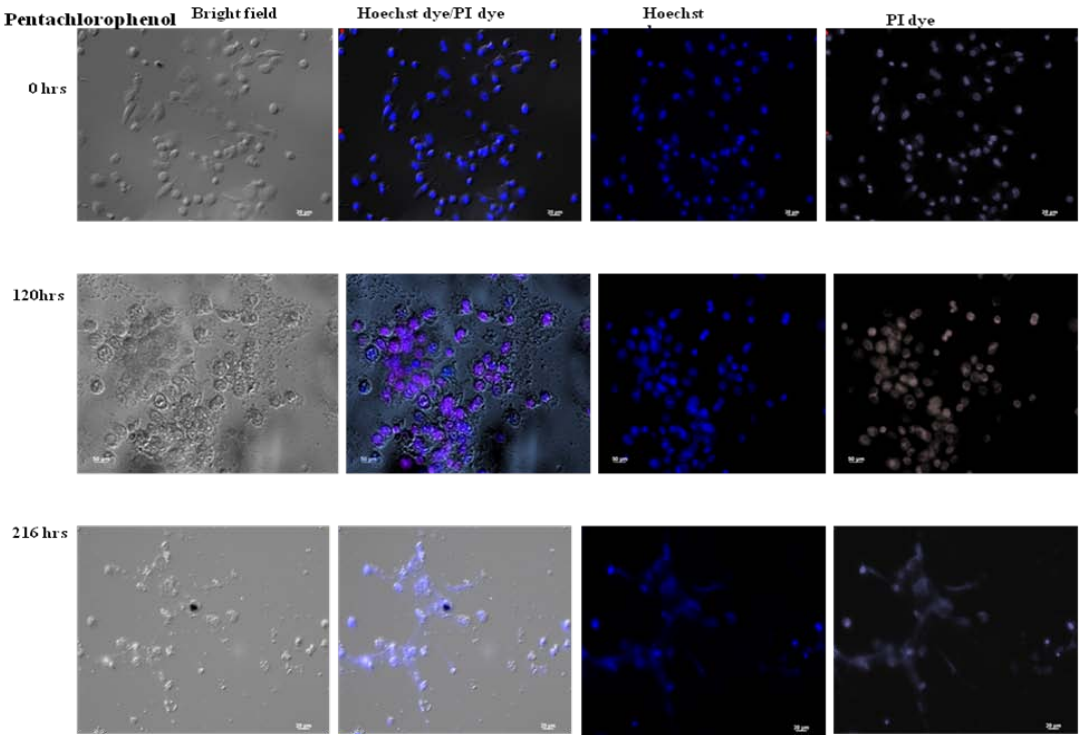

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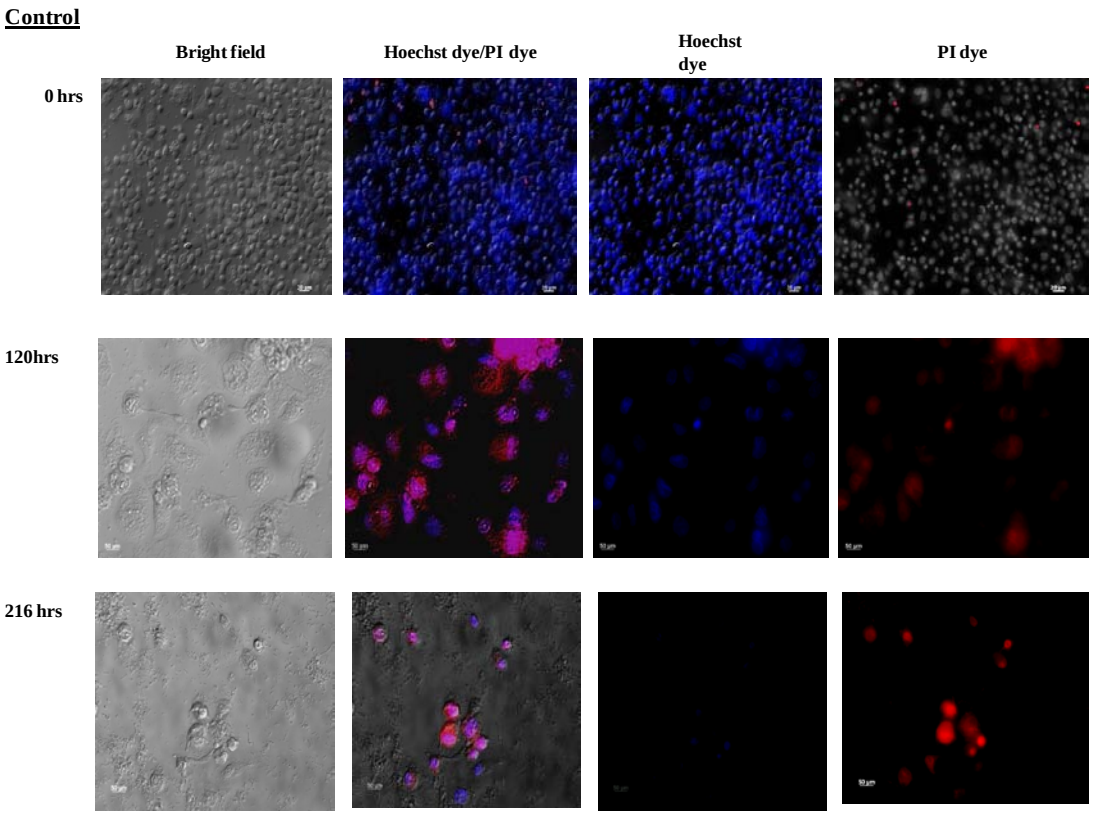

Fig.1.5. **Effect of stage specific inhibitors on death of *M. tuberculosis* infected macrophages**. Infected macrophages treated with rifampicin (A), Pentachlorophenol (B) and vehicle control (C) at their respective MIC and double stained (Hoechst & PI) with

Hoechst (for live cell) and Propidium Iodide (for dead cell) at 0hrs, 120hrs and 216hrs respectively. Superimposed fluorescent microscopic images along with respective bright field, Hoechst field and PI field images (20 x objectives) were used to visualize live (blue in color) and necrotic cells (red in color) by a phase contrast microscope.

images has been taken at different periods of incubation after infection, clearly indicated that the complete host cell death was occurred at after 216hrs of infection in vehicle control. But in presence of rifampicin and pentachlorophenol, host cell death was clearly stopped. This probably indicated that the death of host cell was more associated with the survival and the number of intracellular bacilli. Development of hypoxia possibly reached a threshold due to the number of viable bacilli when the demand for oxygen from the bacilli almost equals with host mitochondria.

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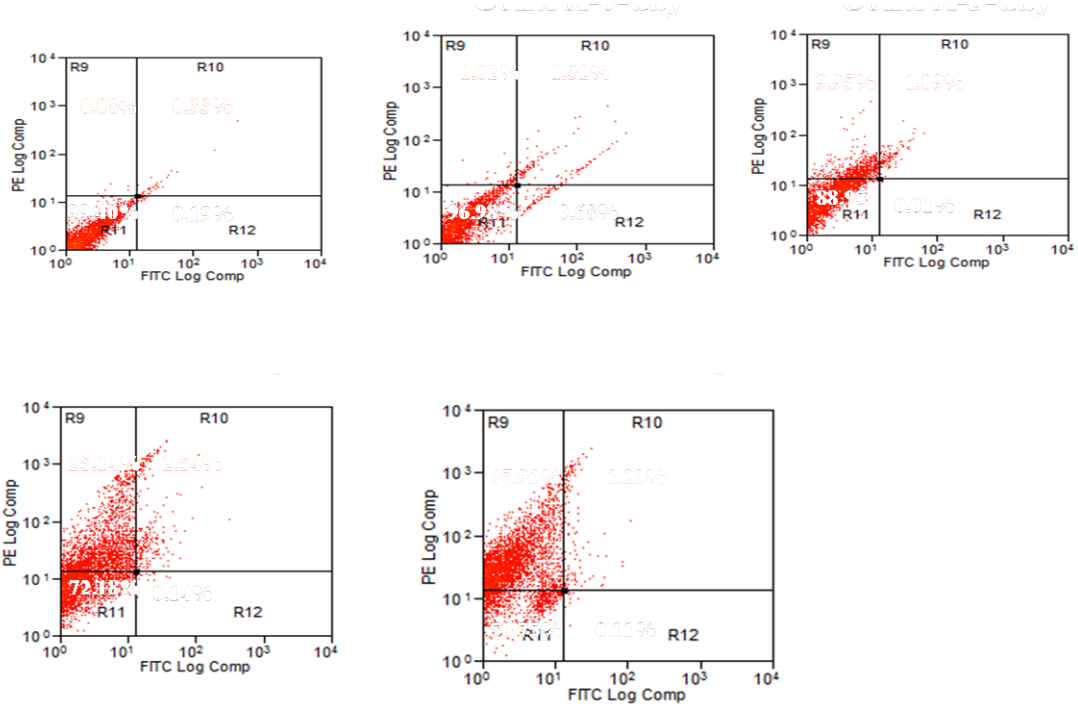

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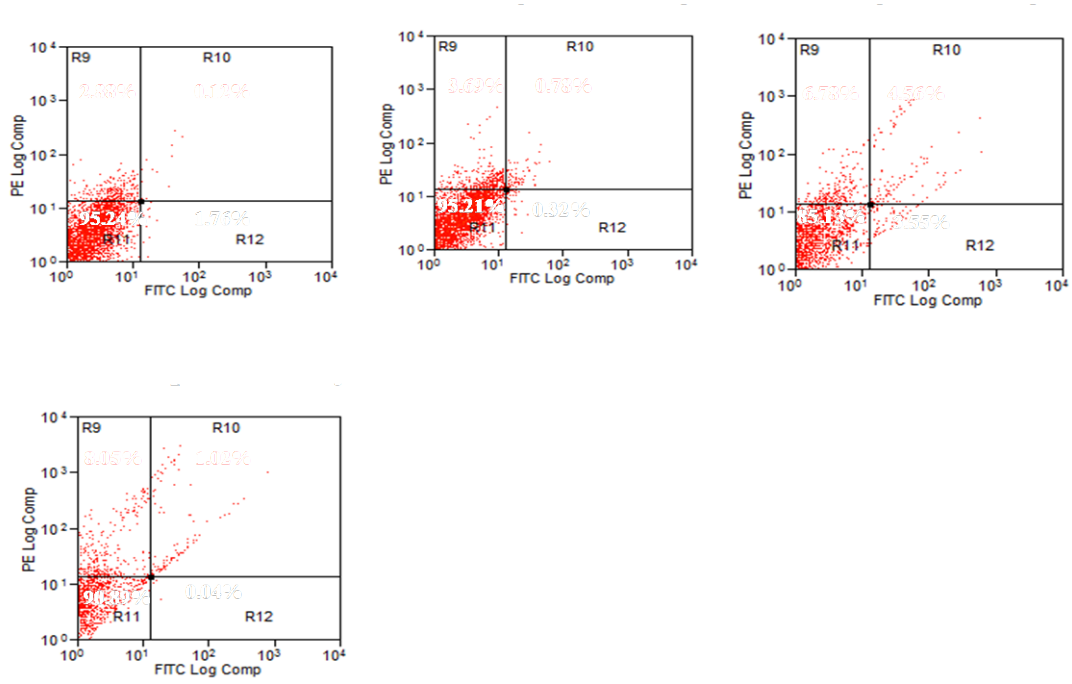
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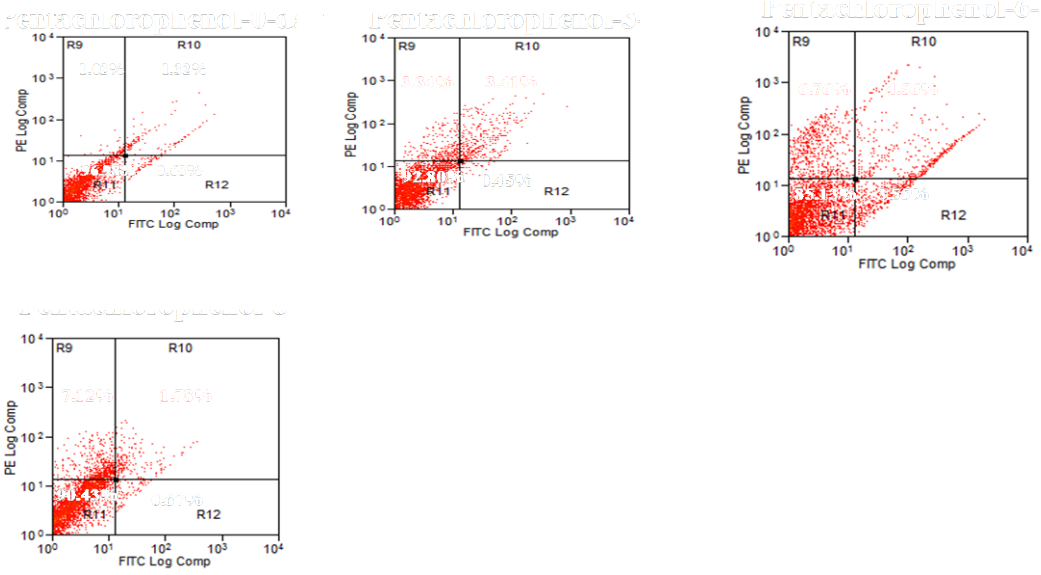
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b)

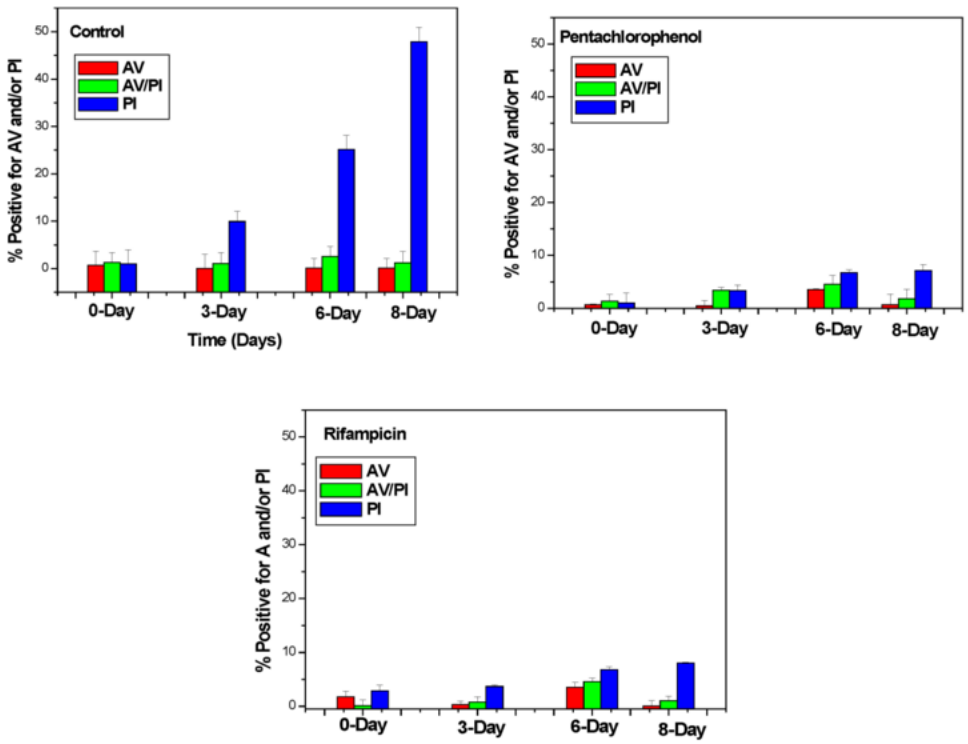


Fig.1.6. **Fate of *M.tuberculosis* infected macrophages by Fluorescence activated cell Sorting (FACS) analysis.** (a) Dot plots were obtained by FACS analysis of infected macrophages treated with vehicle control (A), Rifampicin (B) pentachlorophenol (C)at 0, 3, 6 and 8 days after infection with MTB at their respective MIC. Lower left quadrants represent unstained cells (viable cells) and the upper left quadrants include PI-positive cells (necrotic cells). The lower right quadrants encompass Annexin V-only positive (Apoptotic cells) and the upper right contain the Annexin V-FITC/PI-stained cells.(b) Mean data (± SD) for the fold change in necrosis compared with the corresponding non-necrotic control cells. Each column represents the mean ± SD for three independent experiments.

In order to ascertain the nature of host cell death, its quantitative FACS analysis was carried out at different time points (Fig 1.6). FACS data indicated that almost 48% of host cell death has been occurred due to necrosis in vehicle control whereas only 7% and 8% cells died because of necrosis in presence of rifampicin and pentachlorophenol respectively. The occurrence of cell death due to apoptosis remained at insignificantly low level throughout the

Table 1.1 Sequences of primers used for Real Time-PCR study in mycobacterial and macrophages

Genes	Primer sequences[a]	Amplification product (bp)	PCR parameter[b] Tm (Annealing temp.)
16S	F 5`ATGCATGTCTTGTGGTGGAA 3` R 5`TTCACGAACAACGCGACAA 3`	371	58 °C
devR	F 5` CCGATCTGCGCTGTCTGATC 3` R 5` GTCCAGCGCCCACATCTTT 3`	144	65°C
devS	F 5` TACTGACCGACCGGGATCGT 3' R 5` AGAGCCGCTGGATGACATGG 3`	59	65°C
hspX	F 5` CGCACCGAGCAGAAGGA 3' R 5`CGTGCGAACGAAGGAA3'	64	65°C
nark2	F 5`TGCTTCGTGATGCACCCTACT 3` R 5`CCGCCGAACACGATCGCGTA 3`	120	68°C
Nitrate reductase (narG) (*M. tb)*	F 5`AGCGGCGCACATAGTCGACAAA GAACGGAA 3` R 5`ACTACGCCGACAACACCAAGTT CGCCGACG 3`	149	66 °C

Actin	F 5'ATGGATGACGATATCGCT 3`  R 5`ATGAGGTAGTCTGTCAGGT3 `	700	60 °C
VEGF	F '5 TGGGATCCATGAACTTTCTGCT 3' R 5`CGGAATTCTCACCGCCTCGGC 3`	664	60 °C
IL-8	F 5` TGCTAAAGAACTTAGATGTCA GTGCAT 3` R 5` TGGTCCACTCTCTCAATCACT CTCA 3`	86	68°C

Table 1 footnote.

[a] Primers were designed using the IDTDNA primer designer software.

[b] Initial denaturation of 95 °C for 2 min. followed by 40 cycles of 94 °C (30 sec.), respective annealing temp. (1 min.), 72 °C (1 min.) and final extension of 10 min. at 72 °C were used for amplification of mentioned genes followed by melting curve analysis.

experimental periods. Both microscopic and FACS data together clearly established that the necrosis of host cells is very similar to that observed under physiological conditions and development of hypoxia is probably linked to the escape of bacilli from host macrophages.

