BIOCHEMICAL STUDIES ON SOME ASPECTS OF NITROGEN METABOLISM IN *MYCOBACTERIUM SP.*

Thesis submitted to University of Pune
For the degree of

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

By

ARSHAD KHAN

Research Supervisor
Dr. Dhiman Sarkar

Combichem Bioresource Center
Organic Chemistry Division
National Chemical Laboratory
Pune – 411008
India

October 2008

Dedications

This thesis is dedicated to five people who I couldn't have survived through my stay in Pune without. First, to my father Inayat Ali and mother Jaitoon Bano for never doubting in my ability to achieve a goal and being very supportive of every endeavor I have ventured to take. Second, to my brothers Hanif Khan, Aslam and Iliyas who instilled in me the desire to learn and confidence to achieve.

"My mind is still tender, my thoughts are still young
My dreams are still soar, there are battles to be won"

ACKNOWLEDGEMENTS

Obtaining a PhD is a group effort. While the work presented in this thesis is my own, it would never have been possible without the help of numerous people who have given me immense support in many ways during my long and arduous five years of study. I am deeply indebted to my mentor, Dr. Dhiman Sarkar who has provided me with many gifts, the greatest being a completely different mindset from the one I had at the beginning of my doctoral studies. It was his enthusiasm on mycobacterial research which attracted me to join his laboratory. The ability to think critically, to ask the right questions, and to resolve the problems through experiments are the traits that I didn't possess before meeting him. I thank him for allowing me freedom and resources to pursue different lines of research. I will miss picking his brain for answers to my questions and I just hope I can cultivate a fraction of knowledge that he possesses. Extending this gift from my PhD work to the way I go through life has been an added bonus. I wish Mr. Sarkar a continued success in their lab, which he is sure to achieve.

Another important influence, whose guidance has been wonderful, is Dr. K. N. Ganesh. I cannot thank him enough for all the years of encouragement. He has had a continuous faith in me, pushing me towards academic success, and has been the voice of reason and support during my entire PhD tenure. I owe a huge debt of gratitude to Director NCL, Dr. S. Sivaram and our HOD, Dr. Ganesh Pandey for excellent leadership and an inspirational working environment they provided in the institute. As no one person contains complete wisdom, I have been blessed to be a part of NCL scientific community for which a high level of excellence and commitment to research has been established far and beyond most other institutions.

Dr. Sunita Deshpande, Dr. Shailaja Maybhate and Dr. Anjali Likhite were instrumental for synthesizing and supplying triazoles, analysis of the results and thoughtful discussions for chapter 4. I extend my special thanks to Dr. Vinita Panchanadikar for their invaluable comments and unconditional support. I am also thankful for the guidance and encouragement I received from my PhD research committee members Dr. J. K. Pal, Dr. M. I. Khan and Dr. Vidya Gupta.

Special thanks goes to my lab members Upasana, Sampa, Abhishek, Javed and Shamim for unreserved support I have received countless time from them. They were always helped me during my difficult times and were incredibly supportive. I would like to show sincere gratitude to my four amazing friends Sarvesh, Sampa, Dilip and Sunil for their support, encouragement,

patience and advice during this challenging period in my life. Without them I am not sure I would have made it this far. I owe you all dearly and wish success in your future endeavors. I also owe a big thanks to my friends Ajit, Manoj, Amit, Sapna, Deepak, Minakshi, Nargis who were not here in Pune but always with me silently.

Finally none of this would have been possible were it not for the lifelong love and encouragement of my parents and family. What I am today I owe to them.

Arshad Khan

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "**Biochemical studies on some aspects of nitrogen metabolism in *Mycobacterium sp.***" submitted by **Arshad Khan** 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)

Dr. K. N. Ganesh
(Co- Guide)

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled " **Biochemical studies on some aspects of nitrogen metabolism in *Mycobacterium sp.***", 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) and Dr. K. N. Ganesh (Co-guide). 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.

Arshad Khan

(Research Scholar)

ABSTRACT

The inexorable 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. This thesis describes three approaches for the discovery of new novel therapeutic agents against the disease. First approach is the evaluation and characterization of the biochemical role of enzymes related to nitrate metabolism of Mycobacteria during survival in its dormant or latent stage. The second approach is development of a simple screening assay, which could be used to pick up dormant stage specific inhibitors at large scale. The third approach is screening of diverse chemical library to search biologically active molecules against dormant stage of the bacilli.

A respiratory type of nitrate reductase, NarGHJI was identified as a first report in *Mycobacterium smegmatis* during this study. This enzyme's activity was found to be induced during hypoxic shiftdown of the culture to dormant stage in Wayne's in vitro model. More significantly blockage of the enzyme's function by specific inhibitors led to a steep reduction in viability of the bacilli during hypoxic stage survival in Wayne's in vitro model, which indicated this enzyme as a potential drug target for latent stage. Furthermore, a complete pathway of the assimilation of nitrate was found to be present in *M. smegmatis, M. bovis* BCG and *M. tuberculosis*, which also played significant role during its hypoxic stage persistence. Nitrate reductase, NarGHJI also played a significant role during survival of *M. tuberculosis* within the THP- macrophage environment which indicated the generation of microaerobic environment in the host cells which harbors the pathogen, similar to Wayne's hypoxic model. Interestingly the essentiality of nitrate reductase, NarGHJI was found irrespective of presence or absence of nitrate in the medium during this intracellular survival of the organism, which indicated the production of nitrate and/ or nitrite in the activated macrophages.

We could also use the whole cell activity of nitrate reductase as a reporter system of dormant stage to develop a simple, rapid, and robust cell based high throughput assay for facilitating dormant stage antitubercular screening. An in house compound library of more than 2000 compounds comprising of synthetic and natural products was screened using this high throughput assay. 2-nitroimidazole was discovered from this screening as potential lead molecule having significant antitubercular activity at a very low MIC

against the active stage of the bacilli with promising intracellular efficacy. Three compounds from 1,2,4-triazolethiol class of compounds were discovered with significant antitubercular activity against hypoxic dormant stage of *M. tuberculosis* along with their intracellular efficacy.

The enzyme NarGHJI was also isolated and purified to homogeneity from *M. tuberculosis* H37Ra. Characterization of the certain biochemical properties was also done from the pure NarGHJI in order to better understand the mechanism of the enzyme as well as to use the enzyme for development of enzyme based screening assay in due course to search nitrate reductase specific inhibitors.

TABLE OF COTENTS

ABBREVIATIONS

ADAS:	Active Dormant Antitubercular Screening
ADP:	Adenosine diphosphate
AFB:	Acid Fast Bacilli
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
E. coli:	*Escherichia coli*
EMB:	Ethambutol
ESAT-6:	Early Secretary Antigen Target-6
ETH:	Ethionamide
FAD:	flavin adenine dinucleotide
FDA:	Food and Drug Administration
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
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
MIC:	Minimum Inhibitory Concentration
M. smegmatis:	*Mycobacterium smegmatis*
M. tuberculosis:	*Mycobacterium tuberculosis*
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
NO_3:	Nitrate
NO_2:	Nitrite
NR:	Nitrate Reductase
PAS:	Para amino salicylic acid

PCR:	Polymerase Chain Reaction
PK/PD:	Pharmacokinetics Pharmacodynamics
PPD:	Purified Protein Derivative
PZA:	Pyrazinamide
RIF:	Rifampicin
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
WHO:	World Health Organization
XDR:	Extremely Drug resistant

Publications from thesis

1. **Khan A, Sarkar D.** 2006. Identification of a respiratory-type nitrate reductase and its role for survival of *Mycobacterium smegmatis* in Wayne model. *Microb. Pathog.* **41:** 90-95.
2. **Khan A, Akhtar S, Ahmad JN, Sarkar D.** 2008 Presence of a functional nitrate assimilation pathway in *Mycobacterium smegmatis*. *Microb. Pathog.* **44:** 71-77.
3. **Khan A, Sarkar D.** 2008. A simple whole cell based high throughput screening protocol using *Mycobacterium bovis* BCG for inhibitors against dormant and active tubercle bacilli. *J. Microbiol. Methods* **73:** 62-68.
4. **Khan A, Sarkar S, Sarkar D. 2008.** Bactericidal activity of 2-nitroimidazole against active replicating stage of *Mycobacterium bovis* BCG and *M. tuberculosis* with intracellular efficacy in THP-1 macrophage. *Int. J. Antimicrob. Agents* **32:** 40-45.
5. **Khan A, Sarkar S, Sarkar D. 2008.** NarGHJI dependent survival of *Mycobacterium tuberculosis* in macrophages indicates intracellular microenvironment similar to Wayne's hypoxia model. *Communicated.*
6. **Khan A, Chaudhary PM, Sarkar S, Chavan SR, Likhite AP, Maybhate SP, Deshpande SR, Sarkar D.** 2008. Identification of potent 1,2,4-triazolethiols killing non-replicating *Mycobacterium tuberculosis* and their structure activity relationship. *Communicated.*

CHAPTER 1

Overview of Tuberculosis and Strategies for Combating with a Persistent Organism

1.1. Classification and characteristics of *Mycobacterium tuberculosis*

Kingdom: Bacteria
Phylum: Actinobacteria
Class: Actinobacteridae
Order: Actinomycetales
Suborder: Corynebacterineae
Family: Mycobacteriaceae
Genus: *Mycobacterium*
Species: *Mycobacterium tuberculosis*

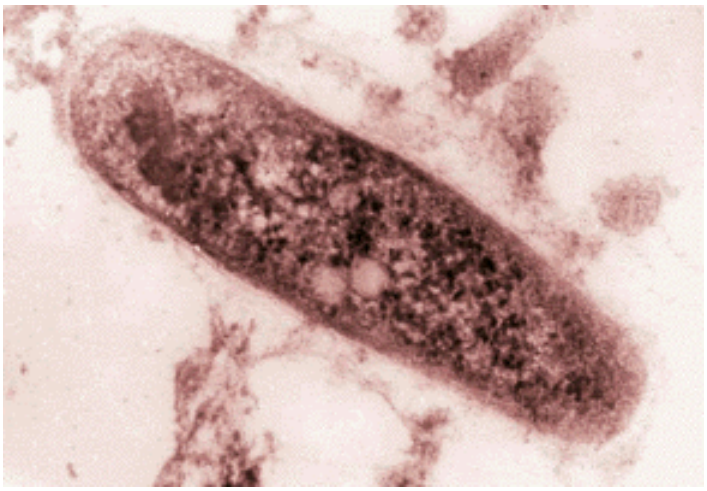


Fig. 1.1 Thin section transmission electron micrograph of *M. tuberculosis* (extracted from www.wadsworth.org/databank/mycotubr.htm).

Mycobacteria are one of the most clinically important and extensively studied of bacterial taxa. They bear a taxonomical relationship to actinomycetes such as the antibiotic producing *Streptomycetes* because of the high guanine cytosine (GC) content in their DNA (1). Analysis of 16S rRNA, highly conserved ribosomal (r) RNA also defines similarities to other families such as Corynebacteriaceae and Nocardiaceae establishing the CMN branch within the Actinomycete genera (2,3). Within this group of organisms many structural and biosynthetically complex systems are related. Mycobacteria bear several notable distinctions, however: (i) resistance to decolorization by acid alcohol following basic fuchsin staining ("acid fast" bacilli) (4); (ii) production of two different siderophores, iron scavangers (exochelins and mycobactins) (5,6);

(iii) complexity of mycobacterial mycolates (cell wall components) (3,7); (iv) extremely slow division compared to other bacteria (2).

Mycobacteria are typically rod-shaped aerobic bacteria as illustrated (Fig.1.1). However, variable morphology can be observed when grown on solid media and some species exist as shorter cocci-bacilli or curved rods on artificial media (8). Although mycobacteria are considered obligate aerobes, metabolic pathways exist to support adaptation to environments with reduced to no oxygen for extended periods of time while maintaining viability (9,10). The ability to adapt extends to numerous environmental stresses, a likely survival tool for their natural habitat mainly soil, water and host cells (in the case of facultative pathogenic species). There are more than 70 distinct *Mycobacterium* species (2). With the exception of *M. leprae*, which can not be cultivated in vitro, mycobacteria are assigned to two groups based primarily on the relative growth rates of the individual species. Few phenotypic properties have been found to distinguish between fast and slow growing mycobacteria but there are differences that are distinctive and have been included in the classification process; neutral red staining (11), composition of lipid structures (7) and sensitivity to high salt concentrations (2). Additionally, the 16S rRNA studies reveal an extended helix at position 451-482 for most slow-growers with the exception of *M. genavense, M. intermedium, M. interjectum, M. simiae* and *M. triviale* (2). In evolutionary terms, phylogenetic analysis seems to suggest that the rapidly growing organisms are older than their slow growing relatives. Several causes have been postulated to explain the growth rate differences. These include differences in the number of rRNA (*rrn*) operons as well as the orientation of genes with respect to the direction of replication. Ribosomes are thought to function at a constant maximum efficiency. Therefore a faster growth rate is equated with a higher ribosome concentration; the number of ribosomes present within a cell is based on the production of rRNA, which, in turn depends on the number of *rrn* operons. Likewise, genes transcribed in the same direction as the replication fork are considered to be expressed more efficiently. Slow growing mycobacteria such as *M. leprae* and *M. tuberculosis* have a single *rrn* operon while rapid growers such as *M. phlei* and *M. smegmatis* have two *rrn* operons (2,12). The amount contrasts to several *rrn* operons found in species such as *Escherichia coli* (7 *rrn* t_d <30 min) (13) or the extremely rapidly growing *Vibrio natriegens* (~13 *rrn* t_d <10 min) (14). Investigations into the macromolecular compositions of *M. bovis* BCG (Bacillus Calmette Guerin) using a chemostat model for mycobacterial growth determined an 82% decrease in the number of ribosomes per cell between *M. bovis* BCG with a t_d of 23 h versus *M. bovis* BCG with a t_d of 69 h supporting a connection between the number of ribosomes and growth rate (15). The ribosomes present in slow growing mycobacteria also appear to function at only 12% of maximal activity and may involve additional regulatory factors (13).

Matsumoto and colleagues discovered a novel mycobacterial DNA binding protein, 'MDPI' localized to the nucleoid, 50S ribosomal subunit and cell surfaces, that was capable of transforming rapidly growing bacteria to slow growing (16). Cell proliferation also requires the uptake of essential nutrients and energy consumption. Another possible explanation for the differences in growth rates could be differences in energy metabolism or transport process for oxygen and essential nutrients across the cellular membrane. Recently, Mailaender and coworkers demonstrated that the uptake of nutrients such as glucose in mycobacteria is 1430-fold slower than other fast-growing bacteria (17). In their work they expressed MspA, the main porin of *M. smegmatis*, in *M. bovis* BCG that led to an increased uptake of nutrients and accelerated the growth rate by 7%.

A unique and complex cell envelope is one of the most characteristic properties of *M. tuberculosis* (Fig. 1.2). The envelope contains abundance of lipids with diversity in lipid structures and exhibits a reduced permeability. This complex wall is thought to confer protection against toxic environments within the host and effectively limit the influx of antibiotics. Almost 60% of the weight of this envelope is lipids (18). *M. tuberculosis* dedicates approximately 250 genes towards lipid biosynthesis as opposed to only about 50 in *Escherichia coli* (19). The cell envelope of *M. tuberculosis* can be divided into three main components (Fig. 1.2); the plasma membrane, the mycolic acid-arabinogalactan-peptidoglycan complex and the surface capsule. The plasma membrane (PM) forms the innermost layer of this cell envelope and is a typical lipid-bilayer, structurally and functionally similar to the PM of other eubacteria. The electron transport chain resides in this layer. External to the PM is the multiple layers of peptidoglycan (PG). Mycobacterial PG is unusual compared to PG from other eubacteria in two main aspects: the location and number of cross-links between its layer and the presence of N-glycollyl muramic acid in place of N-acetylmuramic acid. The tetra-peptide side chain of most eubacterial PG consists of L-alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanine with cross-links occurring between *meso*-diaminopimalic acid (*meso*-DAP) residues of one chain and D-glutamic acid residues of the other. However, in mycobacteria additional cross-links occur such as those between two *meso*-DAP residues and those between *meso*-DAP and D-alanine (20). The PG layer is covalently linked to a layer of sugar residues known as arabinogalactan (AG). AG is a polymer of furanose forms of D-arabinose and D-galactose producing homoarabinan and homogalactan chain respectively that are covalently attached to each other to form the AG unit. AG is a branched structure that is covalently linked at its proximal end via the galactans to N-glycollylmuramic acid residues of the PG layer.

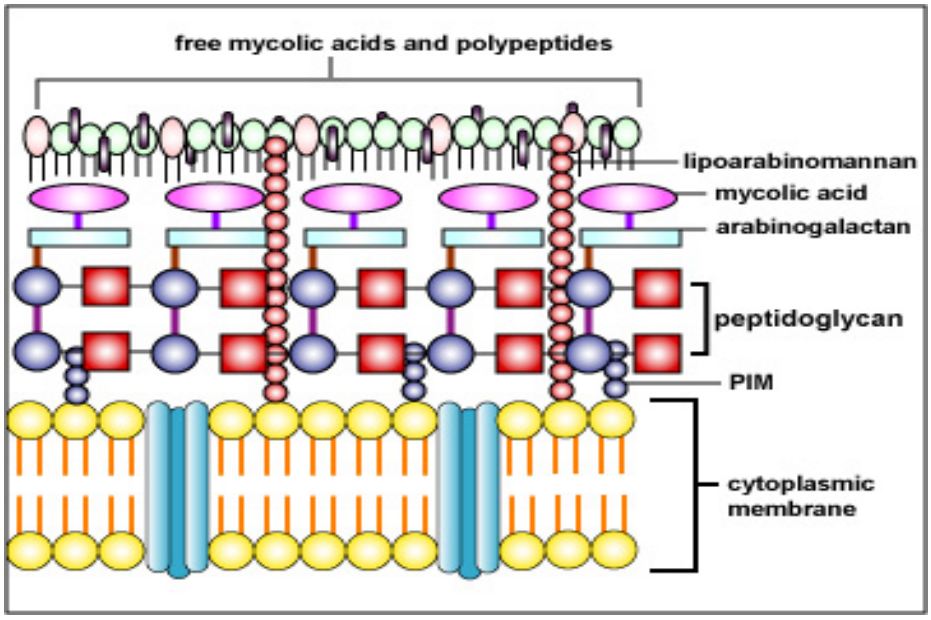


Fig. 1.2 Overview of the structure of the cell wall of *M. tuberculosis* (Extracted from Nature Reviews Microbiology **5**, 883-891).

At the distal end, AG is attached to a special class of lipids found in mycobacteria known as Mycolic acid (MA) through ester-linkage with the arabinans. Mycolic acids are long, branched 2-alkyl-3-hydroxy fatty acids that may be as many as 90 carbon atoms in length (21,22). The mycolic acid chains are perpendicular to the bacterial cell surface and form a monolayer. MAs are often species-specific and are thought to be responsible for the impermeability of mycobacterial cell wall (22). Special classes of MAs are found in *M. tuberculosis* include tehalose-6,6'-dimycolate (TDM) that is commonly known as 'cord factor', sulpholipids (SLs), phthicerol dimycocerosates (PDIMs), and phenolic glycolipids (PGLs). TDM is a potent modulator of the immune response and is also thought to be responsible for the characteristic cording phenotype of *M. tuberculosis* (23). The PG-AG-MA complex forms the core cell wall.

The cell envelope also contains a variety of loosely attached polar lipids and lipoglycans that are unique to mycobacteria. These are thought to be interspersed between the MAs. While the types of lipid present vary from one mycobacterial species to another, some commonly occurring ones include phosphatidylinositol mannosides (PIMs), lipoarabinomannans (LAMs) (20). PIM is the precursor of LM, which in turn is the precursor of LAM (23). PIMs have been shown to have a role in natural killer cell recruitment as well as adhesion to non-phagocytic host cells (24). The type of LAM present in the species correlates with its pathogenic ability. For example, Mannose caps, present on LAM (ManLAM) in *M. tuberculosis* make ManLAM a much stronger

immunosuppressor as compared to PILAM of *M. smegmatis*, which contains phosphatidylinositol caps on the LAM (25).

The outermost layer of the mycobacterial cell envelope is considered to be a loosely attached capsule composed primarily of glucans, arabinomannans and mannans. Mycobacterial glucan is a branched polymer of D-glucosyl residues with branch points consisting of monoglucosyl to pentaglucosyl substitutions. The arabinomannan consists of a heteropolymer of D-arabino-D-mannan. Structurally the arabinan found in the mycobacterial capsule is similar to the arabinan found in the arabinogalactan portion of the cell wall. The capsular mannan is a branched polymer of D-mannosyl subunits with branch points consisting of single D-mannosyl units (20). Lipids and proteins are also known to ornament the capsule, albeit in smaller amounts. Capsular proteins fall in two categories: the 'secreted' proteins and 'resident' capsular proteins. Both types of protein are found to be present in culture filtrate indicating that the 'resident' capsular proteins are likely shed from the cell surface into the culture medium (20). Lipids comprise only 2-3% of the capsular material. Since mycobacteria are cultured in media containing dispersing agents, such as Tween-80, this capsule is frequently lost. Capsular lipids are most commonly glycopeptidolipids (GPLs).

1.2. History and epidemiology of tuberculosis

Consumption, King's Evil, Lupus Vulgaris, and Phthisis are some of the more colorful names for tuberculosis (TB) that have been used in the last several centuries. The disease traces its origins to antiquity when man first domesticated and *M. tuberculosis* evolved from *M. bovis* (26). The history suggests that tuberculosis was a rare disease among early nomadic human populations but became endemic among settled populations 15000 years ago (27). Mummified human remains from Egypt show evidences of TB, including characteristics skeletal deformities and fibrotic lung lesions containing *M. tuberculosis* DNA (28). The rise of the cities with dense populations provided ideal conditions for transmission of the air borne pathogen. Society flourished and trade routes became a mechanism for the spread of disease into new areas.

The TB epidemic reached a peak during the 19th century when it became the leading cause of death in the western world (29). The overcrowded unsanitary cities of the industrial revolution were devastated, especially within the poorer sections of the cities with malnourished populations. By this time the disease was pervasive throughout society and was no longer a disregarded scourge of the poor. In artistic circles TB became a romantically tragic disease, and it was the subject of works of art, literature and music. By the end of the 19th century the incidence

of tuberculosis was steadily declining. Many reasons are cited, including the Pasteur's discovery of microbes as a cause of disease, but perhaps most important was an improvement in the standard of living in many countries (30).

Robert Koch famously identified *Mycobacterium tuberculosis* as the organism that causes TB in 1882. The world press hailed an imminent cure, but an effective treatment remained elusive for over 70 years. While the disease could be diagnosed with accuracy by 1905, largely due to Wilhelm Konrad Rontgen's discovery of X-rays in 1895 and Koch's stain for microscopy, nothing could be done to cure the disease. The standard care of TB was quarantine with a sanitarium pneumothorax, or "lung collapse" therapy twice per week (31). The first effective treatment of TB was discovered by Selman Waxman at Rutgers University in 1939. Waxman observed that certain soil dwelling organisms had an inhibitory effect on mycobacterial growth and coined the term "antibiotic" (32). His laboratory subsequently isolated streptomycin from *Streptomyces griseus*, and Waxman was awarded the 1945 Nobel Prize in medicine. On November 20, 1944 the first human subject received streptomycin: Patricia, a young Minnesotan woman who was near death from progressive pulmonary tuberculosis. Patricia dramatically survived not only to leave the hospital, but to marry, raise 3 children and have an active life (33). Other compounds rapidly followed the discovery of streptomycin, including p-amino salicylic acid (1949), isoniazid (1952), pyrazinamide (1954), cycloserine (1955), ethambutol (1962) and rifampin (1963) (34). The advent of multidrug therapy dropped the rate of TB infection precipitously in the latter half of the 20th century.

The global distribution of TB cases at present is skewed heavily towards low-income and emerging economies (Fig.1.3) (35). The highest prevalence of cases is in Asia, where China, India, Bangladesh, Indonesia, and Pakistan collectively make up over 50% of the global burden. Africa, and more specifically sub-Saharan Africa, has the highest incidence rate of TB, with approximately 83 and 290 per 100,000, respectively (Fig 1.4). TB cases occur predominantly (approximately 6 million of the 8 million) in the economically most productive 15- to 49-year-old age group.

In 2006, around 14.6 million people had active TB disease with 9 million new cases (Table 1.1). The annual incidence rate varies from 356 per 100,000 in Africa to 41 per 100,000 in the Americas. TB has also now become the world's greatest infectious killer of women of reproductive age and the leading cause of death among people with HIV/AIDS.

The emergence of HIV infection, especially within incarcerated populations such as prisons, hospitals and homeless shelters lead to outbreak of infection (36). The rise in HIV infections and the neglect of TB control programs have enabled a resurgence of tuberculosis. The emergence of

drug-resistant strains has also contributed to this new epidemic with, from 2000 to 2004, 20% of TB cases being resistant to standard treatments and 2% resistant to second-line drugs. The rate at which new TB cases occur varies widely, even in neighboring countries, apparently because of differences in health care systems.

The incidence of TB also varies with age. In Africa, TB primarily affects adolescents and young adults. However, in countries where TB has gone from high to low incidence, such as the United States, TB is mainly a disease of older people. There are some other known factors that make people more susceptible to TB infection. Smoking more than 20 cigarettes a day also increases the risk of TB by two to four times (37). Diabetes mellitus is also an important risk factor that is growing in importance in developing countries (38).

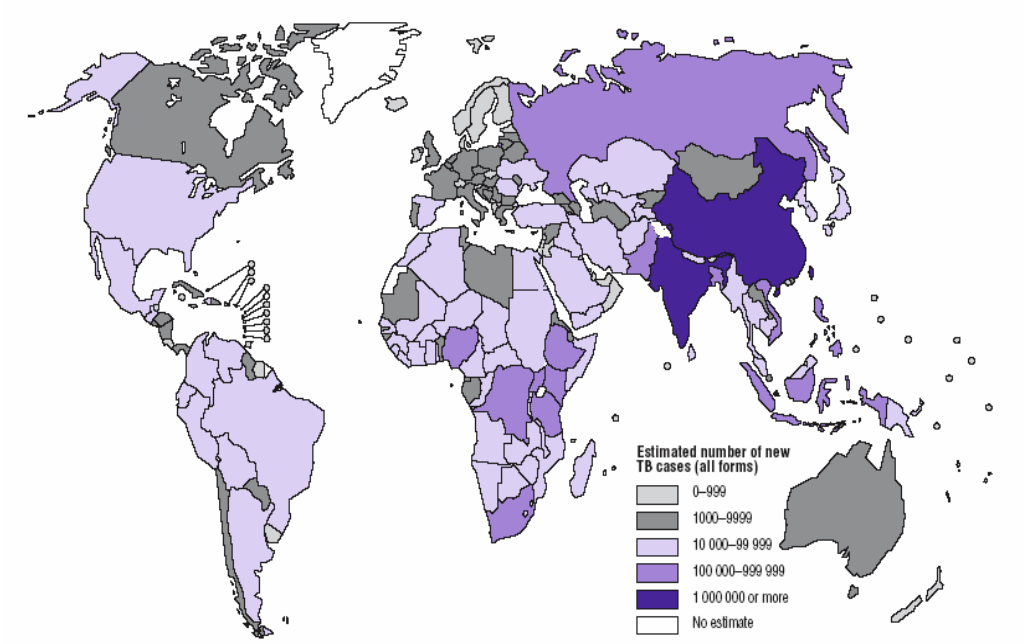


Fig. 1.3 Estimated number of TB cases by country 2006 (Extracted from WHO report 2008, Global Tuberculosis Control: Surveillance, Planning and Financing).

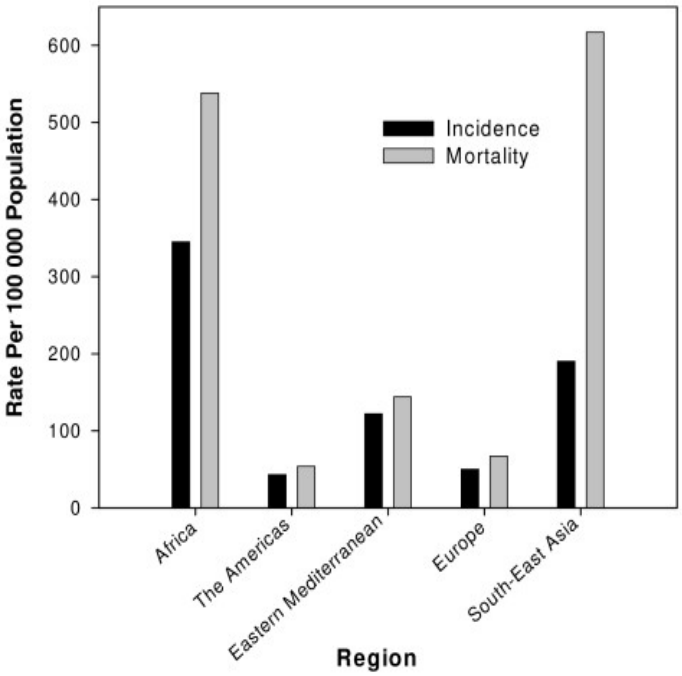


Fig. 1.4 Incidence and mortality rate in various continental TB zones (Extracted from WHO report 2008, Global Tuberculosis Control: Surveillance, planning and financing).

Table 1.1 Summary of estimated epidemiological burden of TB, 2006

(Extracted from WHO report 2008, Global Tuberculosis Control: Surveillance, planning and financing).

		INCIDENCE[a]				PREVALENCE ALL FORMS		MORTALITY ALL FORMS		HIV PREV. IN INCIDENT TB CASES[b]
		ALL FORMS		SMEAR-POSITIVE						
	POPULATION 1000s	NUMBER 1000s	PER 100 000 POP PER YEAR	NUMBER 1000s	PER 100 000 POP PER YEAR	NUMBER 1000s	PER 100 000 POP	NUMBER 1000s	PER 100 000 POP PER YEAR	%
1 India	1 151 751	1 933	168	867	75	3 445	299	325	28	1.2
2 China	1 320 864	1 311	99	590	45	2 658	201	201	15	0.3
3 Indonesia	228 864	534	234	240	105	578	253	88	38	0.6
4 South Africa	48 282	454	940	184	382	482	998	105	218	44
5 Nigeria	144 720	450	311	198	137	890	615	117	81	9.6
6 Bangladesh	155 991	351	225	158	101	610	391	70	45	0.0
7 Ethiopia	81 021	306	378	136	168	520	641	68	83	6.3
8 Pakistan	160 943	292	181	131	82	423	263	55	34	0.3
9 Philippines	86 264	248	287	111	129	373	432	39	45	0.1
10 DR Congo	60 644	237	392	105	173	391	645	51	84	9.2
11 Russian Federation	143 221	153	107	68	48	179	125	24	17	3.8
12 Viet Nam	86 206	149	173	66	77	194	225	20	23	5.0
13 Kenya	36 553	141	384	56	153	122	334	26	72	52
14 UR Tanzania	39 459	123	312	53	135	181	459	26	66	18
15 Uganda	29 899	106	355	46	154	168	561	25	84	16
16 Brazil	189 323	94	50	59	31	104	55	7.6	4.0	12
17 Mozambique	20 971	93	443	39	186	131	624	24	117	30
18 Thailand	63 444	90	142	40	62	125	197	13	20	11
19 Myanmar	48 379	83	171	37	76	82	169	6.1	13	2.6
20 Zimbabwe	13 228	74	557	30	227	79	597	17	131	43
21 Cambodia	14 197	71	500	31	220	94	665	13	92	9.6
22 Afghanistan	26 088	42	161	19	73	60	231	8.3	32	0.0
High-burden countries	**4 150 313**	**7 334**	**177**	**3 265**	**79**	**11 889**	**286**	**1 330**	**32**	**11**
AFR	773 792	2 808	363	1 203	155	4 234	547	639	83	22
AMR	899 388	331	37	165	18	398	44	41	4.5	6.4
EMR	544 173	570	105	256	47	826	152	108	20	1.1
EUR	887 455	433	49	194	22	478	54	62	7.0	3.0
SEAR	1 721 049	3 100	180	1 391	81	4 975	289	515	30	1.3
WPR	1 764 231	1 915	109	860	49	3 513	199	291	17	1.2
Global	**6 590 088**	**9 157**	**139**	**4 068**	**62**	**14 424**	**219**	**1 656**	**25**	**7.7**

[a] All estimates include TB in HIV patients.

[b] Prevalence of HIV in incident TB cases of all ages.

1.3. Pathogenesis of *Mycobacterium tuberculosis*

TB is the example of the interaction between an exogenous agent and the host immune defense system. It may be estimated that while 1900 million people throughout the world are infected with *M. tuberculosis* (representing an enormous reservoir that contributes to perpetuation of this disease), only 8 million actually suffer from the disease each year. This situation is explained by the fact that the human defense mechanism is highly effective and can overcome the disease in most cases.

Primary infection. Primary infection refers to the general biological phenomena that take place when an individual comes into contact with the tubercle bacillus for the first time (Fig. 1.5) (39). During primary infection, 95% of all affected individuals remain asymptomatic or present

with only minimal clinical manifestations similar to those seen with the common cold. Only 5% develop manifest disease. Tuberculin skin test conversion usually occurs in these individuals. This phenomenon typically takes place in childhood, as a result of which primary infection is often associated with childhood TB. Nevertheless, primary infection can occur at any time in life, and is the result of inhalation of bacteria-loaded particles by an individual who has not been previously exposed to the microorganism. Because of the weight of these particles, some of them tend to sediment and are therefore not infective. Other airborne particles, known as Pflüger droplets, with a diameter of 5 to 10μm, either sediment or are cleared by the defense mechanisms of the airways. However, upon condensation of these droplet nuclei and the loss of part of their water content, smaller particles measuring 1 to 5μm are formed, containing approximately three tubercle bacilli each; these droplets are infective. The defence mechanisms of the upper airways (i.e., cough reflex, mucociliary system) non-specifically prevent particles measuring over 5μm from reaching the lung parenchyma. Under infective conditions, however, some particles measuring 1 to 5μm reach the distal airways and are deposited in the alveoli. It is believed that at least 10 to 200 of such microdroplets must reach the alveoli in order for infection to take place. The preferential zone of arrival is the best-ventilated part of the lungs, corresponding to the subpleural region of the inferior lobes. Upon arrival in the alveolar region, the bacteria encounter three types of cells that potentially oppose infection: the alveolar macrophages within the alveolar lumen, the natural killer cells, and the T lymphocytes.

In humans, alveolar macrophages are considered to be the key type of cells involved in the initial interaction with the tubercle bacillus (40). These cells originate in the bone marrow and reach the alveoli after coming into contact with the systemic circulation. As a result, different systemic and local factors can influence their functional characteristics. For example, HIV is able to infect these alveolar macrophages and thereby increase host sensitivity to tuberculous infection. Second, the antigen presenting capacity of alveolar macrophages is low in humans, as compared with other animal species. Third, alveolar macrophages are cells that live in an oxygen-rich environment, as a result of which their free oxygen radical production potential is theoretically great. However and probably to avoid toxicity due to these radicals, alveolar macrophages lack myeloperoxidase (although they do generate superoxide radicals). Fourth, alveolar macrophages contain abundant lysosomal enzymes.

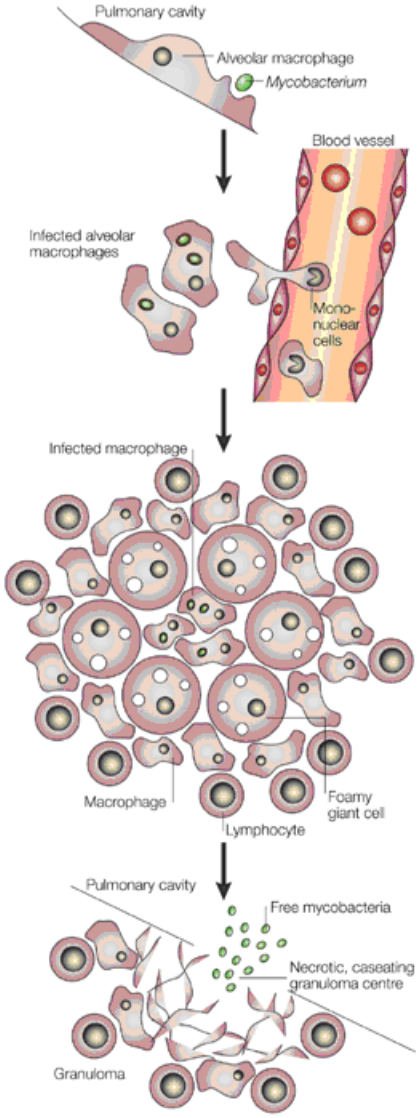


Fig. 1.6 Schematic presentation of the progression of *M. tuberculosis* infection (Extracted from *Nature* Reviews Molecular Cell Biology **2**, 569-586).