2.2.6. Development of intracellular hypoxia linked to depletion of ATP within infected macrophage

Recent studies have clearly established that prolonged exposure to hypoxia leads to cell death by following either of apoptotic or necrotic pathway [36, 37]. Intracellular level of ATP plays an important role as a molecular switch to decide which pathway will be followed [38-40]. Prolonged exposure of cells to hypoxia causes damage to mitochondrial function coupled with lowering of ATP level in the cytoplasm. Our investigation about the level of intracellular ATP in infected

macrophages clearly indicated that the ATP level increased up to the period till hypoxia was achieved on 4th day and then drastically fell to negligible level within 6th day after infection (Fig 1.7). This decrease in ATP level was protected by aerobic (Rifampicin) and dormant (Pentachlorophenol) stage inhibitors. The results clearly suggested that lowering of ATP is responsible for necrosis of infected macrophages.

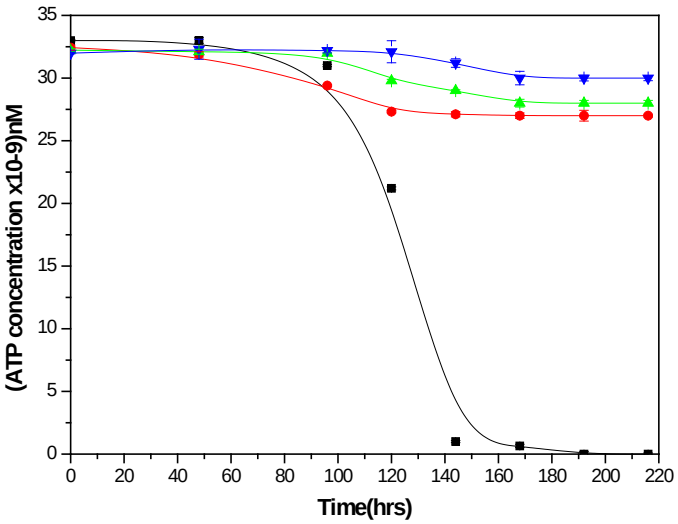


Fig.1.7. **Effect of stage specific inhibitors on intracellular ATP of *M. tuberculosis* infected macrophage.** ATP concentration was measured in presence of stage specific inhibitors control(■), Caspase 3 inhibitor (2.7nM) (▼), Rifampicin (●) and Pentachlorophenol (▲) used at their respective MIC.The intracellular ATP level was monitored up to 216hrs of infection. Results are mean ± SD of three identical experiments.

It was earlier established in tumor cells that malfunctioning of mitochondria leads to release of cytochrome c and activated pro-caspase 9 followed by caspase 3/7. In order to confirm the activation of intrinsic pathway, caspase 8 activity was also monitored. Lack of any change in the activity of caspase 8 clearly indicated that the origin of the signal was released at the intracellular level. Activation of the cascade of caspase enzymes ultimately reaches the level of caspase 3/7. In order to understand it's involvement in this whole process, caspase 3/7 inhibitor (2.7nM) was added immediately after infection and interestingly found that the inhibitor had better protected the fall in intracellular ATP level than even Rifampicin and Pentachlorophenol (44).

In order to confirm the connection between mitochondrial dysfunction and activation of caspase 3/7, we monitored both the activities of caspase 8 and caspase 9 up to 8days after infection of host macrophages (Fig 1.8). For this, caspase 8 and 9 inhibitors were added to the culture at their respective concentrations to completely inhibit the enzyme activities (44). In presence of both the inhibitors, the enzyme activities were reduced to almost negligible level.

A B

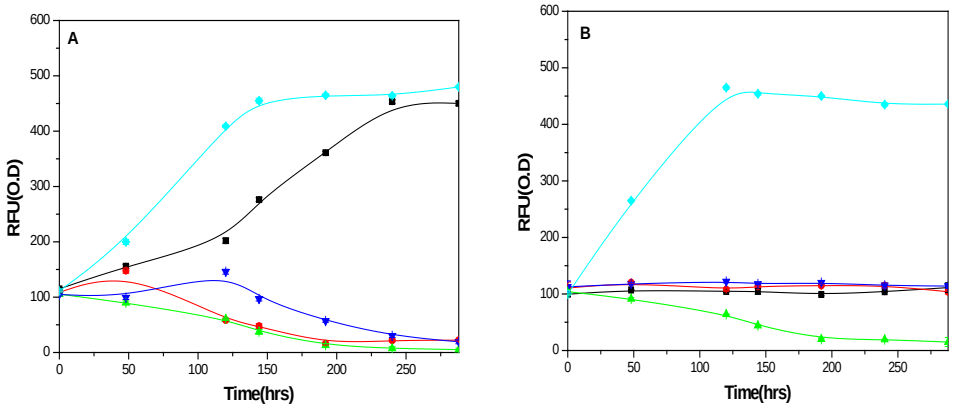
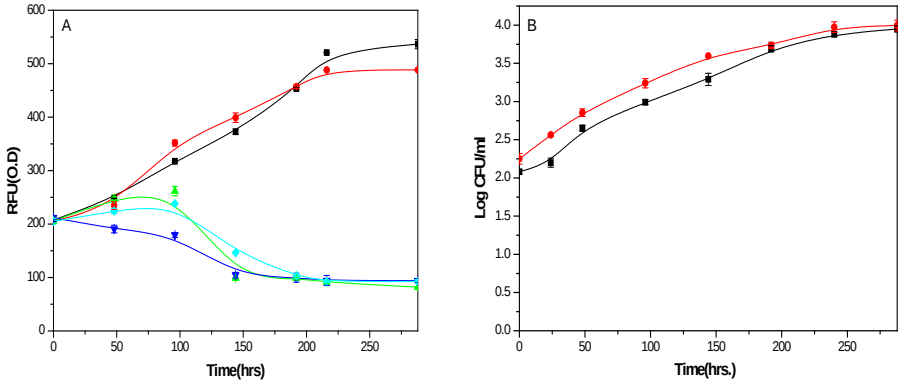


Fig.1.8. **Measurement of caspase 9 and caspase 8 activities in *M.tuberculosis* infected macrophage.** The change in fluorescence intensity (RFU) was monitored as a measure of caspase 9 (A) and caspase 8 (B) within infected macrophage in presence of the respective inhibitors (▲), Pentachlorophenol (▼), Rifampicin (●) and Control (◀) at their MIC. Staurosporine and Digitonin at 200nM (♦) concentration was applied in the uninfected control macrophage cells to induce Caspase 9 and caspase 8 activities respectively. Results are the mean ± SD of 3 identical experiments

In contrast to the caspase 8, only caspase 9 activity within infected macrophages was induced at a rate almost similar to one where Staurosporine, the inducer of caspase 9 in mammalian cells was added. The results clearly established that only caspase 9 was activated during the course of infection of human Thp-1 macrophages confirming involvement of an intrinsic pathway responsible for activation of apoptotic pathway leading to the death of host cells.

A B



C

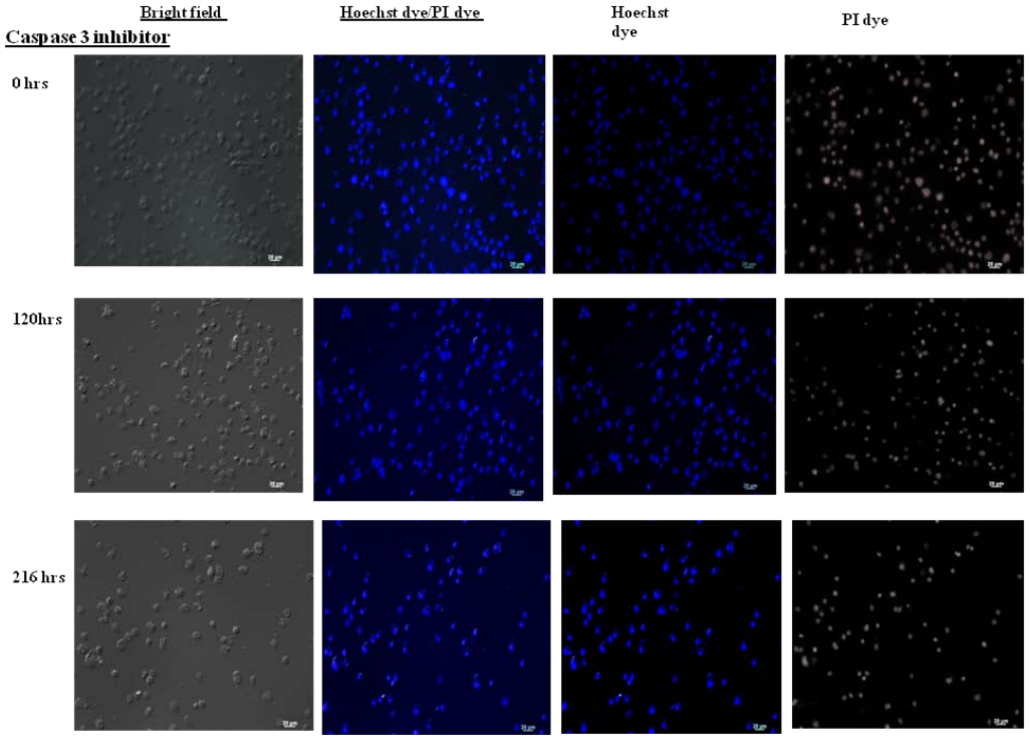


Fig.1.9. **Effect of caspase 3 inhibitor on the viability of intracellular *M. tuberculosis* bacillus and host macrophage.** (A) Measure the caspase 3 activity in presence of Pentachlorophenol (▲), Rifampicin (▼), caspase 3 inhibitor (♦) and control (■) at their MIC. Staurosporine at 200nM (●) concentration was applied in the uninfected control macrophage cells to induce Caspase 3activity.Caspase 3inhibitor was added at 0 hrs(■) and after 120 hrs (●) on cell viability (B). Microscopic evidences demonstrate the effect of caspase 3 inhibitor at 0 hrs, 120 hrs and after 216 hrs (C). Results are mean ± SD of three identical experiments.

Furthermore, to identify the role of caspase 3/7 in the escape mechanism of the bacilli, the enzyme activity was monitored during the course of infection and found that its activity increased steadily to reach a plateau after 7 days of infection which was just opposite in presence of rifampicin and Pentachlorophenol (Fig 1.9). Interestingly, CFU count as well as microscopic pictures suggested that inhibitor of caspase 3/7 was able to protect the death of both the host as well as intracellular bacilli.

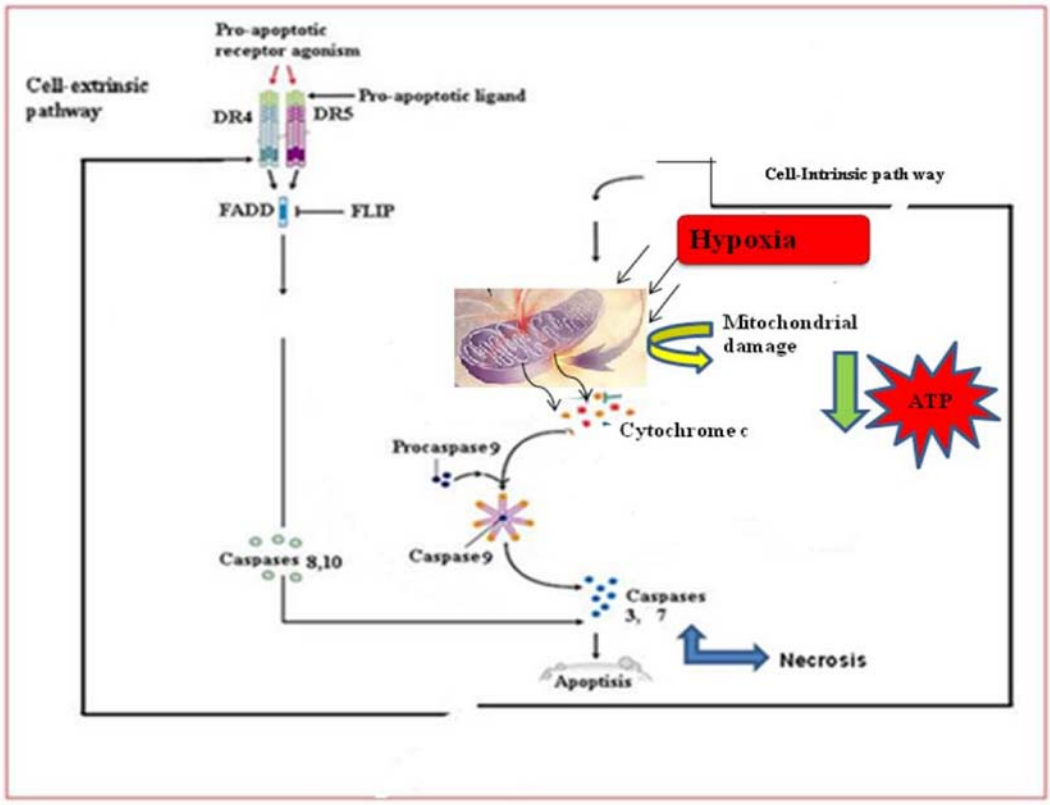


Fig .1.10. **Pictorial diagram of total caspase cascade and turning point of Necrosis from Apoptosis.**

2.3. Materials and methods

2.3.1 Chemicals, Media, Strains and cell line

All chemicals used were of analytical grade and purchased from Sigma unless mentioned otherwise. *M. tuberculosis* H37Ra (ATCC 25177) obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India, was routinely maintained on Dubos albumin agar medium. The stock culture was maintained in Dubos medium with 5% glycerol at -70^{0}C. Thp-1 human monocyte and J774 murrain cell line was obtained from the national cell repository, National Centre for Cell Science, Pune, India. Cells were maintained routinely in RPMI 1640 cell culture medium supplemented with 10% FBS.

2.3.2. Cultivation of the aerobic and dormant *M. tuberculosis* bacilli

For routine experiments, cells were grown in a minimal medium supplemented with 5mM of sodium nitrate unless otherwise mentioned [11]. For aerobic cultivation in the minimal medium, 10ml of the culture was added to a 25 ml tube containing 8mm magnetic bar and incubated at 37^{0}C on a stirrer rotating at 100 rpm. For aerobic cultivation in Dubos medium, culture was grown in 100ml flask having 40ml medium at 150 rpm and 37^{0}C on an orbital shaker (Thermo electron Model No. 481). 1% of 1 O.D$_{580}$ culture was used as inoculum for routine experiments.

For the cultivation of anaerobic dormant bacilli Wayne's 0.5 HSR tube model was followed wherein 20x125 mm tubes 25.5 ml of the Dubos medium was inoculated with 1% v/v of 1 O.D$_{580}$ culture [24]. The culture tubes were incubated at 37^{o}C for 8days after making air tight with rubber septa after inserting an 8mm magnetic

spin bar and gently stirred at 100rpm on a magnetic stirrer. Viable cells were counted by method described earlier [24].

2.3.3. Infection of macrophages by *M. tuberculosis* bacilli

Human acute monocytic leukemia cell line Thp-1 was used to infect and cultivate Mtb within macrophages was carried out by following an earlier method described [42]. Briefly, Thp-1 cells were grown in RPMI 1640 at pH 7.4 at $37^{o}C$ for 4days in presence of 5% CO_2 as well as 95% relative humidity to reach the density up to 6x 10^{6}cells per ml followed by treatment with 100nM of phorbol myristate acetate. The culture was then incubated for 24 hours to allow them converted into macrophages. These macrophages were incubated for 12 hours with MTB at MOI of 1: 100 for infection. At the end of infection, macrophage cells were washed 4 times with PBS to remove extracellular bacilli and the culture was filled with RPMI 1640 as mentioned earlier. In order to check the effect of inhibitors on the growth of intracellular bacilli, compounds were added at 0hr and after 120hrs after infection, Unless mentioned otherwise, the macrophages were lysed after 8days of incubation by treatment with a hypotonic buffer pH 7.4 (10mM HEPES buffer containing 1.5mM $MgCl_2$ and 10mM KCl). The lysate was spread on Dubos albumin agar plates to get the CFU after 3 to 4 weeks of incubation at $37^{o}C$.

2.3.4. Synthesis of Oxygen-Sensitive Lipobeads and its use in measurement of relative level of O_2 within *M. tuberculosis* infected macrophages

Oxygen sensitive Lipobeads were synthesized by following an earlier method [31].Briefly, 4.0 milligrams of polystyrene microspheres was dispersed in 100 µl of ethanol/ hexane (v/v 1:1) mixture by sonication using a 47-kHz, Bran sonic sonicator. A lipid stock solution (50 mM) was prepared with a 5:4:1 molar ratio of dimyristoylphosphatidylcholine, cholesterol and dihexadecyl phosphate in chloroform. A 100µl aliquot of 0.1mM [(bpy) 2Ru (bpy-pyr)] Cl_2 in $CHCl_3$ was added to the lipid solution. The solution was then briefly mixed to ensure homogeneity. A 100 µl aliquot of the microsphere suspension was slowly added while the mixture was sonicated in an ice bath. The solution was kept at room

temperature for 1 h to allow the indicator and the phospholipids molecules to absorb onto the surface of the particles. The sample was then dried overnight under nitrogen. The dried beads were suspended in 1 ml of PBS at pH 7.4. The lipobeads suspension was sonicated for 30 min in an ice bath to break the aggregates and remove loosely bound indicator molecules to ensure even coating of phospholipids on the particles. Excess phospholipids, indicator and uncoated beads are removed by centrifugation (1000*g*, 15 min). Lipobeads evenly coated with indicator and phospholipids were collected at the bottom of the centrifuge tube. The lipobeads were finally suspended and stored in 2 ml of PBS (pH 7.4) at 4 °C.

After each 48 hours of incubation, 10 µl suspensions containing 0.5 mg/ml of lipobeads in PBS was added to each well already fill with infected macrophages. These cells were then incubated with lipobeads for 1 hour at room temperature to allow the phagocytosis of lipobeads to take place. The excess lipobeads were then washed out with PBS. The fluorescence intensity from lipobeads was measured at excitation 495nm and emission 530nm wavelengths respectively by using a Fluoresce Spectrometer (PerkinElmer Model LS 55). Unless otherwise mentioned, inhibitors were added at 0 hour of infection to theThp-1 macrophage cells to monitor their effect on intracellular O_2 level.

2.3.5. RNA extraction and Real time Reverse transcription-PCR

RNA isolation was carried out from intracellular bacilli as well as Thp-1 macrophage cells by following an earlier method [43]. Briefly, spheroplast solution was first added to the infected macrophage culture on 3, 5, and 7 days post infection (PI) and incubated overnight before total RNA was extracted.RNA (1µg/µl) was reverse transcribed to cDNA and further amplification was carried out using gene-specific primers to generate SYBR green-labeled PCR products using RealPlex (Eppendrof Pvt Ltd). For all primers, the following temperature cycling profile was used: 10 min at 95ºC followed by 10 seconds at 95ºCand 1 min at Tm for 40cycles(Table 1). The Tm for each primer set was verified following MIQE guidelines before carrying out Real Time PCR [46]. Transcript levels between various RNA samples were normalized using 16S rRNA and actin specific primers for Mtb and macrophage respectively.

2.3.6. Fluorescence microscopy of intracellular *M. tuberculosis* inside Thp-1 macrophages

The microphotography of infected macrophages was carried out following an earlier method [37]. Briefly, infected macrophages were fixed in 2% paraformaldehyde(PFA) in phosphate-buffered saline (PBS) for 15 min. Cells were gently washed with PBS and then stained with Hoechst 33342 dye (1µM) for 15min in the dark then washed gently with PBS 2-3 times. Another stain Propidium iodide (PI) (3µM) was added and kept it for 15 min. Cells were finally washed with PBS 2-3 times. Cover slips were added by using glycerol solution (70%).Live and Dead cells were determined by a phase contrast fluorescent microscope (Company name and Model). Superimposed fluorescent microscopic images along with respective bright field images (20 x objectives) were used to visualize live (blue in color) and necrotic cells (red in color).

2.3.7. Fluorescence activated cell Sorting (FACS) analysis of *M. tuberculosis* infected macrophages

Infected Thp-1 macrophage cells were seeded in six-well tissue culture plates at a density of 5×10^6 cells per well. Cells were treated with stage specific inhibitors at respective MIC for 0hr, 72hrs, 144hrs and 216hrs at 37°C in 5% CO_2. Adherent macrophage cells were removed by trypsin treatment and washed with 1× PBS and suspended at 10^6cells/ml, in 1× Annexin V binding buffer and stained with FITC-conjugated Annexin V as per the manufacturer's instructions (Promega, USA). Necrotic cells were distinguished from the Annexin-V positive (apoptotic) cells by double-staining with Propidium iodide (1mg/ml) [12, 13]. Data counts for each sample (4000–10,000 events) were acquired using a BD Biosciences FACS caliber flow cytometer and analyzed using SummitV4.3 software.

2.3.8. Measurement of intracellular ATP of *M. tuberculosis* infected macrophages

Intracellular ATP was detected by measuring bioluminescence of luciferin-luciferase reaction [45]. Incubation reaction mixture was added in the microplate

wells containing infected Thp-1 macrophage cells at different time intervals and incubated for another 15 minutes. Intracellular level of ATP was measured from a standard curve by using luminescence spectrometer (PerkinElmer model LS 55). The rate of ATP synthesis (expressed per milligram of whole cell protein) was determined for control and inhibitor treated infected macrophages.

2.3.9. Measurement of Caspase 9, 8 and 3 activities in *M. tuberculosis* infected macrophage

Caspase 9, 8 and 3 activities was measured by using Apo Alert Caspase Fluorescent Assay Kit (Promega, USA) according to the manufacturer's instructions. Infected Thp-1 macrophages cells were quickly washed with chilled PBS and kept at -70ºC for 5 min, thawed at room temperature for 2-3 times. Cells were suspended in 50 µL chilled cell lysis buffer (Promega, USA) for 10 minutes at 4°C [44]. 50µlof 1 mmol/l substrates of DEVD-AFC (for caspase-3), IETD-AFC (for caspase-8), and LEHD-AMC (for caspase-9) were added to the each reaction well. After incubation at 37°C for 30 minutes, samples were transferred to a black 96-well plate. The bioluminescence intensities were measured using a plate reader (model LS55, PerkinElmer.) with 400/500-nm filters for caspase-3 and caspase-8 and 380/460-nm filters for caspase-9.

2.4. Discussion

Tuberculosis is one of the oldest diseases which continue to maintain its influence for centuries in humans. Although latency is in focus for many years to the scientific community, very little progress has been achieved in understanding the development of latency within human host. In vitro dormancy models were developed to explain the sequence of events that might take place during infection of the macrophages. Although from long back macrophages are known as host of Mtb to support their pathogenesis and growth in humans, there was actually no clue so far relating macrophages as the crucial place for development and harboring of dormant bacilli. For the development of a successful disease phenotype in humans, the pathogenic events should follow the steps ultimately leading to necrosis of host macrophage cells. In our experimental conditions, the fate of infected macrophages was found to be necrosis even though attenuated strain of Mtb is used (Fig1.5&1.6). The extent of macrophage death occurred due to infection of attenuated Mtbwas significantly higher than the virulent one reported earlier. This may also be attributed to their difference between host intracellular environments among primary and cell line. In fact, earlier report suggested that growth of bacilli to reach the critical level of at least 20/macrophage needed in developing dormancy within the host system. Earlier study revealed that the formation of foamy macrophages is an important factor favoring the appearance of necrosis. Foamy macrophages play a direct and unique role in necrosis formation [41]. To understand the events that take place inside infected macrophages, the biomarkers of hypoxia (narG, narK2, dos regulon) from Mtb as well as VEGF gene of the host cell were used. The kinetics of expression of all these biomarkers as well as their levels clearly established that a gradual increase in hypoxia was developed within the infected macrophages and reached the plateau in 7days after infection under the condition mentioned (Fig 1.4). Killing of intracellular bacilli by dormant and active stage specific inhibitors maintained the normal physiological condition of macrophages which indicated that development of hypoxia was not only related to bacilli burden, it is also connected to the fate of the host cell. Another important phenomena underlined by our study is the strong correlation between necrosis and lowering of intracellular ATP level from day 4 after infection and its reversal by killing the

bacillus any time before developing acute hypoxia or interfering the caspase cascade (Fig 1.7). Earlier reports suggested the link between long term hypoxia with mitochondrial dysfunction as a result of which release of cytochrome C and ATP depletion occurs in mammalian cells as the presence of ATP favors and promotes the apoptosis. People have already shown that intense nuclear staining by ethidium indicating the loss of cell membrane integrity due to lowering of intracellular ATP level in myocytes [36]. Intracellular ATP play an important role in determining the fate of infected macrophage die by apoptosis or necrosis. It was also reported that during this long period of hypoxia, mycobacterium bacilli remain unaffected because bacterial ATP synthesis is maintained by using alternate ETC through ndh-2 [39]. Released cytochrome C then binds with pro-caspase 9 to activate the intrinsic pathway of apoptosis [38]. Activation of caspase 9 and not caspase 8 supported the involvement of an intracellular signal for mitochondrial dysfunction which could be stopped by killing the intracellular bacilli (Fig 1.8 & 1.10). Finally, execution of either apoptotic or necrotic pathway depends on caspase 3/7 activity, inhibition of which leads to restoring macrophage health comparable to the uninfected controls (Fig 1.7 & 1.9). A broad sketch of intracellular environment of infected macrophage is drown to clearly indicate the key events that take place during Mtb pathogenesis for evolving the escape route from the host (Fig 1.10). Altogether, the results indicate the development of hypoxia inside the host macrophages occur due to the increase in tubercle bacilli leading to significant fall in ATP level to ultimately trigger necrosis of host cells and subsequent escape of bacilli for another round of infection of the host cells.

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CHAPTER 3

Potential use of nitrate reductase as biomarker in identifying active and dormant inhibitors of *Mycobacterium tuberculosis* in THP1 infection model

3.1. Introduction

Long-term host pathogen interaction is the main problem for tuberculosis as a disease. An increase in the number of people having double infections with Mtb and human immunodeficiency virus warns us about the consequences and therefore emphasizes the importance of controlling the infection [1]. Furthermore, the emergence of multidrug-resistant strains of Mtb has led to the expansion of this disease. The World Health Organization has declared as a priority, the need to immediately control tuberculosis infection to prevent the spread of drug-resistant strains [2]. Latency in tubercle bacilli is found as the principal cause for most of the problems associated with the disease [3]. Drug induced latency of the bacilli may be considered as one of the most important hurdles in reducing the duration of therapy, because the actively growing bacilli adapt to the administration of antimicrobial drugs by moving into a persistent state is associated with antibiotic resistance [4]. Therefore, the need to develop new inhibitors or antibiotics for tuberculosis is inevitable.

How the pathogen develops latency within the host as well as reactivates suddenly is still a mystery whose solution is linked to eradication of the disease. It is well recognized that non-pulmonary tissue oxygen concentrations within the human body are far below the oxygen concentration in ambient room air [5, 6]. Further, the oxygen concentration in the phagosome of activated macrophages was shown to be lower than the extracellular oxygen concentration [7]. It is also reported that Mtb cells within lipid-loaded macrophages lose acid-fast staining and become phenotypically resistant to the two frontline anti-mycobacterial drugs, rifampicin and isoniazid as well as induce gene transcripts involved in dormancy and lipid metabolism within the pathogen [8-11]. Wayne's hypoxia and nutrient starvation induced dormancy models were earlier developed to explain certain features in persistent tubercular bacilli obtained from hosts [8]. Earlier studies from our group as well as others have shown that nitrate reductase (NarGHJI), plays important role during transition from aerobic to anaerobic dormant stage. This transition happens during initial exposure to the asymptomatic pathogenesis as well as during exposure to anti-tubercular medicines [14, 15] .Recent reports suggest that nitric oxide (NO) generated inside host macrophages immediately after infection, leads to the development of either

dormancy of the bacilli or able to kill the pathogen by forming peroxynitrite ($ONOO^-$) in association with superoxide (O_2) in the host [9]. So far, there is no report of nitrate reduction that takes place inside infected macrophages.

There are several methods in use to evaluate the antimycobacterial activity of drugs in vitro and in vivo [16-24]. Currently used methods for the evaluation of compounds for antimycobacterial activity in vitro require colony forming unit (CFU) determinations. Such experiments generally require an incubation period of 3 to 4 weeks before colonies can be accurately counted. Because these studies are extremely laborious, require multiple serial dilutions, and use large numbers of agar plates or culture tubes, it is difficult to test more than a few compounds in any one experiment. Nowadays, radiometric methods based on the measurement of $^{14}CO2$ release from a radio labeled metabolic substrate, such as ^{14}C-palmitic acid (BACTEC TB 460 system), or measurement of enzymatic activity of a reporter gene as a measure of killing activity inside macrophage cell line contribute to evaluating drugs rapidly, but these are still of limited use [24, 30-32]. One of the problems associated with these assays is the unavailability of facilities for isotopic measurement or the high cost and inconsistencies in these assays. Methods based on reporter genes, such as β-galactosidase and luciferase, are rapid and sensitive and the results correlate well with those of culture-based methods [21, 24, 29, 30, 33-35]. However, these methods require recombinant bacilli, and various factors affect the measurement of the enzymatic activity. To eliminate the procedure for measuring enzymatic activity, a method using green fluorescent protein gene was recently reported [32, 36]. However, measurement of both the antimycobacterial effect on the bacilli within host cells and cytotoxic effect of drugs on host cells cannot be performed simultaneously. A fibroblast culture based anti-tubercular screening assay was also reported where host cell cytotoxicity was measured to assess the inhibitory activity of compounds against the pathogen. Again, this assay also is not of much use because of inherent problem of using unusual host, very high MOI and finally it reports the death of the host cells which is the last event that happens during infection. Now, there is an urgent need for an anti-tubercular screening protocol to be used in high throughput format using mainly human macrophage infection model which will be advantageous for identifying lead inhibitors.

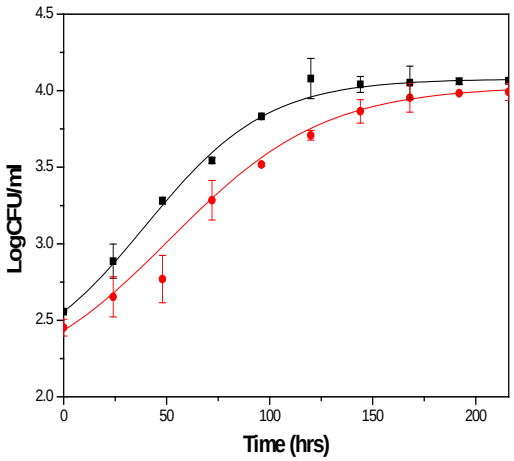
Interestingly, an anti-tubercular screening assay in microplate format was recently established by monitoring NR of *M. bovis* BCG adopted in Wayne's hypoxic model [16]. The authors have described here how nitrate reduction by intracellular Mtb in Thp-1 macrophages has been used to develop an anti-tubercular screening protocol. Altogether, the assay can be of great advantage as it can efficiently pick up inhibitors of both stages of the bacilli within host intracellular environment.

3.2. Result

3.2.1. Kinetics of Nitrate Reduction during growth of intracellular *M. tuberculosis* in flask and micro plate format

Earlier report suggested that NR of *M. bovis* BCG could successfully reflect the viability of dormant bacilli in the micro plate format. The active stage inhibitors of tubercle bacilli could also be identified from the same assay [16]. Recently, data from our laboratory clearly established that pentachlorophenol, a specific inhibitor of nitrate reductase when applied to infected macrophages, was able to efficiently kill the bacilli due to the development of intracellular hypoxia (manuscript under review). Killing of bacteria by using nitrate reductase inhibitor restored the host cells to normal physiological condition.

A



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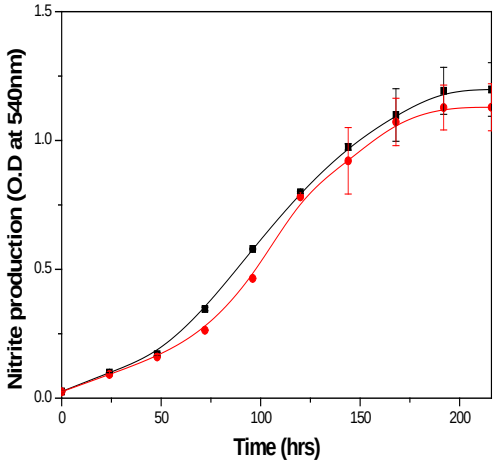


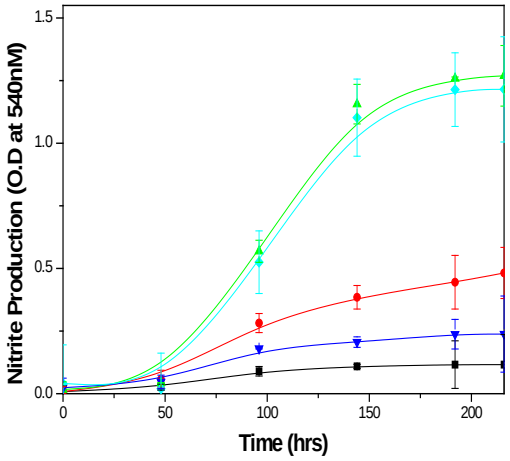
Fig.3.1. Kinetics of *M. tuberculosis* growth inside THP1 macrophages under different conditions. Thp1 macrophage cells was infected with Mtb bacilli and incubated in Tissue culture flask (■) and micro plate (●). The growth of the bacilli was monitored as a function of CFU (A) and nitrite production (B). Other experimental details are described in materials and method section. Experiments were carried out more than three times and results are mean ± SD.

Apart from nitrate reductase being a potential drug target, its activity could also be utilized to develop an anti-tubercular screening protocol. The concept of developing High-throughput screening in macrophage based assay was first tested on tissue culture flask and then transferred into micro plate, where intracellular Mtb was showing basically similar growth kinetics (Fig 1.1A.). During growth of intracellular bacilli, nitrate reductase activity was also found to follow similar pattern in both culture flask as well as microplate (Fig 1.1B).

3.2.2. Optimization of assay signal with substrate concentration as well as incubation period

Although nitrate is known to be produced within infected macrophage, its concentration was not sufficient enough to provide expected S/N ratio from the nitrite estimation. When the dose response effect of nitrate on the kinetics of nitrite production was carried out, optimum production of nitrite was found to occur at both 40mM and 50mM concentrations of nitrate (Fig 1.2 A). The CFU result also clearly indicated reduced viability of the bacilli beyond 50mM of nitrate in the medium (Fig 1.2 B). Currently, we are involved in identifying underlying reason behind this toxicity at higher level of nitrate in the medium.

A



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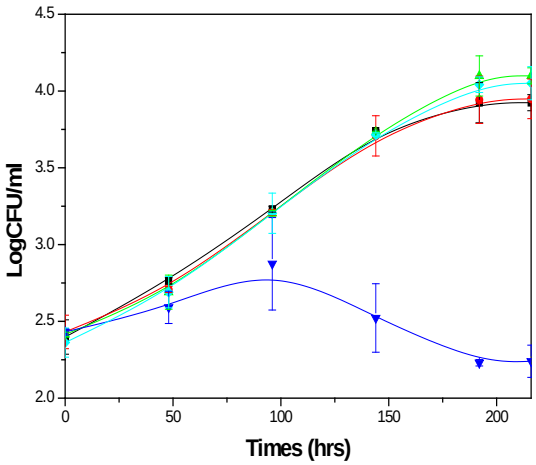


Fig.1.2. Effect of nitrate on the growth of *M. tuberculosis* inside THP1 macrophages**.** Infected Thp1 macrophages with *M .tuberculosis* bacilli was incubated in presence of 10 mM (■), 30 mM (●),40 mM (♦), 50 mM (▲) and 100 mM (▼) nitrate concentrations. The nitrite production (A) and growth of intracellular bacilli was monitored as a function of CFU (B). Rest of the details of the experiment is described in "Materials and Methods" section. Experiments were carried out more than three times and results are mean ± SD.

However, 50 mM of nitrate was selected as the optimum substrate concentration required for the assay. From the kinetics of nitrite production, it was also revealed that NR started at about 144 hours and the activity reached to a maximum level within 192 hours of incubation. Afterwards, the rate of NR reached a plateau.

Therefore, 192 hours (8 days) could be selected as the optimum incubation time for the assay.

3.2.3. Robustness of assay

The robustness of this screening protocol, in microplate format was assessed by determining S/N ratio and Z' factor [29]. When the S/N ratio for NR was determined at different concentrations of nitrate in the medium, the ratio value reached to the maximum level of 5.4 at 50mM of nitrate in the medium (Table 3.1). Z' factor also attained the peak level of 0.965 at the same nitrate concentration.