The initial interaction between *M. tuberculosis* and alveolar macrophages involves non-specific phagocytosis of the bacilli and their inclusion within phagocytic vacuoles. Considering that these alveolar macrophages have not been primed by lymphocytic cytokines and that various mycobacterial components inhibit the bactericidal systems of these cells, it is reasonable that bacterial growth predominates in this initial stage. Practically all bactericidal macrophage systems are inhibited by products derived from the mycobacteria. Thus, glycolipids inhibit phagosome-lysosome fusion, while other less well-known components alter lysosomal acid pH, thereby

complicating enzyme action. Catalase, in turn, destroys hydrogen peroxide, and different mycobacterial components inhibit superoxide production. This phase concludes with destruction of the alveolar macrophages by proliferating intracellular bacilli. Natural resistance to the infection fundamentally occurs during this phase. Tubercle bacilli products such as cord factor and the activation of other chemokine factors exert a potent chemical effect, attracting blood monocytes that ingest the released bacilli. At this point, a symbiotic relation is established in which the bacteria and young macrophages do not destroy each other. The monocytes have not been activated, and the bacteria are not toxic, at least on an acute basis. The tubercle bacilli increase exponentially in a similar manner, killing host cells and spreading locally. In the lung, intense alveolitis takes place at the expense of the young cells of the mononuclear phagocyte system.

The third essential phenomenon in this phase of the disease is mycobacterial spread systemically via the lymphatics towards the regional lymph nodes (41). In this region, the host immune response to tuberculous infection takes place. In some instances, this immune response is sufficient to arrest the progression of infection, although often times the bacilli escape towards the lymphatic duct and penetrate the pulmonary bloodstream, from where there is hematogenous spreading of the bacilli to the other organs. The main metastatic or target zones of such bacterial dissemination are the highly irrigated organs and tissues—the central nervous system, spongy bone, liver, kidneys, and genitals. In each of these zones, the arriving bacilli are phagocytosed by the local cells of the mononuclear phagocyte system. In most cases, this period implies immunologic control of the infection as a result of two mechanisms: cell-mediated immunity and delayed hypersensitivity. From the bacteriological perspective, the consequence of this situation is an abrupt interruption of the bacterial growth curve in both resistant and susceptible individuals. Cellular immunity is not responsible for this growth arrest, since susceptible individuals have only a weak cell mediated immune response and resistant subjects have not yet developed an effective immune reaction. Delayed hypersensitivity is the phenomenon responsible for the destruction of macrophages that contain intracytoplasmic bacteria, thereby forming a characteristic focus of caseous necrosis (42). Although the bacteria may survive within this necrotic focus for years, they are unable to reproduce due to the prevalent acidosis, the lack of oxygen, and the presence of inhibitory fatty acids. The principal factors influencing delayed hypersensitivity reactions are the cytotoxic T lymphocytes, although other factors such as cytokines (tumor necrosis factor), oxygen reactive species, and nitrous oxide may also be involved. Such initial necrosis is therefore beneficial for control of the infection. However, delayed hypersensitivity must be “reinforced” by cell-mediated immunity, since susceptible hosts

with weak immune responses are not only unable to control the infection but also produce granulomas with an increased caseous presence, probably due to the intervention of mycobacterial proteins. Resistant animals, in the same way as immunocompetent humans, avoid tubercle bacilli spread from the tuberculous focus in a second phase, owing to the development of a potent cell-mediated immune response at the expense of the helper T lymphocytes, which activate the macrophage population (43). From the clinical perspective, immunocompetent individuals develop a balance between themselves and the mycobacteria, which persists throughout life until some predisposing event is able to reactivate the infectious focus. An indirect approach to demonstrate this immunologic phenomenon is represented by the tuberculin skin test. In immunocompromised individuals who are unable to control the infection, TB disease develops and the subjects do not usually show a positive tuberculin test. In addition to prior immunodeficiency, one factor that clearly influences the conversion from latent infection to disease is the age at which primary infection takes place. Conversion to disease is more frequent in the very young and very old. Adolescents and early youth also show a tendency towards conversion to disease, although for reasons that remain unclear.

Tuberculous reactivation. Tuberculous reactivation is defined as the development of tuberculous disease in a patient who had already been infected with the tubercle bacillus in the past. Although there are well-documented cases of exogenous reinfection, it is currently accepted that most cases of infection are attributable to endogenous reactivation (44). However, this assumption may change in the coming years, in view of recent information appearing in the literature. It has been calculated that only a minority of people infected with *M. tuberculosis* actually progress to active disease. In general terms, 90% of infected individuals can be expected to keep the tubercle bacilli in a latent state for life, owing to the intervention of the host immune defenses. Five percent will develop progressive primary TB, and the remaining 5% will develop the disease in later stages of life, a condition known as reactivation or post-primary TB (Fig. 1.6). This situation changes drastically in patients with HIV infection, of whom an estimated 50% to 60% who are also infected with *M. tuberculosis* will develop active TB in the course of their lifetime. The number of patients at risk of TB has increased in the course of history, and presently includes some populations that do not demonstrate immunodeficiency but that have other risk factors for TB.

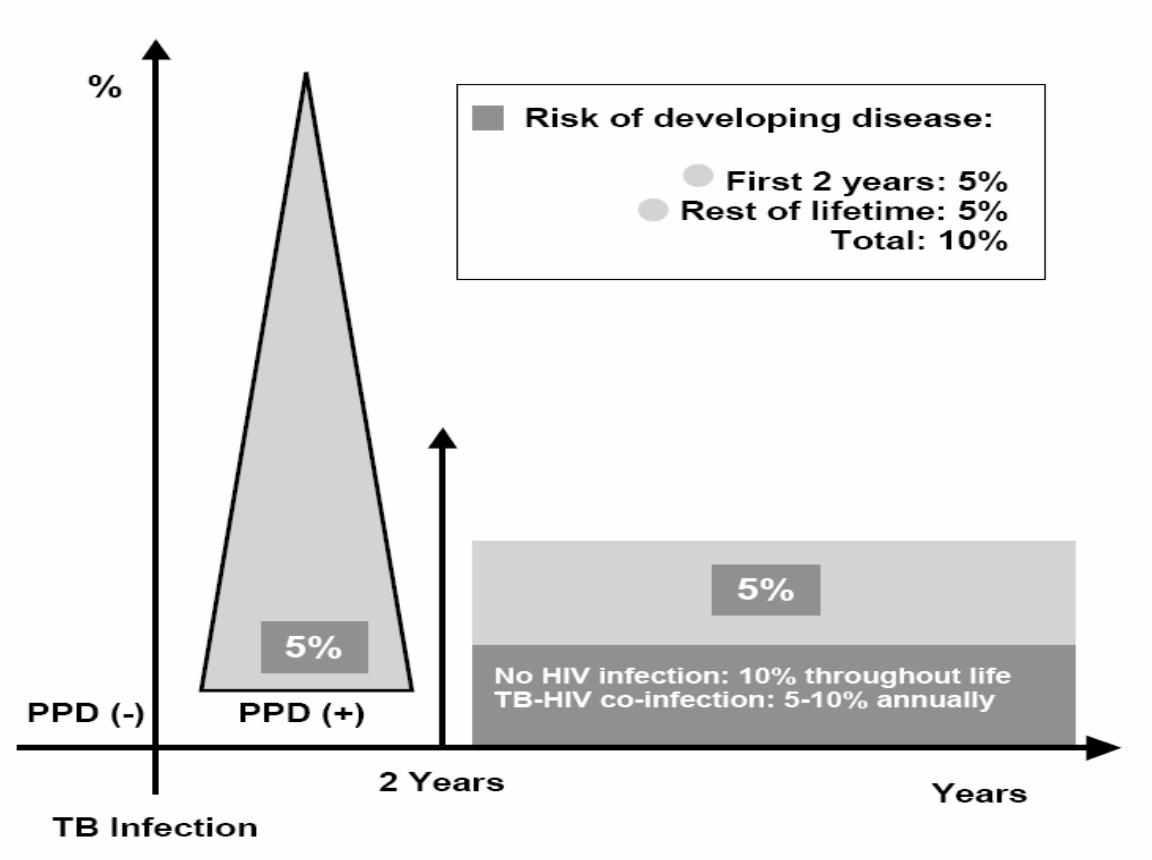


Fig. 1.6 Risk of developing tuberculous disease in a person infected with *M. tuberculosis*. PPD = purified protein derivative.

In resistant individuals, the immune control of haematogenous seeding sites is dependent on local as well as systemic factors. Some systemic factors (e.g., HIV infection, corticotherapy, malnutrition) may account for tuberculous reactivation, although it is less clear how other local factors affect reactivation. It has been speculated that a decrease in interferon production and the intervention of arabinomanans may be involved in this reduction or in the generation of a specific suppressor response. One of the known important pathogenic phenomena of reactivation is caseus liquefaction (45). Although not all the factors implicated in such liquefaction are known, the phenomenon has been attributed to lysosomal enzymes released by the macrophages, and to a delayed hypersensitivity reaction to mycobacterial products. The immediate consequence of caseus liquefaction is the production of an excellent growth medium for the bacteria, which begin to multiply and release products similar to tuberculin that have great toxic potential. In the case of the lungs, these released products rupture the adjacent bronchi, forming cavities and spreading the bacteria via the bronchogenic route.

Based on the above considerations, all TB-infected individuals should be considered patients, and the greatest protection against *M. tuberculosis* corresponds to a subject who has never been infected. The great problem with TB facing us today is that there are a very large number of infected individuals who are never free from the risk of the disease. These individuals, while potentially capable of developing the disease at any time in life, particularly if they develop some form of immunodeficiency, are expected to present with alerted memory lymphocytes in the event of past exposure to tubercle bacilli. Theoretically, this would confer relative protection against such possible exogenous reactivation. In this sense, there has been speculation as to whether the ideal situation is instead represented by the infected subject, who would be more protected against future exogenous reinfections. However, such reasoning is mistaken, since the development of TB due to endogenous reactivation in infected individuals is much more likely than the development of the disease due to exogenous infection in non-infected subject.

1.4. 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.

Medical history. The medical history includes obtaining the symptoms of pulmonary TB: productive, prolonged cough of three or more weeks, chest pain, and hemoptysis (46). 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.

Chest X-ray. Tuberculosis creates cavities visible in X-rays like this one in the patient's right upper lobe (Fig. 1.7). 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) (46). 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.

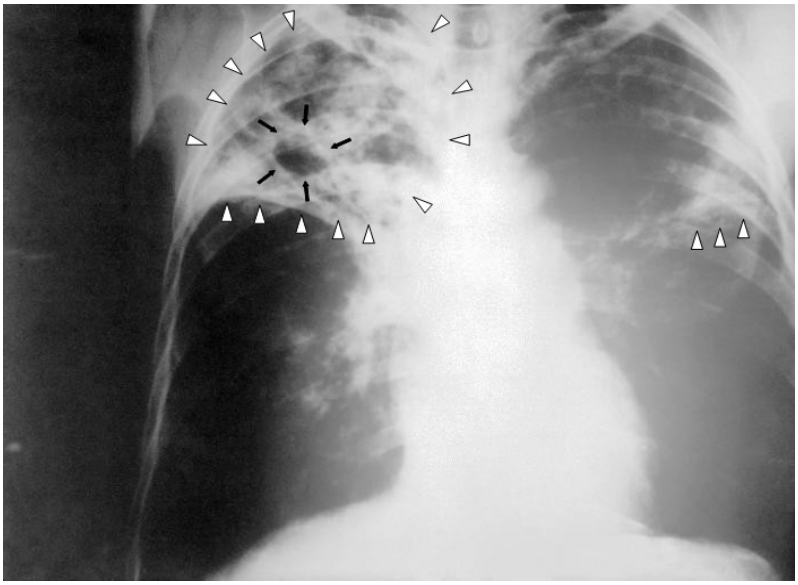


Fig. 1.7 Chest X-ray of a patient suffering from TB.

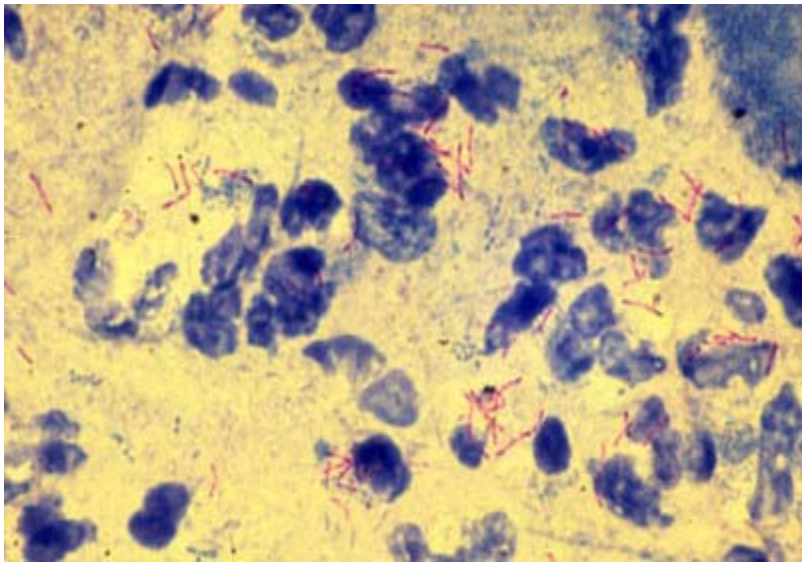


Fig. 1.8 Acid fast stain of sputum containing *M. tuberculosis*. The bacteria are visible as small pink stained rods.

The gold standard of TB diagnostics is confirmation with its growth in selective media (49). 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 (50). 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, *M. tuberculosis* exhibits a slow growth rate, requiring 14-21 days to generate visible colonies and does not produce any pigment (Fig. 1.9). With the advancement in culture system in 1980s BACTEC and biphasic culture methods were developed for faster recovery than traditional culture system (51).

The introduction of nucleic acid amplification assays using polymerase chain reaction (PCR) in 1985 brought the most progress in TB diagnostics (52). 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 *M. tuberculosis* 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 *M. tuberculosis* directly in clinical samples (53). However, some other factors such as an endogenous amplification inhibition factor of *M. tuberculosis* or unreliable quality control can influence susceptibility to both false positives and negatives and have hampered clinical uses of this assay (54). However, the severity of problems began to reduce after development of automated, robust, commercial tests which made clinical risk assessment possible (55).

Another old diagnostic method is the tuberculin skin test developed by Dr. Koch (56). The test is for the identification of the host infected with *M. tuberculosis*. Tuberculin skin tests involve the intracutaneous injection of five tuberculin units of Purified Protein Derivative (PPD) prepared by the Montoux technique (Fig. 1.10). The skin reaction of *M. tuberculosis* infection may discriminate from *M. avium* or other non-tuberculous infections (57). However, the cross reactivity of PPD with *M. bovis* BCG vaccinated individuals represents a major limitation in applying this method to TB patients (58). 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. 1.9 Colonies of *M. tuberculosis* as appeared on selective Dubos medium.

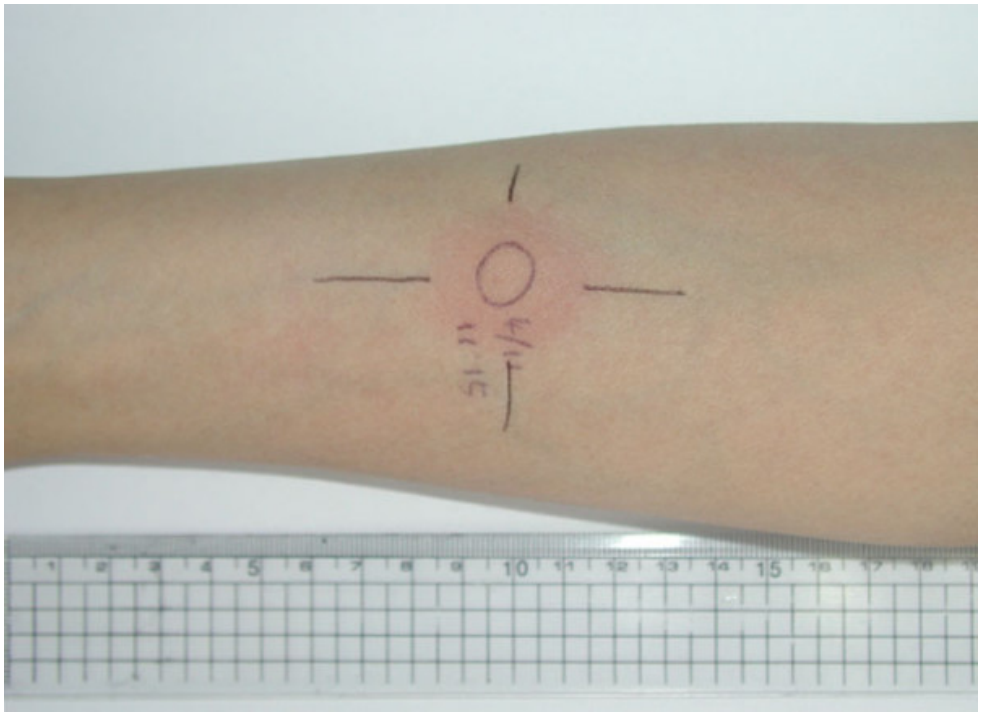


Fig. 1.10 Tuberculin test performed with 2 units of purified protein derivative, yielding a 92-mm induration after 72 hours.

The identification of regions of the *M. tuberculosis* 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 *M. tuberculosis*, *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 *M. tuberculosis* (59). 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 (60). In vitro blood test measuring IFN-γ is very useful to identify contacts of TB cases and show remarkable concordance with the tuberculin skin test (61). 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 (62). However, serological tests alone do not appear to help diagnosis of sputum positive pulmonary TB (63).

1.5. Treatment of Tuberculosis

In the middle of 20th century, the outlook of TB patients and the history of TB dramatically changed with the introduction of chemotherapy. The discovery of p-amino salicylic acid (PAS) by Jorgen Lehmann and of Thiosemicarbazone by Behard Domagk during World War I yielded the first therapeutic agents with efficacy in the treatment of TB. In 1944, Albert Schatz, Elizabeth Bugie and Selman Waksman reported the isolation of streptomycin, the first antibiotic and first bactericidal agent effective against *M. tuberculosis* (64).

With the discovery of isoniazid, the first oral drug in 1952, and rifampin in 1957, a new chemotherapeutic era of TB treatment had dawned and sanatoria were closed. The discovery of the two effective drugs with their application in the armamentarium of anti-tuberculosis strategy in 1966 (65,66) accelerated investigation on reduction of tuberculosis treatment. In the middle of 1970s, comparative studies of different combinations of these drugs demonstrated that the 6-months treating regimens containing rifampin or pyrazinamide showed superior curing efficiency for patients (67). Isoniazid and rifampin were considered as complete bactericidal drugs, being capable of killing bacteria in all environments, while streptomycin and pyrazinamide were of

'half' effective, the former being active in the more alkaline milieu and latter active in acidic intracellular environment. Thus, the combination with streptomycin or pyrazinamide was considered as perfect complementary combination which made a very powerful bactericidal regimen (68). Therefore, the short course regimen began to employ streptomycin, isoniazid and rifampin, which suggested that at least two full bactericidal drugs were required to successfully and dramatically cut treatment duration from 12-18 months down to 6 months.

From the late 1970s, the main issue of TB chemotherapy has been focused on finding the best combination using the available antibiotics. In the earliest study, the 6-month regimen consisted of streptomycin, isoniazid, rifampin and pyrazinamide for four months followed by streptomycin, isoniazid and pyrazinamide for 2 more months, and showed a relapse rate of 6% in patients infected with drug sensitive *M. tuberculosis* bacilli, with an even higher relapse rate with isoniazid resistant strains (69). A subsequent study evaluated another combination of streptomycin, isoniazid, rifampin and pyrazinamide, and had lower than 1% relapse rate, and the other combination, same regimen without streptomycin, around 2% relapse rate. However, without pyrazinamide, the relapse rate strikingly increased to 8% (70). More valuable findings had shown that regimens containing pyrazinamide were highly effective on patients infected with resistant strains against both streptomycin and isoniazid with relapse rate less than 4% (71). A series of previous results had confirmed the importance of sterilizing role of pyrazinamide used together with isoniazid and rifampin, in the reduction phase of chemotherapy.

A series of investigations during the 1980s proposed recommendations to the International Union Against Tuberculosis and Lung Disease (IUATLD), of the regimen including isoniazid, rifampin and pyrazinamide, for a 2-months induction phase followed by isoniazid and rifampin for a 4-months continuation phase (72). Thus, these trial-validated, intermittent short course chemotherapy regimens, DOTS programs became feasible for TB services of high prevalence countries. With the DOTS program developed by the IUATLD together with national TB programs, the WHO recommended directly supervised treatment consisting of a 2-month daily regimen of isoniazid, rifampin, pyrazinamide, ethambutol and streptomycin followed by 4 moths continuation with either daily or intermittent isoniazid plus rifampin for patient with newly diagnosed smear positive disease. As a second option an entirely intermittent 6-month treatment was recommended (73).

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 1.2) (74). D-Cycloserine targets peptidoglycan biosynthesis in various bacteria, including mycobacteria (75). 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) (76). This was shown when *KatG* from *M. tuberculosis* 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 (77). The enoyl reductase is encoded by *inhA*. Ethionamide is now thought to act in a similar manner as isoniazid including the activation step (78). The proposed target of ethambutol was provided by Takayama and Kilburn who showed that it is likely inhibitor of arbinan biosynthesis of arabinogalactan (AG) and LAM (79). 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* (80,81). EMB 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 EMB-resistant strains of mycobacteria and that amino acid 306 of *embB* is the most mutated position in *M. tuberculosis*. From this, it is clear that arabinosyltransferases are the site of action of EMB (82).

RIF 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 RIF in TB treatment, the emergence of different RIF-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*) (83). Quninolone derivatives possess potent antibacterial activities with a broad spectrum organism including *M. tuberculosis*. 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 (84).

PZA is an important sterilizing drug. However, the mode of action is poorly understood. It is a prodrug and activated by *M. tuberculosis* pyrazinamidase which is only active at acidic pH. Pyrazinamidase converts PZA to the active form, pyrazinoic acid. Pyrazinoic acid and PZA de-energize the membrane by collpsing the membrane potential and affect the membrane transport function at acid pH (85).

Table 1.2 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 acid (PASA)	Anti-metabolite interfering with incorporation of para-aminobenzoic acid into folic acid – folate synthesis antagonist.	Inhibits tubercle bacilli at concentrations of 1–5µg/ml.

1.6. Problems in controlling Tuberculosis

The currently popular treatments, DOTS programs, although highly effective are far from ideal. Using the effective combination of available drugs, the curing period cannot be reduced below 6 months. In most developing countries, the treatment duration is still longer, and 8-months regimen. Because of the treatment over a long period of time, it has been difficult to anticipate that patient will complete their therapy on their own. Furthermore, all four of the most effective first-line drugs, INH, RIF, ETH, and PZA, must be taken together and in large quantities during the first 2 months. Therefore, although rates of serious adverse effects are low, many patients have suffered from side effects. Improper implementation of DOTS program also lead to the development and spread of drug-resistant strain of TB, including MDR-TB. Especially, when given under suboptimal dose of drugs, these regimens are associated with high risk of acquired drug resistant cases, defined as the presence of drug resistant strains in a patient that previously had been treated for TB for at least one month (86). As one way to reduce the emergence of drug-resistant TB cases, it is recommended that treatment be directly observed by health care officers, especially during the first two months and whenever RIF is being used. However, this also requires involving a large infrastructure which is labor intensive and expensive.

Development of drug resistance is far more likely when supervised treatment is not given, when the patients are not compliant, when recommended regimens are not used and when drugs with poor bioavailability are used. In the situation where MDR-TB is present, the cure rate using standard DOTS treatment drops from greater than 90% to approximately 50% (87). In curing MDR-TB short course chemotherapy is no longer effective and recommended. The WHO now recommends a modification of DOTS program called DOTS-plus, which consists of DOTS program plus second-line TB drugs for a period of 24 months (88). The second-line drugs for treatment of TB are much more expensive, more toxic and less effective. The problem of drug resistance has become so large, that within some regions such as Ivanova Oblast in Russia, acquired-drug resistance to at least one antitubercular drug is 100% (89). Even more unsettling, the incidence of primary MDR-TB in Estonia increased from 10% to 14% in only four years. In addition, most existing TB drugs are only able to target actively growing bacilli through the inhibition of cell processes such as cell wall biogenesis, protein synthesis and DNA replication. This implies that current TB chemotherapy is characterized by an efficient bactericidal activity but an extremely weak sterilizing activity, which is defined as the activity to kill persisters of dormant bacteria. The weak sterilizing property of available TB drugs is one of the major

drawbacks of current TB chemotherapy. Although RIF and PZA are partially sterilizing drugs and play an important role in shortening the therapy period, there are still populations of persisting bacteria that are not killed by RIF and PZA.

In developing nations, political strife and economic collapse easily lead to an inadequate drug supply, poor infrastructure to support detection and follow-up of TB cases, and a failure to fully or correctly implement DOTS programs. All of these are reasons that DOTS programs are far form ideal for treatment of TB. From a purely epidemiological perspective, ineffective drug therapy is worse than no treatment at all, because it may extend the lives of chronically infected individuals and increase the likelihood of transmission. Not only do these individuals spread TB for a longer period of time but they serve as a reservoir for the dissemination of primary drug resistance within the community (90).

The TB-HIV co-infected patients show a significantly higher risk of presenting with active TB and faster progression of the disease. At this time the usage if antituberculosis drugs are usually limited to patients who are receiving Highly Active Anti-Retroviral Therapy (HAART) due to the drug-drug interactions (91). In addition, chemotherapeutic treatment regimens are less effective and mortality rates can approach upto 80% in the TB-HIV co-infected cases.

With the rapidity of modern international travel and the increase in global immigrations, this will eventually lead to more epidemics of MDR-TB occurring within industrialized nations, compromising once successful anti-tuberculosis programs and resulting in substantial financial and human costs. Therefore, expensive investigative mining for new drug targets have been undertaken. Three crucial purposes for developing novel anti-tuberculosis drugs are as follows: to improve current DOTS programs by shortening the total curing duration (92); to combat MDR-TB (93); and to provide more effective treatment for persistent TB infection (94). The greatest impact would be to current DOTS programs by providing new regimens that shorten the patient curing period and help their compliance. Obviously, a compound that would reduce the length of both the first bactericidal phase and second sterilizing phase would provide the greatest improvement. On the other hand, a novel treatment with the purpose of improving therapy for patients with MDR-TB has been paid a great deal of attention. The final impetus to the development of new antituberculosis drug is for improved treatment of persons with persistent TB infection. INH has been reported to be effective in persons with persistent TB and HIV co-infection and has recently been recommended by WHO for such persons (95). However there are significant limitations to this intervention and new drugs to improve persistent TB treatment have been deemed essential to the elimination of TB in low-incidence countries (96). Unless significant new resources are devoted to the detection and treatment of TB in developing

countries, the worldwide increase in the incidence of TB will continue to escalate at an ever faster rate.

1.7. Latency: The root cause of Tuberculosis problems

In the 21st century, we face the problems of billions of people with latent tuberculosis infection. Latency has been defined as the "presence of any tuberculosis lesion which fails to produce symptoms of its presence" (97). Despite the immune system's ability to clear much of the bacilli and arrest an infection, the lungs may still contain small caseous foci. The first evidence of latent TB in the caseous foci was the reoccurrence of an infection with non drug resistant tubercle bacilli after treatment with a regimen of antibiotics (98). The nature of latency was further elucidated by chemoprophylaxis treatment, which showed that the longer the period of treatment, the lower the chances of reactivation (99). Since susceptibility to antibiotics required some level of growth and metabolism, it was suggested that there was some degree of growth and metabolism of *M. tuberculosis* during latent state. Therefore, persistent bacilli in a lesion are likely to be in a steady state in which intermittent replication is balanced by immune system destruction. The balance of this steady state will determine active disease versus latent infection.

Latency is achieved by cell-mediated immune response which restricts the growth of *M. tuberculosis* bacilli. The restriction, however, does not eliminate the pathogen, leaving the bacilli as a present danger to reactivate years later. Secondary infection occurs as the reactivation of an old lesion with latent bacilli at the apical zone of the lungs (100). Individuals infected with *M. tuberculosis* have a 10% chance during their life time to develop active tuberculosis from a latent infection. 5% of the infected population will develop the disease after 5 years and the others will suffer from it at some point during their lives (101). Often, the precipitating factor for latent tuberculosis reactivation is a waning immunity, which takes place mostly in the elderly at an estimated rate of 5% per year untills complete disappearance of immunity (102). Factors such as corticosteroids, immunosuppressant, HIV and other factors that lower resistance are a danger for reactivation as well.

The contribution of exogenous reinfection to the incidence of secondary tuberculosis has been largely ignored because it was assumed that the primary infection would provide protection against secondary infection. However, there is skepticism about the idea of dormant bacilli waiting to reactivate due to immunosuppression. Often, TB primary complex is sterile within five years, suggesting that secondary infection is an infection with exogenous bacteria (103). It has

also been documented that reinfection of some immunocompetent individuals occur with new strains of *M. tuberculosis*. This indicates that immunity to tuberculosis can be incomplete, and that reinfection, at least in areas where tuberculosis is prevalent, probably has a greater role than previously appreciated (104). The dynamic nature of mycobacteria is highlighted by work showing that exogenously infected *M. marinum* in zebrafish, enters pre-existing granulomas by specific mycobacteria-mediated mechanisms that direct infected macrophages into granulomas (105).

Active *M. tuberculosis* lesions generally contain detectable populations of acid-fast, easily culturable bacilli, but bacilli from tubercles of post-chemotherapy, sputum-negative patients often fail to be cultured (106). Extending culture incubation time from weeks to many months enables fully drug sensitive *M. tuberculosis* from closed lesions of drug-treated patient to be cultured, proving that bacilli from latent tubercles are still viable (107). The difficulty in eradicating *M. tuberculosis* from a latent infection with drugs has also spurred ideas that alternate forms of bacilli may exist, such as protoplast, L-forms (forms without a cell wall), or spores, that may go undetected *in vivo* and are difficult to culture (108,109). Conditions within closed lesions such as a lowered oxygen tension, long chain fatty acids, lactic acid, and other bacteriostatic agents are hypothesized to reduce bacterial metabolism and render the tubercle bacilli resistant to drugs.

Several studies have been carried out to understand the *in vitro* sustainability of *M. tuberculosis* in closed necrotic lesion. Limited amounts of bacilli have been shown to survive twelve year incubation at 37^0C in a sealed culture vessel, suggesting the capability of long term persistence in nutrient limited or anaerobic environment (110). Taking into account that 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. Dormancy as a result of metabolic adaptation to anaerobic conditions was proposed and modeled in a system of limited oxygen tension known as the Wayne's *in vitro* hypoxic model (111,112). Wayne found that *M. tuberculosis* adapts to oxygen restriction by altering its metabolism to obtain energy through other processes (113). The condition of limited oxygen in the granuloma is supported by gas concentration measurements of cavities from the lungs of living tuberculosis patients: blocked cavities, 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 (114).

Latency has been modeled *in vivo* as well to account for additional stresses in a granuloma such as low pH, high concentration of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) in activated macrophages (115). The Cornell model was the first animal

model for dormant bacilli. This model involves partial clearance of *M. tuberculosis* infection by incomplete chemotherapy to induce the latent state (116). However, the granuloma and inflammatory response disappear after chemotherapy and hence the conditions of this model do not resemble those found in humans. The low-dose mouse model of latent tuberculosis, known as the chronic or plateau model, involves aerosol infection or infection by intravenous routes. This model resembles human latency because the host immune response contains the infection, but larger amounts of bacteria accumulate in the mouse than in humans which leads to a steady accumulation of pulmonary damage (117). Although these models have their limitations, they are good sources to learn about the metabolic state of persistent mycobacteria and host immunity.

Mechanism of latency. Formation of granulomas coincides with nutrient deprivation, oxygen restriction and an onslaught of host defenses. Long term survival of *M. tuberculosis* within a granuloma presumably consists of different strategies for persisting in harsh environments and evasion of the host immune response. Many genes that are important for pathogenesis have been identified in expression and mutant screens (104).

M. tuberculosis is likely to have significant alterations in gene expression from its encounter with the host immune response, from its transitions of acute to chronic infection, and from log phase growth to stationary phase growth. *M. tuberculosis* contains 13 sigma factors, including a general house keeping sigma factor SigA and several alternative sigma factors with key roles in pathogenesis at later stages of infection (118). Alternative sigma factors bind to the core RNA polymerase ($\alpha 2\beta\beta'$) and direct it to the promoters that are not normally recognized. Transcription of several sigma factors have been identified to increase during limiting conditions: *sigB*, *rpoS*-like sigma factor, and *sigE* increase during entry into stationary phase, under conditions of low aeration, heat and hydrogen peroxide (119); *sigE* and additionally *sigF*, are expressed during growth of *M. tuberculosis* in a macrophage (120). Disruption of the *M. tuberculosis sigH* gene and a gene encoding a putative transcriptional regulator, *whiB3*, result in attenuation of virulence in mice, while not having an impact on the bacterial load (121). Mice infected with mutants for each of these genes (*sigH* or *WhiB3*) show an altered inflammatory process in the lungs as compared to mice infected with wild-type *M. tuberculosis*. These studies indicate different regulons may be controlled depending on environmental conditions, and altered expression may be essential for TB persistence.

To circumvent nutritional shortages, *M. tuberculosis* has developed alternative means for generating energy. The pathogen increases breakdown and utilization of fatty acids, which are abundant in caseous environment of the granuloma, as a source of carbon and energy during infection in the lungs (122). Among the pathways required for utilization of fatty acids is the

glyoxylate cycle, present in many bacteria but absent in vertebrates. Using bacteria carrying knockout mutations, McKinney has shown that *M. tuberculosis* late-stage persistence in mice was facilitated by isocitrate lyase (ICL), an enzyme that is essential for the metabolism of fatty acids in glyoxylate cycle (123). Disruption of the *icl* gene had no effect on growth in the acute phase of infection in mice, indicating that during late stages of infection, *M. tuberculosis* may reside in an environment with limited carbohydrates and might convert lipids into carbohydrates.

Adaptation to oxygen limitation by *M. tuberculosis* induces enhanced resistance to isoniazid and rifampin, thermotolerance, decreased protein synthesis and thickened cell wall (124). In response to oxygen restriction, microarray analysis identified the induction of two-component system DevR-DevS (125). Inactivation of DevR abolishes the rapid induction of hypoxia induced gene expression, and the *M. tuberculosis* mutant has decreased survival compared to wild type Mtb after 40 days under hypoxic conditions. A microarray study has found that DevR controls the expression of a 48-gene "dormancy regulon," which is induced under hypoxic conditions and by nitric oxide (126). Regulons differentially expressed under hypoxic conditions are likely mechanisms for *M. tuberculosis* adaptation and survival in microaerobic granuloma.

A shift from aerobic to hypoxic conditions also induces the alpha crystallin protein in *M. tuberculosis*, a 16-kD chaperone protein. Decline in α-crystalline protein parallels the loss of *M. tuberculosis* tolerance to anaerobic shock. The protein has also been identified to play a protective role in the survival of bacilli and its growth in macrophages (127).

Morphologic changes are associated with *M. tuberculosis* long-term survival as well. Persistence and virulence of *M. tuberculosis* has been associated with "cording" a formation of rope like tangles of laterally associated bacilli (128). Using a transposon mutagenesis approach, cord formation was found to depend on PcaA, an enzyme responsible for the cyclopropanation of α-mycolates, which are long chain alkyl β-hydroxy fatty acids that are a major constituent of the mycobacterial cell wall. A *pcaA* mutant's enhanced bacterial replication in mice during the acute phase of infection, followed by a later defect in persistence, may indicate that cell wall components can modulate host immunity in a stage-specific manner (129).