Table 3.1 S/N ratio and Z' factor for the macrophage based NR assay

Concentration of No3 (mM) [a]	S/N ratio [b]	Z' Factor [c]
10	3.0 ± 0.78	-0.255 ± 0.007
30	2.3 ± 0.07	0.733 ± 0.005
40	4.5 ± 0.09	0.825 ± 0.001
50	5.4 ± 0.58	0.965 ±0.024
100	1.95 ± 0.35	0.396 ± 0.001

a NR activity measurement at different nitrate concentration

b S/N ratio = Control/Blank.

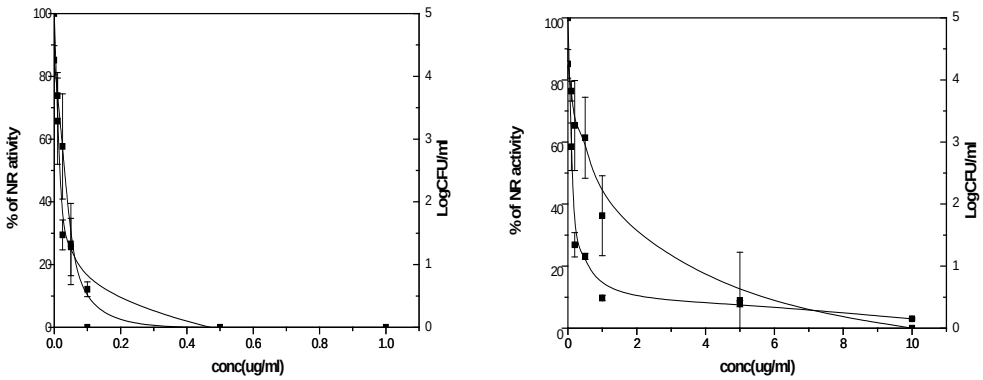
c Z' factor = 1-(3×standard deviation of control+3×standard deviation of blank) /(mean of control-mean of blank).

The compound solutions to be screened for the assay are generally prepared in DMSO. Hence, the maximum tolerable dose (MTD) of DMSO for NR and growth in the assay should be calculated before screening of compounds. The DMSO dose response curve obtained clearly indicated that the growth and nitrite production was not significantly affected up to 1% of DMSO in the assay mix (data not shown). So, the maximum volume of compound solution could be used in the assay was 1% of the total assay mixture.

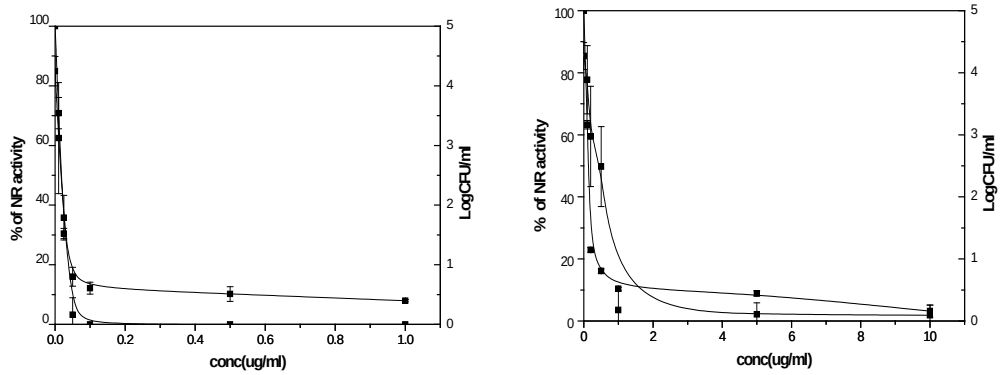
3.2.4. Validation of the assay protocol by using stage specific inhibitors

It was earlier noticed that actively growing bacilli are killed by most of the standard anti-tubercular drugs (rifampicin, isoniazid, streptomycin, ethambutol and pyrazinamid) whereas nitrate reductase (pentachlorophenol) specific inhibitors kill the pathogen more efficiently after the hypoxia is reached within the macrophage (manuscript under review). These known antitubercular drugs were applied in our system and NR activity was measured along with CFU count to confirm that NR activity reflects the viability of active tubercle bacilli (Fig3.3). In order to estimate their effect on nitrite production by Mtb bacilli within macrophage, the IC90 values of the inhibitors were determined by applying them at 0 day of infection and the nitrite production was monitored after 8days of incubation (Table 3.2). The CFU values were also determined using the same set of cultures to obtain a comparative data in parallel with the values reported in simple fibroblast-based assay (SFA). The results obtained from this experiment clearly indicated that the IC90 values for most of the inhibitors are lower than the values reported earlier using CFU based assays.

A. B.



C. D.



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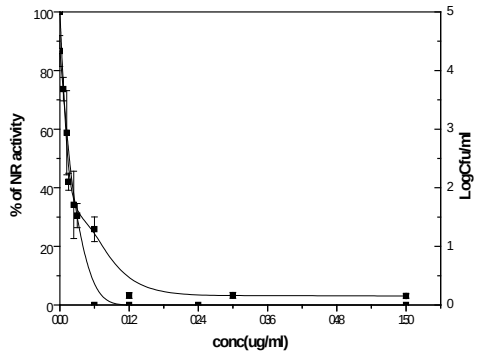

Fig.3. 3. Dose dependent effect of stage specific antimycobacterial drugs on *M. tuberculosis* infected Thp-1 macrophage. (A) Streptomycin and (B) Ethambutol (C) Isoniazid (D) pyrazimid (E) Rifampicin drugs were added on growth (●) and NR activity (■) of the bacilli. The values are shown as mean ± SD of three identical experiments.

Table 3.2 Comparison of IC90 values determined by Nitrate reductase macrophage based assay (NRMQA) with MIC values determined by SFA of standard inhibitors against *M. tuberculosis*.

Antimycobacterial agents[a]	IC90[b](µg/ml) determined by NRMQA	IC90[c] (µg/ml) by CFU on NRMQA	MIC[d](µg/ml) by SFA
Pyrazinamid	1	0.191	3.847
Isoniazid	0.09	0.281	0.428
Streptomycin	0.095	0.918	1.816

Ethambutol	1	0.391	3.346
Rifampicin	0.08	0.511	0.013
Pentachlorophenol	0.08	0.262	ND

a Standard inhibitors were added at the time of inoculation.

b Determined after 8 days of incubation by NRMQA

c Determined after 8 days of incubation on CFU by NRMQA protocol

d Determined after 8 days of incubation by SFA

ND: Not determine

As the intracellular environment of the macrophage was showing very similar pattern of bacilli growth that is observed in Wayne's hypoxia induced dormant culture, inhibition on NR activity could be achieved by any anti-tubercular drug but they should not have any effect on the dormant bacilli once the hypoxia is reached. In order to test this hypothesis, all these inhibitors were added in the assay mix at their respective IC90 values after 5 days of incubation. The kinetics of NR clearly indicated that aerobic stage specific inhibitors like streptomycin, isoniazid and ethambutol had no effect on the viability of the bacilli as expected (Fig 3.4).

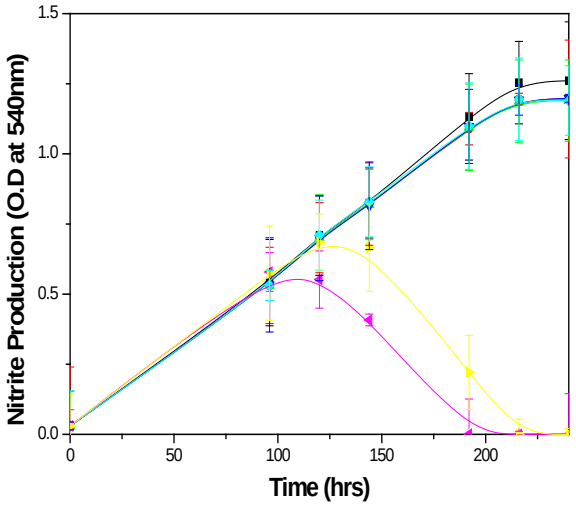

Fig.3.4. **S**tage specificity of standard inhibitors on the viability of *M. tuberculosis* within THP1 macrophages**.** Infected Thp1 macrophages were treated with Rifampicin (▶), Pentachlorophenol (◀), Streptomycin (♦), Ionized (▲), Ethambutol (●), Pyrazinamid

(▼) and Control (■) at their respective IC90 after 5days of infection. Then, the viability of intracellular bacilli was assessed from the kinetics of nitrite production till 8 day of infection. The NR activity values are shown as mean ± SD of three identical experiments.

Pentachlorophenol was most efficient in killing the intracellular bacilli because of the dependence of the bacilli on nitrate reductase in maintaining the respiratory activity at this stage. Rifampicin was also found to inhibit nitrate reduction albeit at a milder level compared to pentachlorophenol which again confirmed its effect on the dormant stage of the bacilli [29].

3.2.5. Assay Protocol

Take 200µl of Thp1 monocyte culture treated with 100nM PMA solution in each well of 96 micro plates and keep it for 12 hrs.

↓

Infect the macrophage culture with 20µl of diluted *M. tuberculosis* at 1:100 MOI and keep it for another 12 hrs. ↓

Wash the infected macrophage culture with 1X PBS solution two to three times.

↓

Add 200µl of fresh medium (MEM) containing 50mM of sodium nitrate.

↓

Transfer inhibitors at different doses in the infected macrophage culture at 0 hrs or after 5days of infection and keep the plate for 8 days incubation.

↓

80 µl cultures is taken out in a separate microplate and 80 µl of sulphanilic acid(1% in 20% HCL) and 80 µl of NEDD solution(0.1% IN d/w) is added and incubate for 15 minutes.

↓

Take the reading at 540nm in plate reader.

3.2.6. Calculation of % inhibition

% Inhibition = 100

$$- \left(\frac{A_{540} \text{ of the culture in presence of compound} - \text{blank}}{A_{540} \text{ of the culture negative control} - \text{blank}} \times 100 \right)$$

Where

Blank = A_{540} of the medium without inoculation

Negative control = A_{540} of culture without having compound

3.3. Materials and Methods

3.3.1 Reagents

Rifampicin, isoniazid, streptomycin, ethambutol, and pyrazinamid were purchased from Sigma, USA. Drugs were solubilizing according to manufacturers recommendations, and stock solutions were filter sterilized and stored in aliquots at −80 °C. Sulphanilic acid, napthyl ethylene diaminedihydrochloride (NEDD) were purchased from Merck, India. Dubos broth base, Dubos albumin supplements were purchased from Difco, USA. Standard sterile flat bottom 96 well plates were purchased from Tarsons, India. Fetal bovine serum (FBS) and minimum essential medium (MEM) (without phenol red) were purchased from GIBCO Biosciences.

3.3.2. Cell culture and their maintenance

Human acute monocyte leukemia cell line Thp1 was obtained from National Center for Cell Science (NCCS), Pune, India, was maintained in MEM medium with 10% heat-inactivated FBS. *Mycobacterium tuberculosis* H37Ra (ATCC 25177) obtained from Microbial Type Culture Collection (MTCC) Chandigarh, India, was routinely maintained on Dubos albumin agar medium at 37^{o}C. The inoculum was prepared by inoculating few colonies from a 21day old slant into Dubos albumin broth followed by incubation at 37^{o}C for 8 days.

3.3.3. Infection of *Mycobacterium tuberculosis* macrophage

Human acute monocytic leukemia cell line Thp1 was used to infect and cultivate Mtb by following a method described earlier [26]. Briefly, Thp1 cells were grown in MEM pH 7.4 at 37^{o}C for 4 days in presence of 5% CO_2 / 95% air as well as 95% relative humidity to reach the density up to 5x 10^4 cells per ml followed by treatment with 100nM of phorbol myristate acetate (PMA). The culture was then incubated for further 24 hours to allow them converted into macrophages. These macrophages were then incubated for another 12 hour with Mtb at 1:100 multiplicity of infection (MOI). At the end of infection, macrophage cells were washed four times with PBS to remove extracellular bacilli and the culture was filled with MEM as mentioned earlier. In order to check the effect of inhibitors on the growth of intracellular bacilli, compounds were added at the start of

incubation. Unless mentioned otherwise, the macrophages were lysed after 8 day of incubation, in hypotonic buffer pH 7.4 (10mM HEPES buffer containing1.5 mM $MgCl_2$ and 10mM KCl). The lysate was spread on Dubos albumin agar plates to get the CFU after 3 to 4 weeks of incubation at 37^oC.

3.3.4. Estimation of nitrite in culture

Nitrite in the culture was determined by following an earlier method [26]. Briefly, the nitrite liberated following the reduction of sodium nitrate in the culture medium at pH 6.8 and 37^oC was used. 1ml of the culture was added with 1ml of 1% sulphanilic acid solution (prepared in 20% v/v HCL) and 1 ml of 0.1% w/v NEDD solution (prepared in distilled water). The tubes were incubated for 15 min to develop pink color. Absorbance of the samples was read at 540nm in SpectraMax Plus384 plate reader.

3.4. Discussion

Our initial objective was first to examine that whether the Thp-1 macrophage model clearly demonstrated that the production of nitrite increases with time as the intracellular bacilli growth occurs. If the growth and nitrate reduction follow a similar pattern when M. tuberculosis bacilli are infected to Thp-1 macrophages and incubated in culture flask as well as micro well plate to monitor both the parameters then this result could be indicated that NarGHJI activity increases as a consequence of growth of bacilli with simultaneous development of hypoxia within intracellular environment.

Our study revealed that the nitrate reduction is directly proportional to the number of viable bacilli inside the infected macrophages (Fig 3.1-3.2). Earlier studies from our laboratory had clearly shown that nitrate reduction in Wayne's model is directly related to the viability of dormant *M. smegmatis* in presence of nitrate in the medium and could be used as reporter system to monitor the dormant bacilli and MTB is a strong reducer nitrate. A respiratory nitrate reductase is responsible for reduction of nitrate to nitrite. This induction of nitrate reduction in dormant stage was found to be associated with the induction in expression of NarK2, a transporter of nitrate present in the medium. Since long back nitrate reductase activity was used as a diagnostic tool to identify different mycobacterial species. This activity has also been used in few drug susceptibility assays [27]. Measurement of NR did not require cell lysis and could be monitored colorimetrically without any specialized instrumental requirement. The color also remained stable at least for 24 hours. Hence, NR activity determination could be considered as most easy, cost saving and simple assay for monitoring the viability of dormant stage specific bacilli. But in case of Mtb, nitrate reduction occurs at a lower rate in the actively growing stage and induced many fold as the availability of O_2 in the environment continue to deplete. Present macrophage based assay demonstrated similar pattern of growth and induction of NR activity in microplate assay representing the transition between aerobic to microaerophilic stage (NRP-1) of the bacilli. The major advantage of this assay is that we could pick up both the aerobic as well as NRP-1 stage inhibitors in the first assay where the inhibitors will be used at 0 day of infection. Once the hits will be identified, they could be used on day 5 after infection of macrophages in the same protocol to

identify the dormant inhibitors (Fig 3.4 and Table 3.2). There was no other assay currently available, could identify inhibitors of both the stages of Mtb. Thus, along with its application in screening of chemical library, it could also be used in biochemical experiments at microplate level to get the advantage of having a lot of data points compared to low throughput tissue culture flask model in cost saving manner. S/N ratio and Z' factor determined clearly indicated the robustness of the assay protocol (Table 1.1). The most valuable information regarding validation comes from the sensitivity of the assay against standard inhibitors. IC90 values for aerobic stage inhibitors determined by this microplate assay were very close and sometimes better than the values determined by other drug susceptibility assays and thus further validated the protocol in favor of its use in screening [19, 21] (Fig 3.3 and Table 3.2) against both actively replicating as well as dormant stage of Mtb. The microplate format of the assay provided screening of compounds in large scale in an automated robotic high throughput-screening platform, which may enormously expedite the drug discovery programs in finding novel antitubercular molecules. Altogether exclusion of CFU counting, minimum number of steps, within 8 days, use of cheaper reagents, with no specialized safety requirements could make the assay most acceptable among all available screening protocols which ultimately will be able to identify both aerobic replicating phase inhibitors as well as NRP-1 stage of Mtb.

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CHAPTER 4

Identification of groEL 2 as potent target of 1, 2, 4-triazolethiols in killing both replicating and non-replicating *Mycobacterium tuberculosis*

4.1 Introduction

Tuberculosis is an important public health problem in both industrialized and developing countries and is responsible for more than two million deaths each year. Therapy is complicated due both to the requirement for prolonged treatment with a combination of drugs and the emergence of drug-resistant strains. However, a major need in global health is to eradicate persistent or nonreplicating subpopulations of bacteria such as *Mycobacterium tuberculosis* (Mtb) [1, 2, 3]. Worldwide, an estimated 1 person in 3 is infected with Mtb and in about 9 of every 10 infected with the bacilli, persist in largely a nonreplicating (‘‘latent’’) state throughout the lifetime of the host. If the immune response flags, Mtb can resume replication and give rise to tuberculosis, a contagious disease that kills more people than any other bacterial infection. Non-replicating Mtb is also problematic in clinically active tuberculosis [4]. The acidic phagosomal environment and ‘‘nitroxidative’’[5] chemistry generated by the macrophages in which Mtb resides and the deprivation of oxygen as well as nutrients may result from the accumulation of inflammatory cells at infection sites can each keep Mtb away from replication [6]. Non-replicating Mtb display non-heritable antibiotic resistance, also called phenotypic tolerance, a phenomenon that pertains to most members of a bacterial population starved for nutrients, as well as to a small, non-replicating fraction of a population undergoing logarithmic expansion [7]. Durable cure of tuberculosis requires eradication of both replicating and non-replicating Mtb [1]. During treatment of tuberculosis, non-replicators termed ‘‘persisters’’ may be responsible for relapse rates that only fall below 5% when chemotherapy is extended for many months. Such prolonged treatment is difficult to sustain and its interruption fosters the emergence of mutants with heritable drug resistance. That most antibiotics act preferentially against replicating bacteria may be a consequence of the way these compounds have been sought: by screening against pathogens in vitro under conditions that sustain rapid growth and assaying for inhibition of that growth [8]. In spite of an enormous amount of work done in understanding the genome sequence of mycobacteria, no new anti-tuberculous drug has been discovered in over 50 years [9]. Hence, there is a pressing need to develop novel chemotherapeutic agents and to change current drug regimens in order to shorten the lengthy treatment, to minimize the

resistance problem in mycobacterial strains, and/or to improve the treatment of latent TB infection.

The 1, 2, 4-triazole nucleus has been incorporated into a wide variety of therapeutically important agents. For example, Ribavirin (antiviral), Rizatriptan (antimigraine), Vorozole, Letrozole and Anastrozole (antitumor) are some examples of drugs containing 1, 2, 4-triazole moiety along with Posaconazole, Fluconazole and Itraconazole used in current treatment as efficient antifungal drugs [10-14]. A number of biological activity such as antibacterial, antifungal, anti-inflammatory, analgesic, anticonvulsant, anticancer, antitumor, antiviral, antileishmanial, potassium channel activators, anti-platelet aggregation and anti-oxidant have been associated with N- substituted triazole attached with different hetrocyclic nuclei [15-27]. Recent reports have described that azoles exert antifungal activity through inhibition of CYP51 and CYP121 by a mechanism in which the heterocyclic nitrogen N-4 of 1, 2, 4-tiazole binds to the sixth coordination of heme iron atom of the porphyrin in the substrate binding site of the enzyme [28, 29, 30]. In the design of new bioactive agents, the development of hybrid molecules through the combination of different pharmacophores in the same structure may lead to compounds having more efficient in biological activity.

Various heterocyclic rings were taken in earlier attempts as a ground to constitute large series of compounds, e.g. imidazoles, tetrazoles, benzimidazoles, pyrazine, quinoxaline, and quinazoline having antitubercular potential [31, 32, 33, 34]. Recently, special attention is being given to synthesize novel triazole derivatives having potent antimycobacterial activity due to their known target of sterol synthesis [35, 36]. SAR studies showed that the presence of the hydrogen bond acceptor subunit, the position in the aromatic ring, the planarity of triazole and phenyl rings in these compounds was important for exhibiting anti-tubercular activity.

Recently, few series of 1, 2, 4 triazoles were reported to have anti-tubercular activity against Mtb keeping in view of azoles and their biological importance. Kucukguzel et al. synthesized a series of N-alkyl/aryl-N'-[4-(4-alkyl/aryl-2,4-dihydro-3H-1,2,4-triazole-3-thione-5-yl)phenyl] thioureas and S-alkylated representatives of Nalkyl/aryl-N'-[4-(3-aralkylthio-4-alkyl/ aryl-4H-1,2,4-triazole-5-yl)phenyl] thioureas. Among all, S-alkylated derivative [37] showed

the best potency of MIC 6.25 μg/ml against *M. tuberculosis* H37Rv [38]. With the similar type of modifications, Klimešová et al. synthesized a series of 3-benzylsulfanyl derivatives of 1, 2, 4-triazole and S-substituted-1, 2, 4-triazoles and evaluated for in vitro antimycobacterial activity against Mtb, *M. avium* and two strains of *M. kansasii*. While, in an almost similar series 2-(4-substituted-5-(pyridin-4-yl)-4H-1, 2, 4-triazol-3-ylthio)-1-phenylethanone, all the compounds exhibited less than 90% inhibition at a concentration of 6.25 μg/ml [39]. Earlier 3, 4, 5-substituted-1, 2, 4-triazole derivatives were evaluated against *M. tuberculosis* H37Rv and two of them have shown the best potency of MIC 0.39 μM and 0.79 μM respectively [40].

Most interestingly, 1, 2, 4-triazole scaffold was found to switch between different molecular targets based on the changes in N-substitutions or incorporating side chains in the heterocyclic ring. 3-Amino-1, 2, 4 Triazole is a competitive inhibitor of the production of HIS3 gene, imidazoleglycerolphosphate dehydratase. It is an enzyme catalyzing the sixth step of the Histidine production and is also a non selective systemic triazole herbicide used on non food crop land to control annual grasses and broad leaf and aquatic weeds [29]. Monazza et al reported 5-aryl-4-(1-phenylpropyl)-2H-1, 2, 4-triazole-3 (4H) -thiones from aryl carboxylic acids as a potent inhibitor of Jack bean urease [41]. Xin Yong Liu et al synthesized a series of novel 3-alkylthio-4-arylideneamino-5-(2-furyl)-1, 2, 4-triazole (98-101) derivatives. ET receptor competitive binding assay showed that some compounds exhibited high selective as potent ET-1 receptor antagonist [42]. Aline Moulin et al synthesized and established the structure–activity relationships concerning 3, 4, 5-trisubstituted 1, 2, 4-triazoles as ghrelin receptor ligands and found that Trp residue was found to lead to the best agonist or antagonist compounds [43].

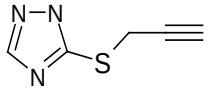
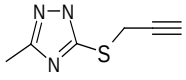
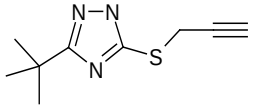
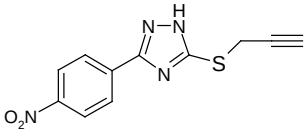
Present communication reports the identification of three derivatives belonging to triazolethiol scaffold from a structure-activity relationship using 54 1, 2, 4-triazolethiol derivatives potentially active against both actively replicative and non-replicative dormant stage of *Mycobacterium bovis* BCG and Mtb. groEL 2 has been identified as potential target for these inhibitors within the bacilli. Indian and PCT applications for obtaining patent on these findings were also filed [44, 45, 46].

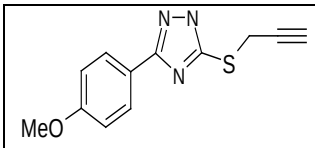
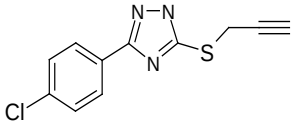
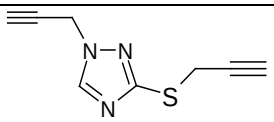
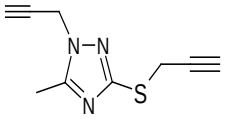
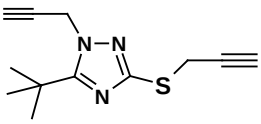
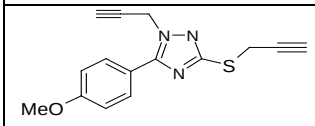
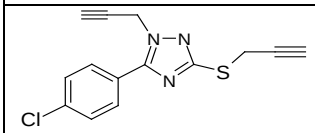
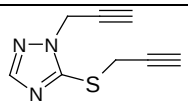
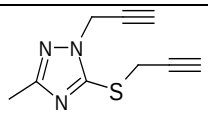
4.2 Results

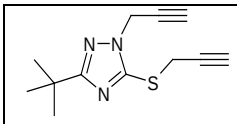
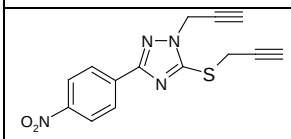
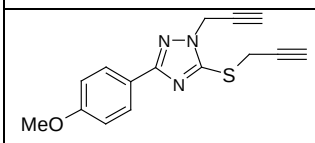
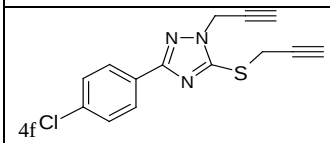
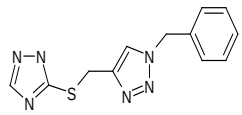
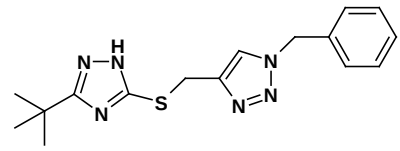
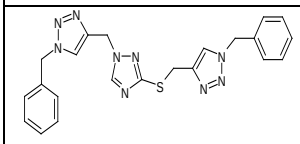
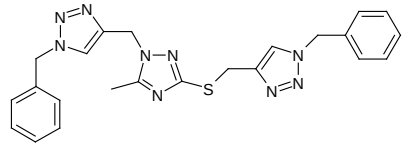
4.2.1 Primary screening of an in-house library of selected triazolethiols against *M.bovis* BCG

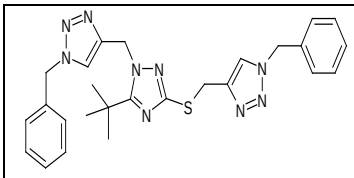
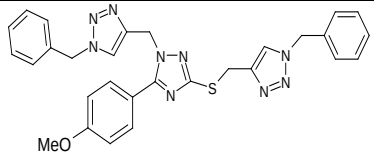
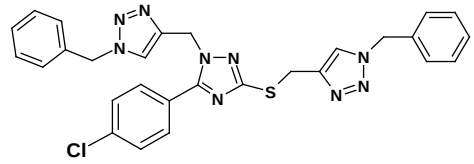
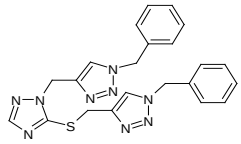
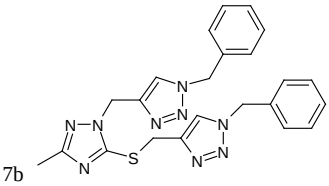
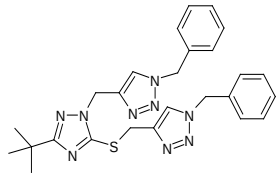
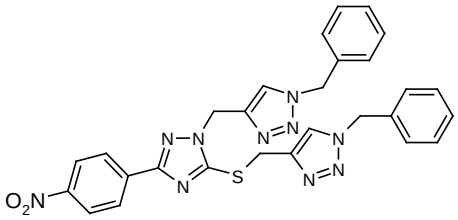
Major lacunae within the ongoing efforts of identifying novel anti-tubercular leads are the lack of sufficient technological support to efficiently screen the small molecule libraries. Interestingly, we had the advantage of having assays capable of screening both actively growing as well as dormant inhibitors [47, 58]. An in-house library of about 54 1, 2, 4-triazolethiol derivatives were screened against *M. bovis* BCG by using ADAS assay protocol developed earlier (Table 4.1) [47]. In primary screening, compounds were initially used at 100µg/ml of concentration to select the actives against the bacilli. The cut off value was fixed at 60% for selecting the actives from the turbidity measurement at 620nm as well as nitrite estimation at 540nm against *M. bovis* BCG.

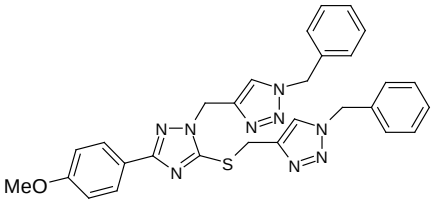
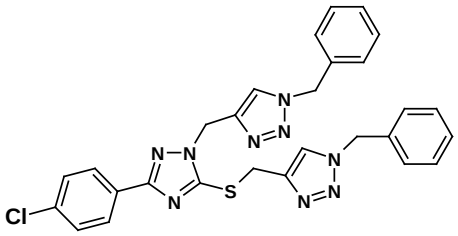
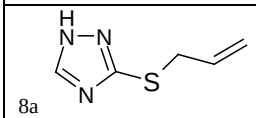
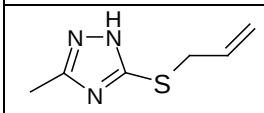
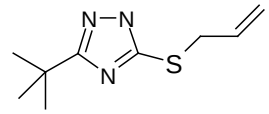
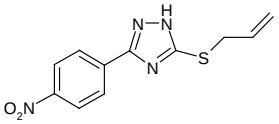
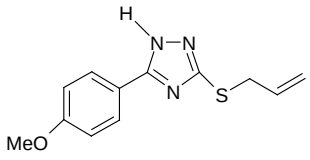
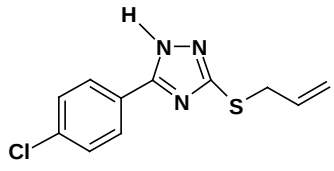
Table 4.1 *In vitro* antimycobacterial activity of synthesized triazoles against *M. bovis* BCG during primary screening

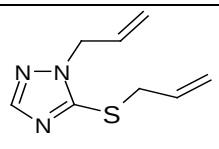
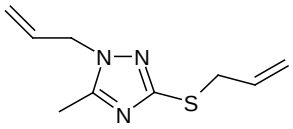
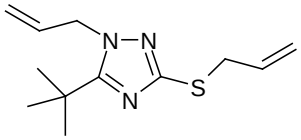
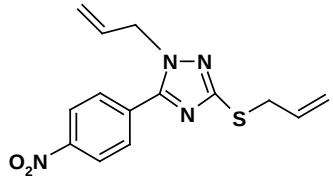
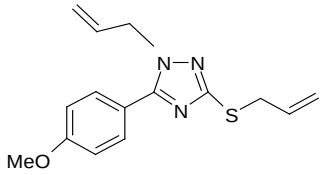
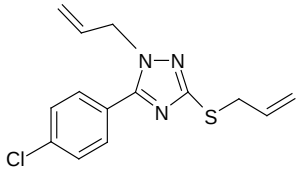
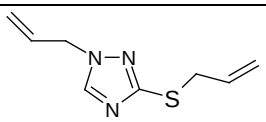
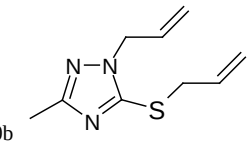
	Antimycobacterial activity determined as % inhibition from [a]	
Compound	Turbidity at 620nm (at µg/ml concentration)	Nitrite estimation at 540nm (at µg/ml concentration)
 2a	10(100)	07(100)
 2b	04(100)	09(100)
 2c	10(100	13(100)
 2d	95 (100), 98 (30), 96 (10), 25 (1)	97 (100), 93 (30), 89 (10), 37 (1)

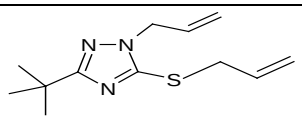
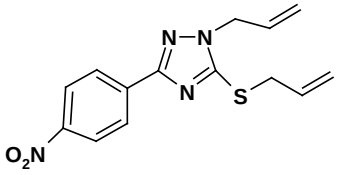
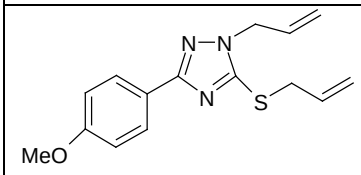
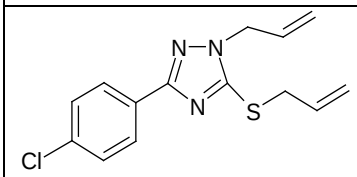
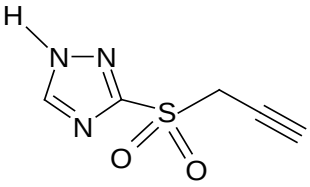
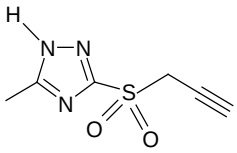
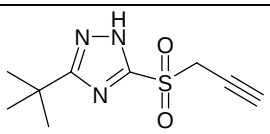
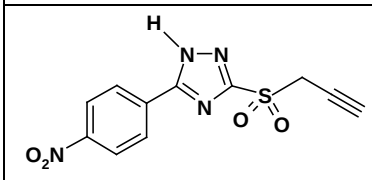
 2e	07(100	12(100)
 2f	09(100)	16(100)
 3a	07(100)	14(100)
 3b	06(100)	09(100)
 3c	16(100)	14(100)
 3e	11(100)	06(100)
 3f	10(100)	17(100)
 4a	83 (100), 12 (30), 16 (10), 11 (1)	74 (100), 22 (30), 12 (10), 02 (1)
 4b	17(100)	12(100)

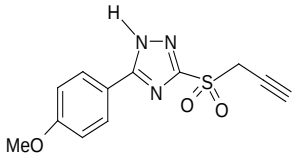
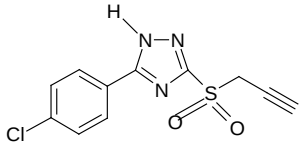
 4c	94 (100), 93 (30), 17 (10), 12 (1)	88 (100), 79 (30), 16 (10), 06 (1)
 4d	03(100)	04(100)
 4e	05(100)	09(100)
 4f	09(100)	17(100)
 5a	03(100)	04(100)
 5c	03(100	11(100)
 6a	11(100)	18(100)
 6b	08(100)	12(100)

 6c	08(100)	15(100)
 6e	01(100)	08(100)
 6f	21(100)	17(100)
 7a	08(100)	03(100)
 7b	85 (100), 46 (30), 12 (10), 05 (1)	76 (100), 41 (30), 11 (10), 09 (1)
 7c	10(100)	13(100)
 7d	13(100)	11(100)

 7e	86 (100), 22 (30), 13 (10), 06 (1)	82 (100), 19 (30), 07 (10), 03 (1)
 7f	31(100)	04(100)
 8a	51(100)	32(100)
 8b	98 (100), 93 (30), 10 (91), 14 (1)	97 (100), 89 (30), 78 (10), 12 (1)
 8c	57(100)	39(100)
 8d	25(100)	14(100)
 8e	94 (100), 57 (30), 07 (10), 06 (1)	93 (100), 48 (30), 19 (10), 06 (1)
 8f	98 (100), 97 (30), 95 (10), 93 (1)	91 (100), 92 (30), 85 (10), 83 (1)

 9a	58(100)	42(100)
 9b	25(100)	15(100)
 9c	45(100)	36(100)
 9d	15(100)	18(100)
 9e	89 (100), 43 (30), 21 (10), 11 (1)	84 (100), 33 (30), 26 (10), 17 (1)
 9f	94 (100), 45 (30), 17 (10), 08 (1)	91 (100), 40 (30), 19 (10), 10 (1)
 10a	65 (100), 04 (30), 15 (10), 12 (1)	60 (100), 14 (30), 11 (10), 02 (1)
 10b	22(100)	10(100)

 10c	30(100)	33(100)
 10d	40(100)	12(100)
 10e	88 (100), 33 (30), 08 (10), 06 (1)	83 (100), 43 (30), 14 (10), 03 (1)
 10f	26(100)	14(100)
 11a	42(100)	23(100)
 11b	92 (100), 45 (30), 23 (10), 06 (1)	82 (100), 33 (30), 12 (10), 09 (1)
 11c	76 (100), 23 (30), 11 (10), 03 (1)	74 (100), 33 (30), 15 (10), 06 (1)
 11d	96 (100), 93 (30), 91 (10), 55 (1)	91 (100), 86 (30), 81 (10), 37 (1)

 11e	88 (100), 39 (30), 12 (10), 10 (1)	82 (100), 33 (30), 11 (10), 04 (1)
 11f	88 (100), 44 (30), 11 (10), 07 (1)	86 (100), 43 (30), 14 (10), 11 (1)
Rifampicin [a]	95 (1), 94 (0.1), 75 (0.05), 29 (0.01)	90 (1), 83 (0.1), 67 (0.05), 15 (0.01)
Isoniazid [a]	98 (1), 97 (0.1), 83 (0.05), 37 (0.01)	93 (1), 91 (0.1), 82 (0.05), 32 (0.01)
Metronidazole [b]	17(100)	56 (100), 39 (30), 23 (10), 11 (1)
Itaconic Anhydride [b]	12(100)	51 (100). 43 (30), 12 (10), 07 (1)

a Antimycobacterial activity was determined at concentrations 100, 30, 10, and 1µg/ml.

b Positive control drugs; std 1) rifampicin, std 2) isoniazid

[c] Concentration of compounds exhibiting 90% inhibition on mycobacterial growth

Among the triazolethiol derivatives used in our screen, 17 actives were identified with >60 % inhibition from both the detection methods on growth of the bacilli (Table 4.1). Subsequently, dose response curves obtained using same protocol produced almost similar responses when a concentration range between 100 and 1µg/ml was applied. The results clearly indicated that three compounds (2d, 8f and 11d) have significant inhibitory effect on the bacilli even at 1µg/ml concentration and pursued for further characterization (Table 4.1).