The discoveries of some of the *M. tuberculosis* survival mechanisms indicate that the pathogen is capable of altering its transcription to adapt changing environment. Persistence is due to its ability to conserve energy by shutting down unused biochemical pathways and utilizing pathways consistent with environmental resource availability. The state of dormancy reflects organims's ability to regulate its cellular metabolic activity.

1.8. Strategies for dealing with persistence of Tuberculosis

In spite of better understanding of the physiology of *M. tuberculosis*, our knowledge about the state of the bacillus during the latent period is far from being complete. Moreover, a true representative model of latent tuberculosis in the laboratory setting is not available. Establishment of such a system would certainly accelerate the efforts to understand the physiology of mycobacteria during the latent period and eventually it will help in the identification of new drug targets that can act on the persistent mycobacteria. Recent advances in modern biology, in combination with bioinformatics tools, proteomics and microarray technology would further facilitate the search of new drug targets against tuberculosis. These exciting techniques are providing new avenues for understanding the biology of mycobacteria. The result of better understanding of the physiology of mycobacteria is manifested by the fact that the list of possible drug targets for tuberculosis is increasing day by day, the utility of these targets, however, cannot be predetermined. The list of potential drug targets encoded in the genome of *M. tuberculosis* include genes involved in persistence or latency, cell wall synthesis, virulence, signal transduction, genes encoding transcription factors and enzymes of other intermediary metabolic pathways (130). All these targets should be explored to identify new drugs against tuberculosis that will overcome the limitations of existing drugs such as, prolonged chemotherapy, failure against persistent infection and multidrug resistance.

Besides the choice of drug targets, there are many different approaches one should consider and use in TB drug development. One is the way drug screens are designed. Current TB drugs were mostly discovered based on their activity against growing bacilli *in vitro*. However, activity against nongrowing persister bacilli is correlated with good sterilizing activity that is responsible for shortening therapy *in vivo*, as shown by PZA and RIF. Thus, novel drug screens that mimic *in vivo* conditions in lesions (i.e. acidic pH and hypoxia) and act against old stationary-phase nongrowing bacilli could be important for identifying drugs that kill persisters and thereby shortening TB treatment. Along this line of combination to screen is the recent interest in the use of systems biology approach for drug discovery. Instead of the conventional reductionist approach of finding a single drug that hits a single target, the systems biology approach proposes using multiple compounds that hit multiple targets in different pathways to achieve the desired outcome. A systems biology approach can be used for identifying novel drug combinations against latent TB.

1.9. Thesis objectives

This thesis was inspired by two major results: determination of the complete nucleotide sequence of *Mycobacterium tuberculosis* and increased nitrate reductase activity during its shiftdown to dormant stage. Given the observation of presence of an operon, *narGHJ*I in *Mycobacterium tuberculosis* genome, which codes for a respiratory type of nitrate reductase (NarGHJI), we investigated the hypothesis that nitrate respiration through NarGHJI could provide energy during latent stage survival of the pathogen. Given that latent stage is assumed to be anaerobic, reduction of nitrate into nitrite may generate necessary ATPs 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.

The first part (Chapter 2) of the study focused on examining the presence of this respiratory nitrate reductase in *M. smegmatis*, as this saprophytic organism could be a most appropriate surrogate strain to study the above hypothesis. This is due to its similarity to pathogenic *M. tuberculosis* in following the dormancy pattern upon gradual transfer to anaerobic environment as well as its added advantage of fast growing nature.

Following the confirmation of presence of nitrate reductase and induction of its activity during gradual shift down of the culture to dormant stage, next objective was to evaluate the dependence of the organism on this nitrate reductase for survival during dormant stage. Extension of this hypothesis to *M. bovis* BCG and *M. tuberculosis* with more thorough investigation within host cell environment was anticipated further to have a better idea and conclusion about taking up nitrate reductase as drug target against latent stage.

Once the results of induction of this enzyme's activity were confirmed in all three species, development of a whole cell based dormant stage specific high throughput screening assay was aimed next to explore nitrate reductase activity as reporter system reflecting the dormant stage of the tubercle bacilli (Chapter 3).

Screening of a diverse chemical library against this assay was also projected to search dormant stage antimycobacterial agents (Chapter 4). 2-nitroimidazole was discovered from this screening as potential lead molecule having significant antitubercular activity at a very low MIC against the active stage of the bacilli with promising intracellular efficacy. Three compounds from 1,2,4-triazolethiol class of compounds were discovered with significant antitubercular activity against hypoxic dormant stage of *M. tuberculosis* along with their intracellular efficacy.

A natural product from *Alpinia galanga* was also identified inhibiting the active as well as dormant stage of *M. tuberculosis* at a very low concentration of 0.5μg/ml.

Another major intent of the thesis was to isolate, purify and characterize the nitrate reductase (NarGHJI), in order to better understand the mechanism of the enzyme as well as to develop enzyme based screening protocol which could be used to search inhibitory molecules of nitrate reductase (Chapter 5).

These studies may help us better understand the fundamental biochemistry and biology of nitrate metabolism of mycobacteria. Furthermore this study may also help in revealing potential drug targets of *M. tuberculosis* against the dormant stage as well as in identification of novel antitubercular compounds.

1.10. References

1. Gey van Pittius NC, Gamieldien J, Hide W, Brown GD, Siezen RJ, Beyers AD. 2001. The ESAT-6 gene cluster of *Mycobacterium tuberculosis* and other high G+C Gram-positive bacteria. *Genome Biol.* **2:** 41-44.
2. Goodfellow M, Magie JG. 1998. Taxonomy of Mycobacteria. In mycobacteria basic aspects. Edited by Gangadharam PRJ, Jenkins PA. Chapman and Hall. **1:** 1-71.
3. Lee RE, Brennan PJ, Besra GS. 1996. *Mycobacterium tuberculosis* cell envelope. In Tuberculosis Edited by Shinnick TM. Springer.
4. Brooks GF, Butel JS, Ornston LN, Javetz E, Melnick JL, Adelberg EA. 1995. Medical Microbiology edition 20, Norwalk CT, Appleton and Lange.
5. De Voss JJ, Rutter K, Schroeder BG, Barry CE III. 1999. Iron acquisition and metabolism by mycobacteria. *J. Bacteriol.* **181:** 4443-4451.
6. Raghu B, Sarma GR. 1993. Isolation and characterization of siderophores and envelope proteins from mycobacteria. *Biochem. Mol. Biol. Int.* **31:** 333-339.
7. Brennan PJ. 1995. The envelope of Mycobacteria. *Annu. Rev. Biochem.* **64:** 29-61.
8. Belisle JT, Brennan PJ. 1989. Chemical basis of rough and smooth variation in mycobacteria. *J. Bacteriol.* **171:** 3465-3470.
9. Boshoff HI, Barry CE, III. 2005. Tuberculosis - metabolism and respiration in the absence of growth. *Nat. Rev. Microbiol.* **3:** 70-80.
10. Wayne LG, Sohaskey CD. 2001. Non-replicating persistence of *Mycobacterium tuberculosis*. *Annu. Rev. Microbiol.* **55:** 139-163.

11. Soto Carlos Y, Nuria A, Isidre G, Marina L. 2002. Simple and rapid differentiation of *Mycobacterium tuberculosis* H37Ra from *M. tuberculosis* clinical isolates through two cytochemical tests using neutral red and nile blue stains. *J. Clin. Microbiol.* **40:** 3021-3024.
12. Cox JS, Chen B, McNeil M, Jacobs WR. 1999. Complex lipid determines tissue-specific replication of *Mycobacterium tuberculosis* in mice. *Nature* **402**: 79-83.
13. Cox RA. 2004. Quantitative relationships for specific growth rates and macromolecular compositions of *Mycobacterium tuberculosis*, *Streptomyces coelicolor* A3(2) and *Escherichia coli* B/r: an integrative theoretical approach. *Microbiology* **150:** 1413-1426.
14. Aiyar SE, Gaal T, Gourse RL. 2002. rRNA promoter activity in the fast-growing bacterium *Vibrio natriegens*. *J. Bacteriol.* **184:** 1349-1358.
15. Beste DJV, Peters J, Hooper T, Avignone-Rossa C, Bushell ME, McFadden J. 2005. Compiling a molecular inventory for *Mycobacterium bovis* BCG at two growth rates: evidence for growth rate-mediated regulation of ribosome biosynthesis and lipid metabolism. *J. Bacteriol.* **187:** 1677-1684.
16. Matsumoto S, Furugen M, Yukitake H, Yamada T. 2000. The gene encoding mycobacterial DNA-binding protein I (MDPI) transformed rapidly growing bacteria to slowly growing bacteria. *FEMS Microbiol. Lett.* **182:** 297-301.
17. Mailaender C, Reiling N, Engelhardt H, Bossmann S, Ehlers S, Niederweis M. 2004. The MspA porin promotes growth and increases antibiotic susceptibility of both *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*. *Microbiology* **150:** 853-864.
18. Brennan PJ, Nikido H. 1995. The envelope of Mycobacteria. *Annu. Rev. Biochem.* **64:** 29-63.
19. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, *et al*. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:** 537-544.
20. Draper P, Daffe M. 2005. The cell envelope of *Mycobacterium tuberculosis* with special reference to the capsule and outer permeability barrier. *In tuberculosis and tubercle bacillus*. (Cole ST, Eisenach KD, Macmurray DN, Jacobs Jr. WR, Editors.) pp. 261-173. ASM press Washington DC.
21. Brennan PJ. 2003. Structure, function and biogenesis of the cell wall of *Mycobacterium tuberculosis*. *Tuberculosis* **83:** 91-97.

22. Draper P. 1998. The outer parts of the mycobacterial envelope as permeability barriers. *Front. Biosci.* **3:** D1253-1261.
23. Kremer L, Besra GS. 2005. A waxy tale by *Mycobacterium tuberculosis*. *In tuberculosis and tubercle bacillus*. (Cole ST, Eisenach KD, Macmurray DN, Jacobs Jr WR. Editors.) pp. 287-305. ASM press Washington DC.
24. Ehlers MRW, Daffé M. 1998. Interactions between *Mycobacterium tuberculosis* and host cells: are mycobacterial sugars the key? *Trends Microbiol.* **6**: 328–335.
25. Khoo KH, Dell A, Morris HR, Brennan PJ, Chatterjee D. 1995. Inositol phosphate capping of the nonreducing termini of lipoarabinomannan from rapidly growing strains of *Mycobacterium*. *J. Biol. Chem.* **270:** 12380–12389.
26. Daniel T, Bates J, Downes K. 1994. In tuberculosis; Pathogenesis, protection and control. Edited by Bloom BR. ASM press Washington.
27. Sreevatsan S, Pan X, Stockbauer KE, Connell ND, Kreiswirth BN, Whittam TS, *et al*. 1997. Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. *Proc. Natl. Acad. Sci. USA* **94:** 9869-9874.
28. Crubezy E, Ludes B, Poveda JD, Clayton J, Crouau-Roy B, Montagnon D. 1998. Identification of *Mycobacterium* DNA in an Egyptian Pott's disease of 5400-year-old, *C.R. Acad. Sci. Paris* **321**: 941-951.
29. Dubos R, Dubos J. 1952. Tuberculosis, man and society. White plague, Brown Co., Boston.
30. World Health Organization Global tuberculosis Programme. 1997. WHO Report of tuberculosis epidemic 1997. World Health Organization, Geneva.
31. Murray JF. 2004. A century of tuberculosis. *Am. J. Respir. Crit. Care Med.* **169:** 1181-1186.
32. Comroe JH Jr. 1978. Pay dirt: the story of streptomycin. I: Fom Waksman to Waksman. Retrospectroscope. *Am. Rev. Respir. Dis.* **117:** 773-781.
33. Daniel TM. 1997. Captain of Death: The story of Tuberculosis, University of Rochester Press, Rockester.
34. Ryan F. 1992. The Forgotten Plague: How the battle against tuberculosis was won and lost. Little and Brown, Boston.
35. WHO report 2008. Global tuberculosis control: surveillance, planning, financing. World Health Organization, Geneva. (WHO/HTM/TB/2008.376).

36. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, *et al*. 2006. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet* **368:** 1575-1780.
37. Davies PDO, Yew WW, Ganguly D, Davidow A, Reichman L, Dheda K, *et al.* 2006. Smoking and tuberculosis: the epidemiological association and pathogenesis. *Trans. R. Soc. Trop. Med. Hyg.* **100:** 291-298.
38. Restrepo BI. 2007. Convergence of the tuberculosis and diabetes epidemics: renewal of old acquaintances. *Clin. Infect. Dis.* **45:** 436-8.
39. Bloom BR. 1994. Tuberculosis: Pathogenesis, protection and control. ASM Press Washington DC.
40. Schorey JS, Carroll MC, Brown EJ. 1997. A Macrophage invasion mechanism of pathogenic mycobacteria. *Science* **277:** 1091-1093.
41. Houben E, Nguyen L, Pieters J. 2006. Interaction of pathogenic mycobacteria with the host immune system. *Curr. Opin. Microbiol.* **9:** 76-85.
42. Dannenberg AN, Jr. 1991. Delayed type hypersensitivity and cell mediated immunity in pathogenesis of tuberculosis. *Immunology today* **12:** 228-233.
43. Schlesinger LS. 1998. *Mycobacterium tuberculosis* and the complement system. *Trend Microbiol.* **6:** 47-50.
44. Flynn JL, Chan J. 2001. Tuberculosis: latency and reactivation. *Infect. Immun.* **69:** 4195-4201.
45. Chan J, Flynn JL. 2000. Latent and reactivation tuberculosis. *Einstein Q. J. Biol. Med.* **17:** 69-77.
46. Kumar V, Abbas AK, Fausto N, Mitchell RN. 2007. Robbins Basic Pathology (8th edition). Saunders Elsevier. pp. 516-522.
47. Koch ML, Cote RA. 1965. Comparison of Fluorescence microscopy with Ziehl Neelsen Stain for demonstration of acid fast bacilli in smear preparation and tissue sections. *Am. Rev. Respir. Dis.* **91:** 283-284.
48. Hobby GL, Holman AP, Iseman MD, Jones JM. 1973. Enumeration of tubercle bacilli in sputum of patients with pulmonary tuberculosis. *Antimicrob. Agents. Chemother.* **4:** 94-104.
49. Golyshevskaia VI, Korneev AA, Chernousova LN. 1996. New microbiological techniques in diagnosis of tuberculosis. *Probl.Tuberk.* **6:** 22-25.

50. Morgan MA, Horstmeier CD, DeYoung DR, Roberts GD. 1983. Comparison of radiometric method (BACTEC) and conventional culture media for recovery of mycobacteria from smear negative specimens. *J. Clin. Microbiol.* **18:** 384-388.
51. Abe C, Hosojima S, Fukasawa Y, Kazumi Y, Takahashi M, Hirano K, *et al*. 1992. Comparison of MB-check, BACTEC and egg based media for recovery of mycobacteria. *J. Clin. Micobiol.* **30:** 878-881.
52. Brisson NA, Gicquel B, Lecossier D, Levy-Frebault V, Nassif X, Hance AJ. 1989. Rapid diagnosis of tuberculosis by amplification of mycobacterial DNA in clinical samples. *Lancet* **2:** 1069-1071.
53. Thiery D, Cave MD, Isenach KD, Crowford JT, Bates JH, Gicquel B, *et al*. 1990. IS 6110 an IS like element of *Mycobacterium tuberculosis* complex. *Nucleic Acid Res.* **18:** 188.
54. Amicosante M, Richeldi L, Trenti G, Paone G, Campa M, Bisetti A, *et al*. 1995. Inactivation of polymerase inhibitors of *Mycobacterium tuberculosis* DNA amplification in sputum by using capture resin. *J. Clin. Microbiol.* **33:** 629-630.
55. Jonas V, Alden MJ, Curry JL, Kamisango K, Knott CA, Lankford R, *et al*. 1993. Detection and identification of *Mycobacterium tuberculosis* directly from sputum sediments by amplification of rRNA. *J. Clin. Microbiol.* **31:** 2410-2416.
56. Huebner RE, Schein MF, Bass JB Jr. 1993. The tuberculin skin test. *Clin. Infect. Dis.* **17:** 968-75.
57. Palmer CE, Edwards LB. 1967. The tuberculin test: in retrospect and prospect. *Arch. Environ. Health* **15:** 792-808.
58. Wang L, Turner MO, Elwood RK, Schulzer M, FitzGerald JM. 2002. A meta-analysis of the effect of bacille Calmette-Guérin vaccination on the tuberculin skin test measurements. *Thorax* **57:** 804–809.
59. Cockle PJ, Gordon SV, Lalvani A, Buddle BM, Hewinson RG, Vordermeier HM. 2002. Identification of novel *Mycobacterium tuberculosis* antigens with potential as diagnostic reagents or subunit vaccine candidates by comparative genomics. *Infect. Immun.* **70:** 6996-7003.
60. Goletti D, Carrara S, Vincenti D, Saltini C, Rizzi EB, Schinina V, *et al*. 2006. Accuracy of an immune diagnostic assay based on RD1 selected epitopes for active tuberculosis in a clinical setting: a pilot study. *Clin. Microbiol. Infect*. **12:** 544-550.
61. Pai M, Gokhale K, Joshi R, Dogra S, Kalantri S, Mendiratta DK, *et al*. 2005. *Mycobacterium tuberculosis* infection in health care workers in rural India: comparison

of a whole-blood interferon gamma assay with tuberculin skin testing. *JAMA* **293:** 2746-2740.

62. Kanaujia GV, Lam PK, Perry S, Brusasca PN, Catanzaro A, Gennaro ML. 2005. Integration of microscopy and serodiagnostic tests to screen for active tuberculosis. *Int. J. Tuberc. Lung Dis.* **9:** 1120-1126.
63. Chan ED, Heifets L, Iseman MD. 2000. Immunologic diagnosis of tuberculosis: a review. *Tuber. Lung. Dis.* **80:** 131-140.
64. Schatz A, Elizabeth B, Waksman S. 1944. Streptomycin: a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. *Proceedings of the Society for Experimental and Biological Medicine* **55:** 66-69.
65. Gallo GG, Magg N, Pasqualucc CR, Ballott R, Sens P. 1966. Rifamycins. XLII. Rifazine, a new phenazinic derivative of rifamycins. *Farmaco Sci.* **21:** 168-175.
66. Youatt J. 1969. A review of the action of isoniazid. *Am. Rev. Respir. Dis.* **5:** 729-749.
67. 1976. Controlled trial of 4 short course (6 month) regimens of chemotherapy for treatment of pulmonary tuberculosis - Second trial. *Am. Rev. Respir. Dis.* **114:** 471.
68. Fox W, Mitchison DA. 1975. Short course chemotherapy for pulmonary tuberculosis. *Am. Rev. Respir. Dis.* **111:** 845-848.
69. 1979. Controlled trial of 6 month and 8 month regimens in the treatment of pulmonary tuberculosis: the result upto 24 months. *Tubercle* **60:** 201.
70. 1982. Controlled trial of four 3 times weekly regimens and daily regimen all given for six months for pulmonary tuberculosis, second report: the result upto 24 months. Tubercle **63:** 89.
71. 1987. Five year follow up of a controlled trial of 5 - 6 months regimens of chemotherapy for pulmonary tuberculosis. *Am. Rev. Respir. Dis.* **136:** 1339.
72. International Union against Tuberculosis and Lung Disease. 1988. Antituberculosis regimen of chemotherapy. Recommendation from the committee on treatment of IUATLD. *Bull. Int. Union. Tuberc. Lung. Dis*. **63:** 60-64.
73. Gbayisomore A, Lardizabal AA, Reichman LB. 2000. Update: prevention and treatment of tuberculosis. *Curr. Opin. Infect. Dis.* **13:** 155-159.
74. Zhang Y. 2005. The magic bullets and tuberculosis drug targets. *Annu. Rev. Pharmacol. Toxicol.* **45:** 529-564.
75. Chopra I, Brennan P. 1998. Molecular action of antimycobacterial agents. *Tuber. Lung. Dis.* **78**: 89-98.

76. Chouchane S, Lippai I, Magliozzo RS. 2000. Catalase-peroxidase (*Mycobacterium tuberculosis* KatG) catalysis and isoniazid activation. *Biochemistry* **39:** 9975-9983.
77. Banerjee A, Dubnau E, Quémard A, Balasubramanian V, Uma KS, Wilson, T, *et al*. 1994. *InhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. *Science* **263:** 227-230.
78. Rozwarski DA, Grant GA, Barton DHR, Jacobs Jr. WR, Sacchettini JC. 1998. Modification of the NADH of the isoniazid target (InhA) from *Mycobacterium tuberculosis*. *Science* **279:** 98-102.
79. Takayama K, Kilburn JO. 1989. Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis*. *Antimicrob. Agents Chemother.* **33**: 1493-1499.
80. Belanger AE, Besra GS, Ford ME, Mikusova K, Belisle JT, Brennan PJ, *et al*. 1996. The *embAB* genes of *Mycobacterium avium* encode an arabinosyl transferase involved in cell wall arabinan biosynthesis that is the target for the antimycobacterial drug ethambutol. *Proc. Natl. Acad. Sci.* USA. **93**: 11919-11924.
81. Telenti A, Philipp WJ, Sreevatsan S, Bernasconi C, Stockbauer KE, Wieles B, *et al*. 1997. The emb operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol. *Nat Med.* **3**: 567-570.
82. Alcaide F, Pfyffer GE, Telenti A. 1997. Role of embB in natural and acquired resistance to ethambutol in mycobacteria. *Antimicrob. Agents Chemother.* **41:** 2270-2273.
83. Marianelli C, Ciuchini F, Tarantino M, Pasquali P, Adone R. 2004. Genetic basis of rifampin resistance phenotype in *Brucella spp. J. Clin. Microbiol.* **42:** 5439-5443.
84. Onodera Y, Tanaka M, Sato K. 2001. Inhibitory activity of quinolones against DNA gyrase of *Mycobacterium tuberculosis. J. Antimicrob. Chemother.* **47:** 447-450.
85. Zhang Y, Wade MM, Scorpio A, Zhang H, Sun Z. 2003. Mode of action of pyrazinamide: disruption of *Mycobacterium tuberculosis* membrane transport and energetics by pyrazinoic acid. *J. Antimicrob. Chemother.* **52:** 790-795.
86. Datta M, Radhmani MP, Selvaraj R, Paramasivan CN, Gopalan BN, Sudeendra CR, *et al*. 1993. Critical assessment of smear-positive tuberculosis patients after chemotherapy under the district tuberculosis programme. *Tuberc. Lung. Dis.* **74:** 180-186.
87. Espinal MA. 1983. The global situation of MDR-TB. *Tuberculosis* **83**: 44-51.
88. Aziz MA, Wright A. 2005. The World Health Orgaization/International Union against Tuberculosis and Lung Disease global project on surveillance for anti-tuberculosis drug resistance: a model for other infectious diseases. *Clin. Infect. Dis.* **41**: S258–S262.

89. Pablos-Méndez A, Raviglione MC, Laszlo A, Binkin N, Rieder HL, Bustreo F, *et al*. 1998. Global surveillance for antituberculosis drug resistance, 1994-1997. *N. Engl. J. Med*. **338**: 1641-1649.

90. Zignol M, Hosseini MS, Wright A, Weezenbeek CL, Nunn P, Watt CJ, *et al.* 2006. Global incidence of multidrug-resistant tuberculosis. *J. Infect. Dis.* **194**: 479-485.

91. Spradling P, Drociuk D, McLaughlin S, Lee LM, Peloquin CA, Gallicano K, Pozsik C, *et al*. 2002. Drug-drug interactions in inmates treated for human immunodeficiency virus and *Mycobacterium tuberculosis* infection or disease: an institutional tuberculosis outbreak. *Clin. Infect. Dis.* **35:** 1106-1112.

92. Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC. 1999. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. *JAMA* **282**: 677-686.

93. 1999. Tuberculosis Elimination Revisited: Obstacles, Opportunities, and a Renewed Commitment. *MMWR*, 8/13/99.

94. Styblo K, Meijer J. 1976. Impact of BCG vaccination programmes in children and young adults on the tuberculosis problem. *Tubercle* **57:** 17-43.

95. WHO/UNAIDS. 1999. Preventive therapy against TB in people living with HIV. *WER* **74:** 385-400.

96. Jereb JA. 2002. Progressing Toward TB Elimination in Low-Incidence Areas of the U.S.: Recommendations of ACET. *MMWR*, 5/3/02.

97. Amberson JB. 1938. The significance of latent forms of tuberculosis. *N. Engl. J. Med*. **219**: 572-576.

98. Fox W, Ellard GA, Mitchison DA. 1999. Studies on the treatment of tuberculosis undertaken by the British Medical Research Council tuberculosis units, 1946–1986, with relevant subsequent publications. *Int. J. Tuberc. Lung. Dis.* **3:** S231–S279.

99. Ferebee SH. 1970. Controlled chemoprophylaxis trials in tuberculosis. A general review. *Bibl Tuberc.* **26:** 28-106.

100. Lucas SB. 1998. Histopathology in Clinical Tuberculosis. Davies PDO, Editor. Chapman and Hall: London, pp. 113-127.

101. Grange JM. 1998. Immunophysiology and immunopathology of tuberculosis, in Clinical Tuberculosis. Davies PDO, Editor. Chapman and Hall: London, pp. 129-152.

102. Stead WW, Lofgren JP. 1983. Does the risk of tuberculosis increase with old age? *J. Infect. Dis.* **147:** 951-955.

103. Canetti G, Sutherland I, Svandova E. 1972. Endogenous reactivation and exogenous reinfection: their relative importance with regard to the development of non-primary tuberculosis. *Bull. Int. Union Tuberc.* **47:** 116-34.

104. Cosma CL, Sherman DR, Ramakrishnan L. 2003. The secret lives of the pathogenic mycobacteria. *Annu. Rev. Microbiol.* **57:** 641-676.

105. Cosma CL, Humbert O, Ramakrishnan L. 2004. Superinfecting mycobacteria home to established tuberculous granulomas. *Nature Immunology* **5:** 828 - 835.

106. Loring WE, Vendiviere HM. 1956. The treated pulmonary lesions and its tubercle bacillus. I: pathology and pathogenesis. *Am. J. Med. Sci.* **232:** 20-29.

107. Vendiviere HM. 1956. The treated pulmonary lesions and its tubercle bacillus. I: The death and resurrection. *Am. J. Med. Sci.* **232:** 30-37.

108. Khomenko AG. 1987. The variability of *Mycobacterium tuberculosis* in patients with cavitary pulmonary tuberculosis in the course of chemotherapy. *Tubercle* **68:** 243-253.

109. Parrish NM, Dick JD, Bishai WR. 1998. Mechanisms of latency in *Mycobacterium tuberculosis*. *Trends Microbiol.* **6:** 107-112.

110. Corper HJ, Cohn ML. 1933. The viability and virulence of old cultures of tubercle bacilli. *Am. Rev. Tuberc*. **28:** 856-874.

111. Wayne LG. 1976. Dynamics of submerged growth of *Mycobacterium tuberculosis* under aerobic and microaerophilic conditions. *Am. Rev. Respir. Dis.* **114:** 807-811.

112. Wayne LG, Hayes LG. 1996. An in vitro model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of non-replicating persistence. *Infect. Immun.* **64:** 2062-2069.

113. Wayne LG, Lin KY. 1982. Glyoxylate metabolism and adaptation of *Mycobacterium tuberculosis* to survival under anaerobic conditions. *Infect. Immun.* **37:** 1042-1049.

114. Haapanen JH, Kass I, Gensini G, Middlbrook G. 1959. Studies on the gaseous content of tuberculous cavities. *Am. Rev. Resp. Dis.* **80:** 1-5.

115. Ulrich T, Kaufmann SHE. 2004. Cell mediated immune response, in tuberculosis. Rom WM, Garay SM, Editors. Lippincott Wiliams and Wilkins. Pheladelphia, pp. 251-262.

116. McCune Jr. RM, McDermott W, Tompsett R. 1956. The fate of *Mycobacterium tuberculosis* in mouse tissues as determined by the microbial enumeration technique. II. The conversion of tuberculous infection to the latent state by the administration of pyrazinamide and a companion drug. *J. Exp. Med.* **104:** 763-802.

117. Orme IM. 1988. Mouse model of the recrudescence of latent tuberculosis in the elderly. *Am. Rev. Respir. Dis.* **137:** 716-718.

118. Mehrotra J, Bishai WR. 2001. Regulation of virulence genes in *Mycobacterium tuberculosis*. *Int. J. Med. Microbiol.* **291:** 171-182.

119. Graham JE, Clark-Curtiss JE. 1999. Identification of *Mycobacterium tuberculosis* RNAs synthesized in response to phagocytosis by human macrophages by selective capture of transcribed sequences (SCOTS). *Proc. Natl. Acad. Sci. USA* **20:** 11554-11559.

120. Manganelli R, Dubnau E, Tyagi S, Kramer Smith FI. 1999. Differential expression of 10 sigma factor genes in *Mycobacterium tuberculosis*. *Mol. Microbiol.* **31:** 715-724.

121. Steyn AJ, Collin DM, Hondalus MK, Jacobs WR Jr, Kawakami RP, Bloom BR. 2002. *Mycobacterium tuberculosis* WhiB3 interacts with RpoV to affect host survival but is dispensable for *in vivo* growth. *Proc. Natl. Acad. Sci. USA* **5:** 3147-3152.

122. Bloch H, Segal W. 1956. Biochemical differentiation of *Mycobacterium tuberculosis* grown in vivo and in vitro. *J. Bacteriol.* **72:** 132-141.

123. McKinney JD, Honer Zu Bentrup K, Munoz -Elias EJ, Miczak A, Chen B, Chan WT, *et al.* 2000. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase *Nature* **406:** 735-8

124. Cunningham AF, Spreadbury C L. 1998. Mycobacterial stationary phase induced by low oxygen tension: cell wall thickening and localization of the 16-kilodalton alpha crystallin homolog. *J. Bacteriol.* **4:** 801-808.

125. Boon C, Dick T. 2002. *Mycobacterium bovis* BCG response regulator essential for hypoxic dormancy. *J. Bacteriol.* **184:** 6760-6767.

126. Voskuil MI, Schnappinger D, Harrell MI, Visconti KC, Dolganov GM, Sherman DR, Schoolnik GK. 2003. Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* persistence program. *J. Exp. Med.* **198:** 705-713.

127. Yuan Y, Crane DD, Simpson RM, Zhu YQ, Hickey MJ, Sherman DR, Barry CE 3rd. 1998. The 16-kDa alpha-crystallin (Acr) protein of *Mycobacterium tuberculosis* is required for growth in macrophages. *Proc. Natl. Acad. Sci. USA* **95:** 9578-9583.

128. Glickman MS, Cox JS, Jacobs WR Jr. 2008. A novel mycolic acid cyclopropane synthetase is required for cording, persistence and virulence of *Mycobacterium tuberculosis. Mol. Cell* **5:** 717-27.

129. Camacho LR, Ensergueix D, Perez E, Gicquel B, Guilhot C. 1999. Identification of a virulence gene cluster of *Mycobacterium tuberculosis* by signature-tagged transposon mutagenesis. *Mol. Microbiol.* **34:** 257-267.

130. Murphy DJ, Brown JR. 2008. Novel drug target strategies against *Mycobacterium tuberculosis*. *Curr. Opin. Microbiol. (In press).*

CHAPTER 2

Investigation of Nitrate Metabolic Pathways for Their Presence, Function and Essentiality during Active and Dormant Stage Survival of *M. Smegmatis, M. Bovis* BCG and *M. Tuberculosis* H37Ra

2.1. Introduction

It is largely unknown how the bacteria survive during the latent stage of infection. Following initial infection, the bacilli typically replicate inside host macrophages until an effective immune response is mounted and the bacilli become restricted to the characteristic tuberculous lesions and the progression of the disease is halted. The bacillus can survive in the caseous necrotic centre of these lesions, but it apparently cannot multiply because of oxygen deprivation and other adverse conditions. It is within this anaerobic environment of the caseous necrotic material that bacterial dormancy probably occurs (1). There is a long-standing interest in the concept that adaptation to an anaerobic microenvironment represents an important stage of latency in the disease.

As all mycobacteria are obligate aerobes, the availability of oxygen seems crucial, yet granulomas and abscesses where these non-replicating bacilli generally reside are believed to be deprived of oxygen (2). Even in the lung, oxygen appears to be limited: super infection with obligatory anaerobic organisms, indicating an anaerobic environment, can occur in cavities of pulmonary tuberculosis (3).

The recent determination of the complete genome sequence of *M. tuberculosis* provided a tremendous opportunity for investigating molecular mechanisms of latency (4). It turned out that within the *M. tuberculosis* genome, genes homologous to the anaerobic nitrate reductase (NR) of *Bacillus subtilis* were present (5).

This anaerobic NR activity is encoded by four genes, *narGHJI*, clustered together in an operon. NarG, H and I are subunits of NR, with NarG probably being the catalytic subunit, whereas NarJ is required for the assembly of the enzyme (Fig. 2.1). NarGHJI, through nitrate respiration, could provide energy for mycobacterial metabolism during their latent stage in an anaerobic environment, because it couples the reduction of nitrate (NO_3) to the generation of ATP in absence of oxygen as a terminal electron acceptor (Fig. 2.2) (6).

Previous studies of mycobacterial nitrate reduction have been limited to its role in classification and identification of the genus *Mycobacterium*, after an extensive study 40 years ago showed that *M. tuberculosis* reduces nitrate to nitrite, whereas *M. bovis* and *M. bovis* BCG have no discernible NR activity (7,8). A single nucleotide polymorphism within the promoter of *narGHJI* operon was later on found responsible for the decreased NR activity in *M. bovis* (9). However, a role of this enzyme in virulence was not established in mycobacteria. Only recent reports revived interest in a possible physiological role by showing upregulation of enzymes involved in nitrate metabolism of *M. tuberculosis* and *M. bovis* BCG under oxygen restriction *in*

vitro (10,11). Some evidences of dependence on NarGHJI were also provided when immunodeficient SCID mice infected with *M. bovis* BCG *narG* mutant showed smaller granulomas with fewer bacteria than those infected with the wild-type strain (12). The mutant although produced tissue damage in the lungs of immunocompetent mice but was cleared from many organs, unlike the wild-type strain (13).

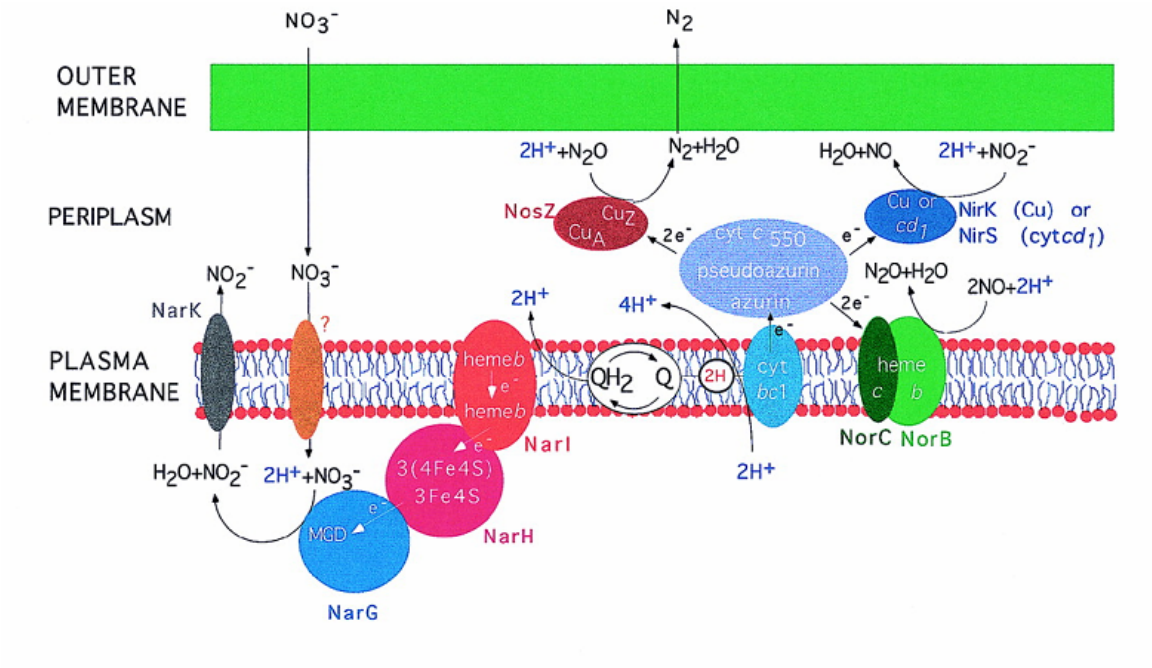


Fig. 2.1 The organization of anaerobic electron transport chain for nitrate respiration by NR (NarGHJI) in bacteria.