4.2.2 Secondary screening of the hits

MIC of the hits against *M. bovis* BCG

The dose dependent effect was examined against actively growing *M. bovis* BCG to find out the MIC values of these 3 hits. The MIC values determined for 2d, 8f

and 11d against aerobically growing *M. bovis* BCG were found to be 2, 0.2 and 2µg/ml respectively (Table 4.2). From the above screening, it was not possible to conclusively identify the inhibitor's action against the dormant phase. For this reason, 3 hits were separately applied on hypoxia induced dormant bacilli in Wayne's tube culture [61]. Tube model of dormancy provided the flexibility of adding compound at any stage of culture without significantly disturbing its oxygen environment. 2d, 8f and 11d reduced the viability of dormant bacilli by 0.75, 1.197 and 0.95 respectively when these inhibitors were added to the culture at their respective MIC obtained against aerobically growing stage (Table 4.2).

Table 4.2 Secondary screening characterization of identified active molecules

	MIC [a](µg/ml) against active stage				Inhibition of dormant stage (by log CFU/ml)	
Actives against	*M. bovis* BCG	*M. tuberculosis*	*M. smegmatis*	*E. coli*	*M. bovis* BCG	*M. tuberculosis*
2d	02	02	>100	>100	0.75	0.82
8f	0.2	0.2	>100	>100	1.197	1.432
11d	02	02	>100	>100	0.95	0.88
Rifampicin [a]	0.1	0.1	0.2	0.5	0.47	0.54
Isoniazid [a]	0.1	0.1	0.3	2	0.46	0.51
Metronidazole [b]	>100	>100	>100	>100	0.56	0.59
Itaconic anhydride [b]	>100	>100	>100	>100	0.44	0.43

[a] these standard compounds were used as positive controls for aerobic stage of *M. tuberculosis*

[b] these standard compounds were used as positive controls for dormant stage of *M. tuberculosis*

[c] Concentration of compounds exhibiting 90% inhibition on mycobacterial growth

Determination of MIC against *M. tuberculosis, M. smegmatis* and *E. coli*

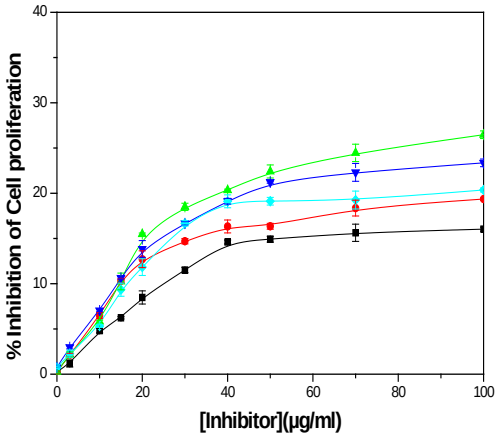
In order to verify the specificity in mode of action, these hits were then applied on Mtb at both the actively growing culture as well as hypoxic culture in Wayne's

tube model. Identical MIC values were obtained in aerobic and hypoxic cultures of *M. tuberculosis* like *M. bovis* BCG (Table 4.2). The estimated reduction of viable cell count due to the addition of 2d, 8f and 11d at MIC values in dormant culture were found to be 0.82, 1.432 and 0.95log values respectively. Then, their effectiveness against non-pathogenic mycobacterium was investigated by applying these inhibitors on *M. smegmatis* culture. Apart from *M. smegmatis*, the hits were also tested on *E.coli* to check their specificity against bacteria from non-mycobacterium origin. None of the hits had any significant effect up to 100µg/ml concentration on growth of either organism (Table 4.2). Hence, the result confirmed their specific action against *M. bovis* BCG and Mtb. Rifampicin and Isoniazid used as standards in these assays gave results identical to that observed earlier [46]. The data obtained so far indicated that all three inhibitors were almost similarly active against MBC group of organisms.

Cytotoxicity against the human THP-1 monocyte and HL-60 cell lines

Hits should be evaluated in terms of its cytotoxicity before taking them ahead into the discovery chain. The results clearly indicated that there was no significant effect of these compounds on proliferation of Thp-1 and HL-60 cell lines even at 10 X MIC levels and at this stage be considered as safe for taking them to the next level of drug discovery program (Fig 4.1).

A



B

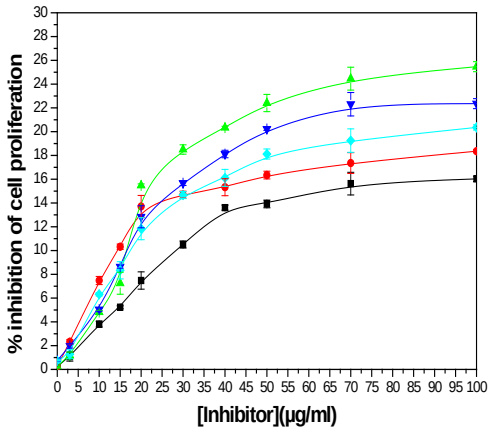


Fig 4.1. **Dose response effect of Inhibitors on cell proliferation of Thp-1 monocyte and HL-60 cell line.** The proliferation of Thp1 (A) and HL-60 (B) cells were monitored in presence of different concentrations of 2d (■), 8f (▲), 11d (●), rifampicin (▼) and isoniazid (♦).The details of experimental procedure for cytotoxicity on Thp1 is described in "Materials and Methods". Results are mean ± SD of three identical experiments.

Efficacy of the hits against *Mycobacterium tuberculosis* inside THP-1 macrophage

Ex vivo infection model provides valuable information about the level of efficacy that could be achieved when the inhibitors will be applied to animal model. For this reason, identified hits were tested against Mtb when residing within Thp-1 macrophage (Fig 4.2). The result clearly indicated drop in the viability of intracellular bacilli started from the very beginning of the incubation in presence of the inhibitors.

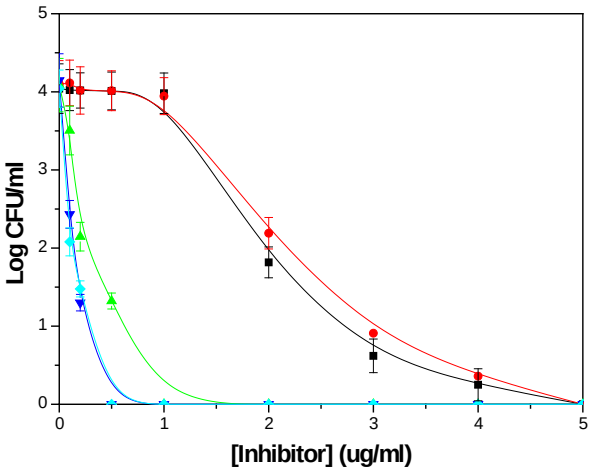


Fig 4.2. **Dose response effect of inhibitors on growth of intracellular *M. tuberculosis*.** The intracellular *M. tuberculosis* bacilli are growing in Thp1 macrophages were monitored in presence

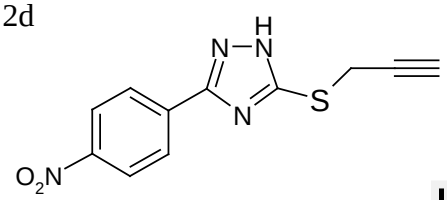
of different concentrations of 2d (■), 8f (▲), 11d (●), rifampicin (▼) and isoniazid(♦).The details of experimental procedure for growth of intracellular *M. tuberculosis* is described in "Materials and Methods". The results described here mean ± SD of three identical experiments.

Both compounds 2d and 11d could completely sterilize the Mtb infected Thp-1 macrophages at concentration of 5µg/ml within 8 days of infection whereas 8f could achieve sterilization at as low as 1µg/ml concentration within same time period. The MBC value of 8f was almost comparable with rifampicin and isoniazid when carried out within same set of experiments. These results indicated that the reduction in colony count was due to the bactericidal activity of compounds and not due to any non-specific lethal effect on Thp-1 macrophage. Altogether, these results showed that the anti-tubercular potency of these triazolethiol derivatives was restored in intracellular environment as well.

Stage specificity of 2d inhibitor on *M. tuberculosis* within infected THP-1 macrophages

Very recently, it has been established that hypoxia generates within infected macrophage during growth of bacilli which makes them resistant to most of the anti-tubercular drugs as a result of achieving dormancy within intracellular environment (manuscript under review). As these hits were also effectively killing the dormant bacilli in Wayne's hypoxia model, we further investigated their stage specificity within macrophage model also (Fig 4.3). For this, we used only one inhibitor (2d) at 0 hr and after 120 hr of infection at its MIC value. The result clearly indicated that effective killing of intracellular bacilli in Thp-1 macrophages took place immediately after addition of the inhibition and the sterilization of both aerobic and hypoxic bacilli occurred within ~70hrs of incubation. This result clearly established these triazolethiol derivatives as potential antitubercular leads for further explorations.

2d



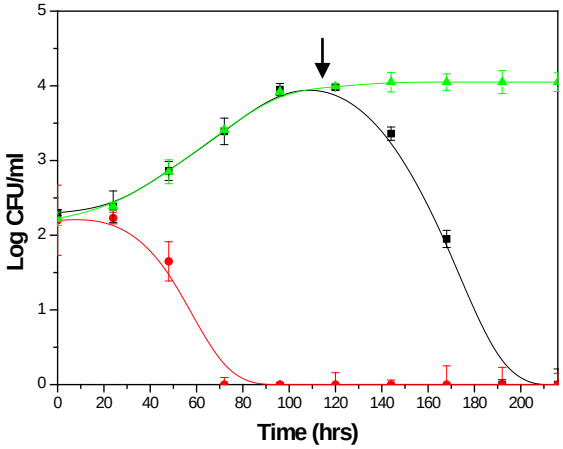


Fig 4.3. **Kinetics of killing of *M. tuberculosis* inside Thp-1 macrophage in presence of inhibitor.** The viable count of the bacilli measured in terms of CFU in presence (■) and absence (▲) of inhibitor (2d) at MIC added immediately after infection and after 5 days of infection (●) at MIC value. The results described here mean ± SD of three identical experiments.

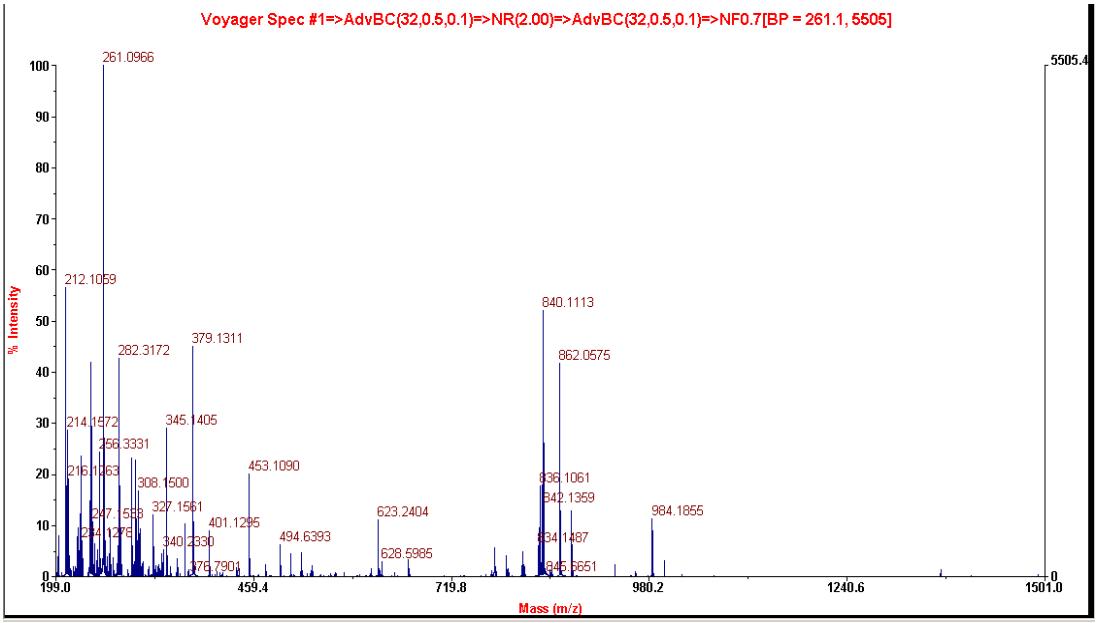
4.2.3 Identification of intracellular target of Triazolethiols within *Mycobacterium tuberculosis*

Identification of the intracellular target of a lead inhibition is an essential criterion for pursuing the scaffold in lead optimization program. Among the different approaches known for identifying target of a novel inhibitor, affinity pooling could be a straight and simple procedure to hit a protein target. As the pharmacophor of this scaffold is still not clearly known, we selected Mts-Atf-Biotin Label Transfer Reagents supplied by Thermo Scientific for labeling the inhibitor with the possibility of interacting at multiple positions. Mts-Atf-Biotin Label Transfer Reagent has a trifunctional cross-linker that contain a biotin, a sulfhydryl-reactive methanethiosulfonate (Mts) moiety and an efficient photoactivatable tetrafluorophenyl azide (Atf) moiety for making covalent linkage with the inhibitor. This biotin cross linker was allowed to react with the inhibitor (2d) and then analyzed the synthesis of the product conjugate.

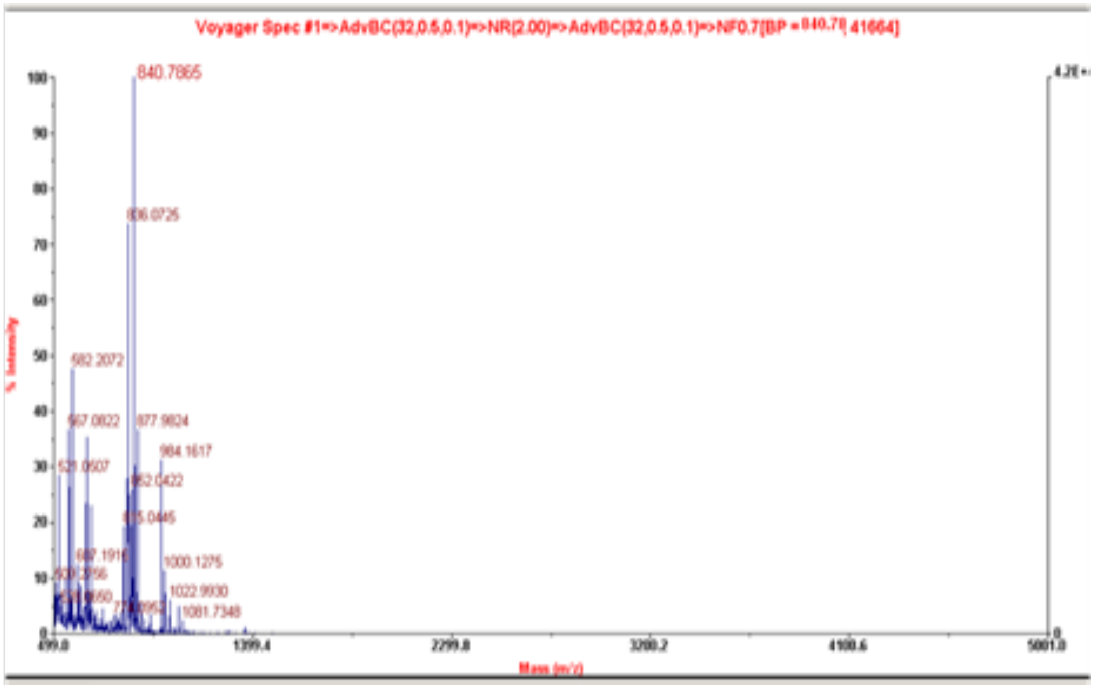
Purification of the product (Inhibitor-biotin linker) by preparative HPLC and MALDI-MS analysis

First, the linker was allowed to interact with a hit compound (2d) under UV light for 72hrs at room temperature. Then, the reaction mixture was subjected to MALDI-MS analysis (Fig 4.4). In this analysis the inhibitor, biotin-linker and one of the products possibly biotin linker- inhibitor conjugate in the reaction mixture were correctly showing their masses 261.09, 840.79 and 1101.3kd respectively. The mass of the product (1101kd) clearly indicated the formation of biotin linker-inhibitor conjugate.