Nitrate reductase (NarGHJI)

$$N0_3^- + ADP + Pi + 2e^- + 2H^+ \longrightarrow N0_2^- + H_20 + ATP$$

Fig. 2.2 Generation of ATP by reduction of nitrate to nitrite through NR (NarGHJI).

It was especially interesting that despite the increase in activity of whole cells of *M. tuberculosis* exposed to hypoxic conditions, NR did not appear to support actual anaerobic growth of this species. Instead, it shifts down to the state of non-replicating persistence as microaerobic conditions develop. Shiftdown appears to be an orderly process, and the cessation

of replication also appears to be part of the cell's adaptation to hypoxia rather than simply energy starvation (14). Since, *M. tuberculosis* does not grow under anaerobic condition, there may be no requirement for induction of NarGHJI. The primary role for NR in *M. tuberculosis* could be redox balancing, or it may serve only a temporary function to provide energy during shiftdown to NRP.

Apparently nitrate as a substrate for this enzyme could also be sufficiently provided in the lungs, liver, and kidneys of infected individuals. The reported estimates of net nitrate synthesis by mammalian tissue vary greatly and range from 0.15 to 1mM day^{-1} (15). Within tissue, nitrate is mainly a product of spontaneous degradation of nitric oxide. Nitric oxide, in contrast, is produced enzymatically by three different nitric oxide synthetases (16). An inducible nitric oxide synthetase is expressed in response to inflammatory and proinflammatory mediators (17). A variety of cells, including hepatocytes, can be induced to synthesize nitric oxide (18). Significant amounts of nitrate are detected in the urine of mice infected with bacteria, suggesting that nitrate is available in the kidney, especially in animals undergoing an inflammatory process (19). It is intriguing to speculate that the inflammatory process due to mycobacterial infection in the lungs, liver, and kidneys might increase the amount of nitrate within granulomas and thereby provide an additional supply of this nutrient for anaerobic metabolism of the pathogen.

Also identified in the *M. tuberculosis* genome during sequencing was a gene designated *narX*, which has been proposed to code for a "fused nitrate reductase" (20). This proposal was made because the predicted product of *narX* would be a protein with homology to parts of the NarG, NarJ, and NarI proteins, although its actual function is unknown. Though, there are two sets of genes in *M. tuberculosis*, *narGHJI* and *narX*, that show homology with prokaryotic NR genes, but only *narGHJI* was found responsible for nitrate reducing activity in culture. This was proved when insertional inactivation of this locus eliminated the production of nitrite, and this activity could be restored by complementation with a plasmid-borne copy of the genes. Insertion in *narX* had no effect on the reduction of nitrate. Consequently, it was also known that the increase in NR activity in hypoxic culture was due not to induction of *narGHJI* but to increased levels of the nitrate and nitrite transporter *narK2* gene present in the *narK2X* operon (21).

Unexpectedly, transcription of both *narGHJI* and *narK2X* were independent of nitrate and nitrite levels. NR activity in *M. tuberculosis* appears to be independent of the substrate concentration as determined by levels of mRNA and assays of cell extracts (11). To be reduced, nitrate must enter the cell where the catalytic site of the enzyme is located. Subsequently, since *M. tuberculosis* is unable to reduce nitrite, which could accumulate to toxic levels, it must then be exported out of the cell. Early work in *E. coli* had suggested that *narK* was involved only in

nitrite export, and so the homologous *narK2* in *M. tuberculosis* was annotated as a "nitrite extrusion protein" (11,22). More recent work with an *E. coli narK narU* double mutant indicated that the two proteins could transport nitrate into and nitrite out of the cell (23). Later on it was shown that *M. tuberculosis narK2* can complement this *E. coli* double mutant, supporting a role for *narK2* in nitrate reduction by coding for a transporter of nitrate into and nitrite out of the cell (21). *M. tuberculosis* RVW3 *narK2*::*aphI*, which lacks the nitrate and nitrite transporter, behaved like the wild-type strain in its NR activity under aerobic conditions. This low level of activity reflects the low rate of diffusion of nitrate into the cell, and this conclusion is supported by evidence that the rate of nitrate reduction by *M. tuberculosis* under aerobic, but not hypoxic, conditions is proportional to the nitrate concentration in the medium. During shiftdown to hypoxic NRP-1, NR activity levels of RVW3 *narK2*::*aphI* lacked the strong induction seen in the wild type but instead continued at aerobic levels. This indicates that NarK2 is responsible for the hypoxic rise in activity by transporting nitrate into the cell.

Eventually to summarize from all the above pieces of evidence which led us to believe that there must be some distinct role of Nitrate metabolic pathways in mycobacterial latency, were, (i) increased NR activity of *M. tuberculosis* during hypoxic shiftdown to dormancy; (ii) correlation of NR activity and virulence between *M. bovis* BCG and *M. tuberculosis* due to SNP in NarGHJI promoter; (iii) smaller granuloma and fewer number of bacilli in the lungs of SCID mice infected with *M. bovis* BCG mutated for NarG compared to wild type strain; (iv) tissue specific dependence of *M. bovis* BCG on NarGHJI in mice; (v) low oxygenic environment in granuloma and abscesses (vi) evidences of availability of nitrate in the tissues where persistent bacilli reside.

These evidences prompted us to investigate the pathways related to Nitrate metabolism especially during the dormant stage survival of mycobacteria. Our major objective of this chapter was to dissect the presence, function and essentiality of enzymes related to nitrate metabolism in *M. smegmatis*, *M. bovis* BCG and *M. tuberculosis* H37Ra. Our findings on nitrate metabolic pathways of these three species have been described in detail in this chapter.

2.2. Results

2.2.1. Identification of a respiratory-type nitrate reductase in *M. smegmatis* and its role for survival during *in vitro* hypoxia induced dormancy

M. smegmatis has a genome size of 6.98 Mb, which is 1.6 times larger than that of *M. tuberculosis* (4,24,25). In spite of the difference in genome size, *M. smegmatis* has been commonly employed as model system to study the metabolism and physiology of the pathogenic *M. tuberculosis*. There are several reasons for use of *M. smegmatis* as a model system for studying physiology of pathogenic mycobacteria. It can be easily cultured in artificial media. It has a much faster generation time as compared to *M. tuberculosis* and also does not require the use of sophisticated containment facilities such as biological safety level -3 (BSL-3) laboratories. The genome of the organism has also been found to contain homologues to multiple virulence genes of *M. tuberculosis* providing further validation to use the *M. smegmatis* as a model system to study *M. tuberculosis.* Besides that *M. smegmatis* also follows similar pattern of oxygen depletion induced dormancy in Wayne model as *M. tuberculosis* and hence can be considered to be as a useful model organism to study the dormancy (26). The Wayne model of dormancy is an *in vitro* cultivation method of dormant tubercle bacilli and is one of the most accepted models among the few models, having close resemblance with the *in vivo* latent stage of tuberculosis in the host tissues. In this model, culture is gradually shifted to anaerobic condition in a sealed tube and when conditions become anaerobic, organism stops multiplying (27). In fact, Wayne model has broadened the scope of developing a thorough understanding about the metabolic pathways operating at anaerobic dormant stage in all mycobacterial species including *M. smegmatis*. There were evidences of the presence and induction of respiratory NR in this *in vitro* dormancy model in *M. tuberculosis* and *M. bovis* BCG. However, the enzyme has not been studied in *M. smegmatis* so far. Since *M. smegmatis* also follows the similar pattern of dormancy, it was tempting to speculate that the similar kind of respiratory NR could perhaps be present in this organism. Therefore we first examined the nitrate reduction capabilities of *M. smegmatis* and identified a respiratory type of NR in this saprophytic organism as evidenced by following observations.

NR activity in aerobic and anaerobic dormant culture of *M. smegmatis.* The essential requirement for these kinds of studies is the availability of a defined medium where comparable growth of the organism/s under study could be seen with respect to changes in composition of the

medium. Most of the media used for growth of *Mycobacterium sp.* are always complex in nature. Here, whole study was carried out using a minimal medium. The growth of *M. smegmatis* in this medium under aerobic condition was comparable with other complex media (Fig. 2.3A) (26). The patterns of growth in Wayne's dormancy model were also comparable with that obtained in complex medium. The stage of non replicating dormancy could be clearly seen after certain time in the Wayne's culture system due to the cessation of oxygen where the organism stopped multiplication but remained viable for extended period of time.

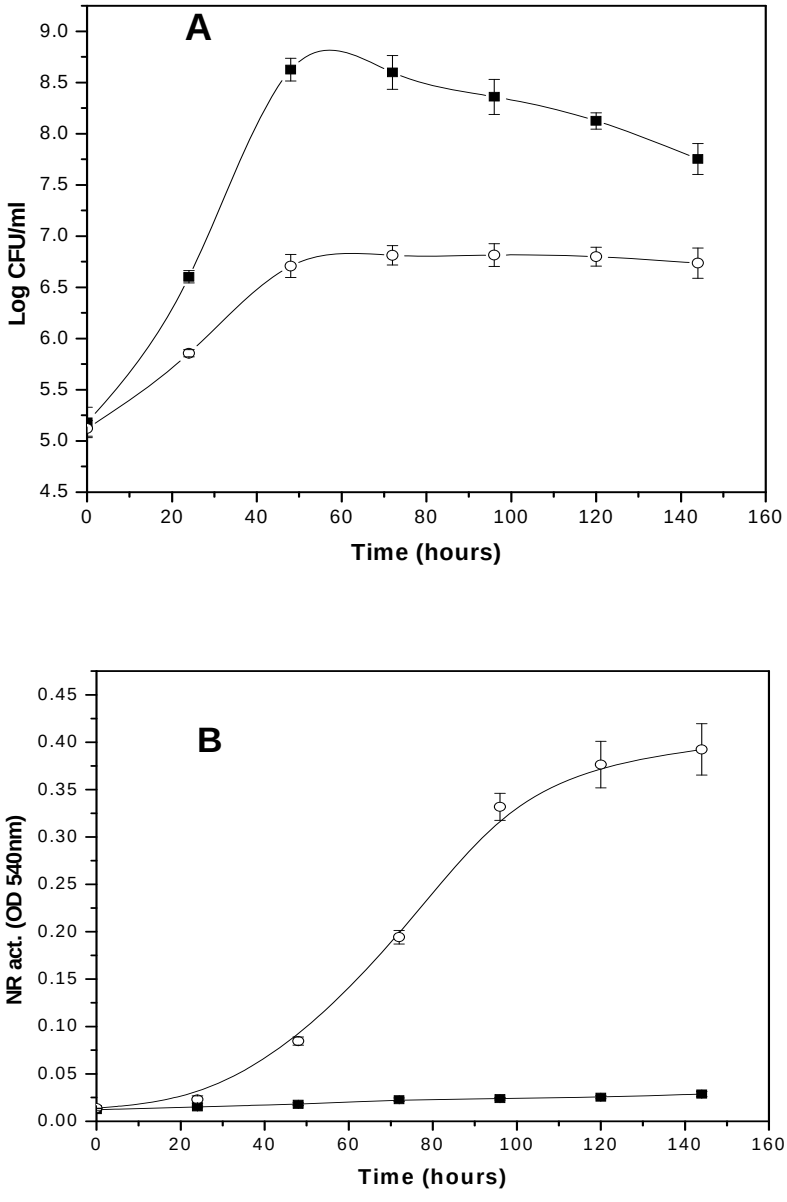


Fig. 2.3 Growth and nitrate reduction in aerobic and Wayne dormancy culture system of *M. smegmatis.* A) Growth of *M. smegmatis* monitored in aerobic (■) and Wayne's dormancy model (○) in minimal medium

in presence of 10mM nitrate. B) Nitrate reduction by whole cell culture of *M. smegmatis* monitored in aerobic (■) and in Wayne's dormancy model (○). Other experimental details are described in materials and method section of this chapter. Experiments were carried out more than three times and results are mean ± SD.

Once it was established that *M. smegmatis* could grow and follow the Wayne's *in vitro* model of dormancy in minimal medium, the organism was examined for its capabilities of reduction of nitrate into nitrite. Nitrate was added in the medium that the organism is supposed to utilize as alternate electron acceptor and nitrite, which is the end product of the enzyme's reaction was estimated in the whole cell of aerobic active as well as anaerobic dormant stage of *M. smegmatis*. The extent of conversion of nitrate to nitrite was an indication of the level of dependence of the cell on the alternate electron transport chain during that stage. The result showed that there is no significant conversion of nitrate to nitrite in aerobic culture of *M. smegmatis* (Fig. 2.3B). Significant NR activity was seen in Wayne culture in spite of having less viable cells in it. These results indicated the presence of a NR in *M. smegmatis*, whose activity is induced in anaerobic dormant stage. Increased NR activity could also be due to increased transport of nitrate inside the cell and/or due to an increase in electron flow to NR under hypoxic condition. The anaerobiosis based induction of the NR activity implied the respiratory nature of the enzyme as well.

Inhibition of NR activity by azide and thiocyante confirmed the respiratory nature of the enzyme. Earlier reports indicated about using sodium chlorate, sodium thiocyanate and sodium azide as inhibitors of anaerobic NR in prokaryotes (28). Azide is a competitive inhibitor of NR with respect to nitrate. By chelating free molybdenum ion from the medium, thiocyanate inhibits NR activity. On the other hand, chlorate inhibits nitrate reduction by competing with nitrate and gets reduced preferentially by the enzyme. End product chlorite leads to the death of bacterial cells through nonspecific manner (29). In order to validate the type of this NR, these inhibitors were examined for their effect on nitrate reduction by whole cell as well as cell free lysate of *M. smegmatis*. These inhibitors were applied at concentrations where no significant effect on growth was observed (Fig. 2.4).

The conversion of nitrate to nitrite was inhibited more than 95 percent by azide and thiocyanate in Wayne's dormant whole cell culture of *M. smegmatis* as well as in cell free lysate prepared from the same culture (Table 2.1). Chlorate could inhibit 67 percent of nitrate reduction in whole cell and 10 percent in cell free lysate with respect to the control value. This result further strengthened the assumption that the conversion of nitrate to nitrite is due to respiratory

type of NR. Apart from using above inhibitors, MTZ and NIT were used in this study as standard dormant stage-specific antitubercular molecules to compare the results (30,31). None of them could exert any significant inhibitory effect on the conversion of nitrate to nitrite in whole cell culture and cell free lysate which indicated that NR should not be the target of these molecules.

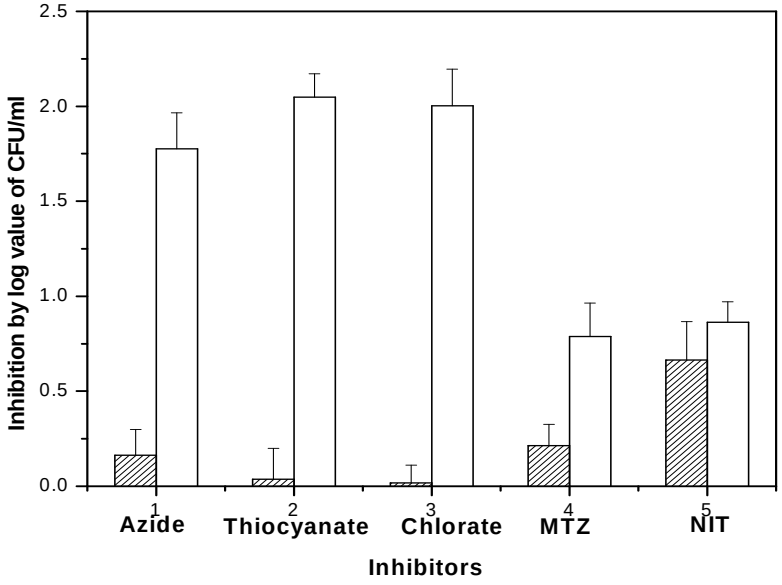


Fig. 2.4 Effect of NR inhibitors, antidormant tuberculosis molecules on viability of *M. smegmatis* during aerobic and Wayne's dormant culture condition. 0.05mM azide, 20mM thiocyanate, 20mM chlorate, 2mM metronidazole (MTZ) and 0.3mM nitrofurantoin (NIT) were added during inoculation of aerobic (dark bars) and Wayne's dormant culture (light bars). The viable counts were taken at 120hours of incubation. Inhibitions were calculated by subtracting log values of control. Experiments were carried out more than three times and results are mean ± SD.

Table 2.1 Effect of inhibitors on nitrate reduction by Wayne's dormant whole cell culture of *M. smegmatis* and its cell free lysate.

Inhibitors[a]	% inhibition of nitrate reduction in whole cell culture	% inhibition of nitrate in cell free lysate[b]
Azide (0.05mM)	99.34 ± 1.89	98.45 ± 3.18
Thiocyanate (20mM)	97.86 ± 2.67	96.74 ± 1.98
Chlorate (20mM)	67.25 ± 4.16	10.22 ± 1.2
MTZ (2mM)	06.67 ± 0.51	Nil
NIT (0.3mM)	19.47 ± 1.11	Nil

[a], inhibitors were added at the time of inoculation.

[b], 96hours old Wayne culture was used to prepare cell free lysate.

Importance of Nitrate reductase for survival of hypoxic dormant bacilli of *M. smegmatis.* In order to examine the importance of this NR for survival of *M. smegmatis* during the dormant stage, the viability of cells in Wayne's dormancy model was also checked in presence of the above mentioned inhibitors. The inhibitors were added in the culture at the time of inoculation and experiment was terminated at 120hours of incubation. It was observed that the cell number was reduced by 1.75, 2.0l and 1.98logs respectively when azide, thiocyanate and chlorate were used at concentrations of 0.05mM, 20mM and 20mM respectively (Fig. 2.4). At these concentrations azide, thiocyanate had a negligible effect of viability of aerobic stage of *M. smegmatis*. The result indicated that the growth was inhibited possibly because of very specific effect of inhibitors on anaerobic NR. Along with those specific inhibitors of NR, anaerobic stage specific inhibitors like MTZ and NIT was also used to understand if they have any effect on the same. MTZ reduced the number of cells in aerobic and Wayne's model by 0.2log and 0.75log respectively. Under similar condition, NIT reduced the cell number by 0.65log and 0.8log respectively. The result indicated that the effect of MTZ and NIT was to some extent independent of either aerobic or anaerobic stage of the bacterium.

In order to get an idea about the phase where azide, thiocyanate and chlorate were most active in Wayne's model, the growth and viability was monitored at every 24hours interval. Initially, growth was seen in all the cultures but the viability reduced with time more drastically after ~48hours of inoculation in those cultures where NR specific inhibitors were applied (Fig. 2.5A). Initial growth seen was due to the availability of dissolved O_2 in the medium and the inhibitors were not affecting their growth at that stage. In order to remain viable at anaerobic stage, the dependence of culture increased on NR, it failed to grow in presence of the inhibitors. This was clearly observed when NR activity was also monitored in same culture samples (Fig. 2.5B). In presence of azide and thiocyanate, NR activity remained at insignificant level. Chlorate inhibition was increased upto 65 percent at the termination of the experiment. This clearly indicated that NR activity plays an important role for the viability of *M. smegmatis* during anaerobic shift down in Wayne's model.

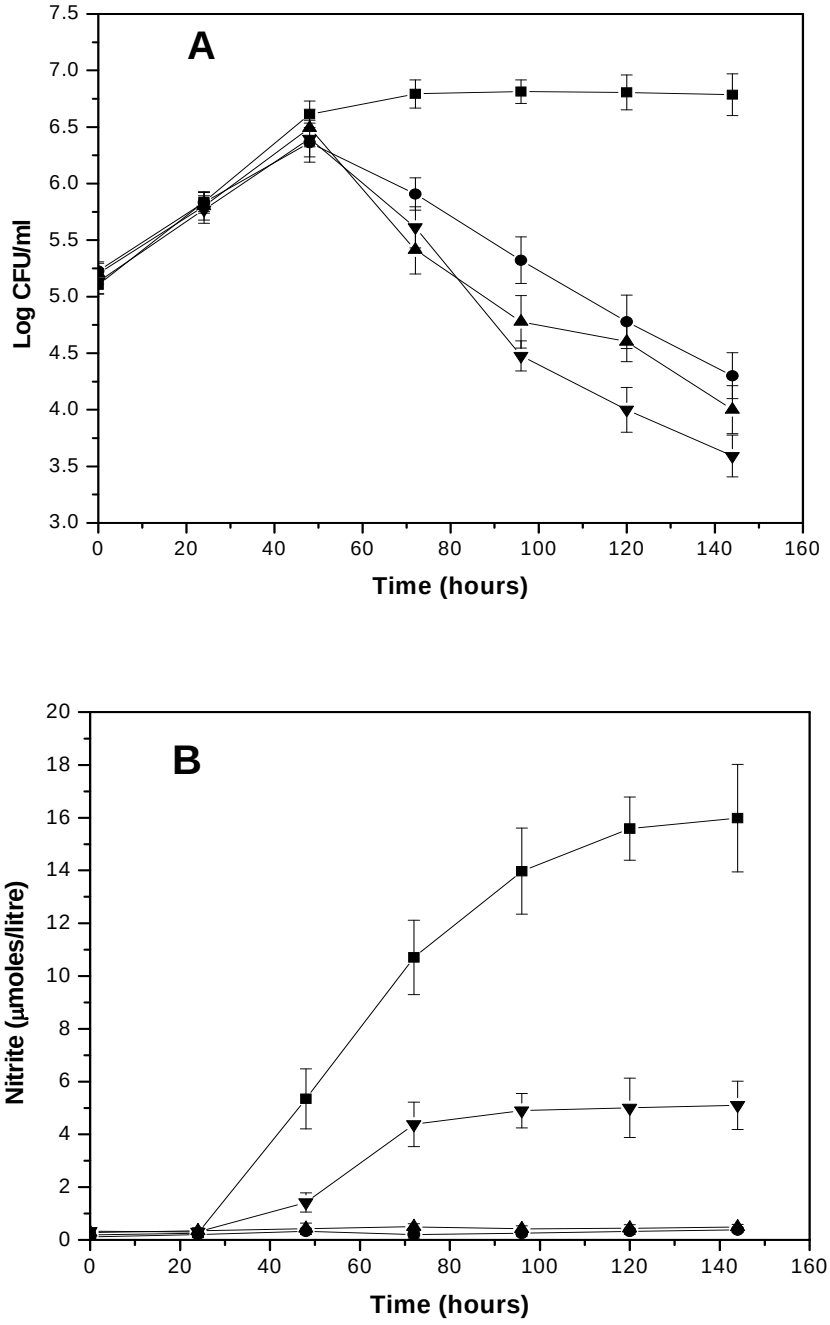


Fig. 2.5 Kinetics of inhibition on nitrate reduction and viability of *M. smegmatis* in Wayne's model by NR inhibitors. 0.05mM of azide (●), 20mM thiocyanate (▲), 20mM chlorate (▼), none (■) were added during inoculation. A) Nitrate reduction and B) viable cell counts were monitored in presence of the inhibitors. The detail of the experiment is described in 'materials and methods' of this chapter. Experiments were carried out more than three times and results are mean ± SD.

In order to find out more precisely the phase in Wayne's model where inhibition of NR has maximum impact on the viability of *M. smegmatis,* inhibitors were added after every 24hours of inoculation. The experiment was terminated at 120hours and the viable counts were taken. Almost similar effect on the viability of *M. smegmatis* was observed when the inhibitors were added at 0 and 96hours (Fig. 2.6). Viable cell count was reduced by ~2 to 2.4logs value in presence of thiocyanate and chlorate compared to the control. Azide reduced the viable count by ~1.75logs value compared to the control. This clearly indicated the dependence of *M. smegmatis* on nitrate reduction not only during its transition to dormant stage but also at the stages of dormancy afterwards. This is evident from the data that interference of NR led to drastic killing of the bacilli even when added at 96hours when the organism had already reached to dormant stage. MTZ reduced the viable count only by 0.7log value at the same time period. NIT has more effect on the viability of aerobic cells than anaerobic cells at the concentrations used. The results also indicated that NR inhibitors had superior inhibitory impact on the viability of anaerobic bacilli compared to MTZ and NIT under experimental condition mentioned.

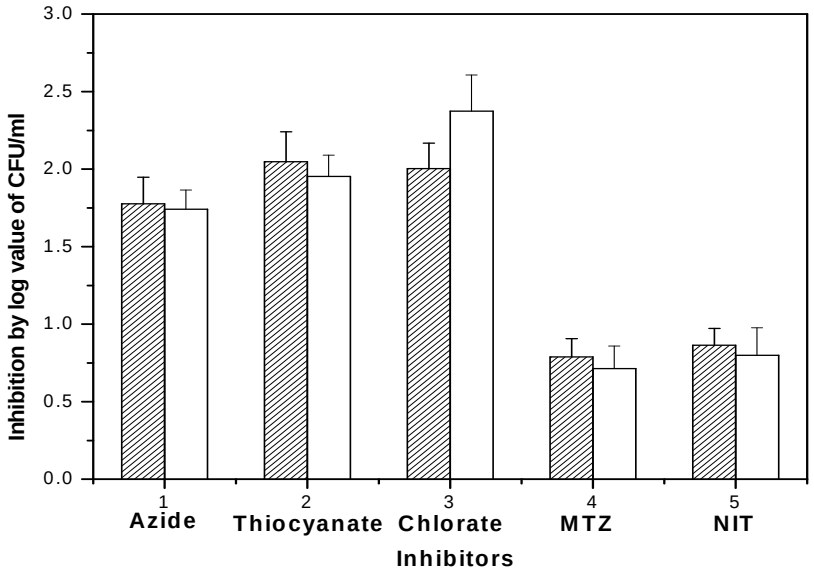


Fig. 2.6 Effect of NR inhibitors and antidormant tuberculosis molecules on the viability of *M. smegmatis* when added at different stages in Wayne's dormancy model. Inhibitors were added at 0hours (dark bars) and 96hours (light bars) into the tubes with *M. smegmatis* culture following Wayne's model terminating simultaneously at 120hours after inoculation and viability were checked as described in 'materials and methods' section of this chapter. Experiments were carried out more than three times and results are mean ± SD.

2.2.2. Presence of a functional nitrate assimilation pathway in *M. smegmatis*

While identifying the respiratory NR in the above mentioned studies it was observed that even though nitrate reduction reaches a plateau after certain period of time in Wayne's dormancy model, the bacilli were still dependent on NarGHJI for their survival. This indicated that nitrite could be subsequently reduced either for assimilation or respiration purpose. Following of *in vitro* dormancy system with added advantage of its non-pathogenic and fast growing nature allowed us to use *M. smegmatis* again as a model organism for this kind of study. Use of a minimal medium where a single nitrogen source could be added at a time according to the experimental requirement provided advantage to study the complete nitrate metabolism. In this section, the capability of nitrate assimilation of *M. smegmatis* were investigated by using three substrates mentioned as sole nitrogen source along with the use of respective inhibitor of the enzymes involved in the pathway.

Growth of *M. smegmatis* in aerobic culture in presence of nitrate/nitrite or ammonia as sole nitrogen source. Assimilation of nitrate in bacteria involves a multistep pathway, which requires a series of enzymes functioning in combination (Fig. 2.7) (32). Generally, nitrate is first converted into nitrite with the help of a NR, which subsequently is converted into ammonia by nitrite reductase (Nir). Ammonia is normally taken up into glutamine via glutamine synthetase (GS). In bacterial system, this nitrogen could then be distributed for various cellular requirements of nitrogen through glutamine 2-oxoglutarate amidotransferase (GOGAT) (33).

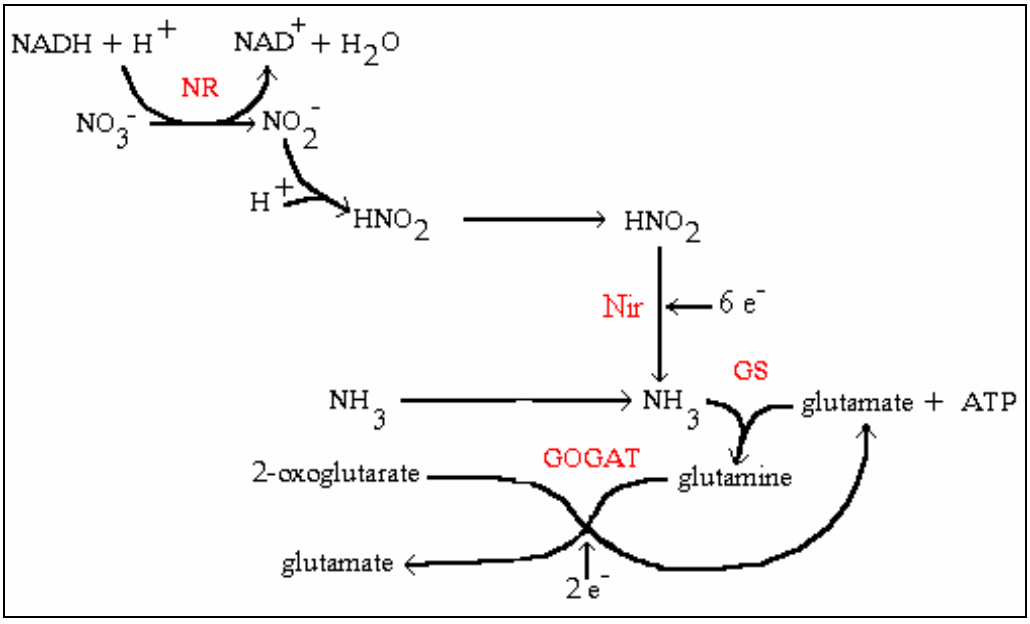


Fig. 2.7 Biochemical pathway of nitrate assimilation through NR (Nitrate reductase), Nir (Nitrite reductase) and GS (Glutamine synthetase) in bacterial system.

In this study, all the experiments were carried out using a minimal medium called as *M. phlei* medium, which normally contain asparagine as sole nitrogen source (37). In order to assess nitrate assimilation pathway in *M. smegmatis*, the growth in presence of nitrate, nitrite and ammonia, and their utilization were monitored using them as sole nitrogen source (Fig. 2.8). In presence of nitrate (10mM) and ammonia (10mM) as sole nitrogen source, *M. smegmatis* grew as good as it grew in a medium with asparagine as sole nitrogen source (Fig. 2.8A). The growth of *M. smegmatis* in presence of nitrite (0.5mM) was proportional to its availability in the medium. Higher concentration of nitrite exerts toxic effect on the bacilli (data not shown). The rate of depletion of nitrate, nitrite and ammonia were almost comparable with growth of the bacilli, which indicates that they were utilized for the growth of the organism (Fig. 2.8B). 10mM of nitrate and 0.5mM of nitrite was completely used up in 144hours whereas 10mM ammonia was depleted in 96hours. These results indicated that this saprophytic bacillus must contain all the enzymes required for assimilation of nitrate. It was also observed that the traces of nitrite and ammonia were not detected in the culture when nitrate was used as sole nitrogen source and similarly ammonia was absent in culture when nitrite was used as nitrogen source (data not shown). It thus indicated that all these three nitrogen sources of the pathway when used separately would be channelised for assimilation purpose in tandem with growth requirements. All these evidences well indicated the presence of functional nitrate assimilation pathway in *M. smegmatis*.

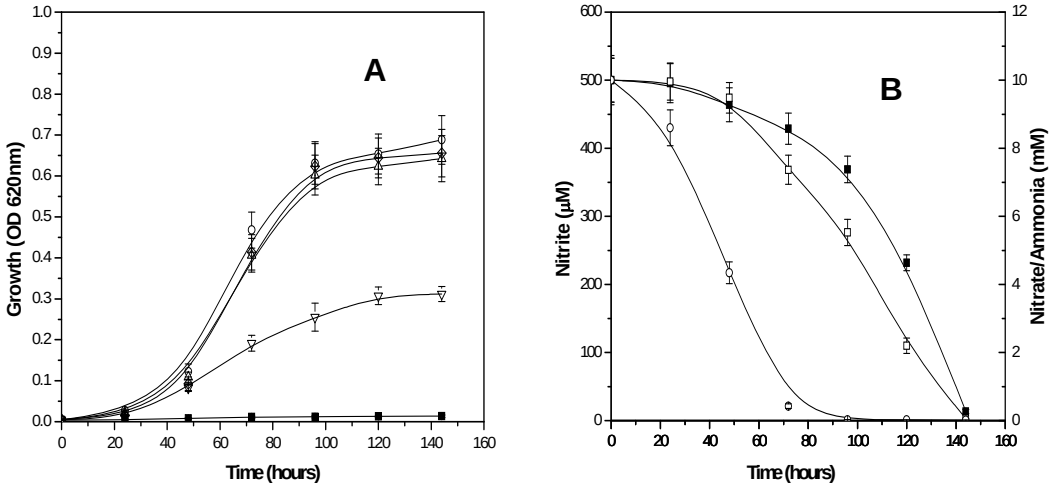


Fig. 2.8 (A**)** Growth of *M. smegmatis* in presence of 34mM asparagine (○), 10mM sodium nitrate (Δ), 500µM sodium nitrite (∇) and 10mM ammonium chloride (◊) and none (■) during aerobic culture condition. (B) Utilization of nitrate (■), nitrite (□) and ammonia (○) by *M. smegmatis* with time during aerobic culture condition. The experiments were carried out more than three times and results are mean ± SD.

Effect of azide and L-methionine sulfoxamine (L-MSO) on growth and nitrate/nitrite/ammonia utilization. It was earlier known that azide inhibits prokaryotic assimilatory NR (28). In order to check the assimilation of nitrate catalyzed by a respective NR in the bacilli, sodium azide was applied in the culture (Fig. 2.9A). At 50µM concentration, sodium azide did not affect the growth of *M. smegmatis* in presence of asparagine as nitrogen source. At same concentration of azide, there was no growth observed in *M. smegmatis* culture in presence of nitrate as sole source of nitrogen. The effect of azide on growth was also not seen when nitrite and ammonia were used as sole nitrogen source. As expected, azide stopped the depletion of only nitrate and not of nitrite and ammonia in the culture (Fig. 2.9A). This evidence proved that conversion of nitrate to nitrite occurred specifically by a NR. Since a respiratory NR is also present in *M. smegmatis,* it was necessary to identify the type of NR involved in the conversion of nitrate to nitrite. In order to find this, a more specific inhibitor thiocyanate (40mM), which inhibits the respiratory NR under such condition, was found to remain ineffective on growth as well as on nitrate reduction (data not shown). This result confirmed that conversion of nitrate to nitrite was catalyzed here by an assimilatory type NR. The genome database of *M. smegmatis* was also found to have *narB* (Gene Accession Number YP_887157) gene, which might function as assimilatory NR.

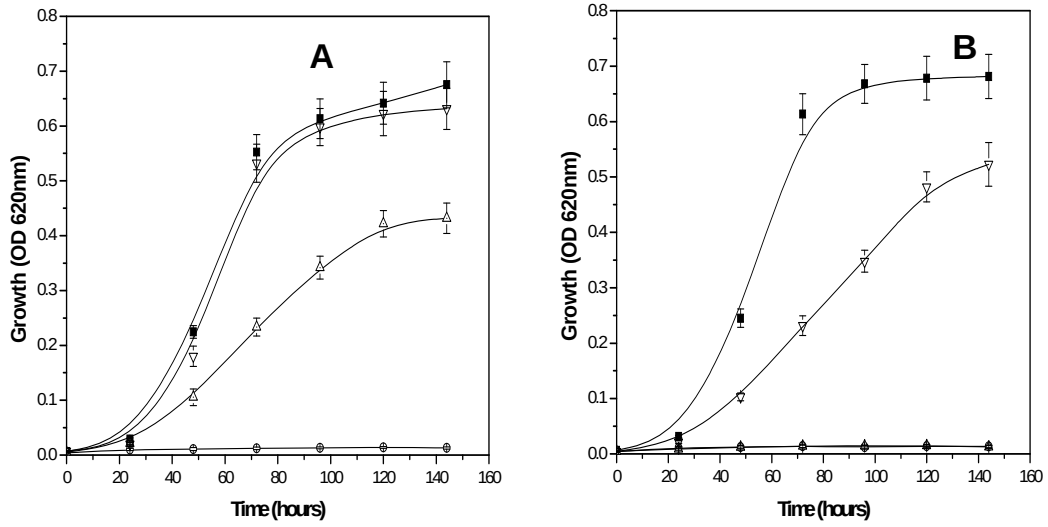


Fig. 2.9 Effect of (A) Azide (50µM) and (B) L-MSO (10µg/ml) on growth of *M. smegmatis* in aerobic culture condition with 34mM asparagine (■), 10mM sodium nitrate (○), 500µM sodium nitrite (Δ) and 10mM ammonium chloride (∇) as sole nitrogen source. The results are the mean ± SD of three identical experiments.