a



b



c

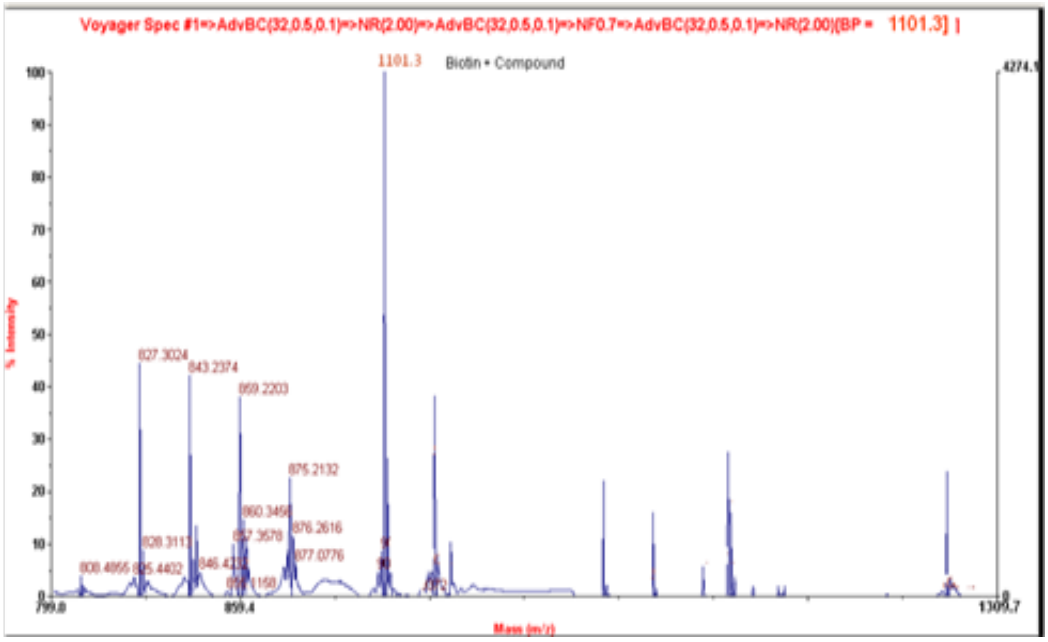
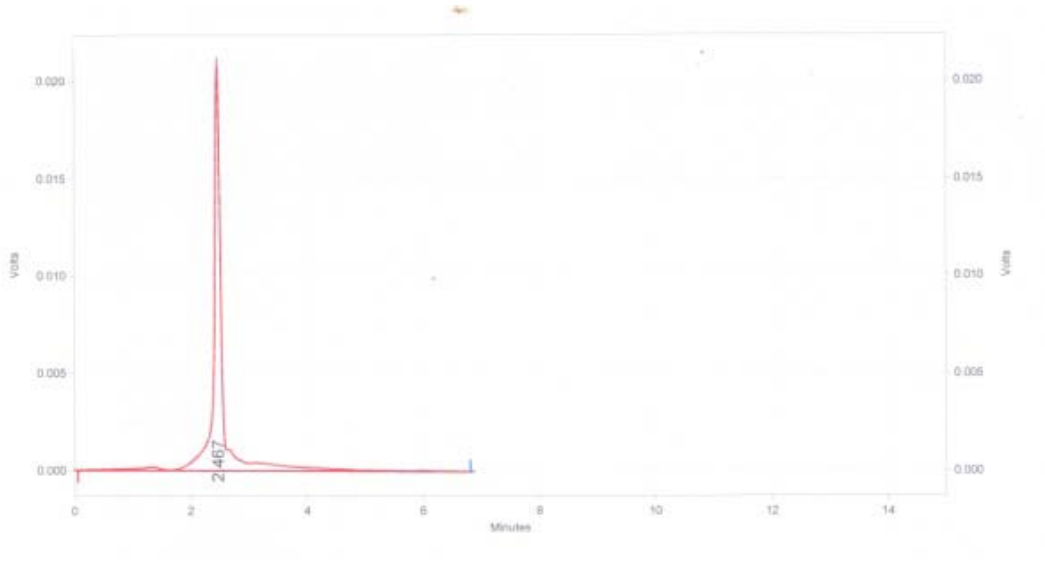


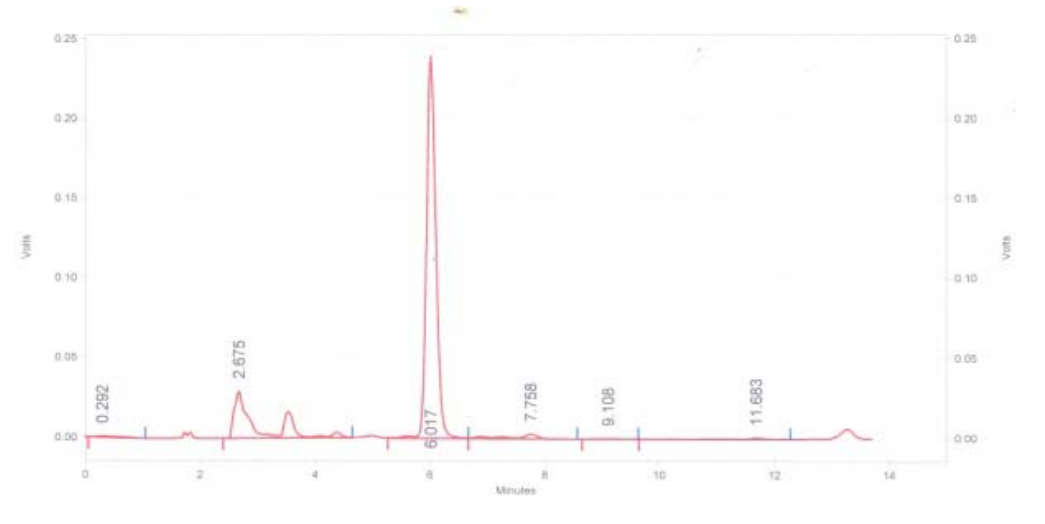
Fig 4.4. **Mass confirmation by MALDI-MS analysis.** MALDI mass spectra were obtained using the dried-drop method or the slow crystallization method of only inhibitor (a), only biotin –linker (b) and biotin-inhibitor (c). Spectra were obtained from a matrix solution consisting of 4HCCA-water/acetonitrile (2:1 v/v).

The product was then purified from the rest of the reagents by carrying out preparative HPLC (Fig 4.5). The retention time of the solvent, biotin-linker and inhibitor were first standarized as 2.46, 6.01 and 11.6 minutes respectively.

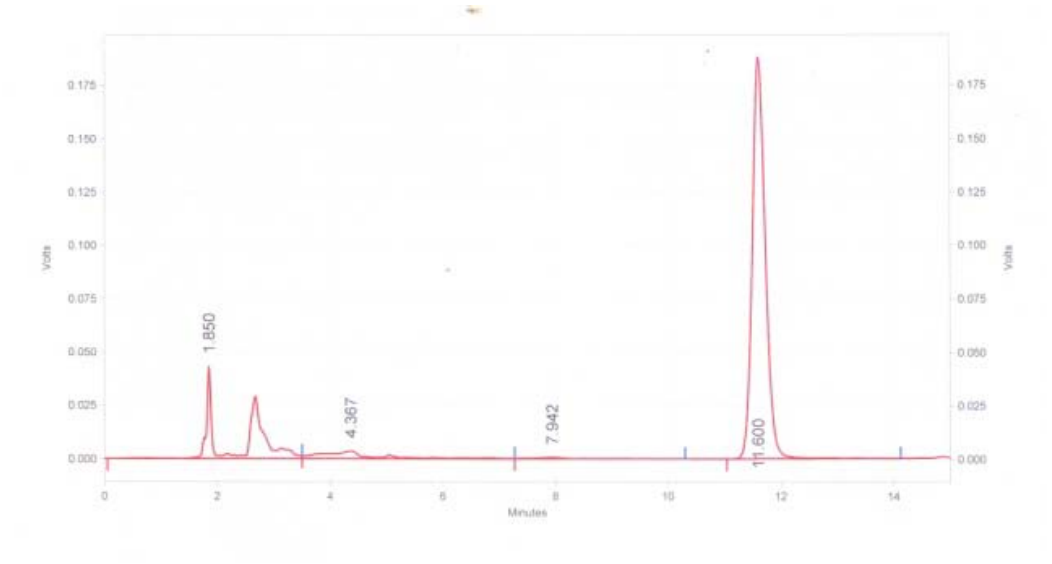
a


Volts
0.020
0.015
0.010
0.005
0.000
2.467
0
2
4
6
8
10
12
14
Minutes

b


Volts
0.25
0.20
0.15
0.10
0.05
0.00
0.292
2.675
6.017
7.758
9.108
11.683
0
2
4
6
8
10
12
14
Minutes

c



d

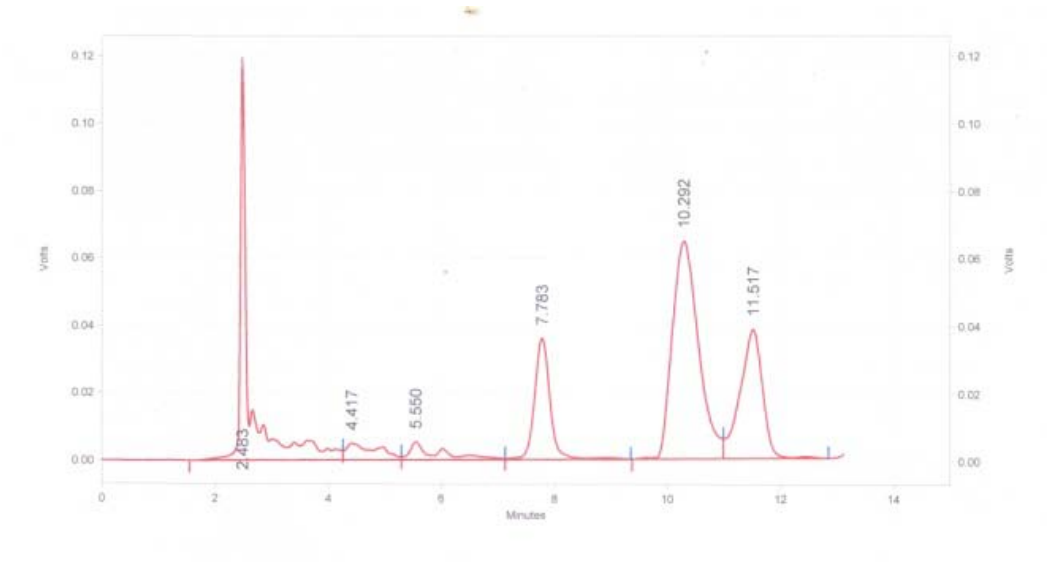


Fig 4.5. **HPLC profile of the product (biotin-inhibitor conjugate).** Chromatogram of solvent (a), biotin at 1mg/ml (b), 2d at MIC (c) and reaction mixture (d) were analyzed by preparative HPLC. The chromatograms are the representatives of result obtained from three identical set of each experiments. The rest of the details about the chromatography are provided in "Materials and Methods" section.

One foreign pick was identified with 10.292 minute retention time when the reaction mixture obtained after incubation was run through the HPLC column using similar conditions. This new peak was collected and subjected to MALDI-MS analysis and found to have Mol. Wt of 1101kd confirming separation of the Biotin linker-inhibitor conjugate from the mixture. Then, the product was collected and pooled for carrying out further experiments.

Identification of target protein by using Biotin linker-Inhibitor conjugate from *Mycobacterium tuberculosis*

Addition of Biotin Linker-Inhibitor conjugate was supposed to bind protein/s in the whole cell extract because of the higher affinity of the inhibitor remained for the target protein. This whole complex could then be pooled down with the help of MagnaBind Streptavidin beads, because of the affinity of streptavidin to biotin, to the bottom by applying a magnetic field. At this stage, the beads were washed with PBS for 2-3 times to remove the unwanted proteins. SDS-PAGE analysis of the target proteins pooled by streptavidin beads clearly indicated presence of a major protein band at ~60kDa molecular weight (Fig 4.6). This band was insignificantly present in the control sample where the extract was pre-treated with the inhibitor alone.

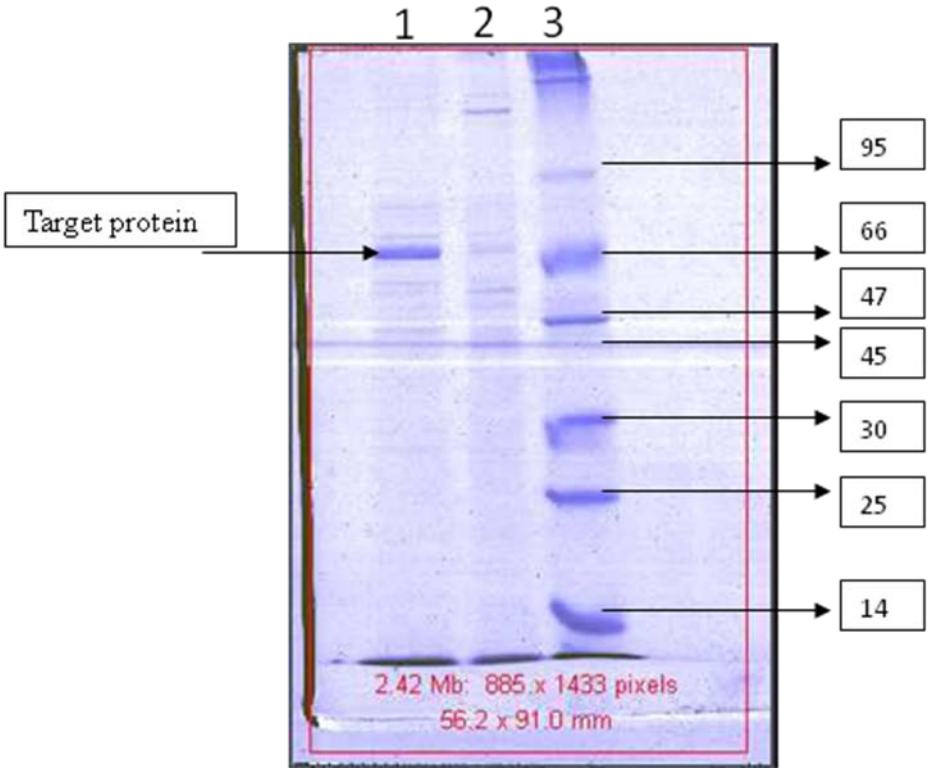


Fig 4.6. **Separation of identified proteins by SDS PAGE on the basis of the molecular weight Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the protein isolated from streptavidin beads**. The gel picture represents the protein samples obtained from the whole cell extract previously treated with (Lane 2) and without (Lane 1) the inhibitor and Lane 3 is represented by high molecular weight protein markers.

The protein bands found in the gel was digested by trypsin and used for mass determination (Fig 4.7). The protein mass was found to be 60 kDa which was further identified as chaperonin 2OS *Mycobacterium tuberculosis* GN groL2 PE 1 SV 2 by using PLGS score.

4.2.4. Validation of Triazolethiol leads inhibiting *Mycobacterium tuberculosis* groEL 2 as target

groEL 1 and 2 are functionally very similar because both belong to the class of protein having 60% sequence similarity representing 60Kd HSP family of chaperonines. Both of these two proteins are reported to have ATPase activity and found to protect citrate synthase agglutination during heat shock [56]. In spite of these facts, enormous differences exist in 3D structure as well as amino acid sequence (Fig4.8) between groEL1 and groEL2 [63]. In order to validate the specific action of these three inhibitors on groEL 2, we took groEL 1 also in our experiments as control. The results clearly indicated that 2d, 8f and 11d inhibited ATPase activity by ~86% at their respective MIC when applied to these purified groEL2 but did not exhibit any significant effect on groEL1 (Table 4.3). We also investigated the effect of these inhibitors on citrate synthase agglutination and found to have no effect on it (data not shown). In fact, interaption of functional role of these chaperonines by this lead inhibitor could best be demonstrated using whole cell.

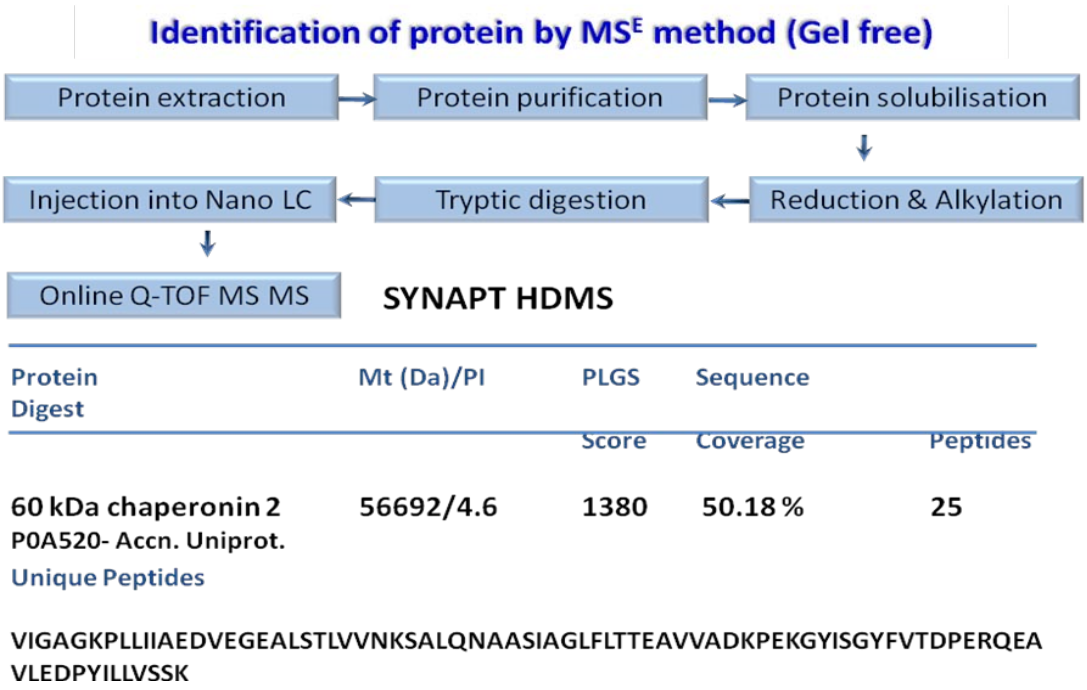


Fig 4.7. **Identification of protein by MS^E method (Gel free) analysis.** This data was analyzed by using the single protein band taken out of SDS-PAGE representing molecular weight of ~60 Kda.

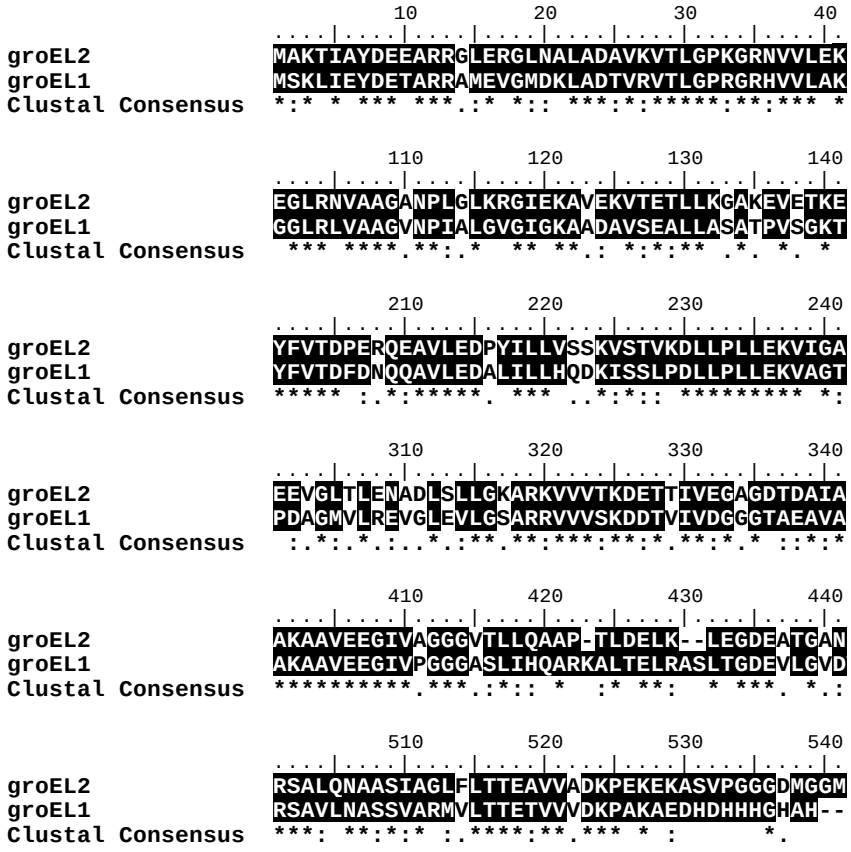
Table: 4.3 ATPase activity on purified groEL-1 and gro-EL-2

Inhibitors	**ATPase activity determined as % inhibition [a] on purified**	
	groEL-1[b]	**groEL-2**
2d	**2.56**	**85.64**
8f	**5.87**	**87.43**
11d	**3.21**	**86.54**

a Concentration of compounds exhibiting 90% inhibition on whole cell growth

b Positive control: Purified groEL-1

a



b

CLUSTAL 2.1 Multiple Sequence Alignments

Sequence 1: groEL2 540 aa

Sequence 2: groEL1 539 aa

Sequences (1:2) Aligned. Score: 60

Fig.4.8. Amino acid Sequence analysis of groEL1 and groEL2 by clustral w. analysis.