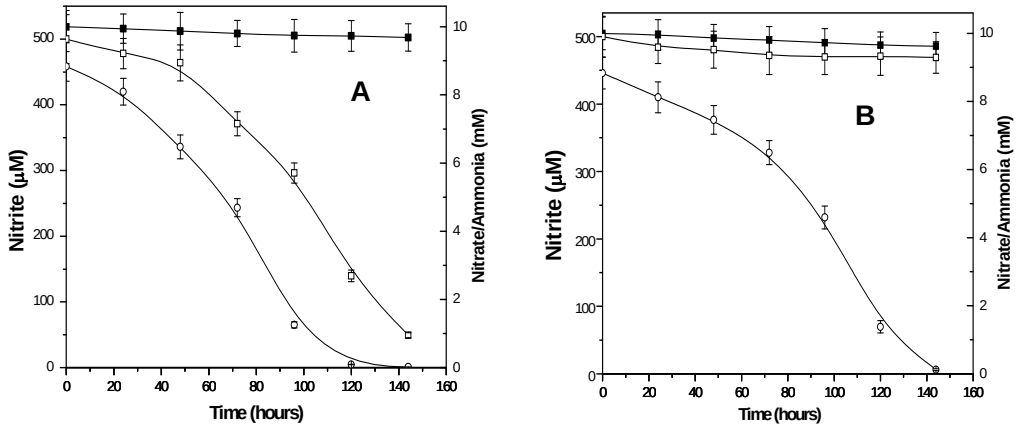


Fig. 2.10 Effect of (A) Azide (50µM) and (B) L-MSO (10µg/ml) on utilization of sodium nitrate (■), sodium nitrite (□) and ammonium chloride (○) as sole nitrogen source by *M. smegmatis* in aerobic culture condition. The results are the means ± SD of three identical experiments.

In step 3 of nitrate assimilation pathway, ammonia reacts with glutamate in presence of ATP and metal ion Mg^{2+} to produce glutamine, ADP and inorganic phosphate in the course of its assimilation. This biosynthetic reaction is catalyzed by glutamine synthetase (GS) (34). L-

methionine sulfoximine (L-MSO), which is a well-known inhibitor of glutamine synthetase, was used here to more precisely elucidate this assimilation pathway (35). There was no effect of L-MSO on growth of *M. smegmatis* observed in medium with asparagine as sole nitrogen source whereas 10μg/ml of L-MSO completely inhibited the growth of *M. smegmatis* when grown in medium with nitrate/nitrite as sole nitrogen source (Fig.2.9). Utilization of nitrate/nitrite from the medium was not seen in the culture as well in presence of L-MSO, which indicated direct involvement of glutamine synthetase in nitrate assimilation process (Fig. 2.10). Unexpectedly, L-MSO did not show any effect on growth of *M. smegmatis* grown in medium with ammonia as sole nitrogen source and depletion of ammonia was also seen to occur with time, indicating that ammonia could be utilized by other enzymes in the pathway. Glutamate dehydrogenase (GDH) has already been reported to bypass the function of glutamine synthetase when higher concentration of ammonia is available in the medium (36). Possibility of the conversion of ammonia into glutamine by a thermolabile glutaminase (EC 3.5.1.2) again could not be denied, as the gene MSMEG_3818 encoding for this enzyme (Gene Accession Number YP_888111) is also present in *M. smegmatis* Genome. Altogether these results confirmed that nitrate assimilation pathway is functional in *M. smegmatis* and inhibition of glutamine synthetase could affect the growth of the bacilli during the assimilation of nitrate/nitrite.

Utilization of nitrate, nitrite and ammonia by *M. smegmatis* in Wayne's *in vitro* dormancy model. The evidences discussed above indicated the presence of nitrate assimilation pathway in this saprophytic mycobacterium during its growth in aerobic culture. The role of this pathway in dormant culture could provide a better insight to understand the nitrogen metabolism operative in latent phase. In this course organism's growth with nitrate, nitrite and ammonia utilization was monitored in Wayne's *in vitro* dormancy model. Interestingly, it was noticed that the depletion of nitrate, nitrite and ammonia did not stop in non-replicating phase (Fig. 2.11). Rate of utilization of nitrate, nitrite and ammonia were found 289μM, 25μM and 354μM per day respectively in this non-replicating stage of the bacilli which was almost 5-8times less than the rate of utilization seen in actively replicating stage (Table 2.2). These results indicated that assimilation of nitrate/nitrite/ammonia, even though at a slow rate, continued in dormant phase.

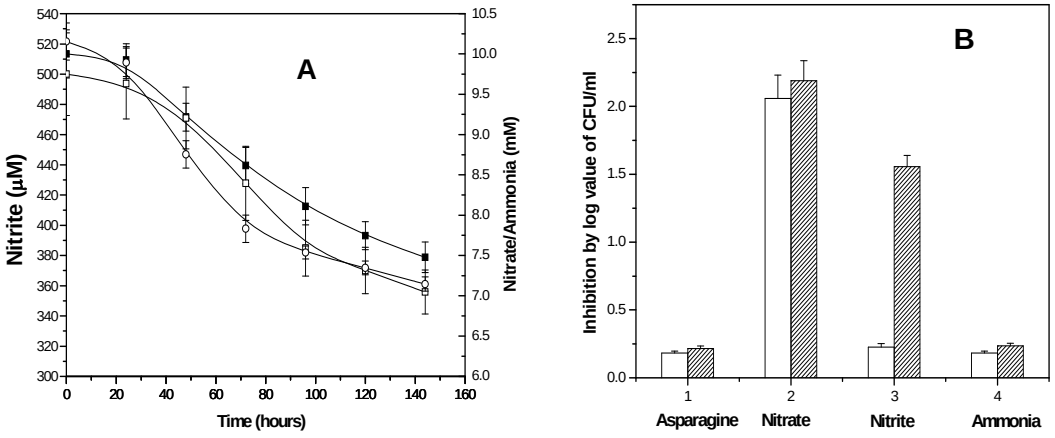


Fig. 2.11 (A) Utilization of sodium nitrate (■), sodium nitrite (□) and ammonium chloride (○) by *M. smegmatis* with time when cultured in Wayne's dormancy system. (B) Effect of Azide (light bars) and L-MSO (dark bars) on viability of *M. smegmatis* during its survival in Wayne's dormancy model when sodium asparagines, sodium nitrate, sodium nitrite and ammonium chloride were used as sole nitrogen source. Azide (50μM) and L-MSO (10μg/ml) were added at 4days old Wayne culture by syringe when all the cells had reached to non-replicating phase and viability was determined after 96hours of addition of these inhibitors. Experiments were carried out more than three times and results are mean ± SD.

Table 2.2 Rate of nitrate, nitrite and ammonia utilization by *M. smegmatis* in aerobic and dormant stage.

Substrates[a]	Rate of utilization during active replicating stage (μM/day)	Rate of utilization during dormant non replicating stage (μM/day)
Nitrate	1966.67±43.57	289.64±11.26
Nitrite	127 ±3.41	25.48±1.35
Ammonia	2890.64± 51.24	354.79± 17.77

[a] these substrates were added with initial concentration of 10, 0.5 and 10mM respectively for nitrate, nitrite and ammonia.

Importance of nitrate assimilation pathway for survival of *M. smegmatis* during *in vitro* hypoxic dormancy. Dependence of the organism for survival in dormant stage on nitrate assimilation pathway was further evaluated by applying azide and L-MSO to the Wayne culture after 96hours of inoculation. It was incubated for another 96hours to examine the effect on viability of the bacilli. A significant reduction of 2.05 and 2.18logs respectively by azide (50μM) and L-MSO (10μg/ml) was observed on viability of dormant bacilli in medium where nitrate was

used as sole nitrogen source (Fig. 2.11B). This reduction in viability of the bacilli might have occurred due to the inhibition of either respiratory or assimilatory type of NR because inhibition of respiratory NR using thiocyanate also reduced the viability of dormant bacilli by ~2logs. Interestingly, L-MSO reduced the viability of bacilli by 1.51logs in presence of nitrite as sole nitrogen source which indirectly proved that whatever may be the nature of nitrate reduction in dormant stage, it will be channelized for assimilation (Fig. 2.11B). In contrast, negligible effect of these inhibitors was seen on viability of the dormant bacilli when asparagine and ammonia were used as sole nitrogen source. This observation indicated that assimilation of ammonia and asparagine does not take separate routes in this dormant phase of *M. smegmatis*. Thus, it could be concluded that nitrate assimilation pathway remains active even in dormant stage of *M. smegmatis* and also plays important role in survival of this non-replicating stage in case when nitrate or nitrite is available as sole nitrogen source.

2.2.3. Intracellular dependency of *M. tuberculosis* H37Ra on NarGHJI indicated microenvironment similar to Wayne's hypoxia model in macrophages

Results of section 1 of this chapter on saprophytic *M. smegmatis* indicated that the respiratory NR could play important role in its survival during dormant stage as inhibition of the enzyme reduced the viability of the bacilli in a significant manner during its survival in Wayne's hypoxic model (37). Yet the role of this respiratory NR in survival and pathogenesis needed to be validated in *M. tuberculosis*, before accepting it as a promising drug target for persistent stage.

Here in this section we investigated the dispensability of this respiratory NR (NarGHJI) during survival of *M. tuberculosis* H37Ra (should be understood as H37Ra strain if mentioned as *M. tuberculosis*, in this study) in *in vitro* dormant culture condition as well as during its intracellular residence in THP-1 macrophage by using specific inhibitors. Effect of availability of nitrate as substrate for NR has also been evaluated in axenic dormant and intracellular culture condition.

Growth and nitrate reductase activity of *M. tuberculosis* in aerobic condition and Wayne's *in vitro* dormancy model. First, growth and NR activity was examined in aerobic as well as in Wayne's oxygen depletion induced *in vitro* dormant culture condition. The concentration of sodium nitrate to be added in the medium for NR activity was determined as 5mM for *M. tuberculosis* based on the toxicity of substrate nitrate as well as product nitrite (data not shown). *M. tuberculosis* showed a similar pattern of growth in aerobic and Wayne culture system as seen in *M. smegmatis* (Fig. 2.12A). Growth of the bacilli stopped once the oxygen availability became limiting in Wayne's tube model, but the bacilli still remained viable. This confirmed that organism is following the dormancy model nicely as described previously in literature (27).

Shifting from growth to NR activity, significant difference was clearly observed between aerobic and anaerobic Wayne culture of *M. tuberculosis* (Fig. 2.12B). Significant amount of nitrite accumulation even in aerobic condition by *M. tuberculosis* suggested that a constant level of basal NR activity is maintained independent of oxygen availability. Slow transport of nitrate inside the cell by diffusion could also be offered as one of the reason for this NR activity in aerobic culture condition. Overall these results indicated that NR activity is increased as soon as the environment became oxygen limiting in Wayne culture. Similar conclusion was also drawn on *M. smegmatis* except the degree of NR activity (37).

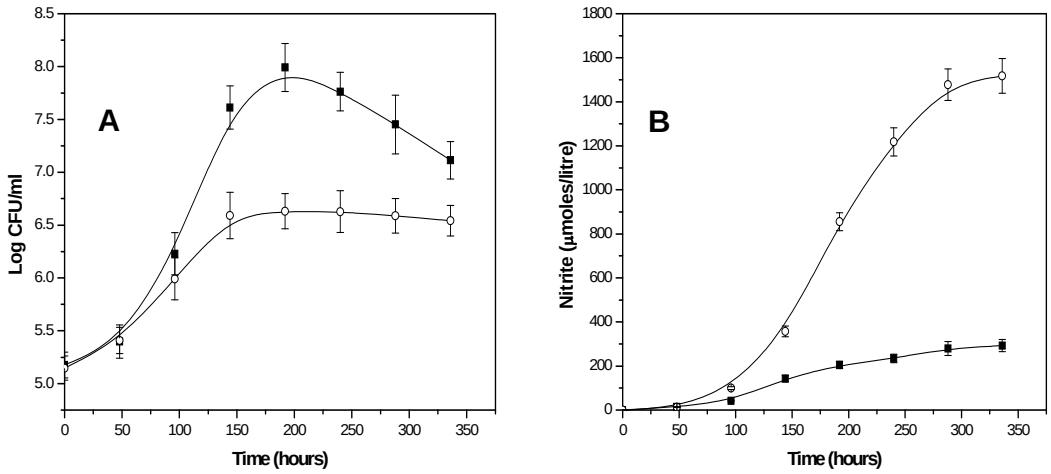


Fig.2.12 Growth (A) and NR activity (B) of *M. tuberculosis* in aerobic (■) and Wayne's dormancy culture system (○). Minimal medium was supplemented with 5mM of Sodium nitrate. Separate tubes were used for each time point CFU determination and discarded after sampling. Experiments were carried out more than three times and results are mean ± SD.

Effect of NR inhibitors on aerobic stage. Azide and thiocyanate, which were used to inhibit NR of *M. smegmatis* in previous section also applied here on *M. tuberculosis* (28). These specific inhibitors were applied to whole cell culture of *M. tuberculosis* H37Ra in order to characterize the functional role of NarGHJI. At certain concentration these inhibitors specifically inhibit NR activity without any non-specific killing of the bacilli (37). At these concentrations, azide (50µM) and thiocyanate (20mM) were applied to aerobic as well as Wayne's dormant culture of *M. tuberculosis*. The effect of azide as well as thiocyanate at above concentrations on aerobic growth of the organism was not found significant when compared with standard antimycobacterial drugs isoniazid and rifampin (Fig. 2.13A). Effect of these inhibitors on NR activity in aerobic culture condition was found variable. Azide inhibited the NR activity of aerobic culture by 75.17% whereas thiocyanate and chlorate did not inhibit the NR activity of aerobic culture in a significant manner (Table 2.3). Inhibition of aerobic NR by azide indicated that NR activity observed in aerobic culture could be of assimilatory nature since azide only can inhibit assimilatory NR activity along with respiratory one. Thiocyanate can inhibit only respiratory NR activity hence were unable to inhibit the NR activity of aerobic stage. Though the organism is reported to contain only respiratory type of NR (NarGHJI), residual assimilatory function of this enzyme could be predicted from the level of NR activity and its inhibition by azide. Aerobic stage NR

activity could also be due to another putative nitrate reductase NarX, which is assumed to be of constitutive nature (20).

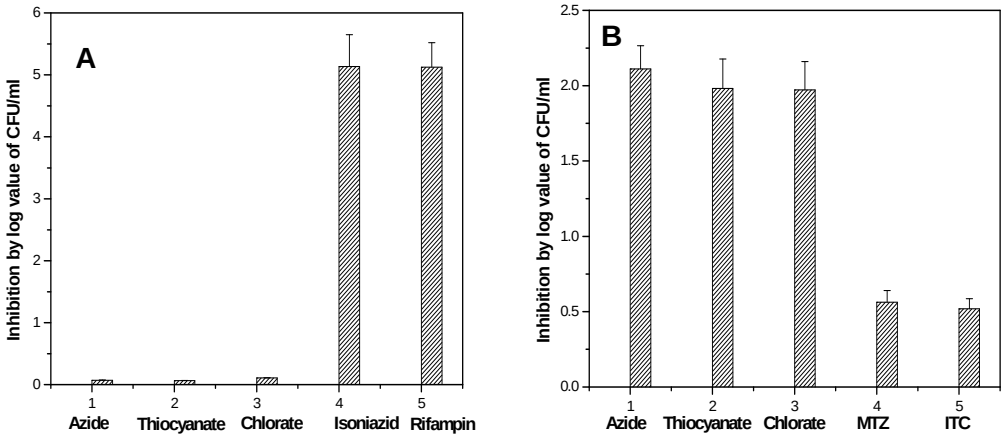


Fig. 213 Effect of inhibitors on aerobic (A) and anaerobic dormant culture (B) of *M. tuberculosis*. Azide, thiocyanate, chlorate metronidazole, itaconic anhydride, isoniazid and rifampin were added at the time of inoculation in concentration of 50µM, 20Mm, 500µM, 1mM, 2mM, 0.1µg/ml and 0.1gµ/ml respectively. CFU was then determined after 8days of incubation. Inhibitions were calculated by subtracting log values from control. Experiments were carried out more than three times and results are mean ± SD.

Table 2.3: Effect of inhibitors on NR activity of aerobic and anaerobic culture of *M. tuberculosis.*

Inhibitors[a]	% inhibition of NR activity[b] of aerobic culture	% inhibition of NR activity[b] of Wayne's dormant culture
Azide (50µM)	75.17 ± 6.23	92.28 ± 9.75
Thiocayanate (20mM)	2.32 ± 0.27	87.57 ± 7.42
Chlorate (500µM)	4.74 ± 0.34	60.23 ± 4.89

[a] added at the time of inoculation

[b] determined after 8days of incubation

[c] in presence of 5mM sodium nitrate supplemented in the medium.

Effect of NR inhibitors on Wayne's dormant culture. Since inducible NR activity in Wayne culture is assumed to be respiratory type, these inhibitors were again applied to this dormancy culture system in order to know the functional role of the enzyme and its dispensability for *M. tuberculosis*. In Wayne's dormant culture, azide and thiocyanate inhibited NR reductase

activity of *M. tuberculosis* significantly by 93.19% and 87.51% respectively when applied at the same concentration used in aerobic culture conditions (Table 2.3). These results proved that NR activity induced during anaerobic condition in Wayne culture must be of respiratory nature. We assumed that similar to *M. smegmatis*, this respiratory NR activity could become important for survival *M. tuberculosis* as well under *in vitro* dormancy stage where oxygen is unavailable. This idea was tested by applying again azide and thiocyanate to the dormant culture of the organism and monitoring its survival through viable cell count. Azide and thiocyanate were added to Wayne culture at the time of inoculation with same concentrations, which did not affect the aerobic growth of the bacilli. Survival of *M. tuberculosis* in dormant stage in presence of azide and thiocyanate was compared with control where no inhibitor was present. This was examined by determining the viability of the bacilli grown in Wayne culture in presence and absence of the inhibitors. A significant reduction in the viability in presence of these inhibitors was seen for Wayne culture *M. tuberculosis* when compared to control without inhibitor (Fig. 2.13B). Azide reduced the viability of *M. tuberculosis* by 2.15logs whereas thiocyanate reduced by 1.85logs during this anaerobic survival. The effect of these inhibitors on viability was almost identical for *M. bovis* BCG also (data not shown). These results suggested that NarGHJI becomes essential for survival of *M. tuberculosis* and *M. bovis* BCG as well in *in vitro* dormant phase. Results also suggested that even after a reduced level of NR activity (due to SNP) than *M. tuberculosis*, NarGHJI of *M. bovis* BCG can also serve efficiently for its survival in dormant stage (38). Metronidazole and itaconic anhydride were also applied to these dormant bacilli for comparative analysis and it was found that NR inhibitors had a much more profound effect on viability of dormant bacilli than these dormant stage inhibitors. This indicated that targeting energy metabolism could result in a much better killing of the dormant bacilli rather than targeting other cellular processes.

Role of NarGHJI in absence of nitrate. It was observed in *M. smegmatis* that azide and thiocyanate did not affect the viability when medium was not supplemented with nitrate in Wayne model (37). We performed similar experiment with *M. tuberculosis* where nitrate was not added in the medium. Like *M. smegmatis,* the effect of azide and thiocyanate was negligible on viability of dormant culture of *M. tuberculosis* grown without nitrate (Fig. 2.14). This can be explained by the fact that in absence of nitrate, presence of nitrate will always control the electron flow through NarGHJI. Nitrate would always be preferred above other possible terminal electron acceptors due to the difference in redox potential even if other alternate energy generation pathways are available (39). When both oxygen and nitrate are absent then only the flow of electron to generate

energy will be diverted to other alternate pathways. Naturally, the result also suggested that dependence on NR for survival of dormant bacilli is dependent on availability of nitrate as terminal electron acceptor.

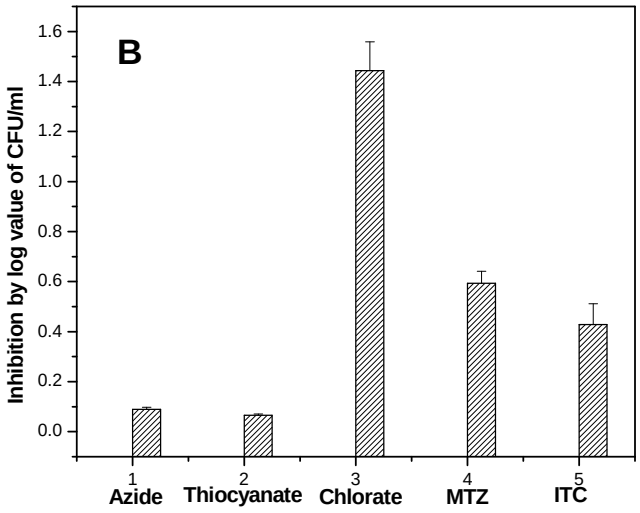


Fig. 2.14 Effect of inhibitors in Wayne's dormancy model in absence of nitrate for *M. tuberculosis*. Azide, thiocyanate, chlorate, metronidazole and itaconic anhydride were added at the time of inoculation in concentration of 50µM, 20mM, 500µM, 1000µM and 2000 µM respectively. CFU was then determined after 12days of incubation. Inhibitions were calculated by subtracting log values of with control. Experiments were carried out more than three times and results are mean ± SD.

Effect of inhibitors on *M. tuberculosis* residing within macrophage. Though inhibitors of NR reduced the viability of dormant bacilli significantly in axenic culture model of Wayne, *M. tuberculosis* may not exactly face the similar oxygen environment when residing in the host cells. Infection profile of *M. tuberculosis* in presence of NR inhibitors could provide an insight about the oxygenic environment prevailing within macrophage and its dependence on NR as well. Azide (50µM) and thiocyanate (20mM) did not show its effect on the viability of *M. tuberculosis* in THP-1 macrophage till the growth reached 3.5logs values after ~90hours of incubation (Fig. 2.15A). Azide and thiocyanate reduced the growth from this peak stage by 2.83 and 1.41logs respectively compared to the control. At these concentrations, both azide and thiocyanate did not have any toxic effect on THP-1 macrophage cells (data not shown). Thus, the reduction in cell count was due to specific killing of intracellular *M. tuberculosis* bacilli by NR inhibitors. The effect of rifampicin (0.121µM), on viability of intracellular bacilli could be seen immediately after its addition in the medium and complete killing was achieved within 72hours. This observation indicated that a microaerobic environment might have been created due to the

overburden of *M. tuberculosis* during the later stage of growth in THP-1 macrophages. Under such conditions, the demand for oxygen exceeds that of supply for continuing aerobic respiration. This could be one of the possible explanations why the bacilli have to depend on NarGHJI for survival in the intracellular environment.

Nitric oxide and superoxide radicals released by activated macrophages generates nitrate in the host (15). Under such condition, addition of sodium nitrate in the medium may not be required to get an effect of NR inhibitors on the viability of intracellular bacilli. Hence, inhibitors were used in macrophage culture infected with *M. tuberculosis* in absence of nitrate in the medium (Fig. 2.15B). Azide and thiocyanate reduced the viability of bacilli surviving within THP-1 macrophages by 1.51 and 1.56logs respectively within 168hours of incubation (Fig. 2.15B). Interestingly, the killing effect azide on the intracellular bacilli was significantly greater than thiocyanate when both were used in presence of added nitrate in the medium. The effects of rifampicin and metronidazole remained same in absence of added nitrate indicating the fact that their action is in no way related to NR activity. It was also observed that under both the conditions, NR inhibitors were more effective than metronidazole in killing the bacilli. These results also suggested that indigenously produced nitrate, by activated macrophages, could be used by *M. tuberculosis* as alternate electron acceptor for respiration (17). The effect of inhibitors on the growth and viability of intracellular bacilli were counter checked by using fluorescent microscopic technique. At the end of incubation for 8days of infected macrophages with inhibitors, the cells were stained with fluorescent dye auramine-rhodamine and viewed under a microscope (Fig. 216). The fluorescent picture indicated a drastic decrease in the number of bright yellow spots in the slides treated with inhibitors compared to the control. The bright yellow spots were represented by *M. tuberculosis* bacilli. The result again confirmed our earlier observation that there were indeed a reduction in intracellular bacilli count because of the treatment of NR inhibitors along with rifampicin and metronidazole.

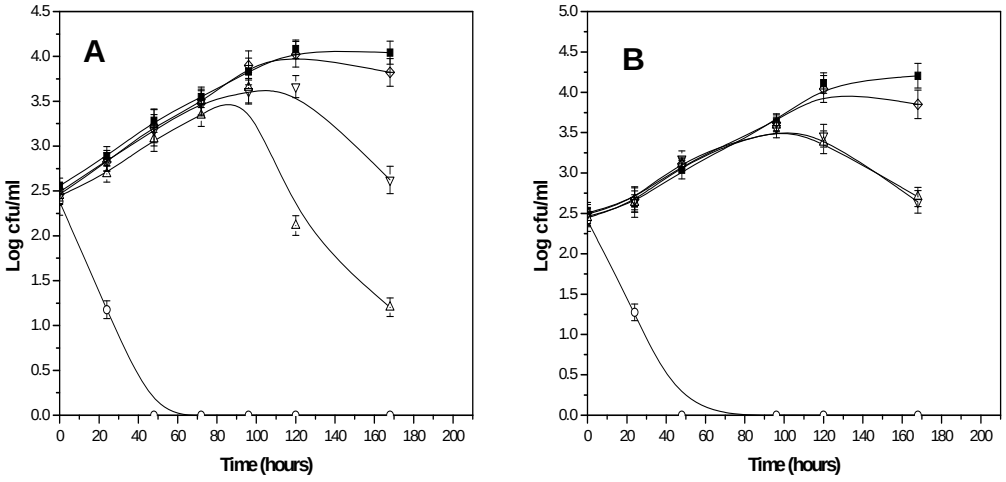
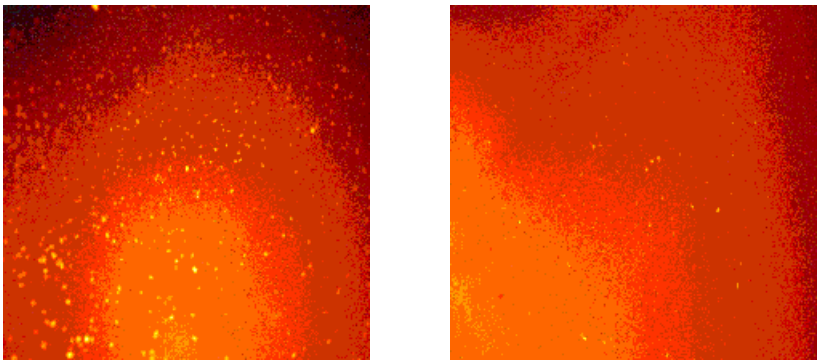
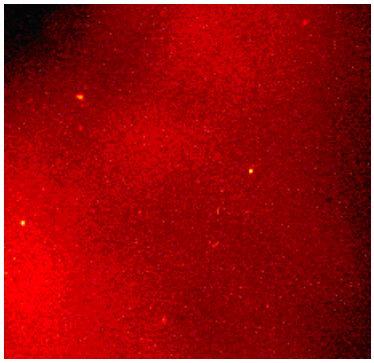


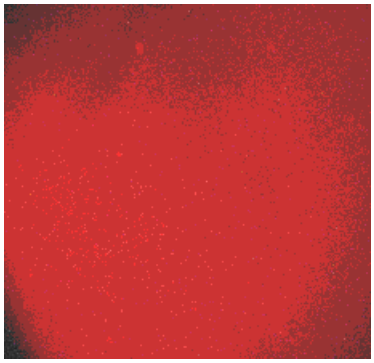
Fig. 2.15 Effect of inhibitors on *M. tuberculosis* when residing in THP-1 macrophage in presence (A) and absence (B) of nitrate. Rifampin (O), azide (Δ) thiocyanate (∇) and metronidazole (◊) were added just after the infection in 0.1µg/ml, 50µM 20mM and 500µM concentration respectively. 5mM sodium nitrate was added in the macrophage culture media and separate flasks of macrophage culture were used for each time point CFU determination and discarded after sampling. Experiments were carried out more than three times and results are mean ± SD.



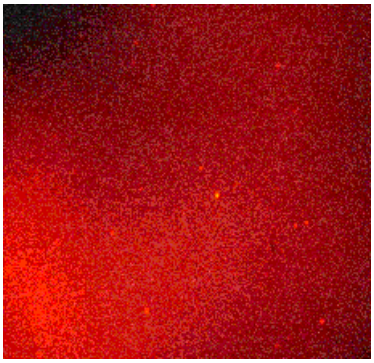
(a) (b)



(c)



(d)



(e)

Fig. 2.16 Photomicrographs of *M. tuberculosis* after 8days of culture in monocytes-derived macrophage cells with none (a), 50µM azide (b), 20mM thiocyanate (c), 500µM metronidazole (d) and 0.1µg/ml rifampicin (e). The photomicrographs of *M. tuberculosis* cells in macrophages after staining with auramine-rhodamine were taken with a fluorescence microscope equipped with a 470- to 490nm filter (Leitz Wetzlar, Germany). Bacilli are bright yellow, and macrophages are dull red.

2.2.4. Assimilation of nitrate and some other nitrogen source in *M. bovis* BCG and *M. tuberculosis* H37Ra occurs via intracellular glutamine synthetase during active and dormant stage

Once it was proved that a complete nitrate assimilation pathway is function in saprophytic strain *M. smegmatis*, our attention was shifted to further investigate the operation of this pathway in *M. bovis* BCG and *M. tuberculosis* H37Ra, which are genetically much closer to pathogenic *M. tuberculosis*. Comparative study of this pathway should allow developing a better understanding about the role of nitrogen metabolism in pathogenesis and physiology of *M. tuberculosis* during both active and dormant stages. Although it has been reported in some previous studies that some of the mycobacteria could use the nitrate and nitrite as sole nitrogen source, this has not been examined during dormant stage of the organism where cell multiplication is held back. Literature also describes only a few studies of the utilization of nitrite by *M. tuberculosis* and that also with reports having incongruity (7,40).

As previously mentioned that assimilation of any nitrogen source which requires GS-GOGAT pathway, is mediated by intracellular GS in bacteria (Fig. 2.7) (32). Therefore, inhibition of growth by GS inhibitor in presence of a nitrogen source indicates the involvement of intracellular GS in its assimilation. While identifying nitrate assimilatory pathway in *M. smegmatis,* it was found that intracellular GS was actively involved in assimilation of nitrate as nitrogen source (41). In order to assess whether this intracellular GS has any general role in growth and survival of mycobacteria, different other nitrogen sources were evaluated for their assimilation. Nitrogen sources, which allowed the growth of organism as sole nitrogen source but were unable to support the organism's growth in presence of GS inhibitor L-MSO, were determined for *M. tuberculosis* (Table 2.4).

These nitrogen sources, used by intracellular pathogen could be assumed to come from this list for their assimilation. MIC of L-MSO for these nitrogen sources were compared with MIC in Dubos medium (which contains BSA as nitrogen source) for *M. tuberculosis*. Inhibitory effect of L-MSO in presence of any one of these nitrogen sources was found much more prominent compared to its effect in Dubos medium on growth of *M. tuberculosis* (Fig. 2.17 A and B).

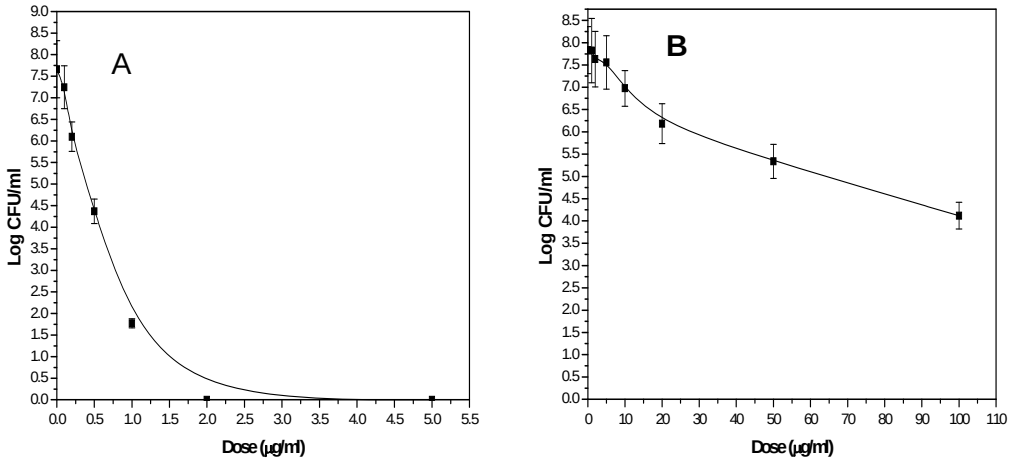
Table 2.4 Effect of L-MSO on assimilation of different nitrogen sources by *M. tuberculosis*.

Unassimilated[a]	Assimilation unaffected[b] by L-MSO	Assimilation affected[c] by L-MSO
Lysine	Asparagine	Arginine
Histidine	Alanine	Aspartic acid
Threonine	Serine	Glutamine
Valine	Glutamic acid	Glycine
Tryptophan		Proline
Methionine		Serine
Phenyl alanine		Isoleucine
Cystine		Leucine
Tyrosine		Nitrate
Hydroxy proline		Nitrite
		Ammonia

[a] did not support the growth when used as nitrogen source.

[b] L-MSO did not inhibit growth in presence of these sole nitrogen sources.

[c] supported the growth as sole nitrogen source but not in presence of L-MSO.



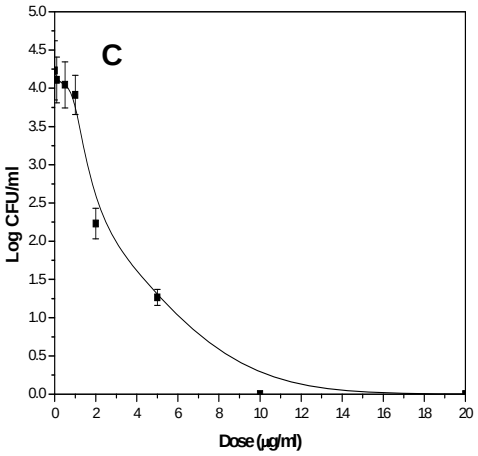


Fig. 2.17 Dose dependent effect of L-MSO against *M. tuberculosis* in (A) minimal medium with Glutamine as nitrogen source, (B) enriched medium and (C) intracellular environment of THP-1 macrophage. Log CFU/ml was determined after 8days of incubation. Results are mean ± SD of three experiments. Other experimental details are given in "materials and methods' section of this chapter.

In order to further clarify the involvement of GS during intracellular growth and survival, dose dependent effect of L-MSO were compared between enriched medium axenic culture and intracellular *M. tuberculosis*. At 2µg/ml concentration of L–MSO, growth of *M. tuberculosis* was completely inhibited in glutamine containing minimal medium as well as within macrophage whereas no inhibition on growth was observed in enriched medium (Fig. 2.17 B and C). These results indicated that *M. tuberculosis* gets access of only few selected amino acids in the phagosome, which belong particularly the third category (Table 2.4) (42). These results also suggested that intracellular GS could also be contributing in the survival of organism.