4.3 Materials and Methods

4.3.1 Bacterial strains, media and inoculum preparation

M. bovis BCG (ATCC 35745), *M. smegmatis* (ATCC 607) was obtained from Astra Zeneca, Bangalore, India and *M. tuberculosis* H37Ra (ATCC 25177) was obtained from Microbial Type Culture Collection, Chandigarh, India. *E. coli* strain DH5α was obtained from National Collection of Industrial Microorganisms (NCIM), Pune, India. Sub culturing of all mycobacterial strains was routinely done in Dubos albumin agar slants or plates. Liquid inoculum was prepared in Dubos tween albumin broth, incubated in a shaker incubator rotating at a speed of 150 rpm at 37^0C. 1% of 1.0 O.D at 620nm of the culture was used as standard inoculum size for all the experiments, yielding final inoculum of approximately 10^5 CFU/ml. Thp-1 human monocyte and HL-60 cell line was obtained from, National Centre for Cell Science (NCCS), Pune, India. Cells were maintained routinely in RPMI 1640 cell culture medium supplemented with 10% FBS.

4.3.2 Screening compounds for anti-mycobacterial activity

Active as well as dormant stage inhibitors against *M.bovis* BCG were identified by using a recently developed protocol to screen the compounds [47]. Briefly 2.5 µl of compound solution in DMSO was aseptically transferred to individual wells of sterile 96-well plates. 247.5µl of bacterial culture containing ~10^5cells/ml, supplemented with 40mM $NaNO_3$ was aseptically transferred to each well to make up the total volume to 250µl and the plate was covered with a sealer. 125µl space is left in each well to make the headspace to culture volume ratio exactly 0.5. After sealing, these culture plates were incubated at 37^0C in an incubator. After 8 days of incubation, culture OD was read using a cut off filter of 620nm after sonication for 2mins in a bath sonicator (Bandelin Electronic, Germany). Then, 80µl of culture was taken out from each well and transferred to a separate 96 well plate. Then, 80µl of 1% sulphanilic acid and 80µl of 0.1% NEDD solution were added in each well and the plate was incubated for 15 minutes at room temperature to develop pink color. The color was read in $Spectra_{max}$ plus ^{384}, Molecular Devices, USA at 540nm to measure NR activity.

Confirmation of inhibitors active against non-replicating *Mycobacterium bovis* BCG

The testing of the inhibitory effect of compounds against dormant bacilli was carried out by using Wayne's 0.5 HSR (head space ratio) in test tubes as described earlier [48]. Briefly, 17.5ml of diluted culture containing ~10^5 bacilli per ml of *M. bovis* BCG was transferred to 20X125mm tubes. Culture tubes were then sealed with rubber septa and gently stirred with the help of 8mm magnetic beads rotating at 100rpm on a magnetic stirring platform at 37^{o}C. After 8 days of incubation when the culture reached to non-replicating phase, compounds were added by using a Hamilton syringe with a 24-gauge needle and incubated for another 4 days. Culture samples were then spread on Dubos agar plates and colonies were enumerated on day 21 to examine the effect of compound on dormant stage.

4.3.3 Specificity of active molecules against *Mycobacterium tuberculosis, Mycobacterium smegmatis* and *E.coli*

M. smegmatis and *Escherichia coli* were used as representatives of non-pathogenic mycobacterial and non-mycobacterial strains respectively to examine the specific action of molecules by following methods described earlier [49]. Briefly, diluted culture containing 10^5cells/ml was transferred to 20mm x 125 mm tubes. Compounds were added at the time of inoculation at doses ranging from 0.1µg/ml to 10µg/ml in *M. smegmatis* and *E. coli* culture and growth was measured by reading the absorbance at 620nm after 6 and 72 hours for *E. coli* and *M. smegmatis* respectively.

4.3.4 Cytotoxicity of the active molecules

Toxicity of the active compounds was done by examining their effect on proliferation of monocyte cell line Thp-1 [49]. Approximately 10000 cells per ml were seeded in MEM medium containing 10% heat inactivated fetal bovine serum along with each 100µg/ml of streptomycin and ampicillin respectively. 100 µl of culture of was aseptically added to each well of sterile 96-well plate and inhibitor was added up to 50 X higher concentration of their MIC along with the inoculums in a dose dependent manner. 10µl of MTT dye solution (5mg/ml) was added after72 hours of incubation and was again incubated for 1 hour. Absorbance of the culture was read at 490nm within 4hrs of adding 200µl isopropanol to examine their effect on proliferation.

4.3.5 Inhibitory effect of actives against intracellular *Mycobacterium tuberculosis* inside THP-1 macrophage

Thp-1 was used to examine the inhibitory activity of the compounds against intracellular bacilli by following a method described earlier [50]. Briefly, Thp-1 cells (~$5x10^4$ cells/ml) were treated with 100nM of phorbol myristate acetate in a culture flask for 24 hrs to convert them into macrophages. These macrophages were then incubated for 12 hrs with *M. tuberculosis* $H_{37}Ra$ at MOI (multiplicity of infection) of 1: 100 for infection. Extracellular bacilli were removed by washing twice with PBS and then adding fresh medium to adhered cells. Compounds were then added to these infected macrophages at different concentrations. In order to check the effect of inhibitors on the growth of intracellular bacilli, compounds were added at 0hr and after 120hrs after infection, Unless mentioned otherwise, The effect of compound was monitored by determining the bacterial load within macrophage by lysing them with hypotonic buffer (10mM HEPES, 1.5mM $MgCl_2$ and 10mM KCl) at different time points and spreading 100 µl of the samples on Dubos agar plates to enumerate colonies after 21days.

4.3.6 Conjugation of Mts-Atf-Biotin linker with the Inhibitor

Mts-Atf-Biotin Label Transfer Reagents are tri-functional cross-linkers that contain a biotin, a sulfhydryl-reactive methanethiosulfonate (Mts) moiety and an efficient photoactivatable tetrafluorophenyl azide (Atf) moiety obtained from Thermo Scientific (Cat.No.33093). 10µl of the Biotin linker (5mg/ml) was mixed with 5µl (1mg/ml) of triozole inhibitor (2d) and kept under Ultra Violet (UV) radiation for 72 hrs. The reaction mixture was then analysed for product formation.

Matrix-Assisted Laser Desorption/ Ionization-Mass (MALDI-MS) analysis of Biotin-Inhibitor conjugate

Matrix Solution Preparation

All of the matrix solutions were saturated with 4HCCA and were prepared by adding 4HCCA (solid) to the organic solvent, followed by the addition of water and acid (as required). Each mixture was thoroughly vortexed and centrifuged, leaving a clear working matrix solution. The solubility of 4HCCA were dependent on the solvent composition, ranging from a low of 5 mM (water/methanol, 2:1 v/v), to an intermediate value of 29 mM (formic

acid/water/2-propanol, 1:3:2 v/v/v), to a high value of 74 mM (water/acetonitrile, 1:1 v/v) [51].

Sample-Matrix Crystallization Procedures

To prepare the sample-matrix solution, an aliquot (0.5 µL) of sample was combined with 15 µL of matrix solution in a small microcentrifuge tube (Tarson, India), Sample-matrix cocrystals were obtained by the following techniques [51].

Dried-Drop Method

An aliquot (0.5-1 µL) of the sample-matrix solution was deposited onto an aluminum 10-sample MALDI probe tip and allowed to air-dry (several minutes) at room temperatures, resulting in a uniform layer of fine granular matrix crystals. Cold water was placed over the crystals for 10 s to help remove involatile salts. The water was subsequently removed with vacuum suction [51].

4.3.7 Preparative HPLC purification of Biotin-Inhibitor conjugate

Chromatographic separation and analysis of product and each component of the reaction was done by using reversed- phase X bridge C_{18} (5µm, 46x250mm) column in preparative high-performance liquid chromatography (HPLC, SCL-10vp-SHIMADZU system controller). The mobile phase composition used was in, 'A' solution - water (60, 60, 45, 40) % and 'B' solution – acetonitrile (40, 40, 55, 60) %. The analysis was carried out in gradient mode as flow rate 1ml/min with column effluent being monitored at 254 nm wave length.

4.3.8 Binding of Biotin-Inhibitor conjugate with crude whole cell extract of *Mycobacterium tuberculosis*

The Biotin-Inhibitor conjugate was allowed to keep for solvent evaporation in speed vac for 30 min, then dissolved in 100µl DMSO and then added to the whole cell extract. For whole cell extract preparation, 5ml spheroplast solution (0.0006%) was added in 100ml log phase Mtb culture and allowed to incubate for 24hrs. This Biotin linker-inhibitor conjugate was also added in the control culture, which already treated with 2d inhibitor, both control and test samples were kept for 30min. in incubator shaker at 37ºC for binding to the target protein. Both the culture were sonicated in presence of protease cocktail and centrifuge the cell extract at 14,000rpm for 1 hr. to releasing the intracellular protein. The supernatant contain the target protein with Biotin-Inhibitor conjugate. Supernatant

was taken out and dialysed against PBS to remove the excess Biotin-Inhibitor conjugate and inhibitor molecules.

4.3.9 Purification of Biotin-Inhibitor conjugate tagged target Proteins by MagnaBind™ Streptavidin Beads

MagnaBind Streptavidin Beads are convenient for affinity purification or separation methods involving of biotin-labelled molecules obtained from Thermo Scientific (Cat no 21344). MagnaBind Streptavidin Beads were added in the supernatant for pulling down the target protein already bound with Biotin-Inhibitor conjugate by using an external magnetic field, the beads were washed with PBS for 2-3 times to remove the unwanted proteins. To release our target protein attached with Biotin-Inhibitor conjugate, we added an excess of free biotin molecule before dialysis against water. Dialyzed sample was collected and gone for protein precipitation.

4.3.10 Chloroform-Methanol Precipitation of Target proteins

For removal of salt and detergents chloroform-methanol method has been followed.100µl sample was added to 400µl methanol, vortex it well, then 100µl chloroform was added and vortex it. 300µl mili Q water was added and again vortex it.2mim @14,000g spin was given for 1 min. Removed the top aqueous layer (protein is between layers) then 400µl methanol was added and vortex it.2min @ 14,000g spin was given and removed the MeOH without disturbing the pellet. Sample was dried in speed-Vac.1X sample buffer was added for SDS PAGE [52].

4.3.11 Protein estimation

Protein quantification was done by following Bradford method (Bio-Rad Protein Assay; cat no. 500-0006) [53]. The measurements were carried out according to the manufactures instructions (Bio-Rad, Hercules, CA).

4.3.12 SDS-PAGE and Proteomic Analysis of Captured Proteins

Individual proteins in sample were separated by carrying out SDS-PAGE [54]. Briefly, protein samples were first mixed with 1X loading (sample) buffer containing 5% β-mercaptoethanol (Sigma, MO, USA). The samples were then incubated for 10 min at 80ºC. 30 µl samples was then loaded onto 12.5% Bis-Tris pre-cast polyacrylamide gels and the SDS-PAGE was carried out using mini-cell system (Invitrogen, CA, USA) [52]. After electrophoresis, the gel was subjected

to Comassie staining for overnight. Protein bands seen within the gel were cut properly and subjected to trypsin digestion, followed by peptide extraction and proteomic analysis in MALDI-ESI-MS.

4.3.13. LC-MS^E analysis

Two micro-liter digested peptides with final concentration of 100 ng/ µL was analyzed by nano LC-MS^E using nanoACQUITY online coupled to SYNAPT HDMS system (Waters Corporation, MA, USA) equipped with a nanolockspray ion source with flow rate of 300 nl/ min (external lockmass standard: Glu-fibrinopeptide). Peptide samples were injected online onto a 5 µm Symmetry C18 trapping column (180 µm x 2 cm length) at a flow rate of 15 µl/ min. Peptides were separated by in-line gradient elution onto BEH (Bridged Ethyl Hybrid) 130 C18 1.7 µM x 75 µM x 150 mm nanoACQUITY analytical column, at a flow rate of 300 nl/ min using a linear gradient from 5 to 40% B over 35 min (A. 0.1% formic acid in water, B. 0.1% formic acid in acetonitrile) shown in Figure 2a and 2b. Acquisition was performed in positive V mode in a mass range of 50-1990 m/z with a scan time of 1 second with alternating low (5 eV) and high (15-40 eV) collision energy. MS^E data were processed with ProteinLynx Global Server (PLGS version 2.4. Waters Corporation, MA, USA). The processed data were allowed to search against human subset of UniProt database containing all 44,987 protein entries for protein identification as described by Silva et al [55].

4.3.14. ATPase activity assay

The ATPase activity of the purified groEL 1 and groEL 2 (gifted by Dr. shekhar mande) was quantified with a colorimetric assay performed as described previously [58]. Briefly, 25 µl of the reaction buffer containing 50mM Tris-HCL (Ph 8.0), 10Mm KCL, 10Mm $Mgcl_2$ and 2.5µM of each groEL was incubated with 1mM ATP at 37ºC for 20min. Enzymatic reactions were terminated by the addition of 100 µl of an acidic solution of malachite green. The amount of inorganic phosphate liberated was measured at 655nm. In control, reaction was performed in absence of ATP and groEL proteins. The estimation of liberated phosphate was done from the standard curve generated by using monobasic potassium phosphate with each experiment.

4.3.15 Prevention of the aggregation of citrate synthase by chaperonins

Pig heart citrate synthase aggregation was performed as reported previously [59]. Briefly, 0.15 µg/ml citrate synthase was incubated at 43°C in the presence or absence of equimolar oligomer ratios of different groEL variants in 40 mM HEPES–KOH buffer (pH 7.5). The ability of the said chaperones to prevent the aggregation of citrate synthase was monitored for 20 min on a LS55 spectro-fluorimeter with emission and excitation wavelengths set at 465 nm and corresponding band passes set at 3.0 nm. The temperature of the sample was maintained with a Julabo circulating water bath (Thermo NESLAB, USA).

4.4. Disscussion

Antifungal, antiviral and plant growth regulatory activities of triazoles were known for long time but their antimycobacterial potential has gained importance in recent years [28]. Fluconazole and tebuconazole are the only triazoles known for their antimycobacterial activity without having higher MIC values with no specificity [60]. Moreover, they are not effective against dormant tubercle bacilli. Here, in this communication alkyl substituted 1, 2, 4-triazolethiols and their hybrids have been synthesized and tested for the antituberculous activity. Out of 54 compounds selected for primary screen, 17 hits were identified based on their effect against aerobic replicating stage of *M. bovis* BCG (Table 4.1). After considering their dose dependent effect, only 3 compounds were identified with significant effect on growing bacilli even at 1.0µg/ml concentration (Table 4.2). Most importantly, these hits were also effective against dormant stage of the bacilli (Table 4.2 & Fig 4.3). Although propargyl or allyl groups were present in other triazole structures, they could not achieve inhibition against bacilli, which pointed the involvement of different substituent present at C-5 position for killing action. Although different groups were tried at C-5 position, only p-nitrophenyl (2d) was found to show desired activity. Sulfone derivative (11d) of this p-nitrophenyl compound was equally potent against actively growing *M. bovis* BCG which indicated that oxidation of sulphur did not produce any noticeable impact on its biological activity (Table 4.2). 2d, 8f and 11d were found inactive against both *M. smegmatis* and *E. coli* which indicated their specific effect against tuberculous bacilli (Table 4.2). The inhibitory effect of these compounds against both *M. bovis* BCG and Mtb indicated that an inductive effect of the group attached to the *para* position of the phenyl ring connected to C-5 position of 1,2,4 triazolethiol is very important in bringing the potent inhibitory effect against the bacilli. In the other compound (8f), the replacement of propargyl moiety by the allyl group was able to retain the extent of inhibition against the bacilli. These three molecules are structurally novel compared to known anti-tubercular agents. In vitro studies on Thp-1 cell line also indicated the compounds as nontoxic in nature. The effect of these three compounds on both the stages of MBC group of organisms has apparently raised question against its acting as 14α-demethylase inhibitor. These compounds were not active against *M. smegmatis* which also

possesses the target enzyme representing cytochrome P450 class of proteins [61]. During analysis of structure activity relationship between these 54 compounds, it was also observed that structures with thiopropargyl group at C-3 position of 1, 2, 4-triazole ring were very active against mycobacterial survival under aerobically growing stage. Oxidation of this S-propargyl compound did not perturb the inhibitory action of the molecule. SAR study also indicated that allylation at 1 and 2 positions were inversely related to the potency of the molecule. A phenyl substitution with an electron withdrawing group at its para position was required at C-5 position in the triazole ring. Replacing $–NO_2$ group by Cl- with simultaneous change of propargyl to allyl group attached to sulphur at position C-3 in1, 2, 4-triazole ring has increased the potency of the molecule by 10 fold as anti-tuberculous agent. Eventually the invention identified three compounds representing basically the same scaffold, which could be used as novel leads for further testing against tuberculosis infection in animal model.

Earlier studies using well known anti-fungal triazole drugs had clearly established that 14-α-demethylase belonging to the Cytochrome P-450 class of proteins are the targets in mycobacterial [62]. Interestingly, our lead inhibitors were not effective on *M. smegmatis* even though *M. smegmatis* has 51 copies of those proteins whereas in Mtb and *M.bovis BCG* only 22 copies are present [62]. Further investigation using pool down technique assisted by HPLC and MALDI-LC-MS approach identified groEL 2, a chaperonine as target of these inhibitors in Mtb (Fig. 4.5-4.7). Inhibitory effect of these leads on ATPase activity of groEL 2 conclusively proved that the potential anti-tubercular action of these triazolethiols was achieved through their binding with chaperonine 2 proteins (Table 4.3). Altogether, this study has clearly established the identification of a novel scaffold in 1, 2, 4-triazolethol as potent anti-tubercular specific inhibitor with its intracellular target protein as groEL2 which could provide an impetus into Anti-TB drug discovery.

4.5. References

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