A non-replicating dormant phase of tubercle bacilli, which is responsible for persistence in the host tissues, was then evaluated for its dependence on GS for survival (2). Wayne's hypoxic model was used to cultivate these dormant bacilli *in vitro* (27). Glutamine, assimilation of which required GS in aerobic replicating phase was used as sole nitrogen source for *M. tuberculosis*. Once the cultures reached to dormant phase, L-MSO was added by syringe without disturbing the oxygenic environment at its MIC (2µg/ml) (Fig. 2.17A). Effect of L-MSO on viability of the dormant bacilli was determined by taking plate counts of the bacilli after every 24hours of incubation in presence of L-MSO. A significant reduction in viability of the dormant bacilli of about 2.2logs after 96hours incubation in presence of L-MSO was seen (Fig. 2.18).

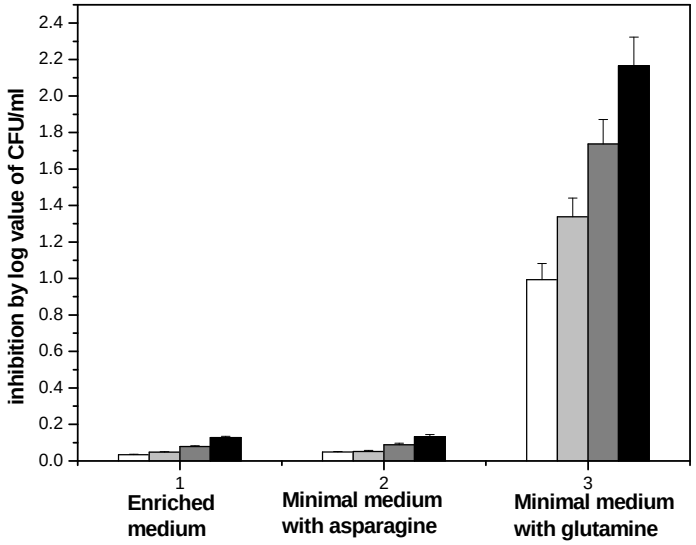


Fig. 2.18 Effect of L-MSO on survival of non-replicating dormant bacilli of *M. tuberculosis* with different nitrogen source conditions in Wayne's hypoxic *in vitro* model. Inhibitions by log value were calculated by subtracting determined log CFU/ml in presence of L-MSO from control's log CFU/ml where L-MSO was not added. Different bars represent the effect on viability after incubation of 24hours (white bars), 48hours (light grey bars), 72hours (dark grey bars) and 96hours (black bars) in presence of L-MSO. Experiments were carried out three times and results are mean ± SD.

The results thus obtained under identical condition were compared between media containing asparagines, glutamine with Dubos medium containing BSA as nitrogen source. The effect of L-MSO was negligible on dormant bacilli of *M. tuberculosis* in medium where asparagine was provided as sole nitrogen source or enriched medium like Dubos with BSA (Fig.2.18). These results suggested that assimilation of nitrogen source continues in dormant and intracellular stage of *M. tuberculosis*.

2.2.5. Nitrate reductase activity of *M. smegmatis*, *M. bovis* BCG and *M. tuberculosis* H37Ra in nutrient starvation induced dormancy model

Earlier evidences suggest that persistent bacilli in lung lesions suffer nutrient deprivation condition, either unaided or in combination with reduced oxygen availability (43). Therefore, we extended our study of nitrate reduction by mycobacteria in nutrient deprived condition to better understand the role of NR. Betts used a nutrient starvation model to investigate the effect of starvation predicted to prevail in granuloma on the metabolism of *M. tuberculosis* (44). In an effort to develop a simple model for such studies we examined first *M. smegmatis* for its ability to sustain the nutrient starvation induced dormancy. There are several reasons for use of *M. smegmatis* as a model system for studying physiology of pathogenic mycobacteria. It can be easily cultured in artificial media. It has a much faster generation time as compared to *M. tuberculosis* and also does not require the use of sophisticated containment facilities such as biological safety level -3 (BSL-3) laboratories. The genome of the organism has also been found to contain homologues to multiple virulence genes of *M. tuberculosis* providing further validation to use the *M. smegmatis* as a model system to study *M. tuberculosis*. On the basis of our earlier studies, we first standardized *M. smegmatis* for its ability to follow the nutrient starvation based dormancy. Some indications of nutrient starvation based dormancy have also been provided when stringent response was required for long term survival of *M. smegmatis* in nutrient starved condition (45). When it was transferred and incubated in a medium (PBS) without having any carbon and nitrogen source, there was initially a moderate decrease in viability (0.5log), which became almost constant within 92hours of incubation (Fig. 2.19). The viable count was found constant even upto 240hours afterward, that suggested that the organism was following the similar dormancy pattern as it was seen in *M. tuberculosis* (46). Owing to its simplicity, reproducibility and ease of handling, the model may also be useful for testing of novel antimycobacterial agents aimed at killing persistent bacteria.

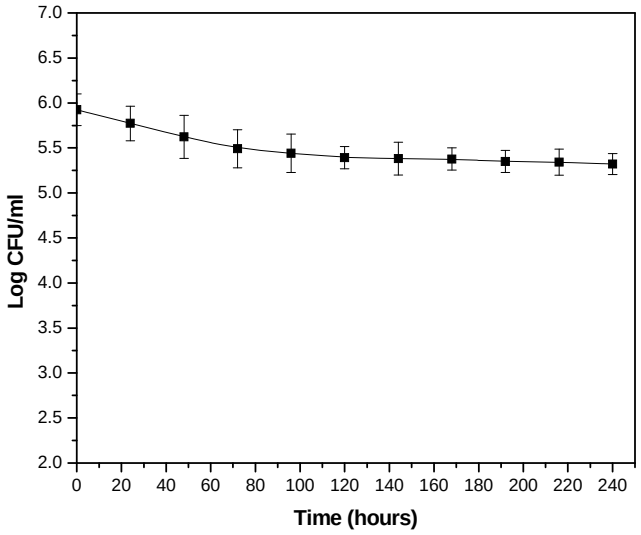


Fig. 2.19 Viability of *M. smegmatis* in nutrient starved culture condition upto 10days of incubation in PBS. CFU was determined after every 24hours by spreading different dilutions of each sample on Dubos agar plates and counting the colonies appeared after 4days of incubation at 37^0C. Experiments were carried out more than three times and results are mean ± SD.

In order to validate the dormant stage, susceptibility pattern to known antitubercular drugs was examined in this model. Drugs at MICs in nutrient rich condition were applied in this model and the effect was monitored by counting the viable cells on agar plates. The organism was found to have resistance against rifampin, isoniazid and ethambutol and pyrazinamide and thus confirmed its adoption to dormant stage (Fig. 2.20). Interestingly, it was also seen that organism was susceptible to drugs like streptomycin and gentamycin, which are known to act as protein synthesis inhibitors as well as nucleic acid inhibitors like ciprofloxacin, and ofloxacin in this nutrient starvation induced dormancy. This result indicated that although cells are not multiplying but protein and DNA synthesis remains active in nutrient starvation induced dormancy of *M. smegmatis*.

As the environment in nutrient starvation based dormancy is not restricted from oxygen and yet the organism was not multiplying, the normal energy generation pathway operative in this type of dormancy could be different from regular oxygen respiration. We examined NR activity to understand its involvement during this nutrient starvation based dormancy stage of tuberculosis. It was surprising that there was an induction of nitrate reduction seen even in this nutrient starvation based dormancy where condition is supposed not to be oxygen restricted (Fig. 2.21).

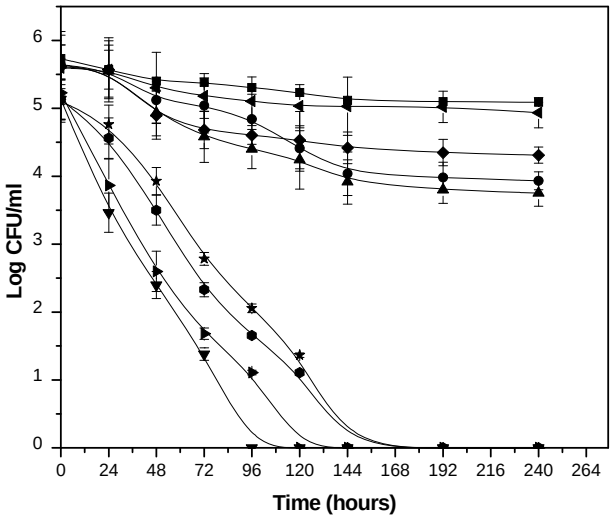


Fig. 2.20 Susceptibility patterns of nutrient starved cultures of *M. smegmatis* to rifampicin (●), isoniazid (▲), streptomycin (▼), ethambutol (♦) pyrazinamide (◄), gentamycin (►), ofloxacin (⌂) and ciprofloxacin (*). In identical condition a triplicate sample without any drug treatment was kept as control (■). The effect on the viability was investigated at reported MICs of the drugs. Cultures were treated with these drugs in triplicates upto 10days. Viable count was done after 4days of incubation of the each sample of different dilutions in triplicates on Dubos agar plates.

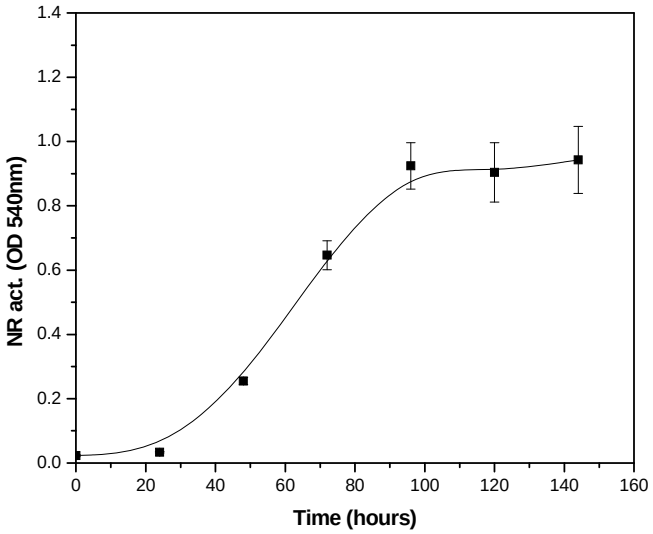


Fig. 2.21 Nitrate reduction by nutrient starvation induced dormant culture of *M. smegmatis* with time. Experiments were carried out more than three times and results are mean ± SD.

Though the rate of nitrate reduction was less compared to that of oxygen depletion induced dormancy yet the induction was clearly visible as compared to aerobic nutrient rich culture condition of the organism (Fig. 2.3B). The respiratory nature of nitrate reduction during this dormancy was also confirmed by specific inhibition by azide and thiocyanate when they inhibited the reduction by more than 80% (Fig. 2.22). This result pointed out towards possible involvement of NR towards energy generation during nutrient starved condition. The inhibition pattern was also followed by using different dormant stage specific inhibitors on the viable count of the bacilli with time to know the dependency on NR during their nutrient starved dormant stage survival. The effect of these inhibitors on viability nutrient starved dormant bacilli of *M. smegmatis* was almost negligible as evident from the cell count (Fig. 2.23). Therefore unlike the oxygen depletion induced dormancy, NR was dispensable here in this model of dormancy.

These results were also corroborated with subsequent analysis in cognate organisms *M. bovis* BCG and *M. tuberculosis* H37Ra. These two organisms also demonstrated the similar results in nutrient starvation induced dormancy model as it was seen in *M. smegmatis* (data not shown).

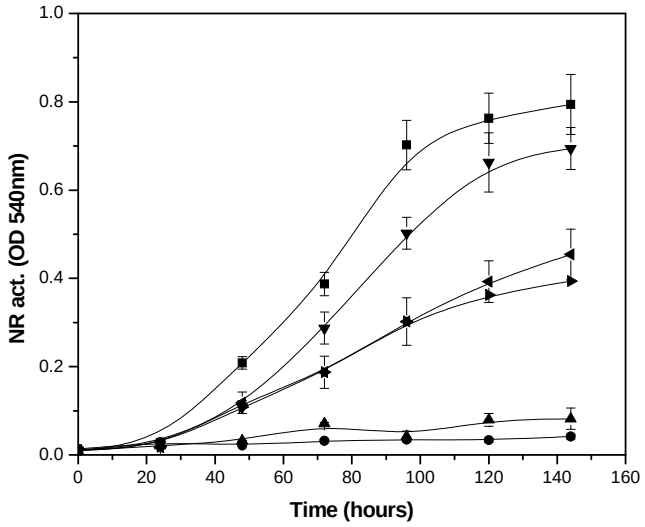


Fig. 2.22 Kinetics of inhibition on nitrate reduction by *M. smegmatis* in nutrient starvation induced dormant culture by NR inhibitors. 0.05mM of azide (●), 20mM thiocyanate (▲), metronidazole (▼), itaconic anhydride(♦), nitrofurantoin (◄), furaltadone (►)were added at the time of inoculation and Nitrate reduction was monitored after every 24hours upto 10days . In identical condition a triplicate sample without any inhibitors addition was kept as control. (■). Experiments were carried out more than three times and results are mean ± SD.

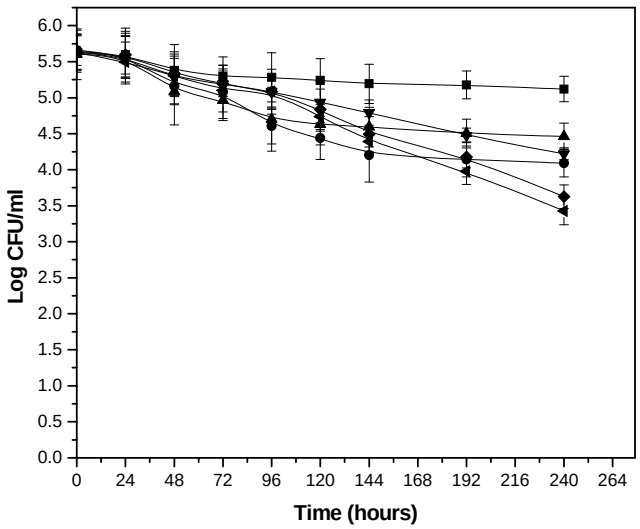


Fig. 2.23 Effect of azide (●), thiocyanate (▲), metronidazole (▼), nitrofurantoin (♦) and furaltadone (◄) on viability of dormant culture of *M. smegmatis* induced by nutrient starvation. A triplicate sample without any inhibitors addition was kept as control. (■). Experiments were carried out more than three times and results are mean ± SD.

2.3. Materials and Methods

2.3.1. Chemicals, strains and Media

All the chemicals were purchased from Sigma-Aldrich, USA. Dubos medium was purchased from DIFCO, USA. *M. smegmatis* strain ATCC 607 was used throughout the present study which was grown in a defined medium containing 0.5 gm KH_2PO_4, 0.2 gm sodium citrate, 60mg $MgSO_4$, 0.5 gm asparagine and 2 ml glycerol in 100ml of distilled water at pH 6.6. The stock culture was maintained at -70^0 C and sub cultured once in liquid medium before inoculation to an experimental culture.

2.3.2. Cultivation of oxygen depletion induced dormant bacilli

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 medium was inoculated with 1% v/v of 1 O.D580 culture (27). The culture tubes were incubated at 37^oC 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 (10).

2.3.3. Cultivation of nutrient starvation induced dormant bacilli

The nutrient starvation induced dormant bacilli were developed by method given by Betts and coworker (44). Briefly, the cells were grown under shaking conditions (150rpm) in *M. phlei* medium up to 72, 144 and 240hours respectively for *M. smegmatis, M. bovis* BCG and *M. tuberculosis* (upto late logarithmic phase). Cells were then harvested by centrifugation and washed twice with phosphate-buffered saline (PBS). The washed cells were then transferred to flasks containing sterile PBS and incubated at 37^0C and 150rpm shaking condition. After a certain period of time (1, 2 and 2weeks for *M. smegmatis*, *M. bovis* BCG and *M. tuberculosis* respectively), dormancy was confirmed by CFU enumeration and drug resistance (27). The effect of inhibitors or compounds on latent bacilli was evaluated by the adding them at the time of incubation to flasks containing these bacilli in PBS and incubated at 37^0C followed by CFU enumeration after plating different aliquots (50mL of undiluted, 1:100 and 1:1000 diluted samples) from these flasks on Dubos agar plates. The CFU enumeration was performed 4, 21 and 28days postplating for *M. smegmatis*, *M. bovis* BCG and *M. tuberculosis* H37Ra.

2.3.4. Infection and cultivation of *M. tuberculosis* in macrophages

Human acute monocytic leukemia cell line THP-1 was used to infect and cultivate *M. tuberculosis* in the macrophage system by following an earlier method described (47). Briefly, THP-1 cells were grown in MEM at pH 7.4 at 37^{o}C for 4days 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. The culture was then incubated for 24hours to allow them converted into macrophages. These macrophages were incubated for 12hours with *M. tuberculosis* 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 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 this incubation. Unless mentioned otherwise, the macrophages were lysed after 8days of incubation, in 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.

2.3.5. Estimation of nitrate, nitrite and ammonia in whole cell

Concentration of the nitrate in the whole cell culture was estimated by a method based on salicylic acid nitration (48). Briefly, 50 µL of the culture was added with 200µL of 5% salicylic acid solution prepared in conc. H_2SO_4. The solution was incubated for 20minutes and 4.75ml of 2N NaOH was added to develop a yellow color. Absorbance of the sample was read at 410nm and nitrate concentration was calculated by comparing with a standard nitrate curve. Cell culture and nitrite did not interfere in the estimation of nitrate.

Nitrite concentration in the culture was determined by using Griess reaction (49). Briefly, 1 ml of the culture was added with 1ml of 1% sulphanilic acid solution (prepared in 20% HCL) and 1 ml of 0.1% NEDD solution (prepared in distilled water). The tubes were incubated for 15minutes to develop pink color. Absorbance of the samples was read at 540nm and nitrite concentration was calculated by using nitrite standard curve.

Ammonia concentration in the culture was determined by following salicylate-hypochlorite method (50). Briefly, 3.5 ml of sample were added with 0.5 ml of salicylate solution (40% in D/W), 0.5 ml of potassium ferrocyanide solution (2% $K_4Fe(CN)_6.2H_2O$ and 10% Na3 citrate.$2H_2O$ (in 0.1 N NaOH) and 0.2 ml of Hypochlorite solution (1.93% in 0.1 N NaOH). After covering the tubes with parafilm, sample was vortexed and placed in water bath at 37^{0} C for 45 minutes. Samples were then cooled for 15minutes, vortexed and absorbance at 650nm was

measured. Standard curve made of ammonia was used to estimate the ammonia level in culture samples.

2.3.6. Preparation of cell extracts and cell free nitrate reductase assay

In order to make the cell free mycobacterial extract, cells were first washed twice with 5 × volume of cell pellet using Tris buffer (0.1 M pH 7.5). The cell pellet was resuspended in 10 ml of same buffer for each gram of cell pellet. The cells were then lysed by bionebulization (BioNeb Glas-Col, USA). Briefly, 10 continuous cycles of 3 minute each were applied at 200psi to efficiently lyse the cells. The suspension was then freeze-thawed thrice to further increase the extent of lysis. The suspension was centrifuged at 4,500x g for 15minutes to remove the cell debris. The supernatant was further centrifuged at 25,000xg for 2hours. The pellet was resuspended in sodium phosphate buffer (20mM pH 7.2) to get the membrane fraction which was used as source of NR. NR assay was carried out by following a method described earlier (51). Protein concentration was determined by following Bradford method (52).

2.3.7. Rhodamine-auramine staining and fluorescence microscopy of intracellular *M. tuberculosis* in THP-1 macrophages

The microphotography of infected macrophages was carried out following an earlier method (53). Briefly, the same infection procedure as in 96 well plates was carried out on slides in removable chambers (Sonic seal Slide wells; Nunc Inc., Naperville, Ill). After incubation of infected macrophages in presence of inhibitors for 8days, cultures were washed with PBS for 3 times. The slides were flooded with auramine-rhodamine stain for 20minutes. Subsequently, the slides were ringed with water for 5 times and then flooded with 0.5% HCl in ethanol to decolorize the stain for 5minutes. Again, rinse off 0.5% acid alcohol with water for treatment with potassium permanganate for 1 minute. Permanganate was washed off from the slides by water to view the bacilli under a fluorescent microscope. The photomicrographs were taken with a fluorescent microscope equipped with a 470- to 490-nm filter (Leitz Wetzlar, Germany). Bacilli were viewed as bright yellow, and macrophages were dull red under the microscope.

2.4. Discussion

Nowadays, persistence has gained a lot of attention with respect to developing novel antitubercular drug, which could drastically reduce the duration of tuberculosis therapy. A persistence specific drug should ideally work at anaerobic stage like NRP2 shown in *M. tuberculosis* (54,55). Identification of a target specific for this stage as well as having an easy assay system for screening a chemical library could provide a novel anti TB inhibitor. From earlier studies, it was very difficult to predict any target worth pursuing for drug development against this persistent bacillus. Looking at different options, targeting the electron transport chain of mycobacteria could be a very novel approach to control the latency of *M. tuberculosis*. This is due to the fact that oxygen limitations are involved in establishing and maintaining the dormant stage of the pathogen. There could be a few alternate respiratory mechanisms available for the pathogen to generate energy in absence of oxygen. Cytochrome *bd* oxidase system, encoded by *cydABDC* operon has been described to be present in many pathogenic and nonpathogenic mycobacteria and provides an option to produce energy through an alternate pathway when oxygen availability to cells is low (56). Since, cytochrome *bd* oxidase has more binding affinity to oxygen compared to cytochrome *a1a3* oxidase; it could be one of the possible options for providing necessary energy during the shiftdown of *M. tuberculosis* to anaerobic dormant stage when oxygen concentration decreases to negligible level. However, cytochrome *bd* oxidase could not successfully prove itself a potential drug target based on the observation that *cydA* mutant of *M. smegmatis* could show only a moderate disadvantage of viability compared to wild type strain. Looking at the other alternate respiratory chains, NarGHJI encoded by *narGHJI* operon present in *M. tuberculosis* genome appeared to be the better choice of target for latent stage. This is due to some initial evidences of its increased activity during shiftdown and presence of fewer bacilli and smaller granuloma formation in lungs of SCID mice infected by *narG* mutant of *M. bovis* BCG compared to wild type strain (12). In spite of genome size difference of *M. tuberculosis* and *M. smegmatis*, the later has been commonly employed as a model system to study the physiology and pathogenesis of mycobacteria. Presence of the gene for NR in *M. smegmatis* was predicted in an earlier report by sequence homology analysis showing two genes *narG* and *H* having ~80 % sequence homology with *M. tuberculosis* genes (24). Our study confirmed the presence of the functional respiratory NR in *M. smegmatis*, which was also corroborated later on with availability of genome draft showing a complete set of genes coding for respiratory type of NR in this fast growing organism (25,37). A thorough study on the nitrate reduction in *M. smegmatis* has helped

in understanding the alternate respiratory pathways operative during latent stage survival of mycobacteria. The possibility of this anaerobic NR becoming *narGHJI* was further strengthened by its almost complete inhibition in presence of azide, thiocyanate and in major extent by chlorate under anaerobic condition (Fig. 2.4, 2.5 and 2.6). The inhibition of nitrate reduction by whole cell lysate in presence of azide, thiocyanate and to a negligible extent by chlorate further strengthened earlier prediction (Table 2.1). As the stages were not clearly defined from growth curve of *M. smegmatis* in Wayne's model, it could be divided into aerobic and anaerobic stages based on the rapid change observed at ~48hours after inoculation (Fig. 2.3A). Therefore, the period between 96hours to 120hours in Wayne's model could be assumed as clearly an anaerobic stage. The inhibitory action of azide, thiocyanate and chlorate was exhibited at later stage (after 48hours) (Fig. 2.4 and 2.5). The presence of nitrate in the medium is an obligatory requirement for the functioning of NR during anaerobic respiration (37). In presence of nitrate in the medium, whole respiratory mechanism becomes completely dependent on NR. The presence of an alternate respiratory mechanism and fermentation pathway also cannot be ruled out in mycobacterial system because it could survive at anaerobic stage in absence of nitrate also (data not shown). The presence of nitrate in host system is quite evident because of the release of NO by the immune cells and its subsequent conversion into nitrate (15,17). The relevance of NR in *in vivo* survival also could be predicted from tissue specific growth of a *narG* mutant of *M. bovis* BCG (13). Applying specific inhibitors in the host system could only make possible of understanding the exact role of NR in the development and maintenance of persistence of the bacilli. The results further indicated that NR specific inhibitors have reduced cell count by ~2logs compared to only ~0.8log by MTZ (Fig. 2.4 & 2.6). This data clearly indicated that NR inhibitors have most dramatic effect on the survival of bacilli at late anaerobic stage compared to metronidazole (MTZ) and nitrofurantoin (NIT) (Fig. 2.5 & 2.6). Even though MTZ and NIT were identified as persistent stage specific antitubercular molecules and the former is also used as medicine for TB treatment, our studies on *M. smegmatis* indicated that these two molecules are not specific for either of the stages (30,31,58). As the bacilli could survive with or without nitrate during persistent stage, to have an impact on NR specific inhibitors, nitrate should be present as alternate electron acceptor in the vicinity of its residence. It remained to be clear the role of NR in the development and spreading of persistent bacilli under *in vivo* condition. If the *in vivo* development occurs in the same way as predicted by Wayne's model, then complete sterilization could be possible through the inclusion of NR inhibitors in the combination therapy. Altogether the results indicated that induction of anaerobic stage specific respiratory NR activity is observed in *M. smegmatis* and abrogation of the activity dramatically kills dormant bacilli. Therefore, NR

could be adopted as a better target than others for the development of persistent stage specific inhibitor.

The capability of assimilating nitrate by *M. smegmatis* has been shown from this study by using three substrates mentioned as sole nitrogen source along with the use of respective inhibitor of the enzymes involved in the pathway. Growth of *M. smegmatis* in presence of nitrate, nitrite and ammonia as sole nitrogen source along with its depletion from the culture provided a strong evidence in favor of an active nitrate assimilation pathway present in the bacilli (Fig. 2.8 and 2.9). Total inhibition on growth and utilization of nitrate by *M. smegmatis* in presence of azide as well as mention of *narB* gene (Gene Accession Number YP_887157) in genome database confirmed the existence of an active nitrate assimilatory pathway (Fig. 2.9). Another characteristic of the pathway noticed was that the intermediates of nitrate metabolism did not appear in the medium (data not shown). L-MSO blocked the utilization of both nitrate and nitrite as sole nitrogen source for growth, further confirmed the presence and utility of this pathway in *M. smegmatis* (Fig. 2.9 and 2.10). The lack of inhibition observed by L-MSO on the assimilation of ammonia as well as growth of the bacilli could be justified by the presence of genes for glutaminase (Gene Accession Number YP_888111) and glutamate dehydrogenase (Gene Accession Number YP_889681) making a bypass of glutamine synthetase for the survival of the organism (36). Most significantly, this assimilation pathway remained active even when the organism was shifted to oxygen depletion induced dormant stage (Fig. 2.11). Inhibitor of the enzymes involved in the pathway also had significant effect on the viability of the dormant bacilli and hence indicated the importance of this pathway during persistent stage. These results also provided an indication, in contrary to an earlier report, that L-MSO inhibits intracellular GS (59). Analysis of this nitrate assimilation pathway could also help in finding new drug targets, which could be equally useful against active and persistent bacilli of the pathogen.

Though the results clearly indicated about the utility and dependence of the organism on this pathway under certain conditions when nitrate/nitrite is available as sole nitrogen source, presence of nitrate/nitrite in nutrient deprived host cells harboring these mycobacteria may not be at all surprising (18). Presence of similar type of nitrate assimilation pathway in *M. bovis* BCG and *M. tuberculosis* was also observed during initial experiments in our laboratory. Studies regarding the availability and utilization capabilities of other nitrogen sources by these mycobacterial species are needed to further provide a deeper insight in relating a possible link between the different enzymes involved in the pathway with the manifestations of the disease by different mycobacterial species to identify a futuristic drug target.

Dependence of tubercle bacilli on NarGHJI activity was also evaluated during its growth within infected human (THP-1) macrophages by using specific inhibitors. Interestingly, the survival kinetics of *M. tuberculosis* bacilli drawn by estimating CFU coupled with microscopic data obtained in presence of NR inhibitors in intracellular macrophages showed similarity with *in vitro* Wayne's hypoxia culture model (Fig 2.15). The survival kinetics of intracellular bacilli within human macrophage (THP-1) cells in presence of azide and thiocyanate clearly suggested that hypoxic condition achieved within the cytoplasm of the host before reaching the growth to plateau. Very recently, non-replicating stage specific inhibitors of *M. tuberculosis* have been identified from screening against macrophage infected bacilli (60). Since, earlier studies suggested non-specific inhibition by azide and thiocyanate on general cellular functions; they were tested on membrane bound enzyme as well as growth of *M. tuberculosis.* Almost ~85% inhibition of membrane bound enzyme without perturbing the growth of the bacilli in aerobic culture clearly indicative of specific inhibition obtained from NR inhibitors (Fig. 2.12, 2.13, Table 2.4). The effect of azide and thiocyanate in Wayne's dormant culture indicated that specific inhibition of NarGHJI was responsible for the reduction of viability of the bacilli. Under similar conditions, azide was earlier found to inhibit assimilatory type NR in *M. smegmatis* (37). The inability of thiocyanate to stop nitrate reduction in aerobic culture and also reduced killing of the bacilli in infected macrophages compared to the effect of azide indicated the presence of active assimilatory nitrate reduction pathway functional even in intracellular bacilli (Fig. 2.12, 2.13, 2.15A and B). The ability of azide in enhanced killing of intracellular bacilli also showed that both assimilatory and respiratory NR are important for the survival and growth during infection in human macrophages. Further biochemical research will help in evaluating the consequence of generating hypoxic condition in the host milieu during intracellular growth of the bacilli. Altogether, experimental results suggested that hypoxic condition generates within human macrophages due to the growth of the bacilli, which may be helpful in pursuing respiratory NR as potential anti-tubercular drug target.

During our investigation on nitrate assimilation by these organisms, the study also indicated that intracellular glutamine synthetase plays important role during dormant stage of the bacilli. It was earlier reported that GS is extracellularly released in great abundance by *M. tuberculosis* along with 11 other proteins (61,62). The belief in targeting glutamine synthetase for drug development gained momentum with the evidence of L-methionine sulfoximine (an irreversible inhibitor of GS) blockage of multiplication of the pathogen in axenic culture as well as in human mononuclear phagocytes (59). The blockage of growth was found to be correlated with marked reduction in the amount of virulence associated cell wall component poly L-glutamate-L-

glutamine hence indicated towards the importance of extracellular function of the enzyme. More remarkably, this inhibitor had no effect against non-pathogenic bacteria. The results were further confirmed when application of antisense (PS-ODN) against mRNA of *M. tuberculosis* GS reduced its growth by 0.75 to 1.25logs (63). Gene disruption studies also showed that glutamine synthetase is essential as *GlnE* (regulator of GS-1) mutant could not grew and *GlnA1* (GS-1) mutant became attenuated for intracellular growth in THP-1 macrophage (64,65). Since L-MSO is assumed as not being able to cross mycobacterial cell wall and decreased amount of poly L-glutamate- glutamine complex in *GlnE* and *GlnA1* mutant of *M. tuberculosis,* it was correlated with their reduced virulence. It could also be predicted that disruption of extracellular GS is responsible for blockage of growth (66). Thus, the effect of L-MSO seen on growth of *M. tuberculosis* was only because of inhibiting extracellular function of this enzyme as the same enzyme is catalyzing these two different functions. High extracellular amount of this enzyme was due to high expression and extracellular stability rather than to protein specific export mechanism (67). It was also suggested that the release of protein was due to the increased rate of autolysis in *M. tuberculosis* when compared to other bacteria (68). Thus it became essential to analyze the function of this enzyme more precisely in order to validate the target finally. In order to clarify the intracellular function of the enzyme, *M. smegmatis* was allowed to grow in conditions where single nitrogen source was used in presence of L-MSO. Thus, a single nitrogen source at a time in a defined medium provides the opportunity to evaluate the function of intracellular GS in mycobacteria. This was based on our earlier study where we used nitrate as sole nitrogen source and it was found to be assimilated via GS in *M. smegmatis* (41). Here, for *M. tuberculosis* the results were grouped to the utilizable, non-utilizable and L-MSO responsive nitrogen sources (Table 2.4). These results have certain implications for the intracellular function of GS as the assimilation of nitrogen is catalyzed via intracellular GS for certain sources. The intracellular function of GS gained more importance when the dose dependent effect of L-MSO on growth of *M.smegmatis* was compared between nitrogen rich medium and single nitrogen source defined medium which requires GS for its assimilation (Fig. 2.17). L-MSO could completely inhibit the growth of *M. tuberculosis* at very low concentration (2µg/ml) when glutamine was used as sole nitrogen source in defined medium. In contrast, the effect of L-MSO was found sub-inhibitory even at 20µg/ml concentration to *M. tuberculosis* when they it was grown in enriched medium containing multiple nitrogen sources. The result suggests that intracellular function is for survival of the pathogen depending on the availability of suitable nitrogen source. Although the host cells harboring *M. tuberculosis* are supposed to be nitrogen source rich, more prominent effect of L-MSO on growth of *M. tuberculosis* during residence in THP-1 macrophage gave clue of fewer

than expected nitrogen source/s available in the phagosome of infected macrophages. L-MSO was also used at NRP-2 stage of mycobacteria culture in Wayne's model and observed that the inhibitor reduced the viability of the bacilli by almost 2logs for both *M. tuberculosis* when proper nitrogen source was used in the medium (Fig. 2.18). In contrast, L- MSO did not have any significant effect on the viability of dormant bacilli when a nitrogen source which does not require GS for assimilation was used as sole nitrogen source in the medium. Results show that GS is required even in the dormant stage of the pathogen though it again depends on available nitrogen sources. Thus, it can be concluded that intracellular function of GS might play a much more important role than its extra cellular function during survival of *M. tuberculosis* and most importantly this enzyme can be a good drug target for latent stage as well.

Dormancy induced by nutrient starvation in *M. smegmatis* provided another advantage of assessing different enzymes and pathways for their functional role. Although NR was not found indispensable during this nutrient starvation induced dormancy, yet its increased activity in this condition provided the clues about its impossible link with the stress response pathway (Figure 2.18 -2.22).

2.5. References

1. Honer K, Bentrup Z, Russel GD. 2001. Mycobacterial persistence: adaptation to a changing environment. *Trends Microbiol.* **9:** 597-605.
2. Wayne LG, Sohaskey CD. 2001. Non-replicating persistence of *Mycobacterium tuberculosis Annu. Rev. Microbiol.* **55:** 139-163.
3. Garay SM. 1996. Pulmonary tuberculosis. In *Tuberculosis*, 1st edn. Rom WN, Garay SM, Editors. Boston: Little, Brown and Company, pp. 373-412.
4. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, *et al*. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393:** 537-544.
5. Hoffmann T, Troup B, Szabo A, Hungerer C, Jahn D. 1995. The anaerobic life of *Bacillus subtilis*: cloning of the genes encoding the respiratory nitrate reductase system. *FEMS Microbiol. Lett.* **131:** 219-225.
6. Hochstein LI, Tomlinson GA. 1988. The enzymes associated with denitrification. *Annu. Rev. Microbiol.* **42:** 231-261.
7. Virtanen S. 1960.A study of nitrate reduction by mycobacteria. *Acta Tuberc. Scand.* **47:** 1-116.
8. Master RN. 1992. Mycobacteriology. In Clinical microbiology procedures handbook. Isenberg HD, Editor. ASM Press, Washington DC: 1992. pp 3.0.1-3.16.4.
9. Stermann M, Sedlacek L, Maass S, Bange FC. 2004. A Promoter Mutation Causes Differential Nitrate Reductase Activity of *Mycobacterium tuberculosis* and *Mycobacterium bovis*. *J. Bacteriol.* **186:** 2856-61.
10. Wayne LG, Hayes LG. 1998. Nitrate reduction as a marker for hypoxic shift down of *Mycobacterium tuberculosis*. *Tubercle Lung Dis*. **79:** 127-32.
11. Sohaskey CD, Wayne LG. 2003. Role of *narK2X* and *narGHJI* in Hypoxic Upregulation of Nitrate Reduction by *Mycobacterium tuberculosis. J. Bacteriol.* **185:** 7247-7256.
12. Weber I, Fritz C, Ruttkowski S, Dreft A, Bange FC. 2000. Anaerobic nitrate reductase (NarGHJI) activity of *Mycobacterium bovis* BCG *in vitro* and its contribution to virulence in immunodeficient mice. *Mol. Microbiol.* **35:** 1017-1025.
13. Fritz C, Maass S, Kreft A, Bange FC. 2002. Dependence of *Mycobacterium bovis* BCG on anaerobic nitrate reductase for persistence is tissue specific. *Infect. Immun*. **70:** 286-291.
14. Boon C, Dick T. 2002. *Mycobacterium bovis* BCG response regulator essential for hypoxic dormancy. *J. Bacteriol.* **184:** 6760-6767.
15. Kelm M. 1999. Nitric oxide metabolism and breakdown. *Biochim. Biophys. Acta* **1411:** 273-

289.

16. Stuehr DJ. 1999. Mammalian nitric oxide synthases. *Biochim. Biophys. Acta* **1411:** 217-230.
17. Bogdan C, Rollinghoff M, Diefenbach A. 2000. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. *Curr. Opin. Immunol.* **12:** 64-76.
18. Brown GC. 1999. Nitric oxide and mitochondrial respiration. *Biochim. Biophys. Acta* **1411:** 351-369.
19. Flesch IE, Kaufmann SH. 1991. Mechanisms involved in mycobacterial growth inhibition by gamma interferon-activated bone marrow macrophages: role of reactive nitrogen intermediates. *Infect. Immun.* **59:** 3213-3218.
20. Hutter B, Dick T. 1999. Upregulation of *narX*, encoding a putative "fused nitrate reductase" in anaerobic dormant *Mycobacterium bovis* BCG. *FEMS Microbiol. Lett.* **178:** 63-69.
21. Hutter, B. and Dick, T. 2000. Analysis of the dormancy-inducible *narK2* promoter in *Mycobacterium bovis* BCG. *FEMS Microbiol. Lett.* **188:** 141-146.
22. Rowe J J, Ubbink-Kok T, Molenaar D, Konings WN, Driessen AJM. 1994. NarK is a nitrite-extrusion system involved in anaerobic nitrate respiration by *Escherichia coli*. *Mol. Microbiol.* **12:** 579-586.
23. Clegg S, Yu F, Griffiths L, Cole JA. 2002. The roles of the polytopic membrane proteins of NarK, NarU and NirC in *Escherichia coli* K-12: two nitrate and three nitrite transporters. *Mol. Microbiol.* **44:** 143-155.
24. Tyagi JS, Sharma D. 2002. *Mycobacterium smegmatis* and tuberculosis. *Trends Microbiol.* **10**: 68-69.
25. *Mycobacterium smegmatis mc^2 155* Genome Page [http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?database=gms).
26. Dick T, Lee, BH. Murugasu-Oei B. 1998. Oxygen depletion induced dormancy in *Mycobacterium smegmatis*. *FEMS Microbiol. Lett.* **163:** 159-164.
27. Wayne LG, Hayes LG. 1996. An in vitro model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of non-replicating persistence. *Infect. Immun.* **64:** 2062-2069.
28. Moreno-Vivián C, Cabello P, Martínez-Luque M, Blasco R, Castillo F. 1999. Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. *J. Bacteriol.* **181:** 6573-6584.
29. Callaway TR, Anderson RC, Genovese KJ, Poole TL, Anderson TJ, Byrd JA, et al. 2002. Sodium chlorate supplementation reduce *Escherichia coli* 0157:H7 population in cattle. *J. Anim. Sci.* **80:** 1683-1689.

30. Wayne LG, Sramek HA. 1994. Metronidazole is bactericidal to dormant cells of *Mycobacterium tuberculosis*. *Antimicrob. Agents. Chemother.* **38:** 2054-2058.
31. Murugasu-Oei B, Dick T. 2000. Bactericidal activity of nitrofurans against growing and dormant *Mycobacterium bovis* BCG. *J. Antimicrob. Chemother.* **46:** 917-919.
32. Merrick MJ, Edwards RA. 1995. Nitrogen control in bacteria. *Microbiol. Rev.* **59:** 604-622.
33. Harth G, Clemens DL, Horwitz MA. 1994. Glutamine synthetase of *Mycobacterium tuberculosis*: extracellular release and characterization of its enzymatic activity. *Proc. Natl. Acad. Sci. USA* **91:** 9342-9346.
34. Singh U, Sarkar D. 2006. Development of a Simple High-Throughput Screening Protocol Based on Biosynthetic Activity of *Mycobacterium tuberculosis* Glutamine Synthetase for the Identification of Novel Inhibitors**.** *J. Biomol. Screen.* **11:** 1035-1042.
35. Manning JM, Moore S, Rowe WB, Meister A. 1969. Identification of l- methionine-*S*-sulfoximine as the diastereomer of l-methionine-*S, R*-sulfoximine that inhibits glutamine synthetase. *Biochemistry* **8:** 2681-2685.
36. Kanamori K, Weiss RL, Roberts JD. 1987. Role of glutamate dehydrogenase in ammonia assimilation pathways in nitrogen-fixing *Bacillus macerans*. *J. Bacteriol*. **169:** 4692-4695.
37. Khan A, Sarkar D. 2006. Identification of a respiratory-type nitrate reductase and its role for survival of *Mycobacterium smegmatis* in Wayne model. *Microb. Pathog.* **41:** 90-95.
38. Stermann M, Bohrssen A, Diephaus C, Maass S, Bange FC. 2003. Polymorphic nucleotide within the promoter of nitrate reductase (*narGHJI*) is specific for *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* **41:** 3252-3259.
39. Berks BC, Ferguson SJ, Moir JW, Richardson DJ. 1995. Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. *Biochim. Biophys. Acta* **1232:** 97-173.
40. Ramakrishnan T, Murthy PS, Gopinathan K P. 1972. Intermediary metabolism of mycobacteria. *Bacteriol. Rev.* **36:** 65-108.
41. Khan A, Akhtar S, Ahmad JN, Sarkar D. 2008. Presence of a functional nitrate assimilation pathway in *Mycobacterium smegmatis*. *Microb. Pathog.* **44:** 71-77.
42. Lyon RH, Hall WH, Costas-Martinez C. 1970. Utilization of amino acids during growth of *Mycobacterium tuberculosis* in rotary cultures. *Infect. Immun.* **1:** 513-520.
43. Hampshire T, Soneji S, Bacon J, James BW, Hinds J, Laing K, *et al***.** 2004. Stationary phase gene expression of *Mycobacterium tuberculosis* following a progressive nutrient depletion: a model for persistent organisms? *Tuberculosis* **84:** 228-238.
44. Betts JC, Lukey PT, Robb LC, McAdam RA, Duncan K. 2002. Evaluation of a nutrient

starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol. Microbiol.* **43:** 717-731.

45. Primm TP, Andersen SJ, Mizrahi V, Avarbock D, Rubin H, Barry III CE. 2000. The stringent response of *mycobacterium tuberculosis* is required for long-term survival. *J. Bacteriol.* **182:** 4889-4898.
46. Voskuil MI, Visconti KC, Schoolnik GK. 2004. *Mycobacterium tuberculosis* gene expression during adaptation to stationary phase and low-oxygen dormancy. *Tuberculosis* **84:** 218-227.
47. McDonough KA, Kress Y, Bloom BR. 1993. The interaction of *Mycobacterium tuberculosis* with macrophages: a study of phagolysosome fusion. *Infect. Agents Dis.* **2:** 232-235.
48. Caltado DA, Haroon M, Schrader LE, Youngs VL. 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun. Soil. Science Plant analysis* **6**: 71-80.
49. Nicholas DJD, Nason A. 1957. Determination of nitrate and nitrite. *Methods Enzymol.* 3**:** 981-984.
50. Bower CE, Holm-Hansen T. 1980. A salicylate-hypochlorite method determining ammonia in sea water. *Can. J. Fish. Aquat. Sci.* **37:** 794-798.
51. Macgregor CH, Schnitman CA, Normansell DE. 1974. Purification and properties of nitrate reductase from *Escherichia coli* K12, *J. Biol. Chem.* **249:** 5321-5327.
52. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
53. Ming Z, Jianhua G, Yuanguang L, Peter FB. 1998. Growth of virulent and avirulent *Mycobacterium tuberculosis* strains in human macrophages. *Infect. Immun.* **66:** 794-799.
54. Dick T. 2001. Dormant tubercle bacilli: the key to more effective TB chemotherapy? *J. Antimicrob. Chemother.* **47:** 117-118.
55. Stewart GR, Robertson BD, Young DB. 2003. Tuberculosis: a problem with Persistence. *Nat. Rev. Microbiol.* **1:** 97-105.
56. Kana BD, Weinstein EA, Avarbock D, Dawes SS, Rubin H, Mizrahi V. 2001. Characterization of the *cydAB*-Encoded Cytochrome *bd* Oxidase from *Mycobacterium smegmatis. J. Bacteriol.* **183:** 7076-7086.
57. Gunsalus RP. 1992. Control of electron flow in Escherichia coli: coordinated transcription of respiratory pathway genes. *J. Bacteriol.* **174:** 7069-7074.
58. Brooks JV, Furney SK, Orme IM. 1999. Metronidazole therapy in mice infected with tuberculosis. *Antimicrob. Agents. Chemother.* **43:** 1285-1288.

59. Harth G, Horwitz MA. 1999. An inhibitor of exported *Mycobacterium tuberculosis* glutamine synthetase selectively blocks the growth of pathogenic mycobacteria in axenic culture and in human monocytes: extracellular proteins as potential novel drug targets. *J. Exp. Med.* **189:** 1425-1436.
60. Bryk R, Gold B, Venugopal A, Singh J, Samy R, Pupek K, *et al*. 2008. Selective killing of non-replicating mycobacteria. *Cell Host Microbe* **13:** 137-45.
61. Weldingh K, Rosenkrands I, Jacobsen S, Rasmussen PB, Elhay MJ, Andersen P. 1998. Two-Dimensional Electrophoresis for Analysis of *Mycobacterium tuberculosis* Culture Filtrate and Purification and Characterization of Six Novel Proteins. *Infect. Immun.* **66:** 3492-3500.
62. Raynaud C, Etienne G, Peyron P, Laneelle M A, Daffe M. 1998. Extracellular enzyme activities potentially involved in the pathogenicity of *Mycobacterium tuberculosis*. *Microbiology* **144:** 577-587.
63. Harth G, Zamecnik PC, Tang JY, Tabatadze D, Horwitz MA. 2000. Treatment of *Mycobacterium tuberculosis* with antisense oligonucleotides to glutamine synthetase mRNA inhibits glutamine synthetase activity, formation of the poly-L-glutamate/glutamine cell wall structure, and bacterial replication. *Proc. Natl. Acad. Sci. USA* **97:** 418-423.
64. Parish T, Stoker NG. 2000. *glnE* is an essential gene in *Mycobacterium tuberculosis. J. Bacteriol.* **182:** 5715-5720.
65. Tullius MV, Harth G, Horwitz MA. 2003. Glutamine synthetase *glnA1* is essential for growth of *Mycobacterium tuberculosis* in human THP-1 macrophages and guinea pigs. *Infect. Immun.* **71:** 3927-3936.
66. Harth G, Horwitz MA. 2003. Inhibition of *Mycobacterium tuberculosis* glutamine synthetase as a novel antibiotic strategy against tuberculosis: demonstration of efficacy *in vivo*. *Infect. Immun.* **71:** 456-464.
67. Harth G, Horwitz MA. 1997. Expression and efficient export of enzymatically active *Mycobacterium tuberculosis* glutamine synthetase in *Mycobacterium smegmatis* and evidence that the information for export is contained within the protein. *J. Biol. Chem.* **272:** 22728-22735.
68. Tullius MV, Harth G, Horwitz MA. 2001. High extracellular levels of *Mycobacterium tuberculosis* glutamine synthetase and superoxide dismutase in actively growing cultures are due to high expression and extracellular stability rather than to a protein-specific export mechanism. *Infect. Immun.* **69:** 6348-6363.

CHAPTER 3

Exploration of Nitrate Reductase (NarGHJI) Activity as a Marker to Develop Dormant Stage Specific Screening Assay: A Novel Tool for Tuberculosis Drug Discovery

3.1. Introduction

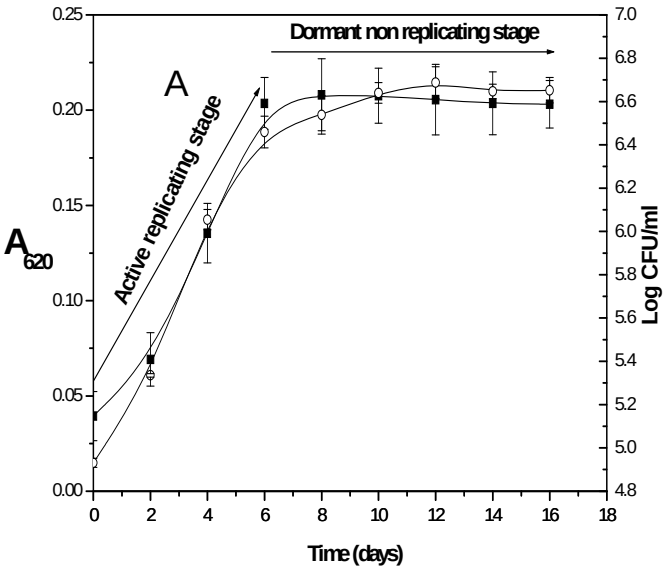
Major obstacle in the development of novel drugs is the lack of a screening system, which can pick inhibitors of latent bacilli of tuberculosis. The screening assays available so far do not also meet certain criteria like high throughput and low cost to run a mass-screening program (1-8). Nutrient starvation and gradual oxygen depletion based models have been established in recent past to cultivate dormant tubercle bacilli *in vitro* (9,10). Oxygen depletion induced dormancy model has gained more acceptances among these *in vitro* models to resemble the latent bacilli present in the host tissues. One of the major difficulties in screening the compounds using this *in vitro* model is its low throughput nature where CFU based determination of inhibition takes almost one month. Identification of NarGHJI as a marker of dormancy and its induction in *M. smegmatis* in the Wayne's *in vitro* hypoxic model prompted us to use this enzyme for developing dormant stage specific screening assay (11). The enzyme has also been reported earlier to be present in virulent strain of *M. tuberculosis* along with its suggested role in the pathogenesis of the bacilli (12,13).

Here in this chapter we have developed a dormant stage specific antitubercular screening assay by adopting the Wayne's hypoxic model in microplate format and monitoring nitrate reduction in *M. bovis* BCG culture. Nitrate reduction by *M. bovis* BCG could successfully reflect the viability of dormant bacilli in microplate format. The active or replicative stage inhibitors of tubercle bacilli could also be identified from the same assay. The assay altogether could provide the advantage of a simple, robust, rapid, safe and low cost screening to identify both active and dormant stage inhibitors against tubercle bacilli.

3.2. Results

3.2.1. Selection of organism and medium

The concept of developing dormant stage specific screening assay in microplate format was first tested on different organisms to compare their ability of nitrate reduction in both the stages (9,14,15). *M. bovis* BCG (ATCC 35745) was considered as the most suitable organism to develop such screening assay due to the advantages of its non pathogenic nature and more genetic closeness to *M. tuberculosis* (16,17) as well as negligible NR activity during aerobic condition (data not shown) (12,18). The strain was first examined for its ability of following dormancy model in microplate format. A_{620}, CFU/ml and decolorization/fading of methylene blue dye within the microplate wells containing *M. bovis* BCG culture was followed at different time intervals. No increase in A_{620} of the culture, constant CFU/ml and fading of methylene blue dye after 6 days of incubation confirmed that Wayne's dormancy model was nicely followed by *M. bovis* BCG culture in microplate well (Fig. 3.1 A) (9).



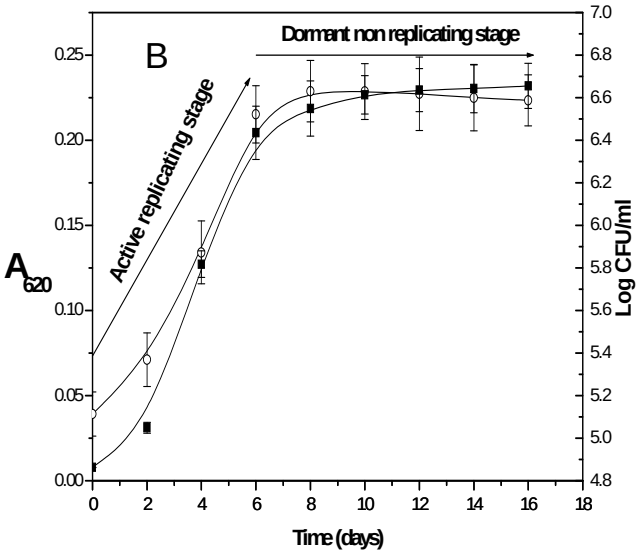


Fig. 3.1 Growth of *M. bovis* BCG in Dubos medium with (A) and without ADS supplement (B) in microplate format of Wayne's dormancy model. A_{620} is represented by closed rectangle and CFU/ml is represented by open circle (○). Experiments were carried out three times and results are mean ± SD using 8 well replicates in each experiment.

The medium used for the growth of bacilli was very complex due to the addition of albumin and dextrose, which brings certain disadvantages for a screening assay as 1) quenching of inhibition due to protein binding, 2) increased possibility of contamination by dextrose and albumin enrichment and 3) increased assay cost. When ADS (albumin, dextrose, saline) supplement was withdrawn from the medium, *M. bovis* BCG still followed the dormancy model in microplate in a similar fashion what was seen in presence of ADS supplement (Fig. 3.1). Thus, ADS could be excluded from the medium to solve the problems associated with it.

3.2.2. NR activity as a marker of dormant stage in microplate assay

While the concept of using activity of an inducible NR to monitor dormancy was initiated by observation from earlier studies on *M. smegmatis*, it was essential to test the same in *M. bovis* BCG to prove the concept (11). Although NarGHJI was already known to be present in genome of *M. bovis* BCG yet the activity profile of the enzyme in this organism was not tested before in Wayne's dormancy model. Therefore, NR activity in microplate format of Wayne model was

monitored with time. Negligible amount of nitrate was reduced when the cells were multiplying in the initial aerobic phase of Wayne model (Fig. 3.2).

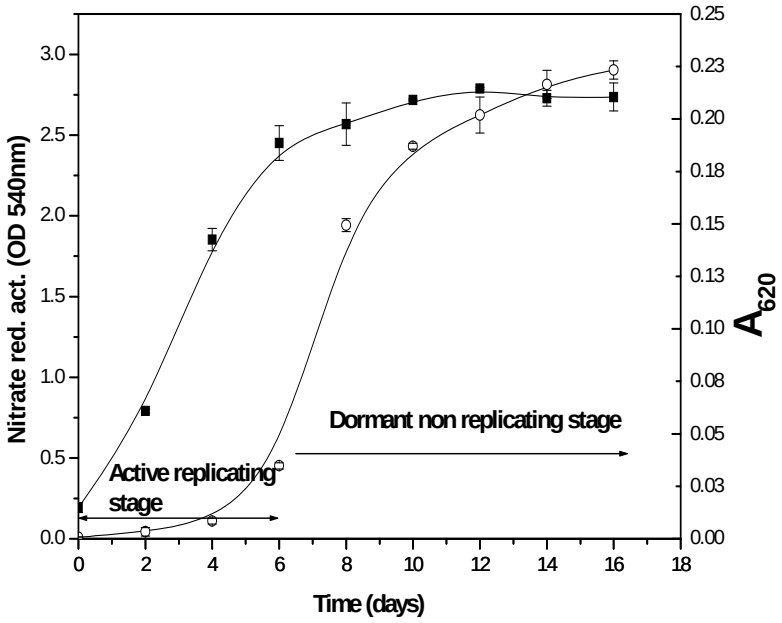


Fig. 3.2 Growth (■) and NR activity (○) of *M. bovis* BCG with time in microplate format of Wayne's dormancy model. Experiments were carried out three times and results are mean ± SD using 8 well replicates in each experiment.

An increased nitrate reduction was clearly seen in the second hypoxic/anoxic phase of Wayne culture of *M. bovis* BCG with simultaneous cessation of cells multiplication. Maximum signal for NR activity was seen at 40mM of nitrate concentration in medium above which it started exerting toxic effect on the cells (data not shown). Therefore, 40mM of nitrate was selected as the optimum substrate concentration for assay. Nitrate reduction started at about 144 hours and maximum signal was achieved within 192 hours incubation. Afterwards, the rate of nitrate reduction though continued but at a decreased rate. Therefore, 192 hours (8 days) was selected as the optimum incubation time for the assay. Known inhibitors of dormant stage were then applied in the microplate assay and NR activity was monitored along with CFU/ml to determine whether NR activity reflects the viability of dormant tubercle bacilli or not (19,20). Concomitant inhibition of NR activity (44 and 38 %) and viability (0.56 and 0.44 logs) of dormant bacilli by

dormant stage inhibitors metronidazole and itaconic anhydride respectively happened with time in this microplate model of dormancy (Table 3.1).

In other words, NR inhibitors were applied to check their effect on viability of dormant stage bacilli as well as to validate the results (21,11). Azide and thiocyanate inhibited NR activity of *M. bovis* BCG by 98 and 97% with a concomitant reduction in viability of dormant bacilli by 2.17 and 2.11 logs respectively (Table 3.1). Both these inhibitors did not show any effect on viability of active aerobic stage bacilli at these concentrations (data not shown). These results proved the utility of this assay in picking dormant stage inhibitors by monitoring just NR activity without waiting for a month for viable cell count on agar plates. Based on NR activity, inhibitory action of a compound against non replicating dormant stage could be calculated by following equation.

Table 3.1 Effect of dormant stage and NR inhibitors on nitrate reduction and viability of dormant *M. bovis* BCG in Active Dormant Antituberculosis Screening (ADAS) Assay.

Inhibitors	Inhibition of viability By log value	% inhibition of nitrate reduction
Metronidazole[a] (0.5M)	0.56 ±[c] 0.04	44.76 ± 4.23
Itaconic anhydride[a] (0.5M)	0.48 ± 0.03	38.27 ± 3.17
Azide[b] (50µM)	2.17 ± 0.15	98.11 ± 2.14
Thiocyanate[b] (40mM)	2.11 ± 0.17	97.22 ± 3.86

[a] dormant stage inhibitors, Concentration of these were selected based on their previous reports. No significant effect on aerobic growth and viability was seen at these concentrations.

[b] NR inhibitors, Concentration of these were selected based on their previous reports. No significant effect on aerobic growth and viability was seen at these concentrations.

[c] SD was calculated from three experiments, each experiment representing 8 wells mean.

$$\% \text{ inhibition of dormant non replicating stage} = 100 - \left[\frac{\text{NR activity in presence of compound - blank}}{\text{NR activity of negative control - blank}} \text{ X } 100 \right]$$

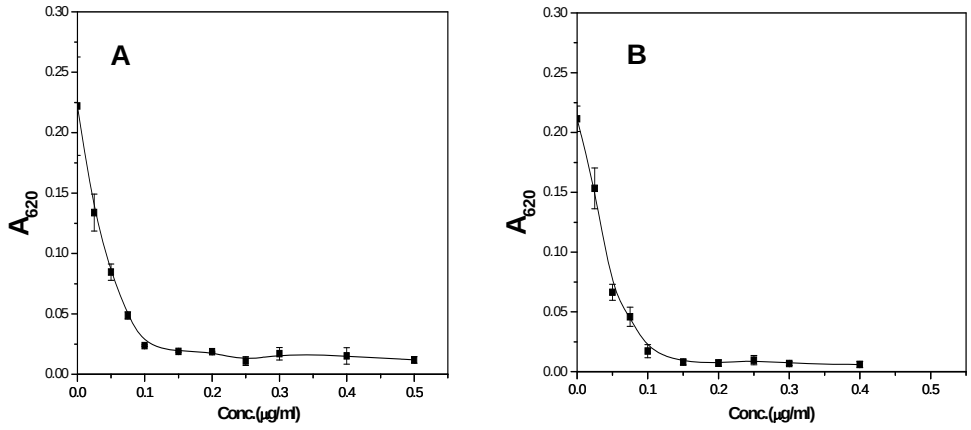
Where

Blank = NR activity of the medium without inoculation

Negative control = NR activity in culture without having compound

3.2.3. Validation of assay using standard active and dormant stage specific antitubercular drugs

Since, Wayne's dormancy model also consists of a distinct aerobic replicating phase along with the dormant non-replicating phase, the assay could be useful in screening the active stage inhibitors as well. This could be done by measuring the absorbance of the culture which is mainly due to the multiplying cells. Reliability of absorbance based identification of aerobic stage inhibitors was further assessed by testing susceptibility of standard antimycobacterial drugs like rifampin, isoniazid, streptomycin and ethambutol which kill the bacilli in aerobic stage. The susceptibility pattern of these agents, when measured by absorbance of the culture, confirmed their action on aerobic replicating phase in a dose dependent manner (Fig. 3.3A, B, C and D). MIC values of these drugs were also comparable with values previously determined by and *Bio-Siv* and BACTEC assay thus validated the assay for testing of aerobic stage inhibitors (Table 3.2) (1,4). Therefore, monitoring A_{620} of the same assay culture after same incubation time (8days) allowed calculation of % inhibition of aerobic replicating stage by compounds using the following equation.



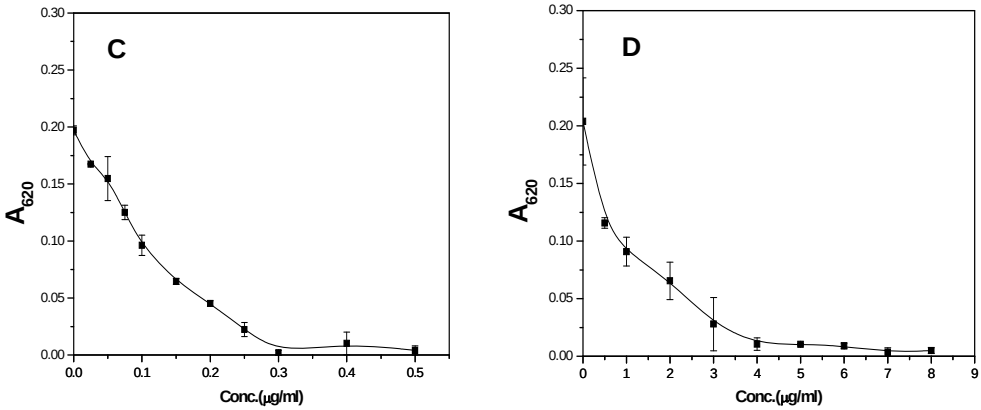


Fig. 3.3 Dose dependent effect of antimycobacterial drugs (A) rifampin, (B) isoniazid, (C) streptomycin and (D) ethambutol on growth of *M. bovis* BCG measured by A_{620} after 8 days of incubation. Experiments were carried out three times and results are mean ± SD using 8 well replicates in each experiment.

Table 3.2 Comparison of MIC values determined by ADAS assay with previously determined by BACTEC and Bio-Siv assays of four antimycobacterial drugs against *M. bovis* BCG.

Antimycobacterial agents[a]	MIC (µg/ml) ADAS assay [b]	previously reported by BACTEC assay	previously reported by Bio-Siv assay
Rifampin	0.08	0.03	0.06
Isoniazid	0.075	0.06	0.06
Streptomycin	0.3	0.25	0.25
Ethambutol	3.0	2.0	4.0

[a]Antimycobacterial agents were added at the time of inoculation.

[b] Determined after 8 days of incubation.

$$\% \text{ inhibition of active replicating stage} = 100 - \left[\frac{A_{620} \text{ of the culture in presence of compound - blank}}{A_{620} \text{ of culture negative control - blank}} \text{ X } 100 \right]$$

Where

Blank = A_{620} of the medium without inoculation

Negative control = A_{620} of culture without having compound

The possibility of same inhibitor acting on both active as well as dormant stage can not be denied. Such dual inhibitors could also be identified by applying active stage inhibitors to the cells already reached to dormant stage in Wayne tube with the use of syringe. Although addition of the compound by syringe has to be done in tube format of dormancy yet this could be quite feasible for low number of active compounds and monitoring of NR activity itself would be easy on non-replicating stage. Thus, the type of inhibitor identified from the assay will be 1) active stage inhibitor, only reducing absorbance of the culture, 2) dormant stage inhibitor, only reducing the NR activity and 3) dual inhibitor, reducing both absorbance and NR activity of the culture.

3.2.4. Robustness and DMSO tolerance of assay

Robustness of ADAS assay was determined by estimating the S/N and Z' factor values. The S/N for NR activity reached maximum at 8.5 for 80µl of culture (Table 3.3). It was not possible to increase the culture volume further because the total volume of assay mixture after addition of the reagents comfortably filled the wells in microplate format. So, the NR activity was checked by transferring 80µl of culture to a separate plate where 80µl of each reagent was added to develop the color. The S/N ratio obtained for NR activity in the assay would be actually 3 fold higher if the whole culture volume (250µl) is considered. Apart from S/N, the Z' factor obtained a 0.81 value of NR activity using 80µl of culture indicated the robustness of the assay protocol (Table 3.3). S/N and Z' factor for absorbance based testing of active stage inhibitors could reach values maximum upto 3.2 and 0.73 respectively.

Table 3.3 S/N ratio and Z' factor for the ADAS assay.

Volume	S/N ratio [b]		Z' factor [c]	
of culture used [a] (µl)	dormant stage [d]	active stage [e]	dormant stage	active stage
50	4.85±0.17	2.45±0.08	0.6212±0.039	0.48±0.043
60	6.02±0.22	2.79±0.07	0.7141±0.031	0.59±0.039
70	7.26±0.19	3.08±0.11	0.7824±0.028	0.65±0.033
80	8.55±0.24	3.23±0.13	0.8115±0.034	0.73±0.037

a No adverse effect of culture was seen on NR activity measurement.

b S/N ratio = control/ blank X 100

[c] Z' factor = 1- (3X SD of control + 3X SD of blank)/mean of control- mean of blank

[d] calculated based on A_{620} of the culture.

[e] calculated based on NR activity

DMSO tolerance. The compounds solutions to be screened for the assay are generally prepared in DMSO. Hence, the maximal tolerable dose (MTD) of DMSO for NR activity and growth in the assay should be calculated before screening of compounds. Therefore, DMSO was added in dose dependent manner to find out its tolerance level of the NR activity in this assay. There was no effect on growth and NR activity observed upto 1% DMSO concentration in the assay (Fig. 3.4). So, the maximum volume of compound solution could be used in the assay was 1% of the total assay mixture.

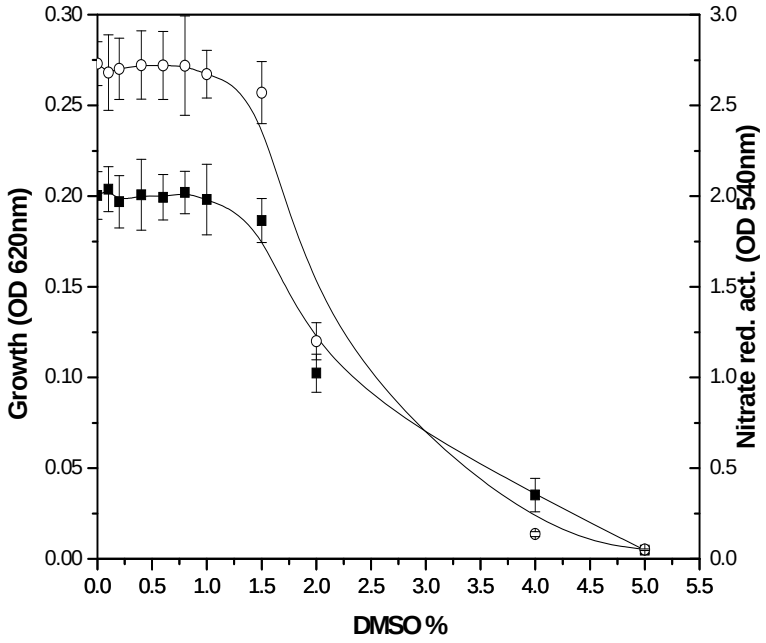


Fig. 3.4 Tolerance of different conc. of DMSO for growth (■) and nitrate reductase activity (○) of *M. bovis* BCG in microplate format of Wayne's dormancy model. Growth and nitrate reductase activity was measured after 8 days of incubation. The results are the means ± SD of 8 wells. Experiments were carried out more than three times and representative data are shown.

3.2.5. Adoption of the assay protocol on HTS platform

In order to carry out High Throughput screening, the ADAS assay protocol was integrated with the controlling SAMI software operative at Beckman Coulter HTS platform. Using this software, assay plates containing 2.5µl of compound solution in DMSO was aseptically transferred from hotel carousal to Biomek 2000. Then, 247.5µl of *M. bovis* BCG culture supplemented with $NaNO_3$ was aseptically added to each well to make up the total volume to 250µl. The plates were transferred to the shaker for 30sec mixing. Then, the plates were moved to sealer. After sealing, the plates were moved to CO_2 incubator for 8days incubation at 37^0 C. After 8 days of incubation, plates were moved from CO_2 incubator to carousal where the seal was manually removed from the plates. From carousal the plates were taken to BMG Polar star for reading at 620nm. After reading plates were moved to Biomek 2000 and 80µl of culture was taken out from each well and transferred to a separate 96 well plate. 80µl of 1% sulphanilic acid solution and 80µl of 0.1% NEDD solution were added in each well of the new plates and moved to carousal for 15 minutes incubation at room temperature to develop pink color. Then, the plates were moved to BMG polar star and read the color at 540nm to measure NR activity. In order to run the program, the HTS system should be operated under Biosafety hood. To make the screening program simple, the program could be divided into two parts. The first part could be terminated after initial incubation for 8days. The second part will start since transferring plates from CO_2 incubator. The throughput capacity could be increased by increasing the capacity of the CO_2 incubator.

3.3. Materials and Methods

3.3.1. Bacterial strains and growth conditions

M. bovis BCG (ATCC 35745) was obtained from AstraZeneca, India. Sub culturing of the strain was routinely done in Dubos albumin agar slants or plates. Liquid inoculum of the organism was prepared in Dubos tween albumin broth medium at 37^0 C and 150 rpm shaking incubation conditions. One percent of 1.0 A_{620} of the culture was used as standard inoculum size for all the experiments, yielding a final inoculum of approximately 10^5 CFU/ml. Viable cell counts were measured by following an earlier described method (9).

3.3.2. Antimycobacterial agents, chemicals, media, apparatus and instrumentation

Rifampin, isoniazid, streptomycin, ethambutol, metronidazole and itaconic anhydride were purchased from Sigma, USA. Drugs were solubilized according to manufacturers' recommendations, and stock solutions were filter sterilized and stored in aliquots at -80^0 C Sulphanilic acid, napthyl ethylene diaminedihydrochloride (NEDD), sodium azide and sodium thiocyanate 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 form Tarsons, India. Well plate sealer of adhesive type was purchased from Nunc, USA. Beckman Coulter platform integrated with Thermo Forma CO_2 incubator, Shaker, Sealer and BMG POLAR star was used to carry out the experiments for assay adoption to high throughput format.

3.3.3. Cultivation of hypoxia based dormant bacilli in microplate

Wayne's *in vitro* 0.5 HSR (head space ratio) model, based on gradual depletion of oxygen from mycobacterial cells to achieve the non-replicating dormant stage was followed to cultivate dormant bacilli in microplate format (9). In order to adapt the model in microplate format, 250µl of the culture containing ~10^5 cells/ml was added to each well of 375µl capacity of 96 well plates and thus exactly maintaining the head space is to culture volume ratio of 0.5. Air supply of the culture in microplate was blocked by applying adhesive type of microplate sealer to allow self generation of hypoxia in the culture. Sealing of plates could be done manually or using a sealer already attached through SAMI software placed on Beckman Coulter HTS platform. Plates were incubated in a CO_2 incubator at 37^0 C. Fading/decolorization of methylene blue (at final concentration of 1.5 mg/L) was used to determine hypoxia/anoxia.

3.3.4. Determination of growth and nitrate reductase activity in microplate

Growth of *M. bovis* BCG in microplate format of dormancy model was measured by reading A_{620} as well as by determining CFU/ml of the culture at different time intervals. The lowest concentration of drugs yielding a differential absorbance (A_{620}) of approximately zero or less was defined as MIC. NR activity of *M. bovis* BCG in microplate dormancy model was measured by following Griess reaction method described earlier (Nicholas and Nason, 1957). Briefly, 1% solution of sulphanilic acid (in 20% HCL) and 0.1% of napthyl ethylene diamine dihydrochloride solution (in distilled water) was added to whole cell culture in 1:1:1 ratio and then reading absorbance at 540nm after 15 minutes of incubation.

3.4. Discussion

Identification of anaerobic stage inhibitors from high throughput screening might help in developing better understanding about persistent stage as well as hopefully a novel drug in future to reduce latent and MDR tuberculosis. So far, pharmaceutical companies could not start antitubercular drug discovery program in large scale due to the lack of an easy whole cell based screening system against persistent and replicating bacilli even though millions of compounds are readily available in their single molecule library. Wayne's dormancy model based on gradual oxygen depletion in *in vitro* culture is considered to represent the persistent stage of bacilli in human better than other models (9). 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 (11). Measurement of NR activity 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 could be considered as most easy and simple marker for monitoring the viability of dormant stage specific bacilli. Selection of *M. bovis* BCG for this assay was justified by the facts that it demonstrated similar pattern of growth and induction of NR activity like *M. tuberculosis* in Wayne's model (Fig. 3.1) (9).The pattern of growth and NR activity in microplate assay was exactly mimicking with Wayne's tube model of dormancy, which indicated the possibility of its adoption for screening protocol (Fig.3.1 and 3.2). 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 tube model in cost effective manner. S/N ratio and Z' factor determined clearly indicated the robustness of the assay protocol (Table 3.3). The most valuable information regarding validation comes from the sensitivity of the assay against standard inhibitors. Dormant stage inhibitors and NR inhibitors could show a correlation between NR activity and viability of bacilli in hypoxic stage of the assay (Table 3.1). MIC values for aerobic stage inhibitors rifampin, isoniazid, streptomycin and ethambutol determined by the ADAS assay were very close with the values determined by other drug susceptibility assays and thus further validated the protocol in favor of its use in screening (Fig 3.3 and Table 3.2) (1,4) against active replicating stage as well. 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 dormant stage specific novel antitubercular molecules. Altogether

exclusion of ADS supplement from medium, minimum number of steps of addition, 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 dormant as well as aerobic replicating phase inhibitors of *M. tuberculosis*.

3.5. References

1. Arain TM, Resconi AE, Hickey MJ. 1996. Bioluminescence screening in vitro (Bio-Siv) assays for high-volume antimycobacterial drug discovery. *Antimicrob. Agents Chemother.* **40:** 1536-41.
2. Changsen C, Franzblau SG, Palittapongarnpim P. 2003. Improved Green Fluorescent Protein Reporter Gene-Based Microplate Screening for Antituberculosis Compounds by Utilizing an Acetamidase Promoter. *Antimicrob. Agents Chemother*. **47:** 3682-87.
3. Chung GAC, Aktar Z, Jackson S, Duncan K. 1995. High-throughput screening for detecting antimycobacterial agents. *Antimicrob Agents Chemother*. **39:** 2235-38.
4. Collins L, Franzblau SG. 1997. Microplate Alamar Blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. *Antimicrob. Agents Chemother*. **41:** 1004-1009.
5. Roberts GD, Goodman NL, Heifets L. 1983. Evaluation of the BACTEC radiometric method for recovery of mycobacteria and drug susceptibility testing of *Mycobacterium tuberculosis* from acid-fast smear-positive specimens. *J. Clin. Microbiol*. **18**: 689-696.
6. Shawar RM, Humble DJ, Van Dalfsen JM, Stover CK, Hickey MJ, Steele S, *et al*. 1997. Rapid screening of natural products for antimycobacterial activity by using luciferase-expressing strains of *Mycobacterium bovis* BCG and *Mycobacterium intracellulare*. *Antimicrob. Agents Chemother.* **41:** 570-74.
7. Srivastava R, Kumar D, Srivastava BS. 1997. Recombinant *Mycobacterium aurum* expressing Escherichia coli ß-galactosidase in high throughput screening of antituberculosis drugs. *Biochem. Biophys. Res. Commun.* **240:** 536-39.
8. Takii T, Yamamoto Y, Chiba T, Abe C, Belisle JT, Brennan PJ, *et al*. 2002. Simple fibroblast-based assay for screening of new antimicrobial drugs against *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* **46:** 2533-2539.
9. Wayne LG, Hayes LG. 1996. An *in vitro* model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of non-replicating persistence. *Infect. Immun.* **64:** 2062-2069.
10. Betts JC, Lukey PT, Robb LC, McAdam RA, Duncan K. 2002. Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol. Microbiol.* **43:** 717-731.
11. Khan A, Sarkar D. 2006. Identification of a respiratory-type nitrate reductase and its role for survival of *Mycobacterium smegmatis* in Wayne model. *Microb. Pathog.* **41:** 90-95.

12. Weber I, Fritz C, Ruttkowski S, Dreft A, Bange FC. 2000. Anaerobic nitrate reductase (*narGHJI*) activity of *Mycobacterium bovis* BCG *in vitro* and its contribution to virulence in immunodeficient mice. *Mol. Microbiol.* **35:** 1017-1025.
13. Fritz C, Maass S, Kreft A, Bange FC. 2002. Dependence of *Mycobacterium bovis* BCG on anaerobic nitrate reductase for persistence is tissue specific. I*nfect. Immun.* **70:** 286-291.
14. Dick T, Lee BH, Murugasu- Oei B. 1998. Oxygen depletion induced dormancy in *Mycobacterium smegmatis*. *FEMS Microbiol. Lett.* **163:** 159-164.
15. Lim A, Eleuterio M, Hutter B, Murugasu-Oei B, Dick T. 1999. Oxygen depletion-induced dormancy in *Mycobacterium bovis* BCG. *J. Bacteriol.* **181:** 2252-2256.
16. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, *et al*. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393:** 537-544.
17. Garnier T, Eiglmeier K, Camus JC, Medina N, Mansoor H, Pryor M, *et al*. 2003. The complete genome sequence of *Mycobacterium bovis*. *Proc. Natl. Acad. Sci. USA* **100:** 7877-7882.
18. Wayne LG, Hayes LG. 1998. Nitrate reduction as a marker for hypoxic shift down of *Mycobacterium tuberculosis*. *Tubercle Lung. Dis.* **79:** 127-132.
19. Wayne LG. 1994. Dormancy of *Mycobacterium tuberculosis* and latency of disease. *Eur. J. Clin. Microbiol. Infect. Dis.* **13:** 908-914.
20. Honer Zu Bentrup K, Miczak A, Swenson DL, Russell DG. 1999. Characterization of Activity and Expression of Isocitrate Lyase in *Mycobacterium avium* and *Mycobacterium tuberculosis*. *J. Bacteriol.* **181:** 7161-7167.
21. Moreno-Vivián C, Cabello P, Martínez-Luque M, Blasco R, Castillo F. 1999. Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. *J. Bacteriol.* **181:** 6573-6584.

CHAPTER 4

Screening of Diverse Compound Library against Active and Dormant Tuberculosis and Appraisal of Identified Hits for Their Antitubercular Drug Perspectives: A Search for Novel TB Drug

4.1 Introduction

The need for new drugs to extend the range of TB treatment options is acute. New chemical entities with novel mechanisms of action will most likely possess activity against MDR-TB (1). However, these alone will not provide the breakthrough that is needed. The key to improving therapy is to develop new agents with potent sterilizing activity that will lead to shortening of the duration of chemotherapy (2). In order to meet the goal of finding the next generation TB medicine within the next decade, a significant increase in effort is required at early stage of the drug discovery process. The known pipeline is relatively sparse and many potential new drugs will be lost by attrition as they proceed through development. It is therefore critical that new leads are identified and then properly resourced lead optimization programmes established. A few new molecules have been disclosed as potential leads for TB drug discovery. These have been identified in complementary screening strategies to secure active entities, which are based on either whole-cell evaluation or profiling against specific biochemical targets. As the treatment of TB infections typically necessitates extended oral dosing regimens, an agent is needed that is both economical to produce and preferably highly specific for mycobacteria to minimize unwanted side effects associated with disturbance of the normal gut flora (3). Unfortunately, most of the compounds described are interesting only because of their activity against growing *M. tuberculosis*. Further effort must be made to identify compounds acting on key targets that are essential for persistence of *M. tuberculosis* if a real breakthrough in therapy is to be made.

The slow growth rate and, more importantly, the highly infectious nature of *M. tuberculosis* preclude its use in a high-throughput screen. Traditional assays and models for early preliminary screening against *M. tuberculosis* are lengthy and also very costly (4-7). Since, we developed a rapid, sensitive, robust and multicontent screening protocol using a non pathogenic organism *M. bovis* BCG (Chapter 3), we seek out to identify novel molecules which possess antimycobacterial activity against the non growing dormant tubercle bacilli (8). The high throughput format of the assay also offers the possibility of screening large numbers of compounds in a system which is highly predictive of activity against *M. tuberculosis.*

Our in-house compound library was equipped with the random classes of chemical structures as well as some derivatives of known antitubercular molecule with known cellular targets. Many pure natural products, fractions and extract from plant sources, were also included in the library to explore the biodiversity of Indian plants. Screening methodology and the characterization of identified antitubercular hits has been described in this chapter in detail.

4.2 Results

4.2.1. Screening results and hits identified

In order to assess the reliability of the assay as well as to search the novel antitubercular molecules, screening was done of a set of diverse chemical library consisting of 1980 extract/fractions from medicinal plants, 124 β-lactams, 20 nitroimidazole derivatives, 96 triazole derivatives and 200 other synthetic compounds for random screening (Table 4.1).

Due to the varied composition of the active ingredients in the crude extracts and fractions obtained from medicinal plants, high concentration (100μg/ml) of the samples was used for screening. It was observed that at this concentration none of the samples in the library developed any significant interference due to its color in either of the read out (data not shown). The histogram of inhibition profile obtained from the screening of 2420 samples separately on growth and NR activity of *M. bovis* BCG followed a typical pattern, which indicated the robustness of the assay protocol (Fig. 4.1A and 4.1B). The samples, which achieved >50% inhibition in either of growth or nitrate reduction were selected as actives. A total of 76 samples were identified as actives against growth of aerobic phase and 21 actives against NR of dormant phase from the screening (Table 4.2).

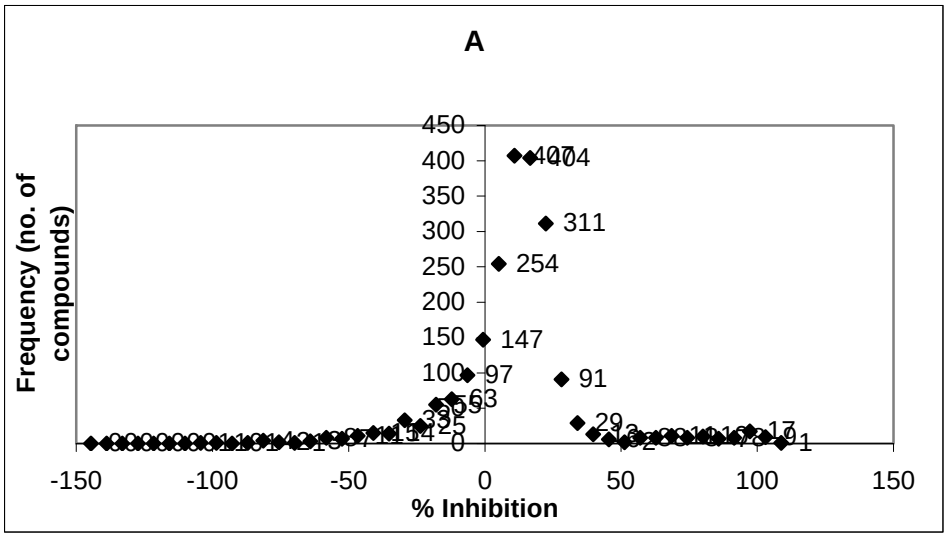
Table 4.1 details of compound library used for screening against ADAS assay.

Compound source	Number
β- Lactams targeted for cell wall inhibitors	124
Nitroimidazoles targeted for DNA synthesis	20
Triazoles targeted for 14 α- demethylase involved in Sterol synthesis	96
Plant extracts and fractions	1980
Other compounds for random screening	200
Total	2420

Table 4.2 Hits identified from screening by ADAS assay

Hits classification	Number
Total no of hits identified	97
No of hits against active stage	76
No of hits against latent stage	21
No of hits confirmed on *M. tuberculosis* H37Ra active stage	74
No of hits confirmed on *M. tuberculosis* H37Ra dormant stage	8
No of hits from synthetic roots	10
No of hits from natural products	72

The confirmation experiment was carried out against *M. tuberculosis* H37Ra using fresh stock solutions of the actives obtained from the primary screen. Finally, 74 samples for growth and 8 samples for NR activity were identified as confirmed hits against *M. tuberculosis* H37Ra. Out of these hits, five compounds (2-nitroimidazole, 3 compounds from 1,2,4-triazole thiols and one natural product; acetoxy chavicol acetate) were identified as hits from their dose response effect and then purdued for secondary stage screening and characterization for promising antitubercular activity (data not shown). This has been described in detail below.



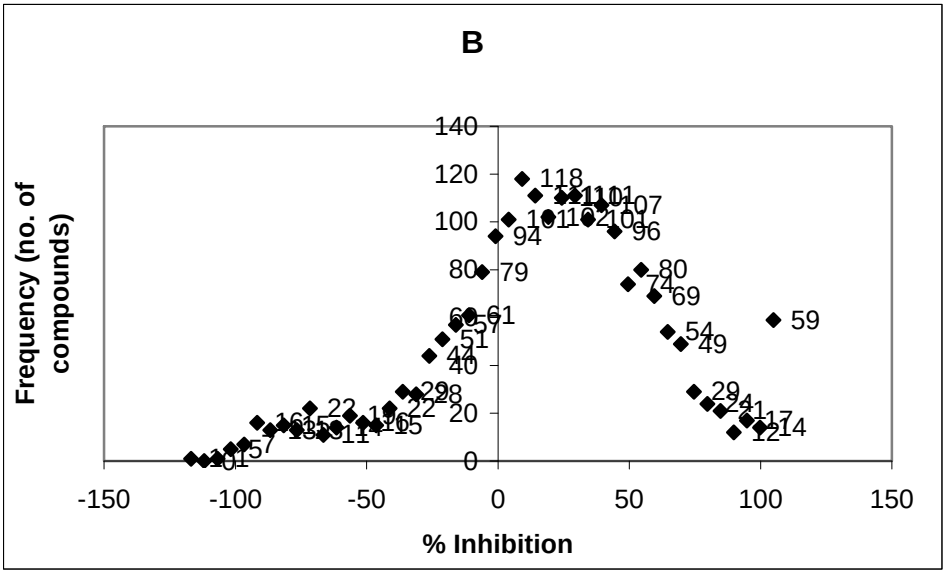


Fig. 4.1 Histogram of percent inhibition of growth (A) and NR activity (B) obtained from screening of the compound library. Screening data of 2420 test compounds obtained from natural products of plants and synthetic compounds against *M. tuberculosis* by using optimized ADAS assay protocol. All the samples screened were used at 100µg/ml concentration as described in materials and methods. Percent inhibition was obtained after normalization of the data obtained in the assay plate. The distribution pattern indicated the robustness of the data.

4.2.2. Bactericidal activity of 2-nitroimidazole against active replicating stage of *M. bovis* BCG and *M. tuberculosis* with intracellular efficacy in THP-1 macrophage

New drug development for tuberculosis has been directed towards the use of nitroimidazole class of compound because of their novel mechanism of action and lesser chances of developing resistance (9). Metronidazole, nitrofurantoin, furaltadone, nitrofurazone, nitroimidazopyran PA-824, CGI 17341 are the active molecules identified in the recent past against TB, which all belong to 5-nitroimidazole series (10-13). A recent finding in *Helicobacter pylori* has clearly shown that 2-nitroimidazole derived molecules are highly effective on strains resistant to even 5-nitroimidazole derivatives (14). Attention has not been paid so far to the evaluation of the effectiveness of 2-nitroimidazole series compounds for their anti-tuberculous potential despite of their known antibacterial activity (15). Nitroimidazoles also remained as major attraction of medicinal chemists to work with, because of their very low level of *in vivo* toxicity (16). These observations prompted us to more precisely evaluate the anti-tuberculous potential of 2-nitroimidazoles.

The present study evaluated the effectiveness and specificity of 2-nitroimidazole against *in vitro* actively replicating and dormant non-replicating tubercle bacilli along with its efficacy in intracellular environment.

Effect of 2-nitroimidazole on active replicating stage of *M. tuberculosis* and *M. bovis* BCG. Most of the nitroimidazole compounds are poorly soluble in aqueous solution. The hydrophobic character of these compounds results in a general tendency to protein binding in solution, which ultimately could affect their bactericidal efficacy even under *in vitro* conditions. In order to properly get a better estimate of the inhibitor, MIC and MBC values were determined in both minimal and ADS (albumin, dextrose and saline) enriched Dubos medium. Different doses of 2-nitroimidazole ranging from 0.1 to 10µg/ml were added at the time of inoculation in the culture flasks rotating at 150 rpm in a shaker incubator at 37^0C. Absorbance at 620nm and CFU/ml was measured after 8 days incubation to determine MIC and MBC values for the compounds, respectively. For both *M. tuberculosis* and *M. bovis* BCG, identical MBC value of 2.26 and 0.556µg/ml were found in enriched and minimal medium respectively (Fig. 4.2A and B).

A sharp decrease in MBC value of about 5 times in minimal medium could be attributed to the availability of limited nutrients to the cells when compared with enriched medium. These results also indicated that medium with minimal nutrient support could be more useful in

evaluating the anti-tuberculous activity of a compound. A higher MIC of 2-nitroimidazole, described in an earlier report could have been observed due to the use of enriched medium in the study (17). This MIC value of 2-nitroimidazole was almost on per with other frontline anti-tuberculous drugs rifampicin, isoniazid, streptomycin and ethambutol (Table 4.3) (18). MIC values for some of the known anti-tuberculous nitroimidazoles; nitrofurantoin, furaltadone as well as the representative 4 and 5-nitroimdazole compounds were also determined to compare the data with 2-nitroimidazole (Table 4.3) (19).

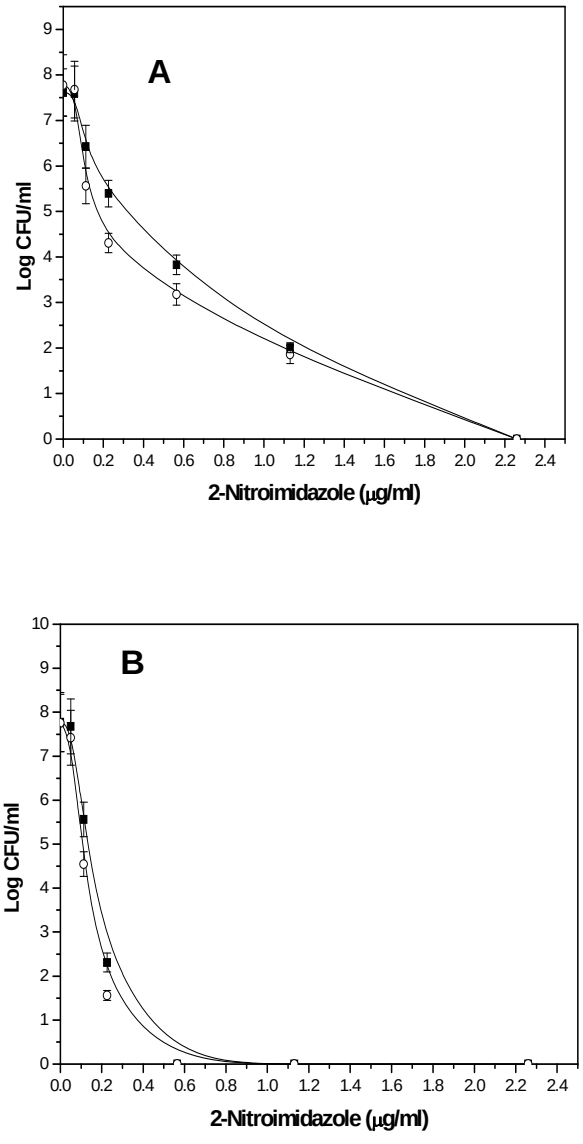


Fig. 4.2 Dose dependent effect of 2-nitroimidazole on growth in enriched (A) and minimal medium (B). Closed rectangle (■) represents *M. bovis* BCG and open circle (○) represents *M. tuberculosis*. Doses of 2-nitroimidazole dissolved in DMSO ranging from 0.1 to 10μg/ml were added at the time of inoculation and

CFU/ml was measured after 8 days of incubation as described in materials and methods section 2.3. Experiments were carried out three times with duplicate cultures and results are mean ± SD.

Table 4.3 MIC values of 2-nitroimidazole compared with other antimycobacterial nitroimidazole compounds.

Compounds[a]	MIC (μg/ml)[b]	
	M. bovis BCG[c]	*M. tuberculosis*[c]
2- nitroimidazole	0.226	0.226
4- nitroimidazole	>50	>50
1,2-dimethyl 5- nitroimidazole	>50	>50
Nitrofurantoin	48	32
Furaltadone	32	32
Rifampin	0.08	0.05
Isoniazid	0.075	0.05
Streptomycin	0.3	0.2
Ethambutol	3	2

[a] all these compounds were dissolved in DMSO.

[b] determined in minimal medium

[c] determined as described in materials and method section..

It was interesting to note that the MIC value of 2-nitroimidazole was at least 100 times less than other nitroimidazoles used in this study. Specificity of the molecule for its antimycobacterial activity was also examined by applying it to a saprophytic mycobacterial strain *M. smegmatis* and representative bacterial strain *E. coli* DH5α (Fig. 4.3). 2-nitroimidazole showed negligible effect on growth of these organisms even up to 50 fold higher concentrations than its determined MIC and MBC against *M. bovis* BCG and *M. tuberculosis*. These results indicated that 2-nitroimidazole is specifically active against tuberculous bacilli.

Effect of 2-nitroimidazole against dormant non replicating bacilli. 2-nitroimidazole was tested against *M. tuberculosis* and *M. bovis* BCG in an *in vitro* dormancy model to evaluate its activity against persistent bacilli as well. Wayne's model which is the most accepted one to mimic dormancy *in vitro*, was selected to assess the effect of 2-nitroimidazole on persistent

bacilli (20). Compound was added to 8 day old Wayne culture, without disturbing the oxygenic environment inside the container. Doses for the compound were kept in range of their determined MIC/MBC (0.226µg/ml) to 20 times of MIC/MBC (4.52µg/ml). The effect of the compound on viability of these dormant bacilli was then examined by CFU enumeration after 96hours of incubation in presence of the compound. Interestingly, the effect of 2-nitroimidazole was found to be very different on these hypoxic dormant bacilli. The compound did not affect the viability of dormant bacilli of both *M. tuberculosis* and *M. bovis* BCG even upto its 20X MIC (4.52µg/ml) (Fig. 4.4).

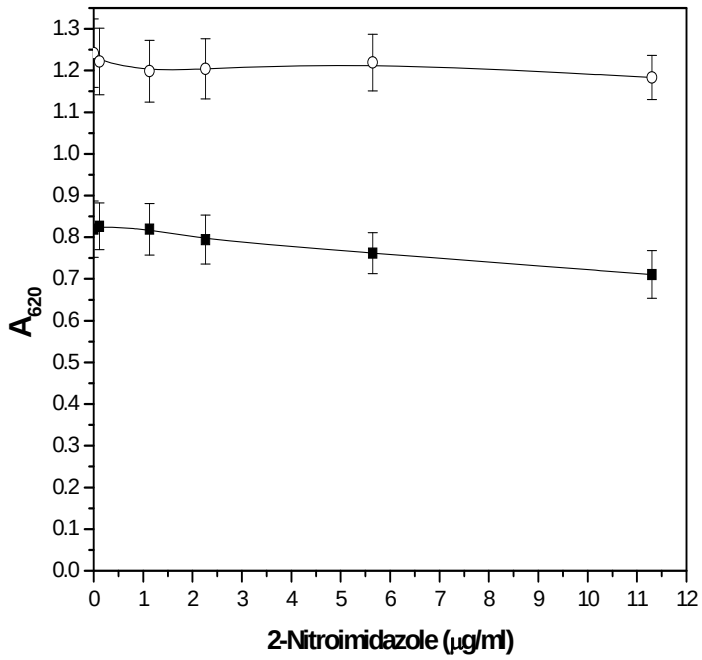


Fig. 4.3 Dose dependent effect of 2-nitroimidazole on *M. smegmatis* (■) and *E. coli* (○). A_{620} represented the growth of the organism which was measured after 72hours and 15hours incubation in presence of the compound for *M. smegmatis* and *E. coli* respectively. Experiments were carried out three times with duplicate cultures and results are mean ± SD.

Nitrofurantoin and furaltadone on the other hand had comparatively better effect (1.1 and 1.3 logs reduction) on viability of these dormant bacilli under such condition. Consistent with previous results, metronidazole showed only moderate bactericidal activity against these hypoxic dormant bacilli (10). These results indicated the inefficacy of 2-nitroimidazole against non-replicating dormant bacilli. Differential anti-tuberculous activity of 2- nitroimidazole (only

against replicating stage), metronidazole (only against dormant bacilli) and nitrofurans (against both active and dormant stage) suggested a thorough evaluation of the proposed mechanism of action of RNI based killing of bacilli through DNA damage by a nitroheterocyclic compounds (11).

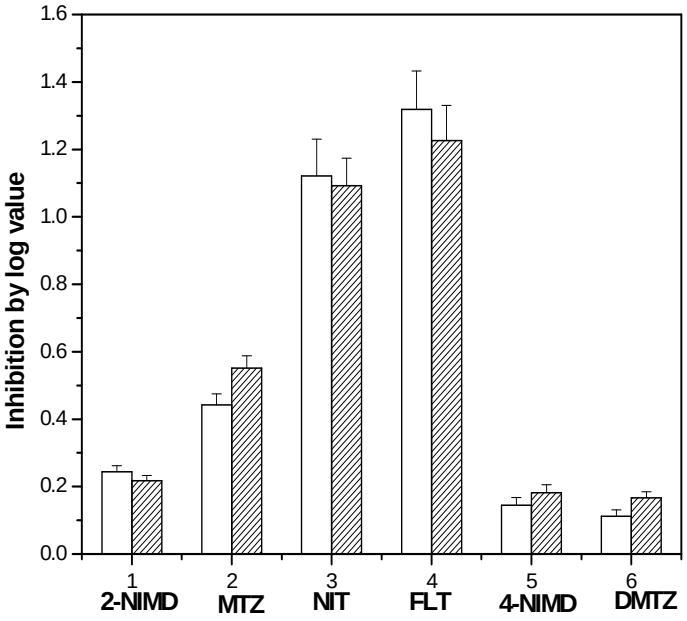


Fig. 4.4 Effect of 2-nitroimidazole (2-NIMD), metronidazole (MTZ), nitrofurantoin (NIT) and furaltadone (FLT), 4-nitroimidazole (4-NIMD) and dimteridazole (DMTZ) on the viability of dormant bacilli in minimal medium at doses of 4.52, 85.5, 48, 32, 50 and 50µg/ml respectively. Dark bars represent *M. bovis* BCG and light bars represent *M. tuberculosis*. Inhibitors were added to 8 days old Wayne culture through syringe and effect was seen after 96hours of incubation by measuring the viable cell count. Inhibitions were calculated by subtracting log values of inhibitors with control. Experiments were carried out three times with duplicate culture and results are mean ± SD.

Effect of 2-nitroimidazole against Intracellular bacilli. Antimycobacterial activity of any molecule under *in vitro* axenic culture condition may lead to false conclusions unless evaluated under the condition that resembles the condition *in vivo*. Considering this factor, 2-nitroimidazole was tested against *M. tuberculosis* when residing within the macrophage. 2-nitroimidazole was applied to THP-1 macrophage infected with *M. tuberculosis*, at concentrations ranging from 0.113 µg/ml to 1.13µg/ml. Killing kinetics was followed by enumerating CFU/ml of the bacilli after lysing the THP-1 cells (Fig. 4.5).

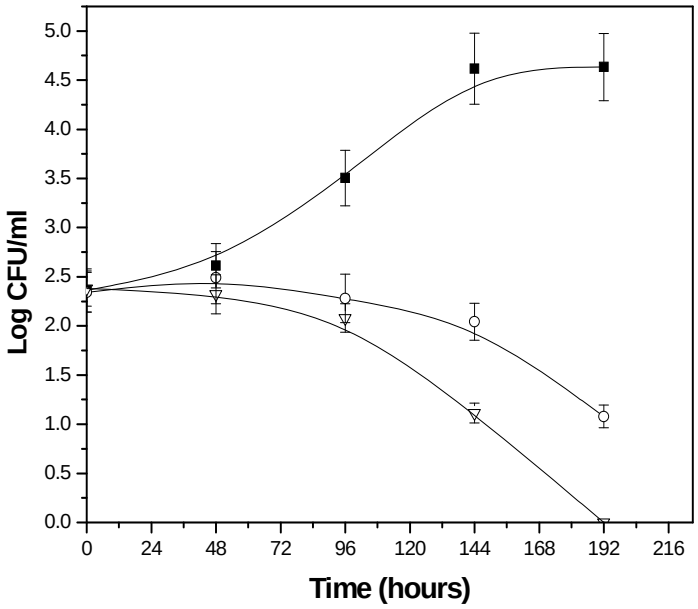


Fig. 4.5 Killing kinetics of 2-nitroimidazole on intracellular *M. tuberculosis* H37Ra. DMSO control (■), 0.113 µg/ml 2-nitroimidazole (O) and 0.565 µg/ml 2-nitroimidazole (∇) was added just after the infection of THP-1 with *M. tuberculosis*. Separate flask of macrophage culture was used for each time point CFU determination. Experiments were carried out three times and results are mean ± SD.

The result clearly indicated the drop in viability of intracellular bacilli started from the very beginning of the incubation. At half of MIC (0.113µg/ml) level of 2-nitrimidazole, 2.5logs reduction in viability of *M. tuberculosis* residing in THP-1 macrophages occurred within 144hours. The inhibitory effect reached its maximum at 2.5 X MIC (0.556µg/ml) of the inhibitor, where complete sterilization occurred within 192hours. The killing kinetics of 2-nitroimidazole clearly supported the earlier observation from axenic cultures that the inhibitory action is restricted to actively growing bacilli. Furthermore, it was also observed that 2-nitroimidazole had

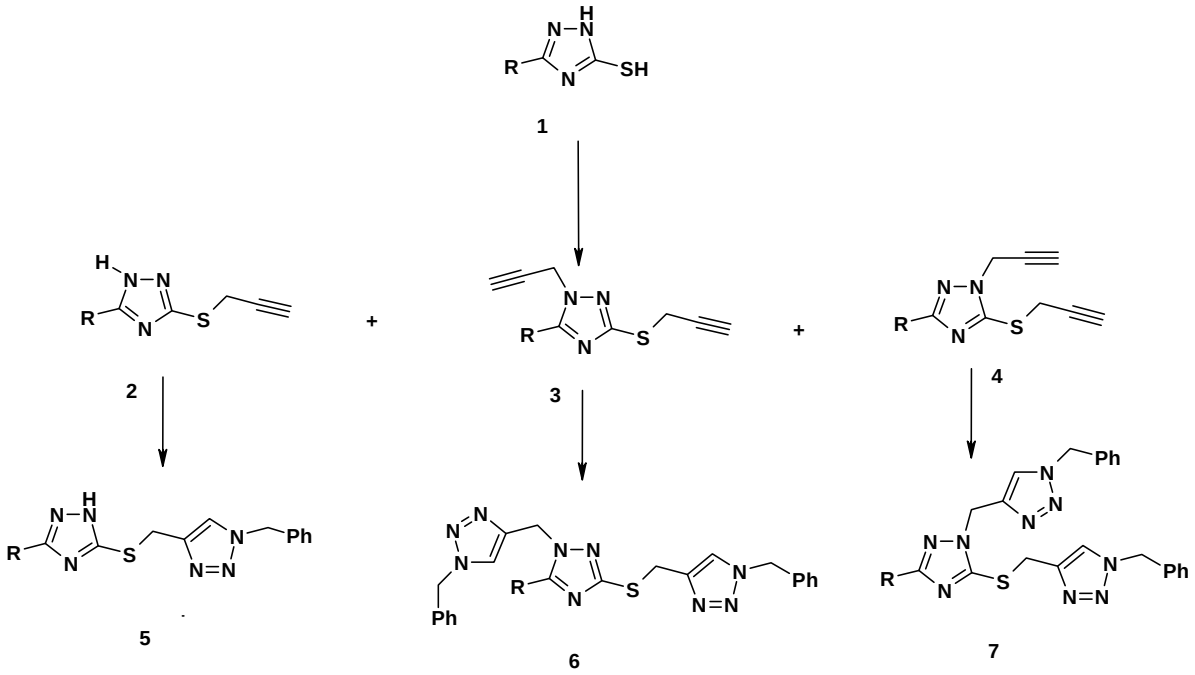
no significant effect on the viability of macrophage THP-1 even upto 100 times of its MIC against *M. tuberculosis* and *M. bovis* BCG (data not shown). These results indicated that the reduction in colony count was due to the bactericidal activity of 2-nitroimidazole and not due to any direct non-specific lethal effect on monocytes THP-1. Altogether, these results showed that the anti-tuberculous potency of 2-nitroimidazole was restored in intracellular environment as well.

4.2.3. Identification of potent 1,2,4-triazolethiols killing non-replicating *M. tuberculosis* and their structure activity relationship

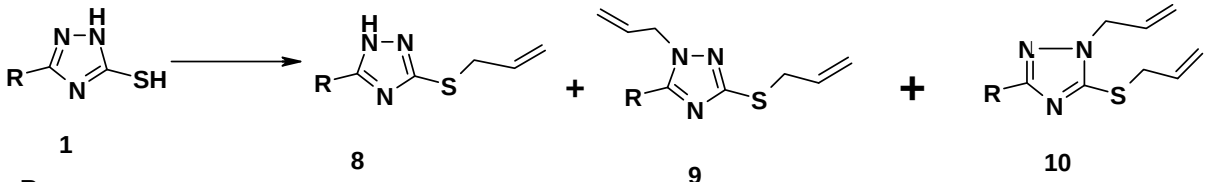
Special attention was given in recent past to synthesize triazole derivatives due to their potent antimycobacterial activity and known target of sterol synthesis (21-23). Commercially important antifungal derivatives fluconazole, hexacoazole are N1 substituted 1,2,4-triazole compounds, which are Ergosterol Biosynthesis Iinhibitors. While antitubercular activity is associated with the derivatives of 1,2,4-triazolethiols in which N4 is substituted (24-26). Based on above observations, we selected 1,2,4-triazole-3-thiols as novel scaffold and substitutions at C5, N1 and N2 positions for their anti-tuberculous activity. Propargyl substitution was chosen so as to enable us for its conversion to 1,2,3-triazole moiety. In particular, we prepared allyl and propargyl derivatives of alkyl, aryl and heteryl substituted 1,2,4-triazolethiols. Hybrid molecules of 1,2,4-triazoles and 1,2,3- triazoles were also prepared from propargylated derivatives adopting "Sharpless click chemistry' conditions. *In vitro* antimycobacterial activity of these propargylated triazole derivatives has been examined against replicating active as well as non replicating dormant phase of *M. bovis* BCG and *M. tuberculosis*.

Schematic presentation of compounds prepared for SAR studies. Synthesis of different derivatives was shown in the schemes 1-3 of Figure 4.6. The starting Compound 1 was substituted with R- at position 5 of the triazole ring where R= H, CH_3-, t-butyl-, p-NO_2-C_6H_4- , p- OCH_3-C_6H_4- and p-Cl-C_6H_4- were prepared from thiosemicarbazide. Propargylation and allylation of these thiols furnished N and S substituted propargyl and allyl derivatives 2,3,4 and 8,9,10 respectively. Hybrid molecules 5, 6 and 7 were prepared by Sharpless click reaction on propargyl derivatives. Oxidation of thiopropargyl compounds gave novel sulphone analogues 11 (scheme 3). The structures were confirmed by IR, NMR and Mass spectral analysis (data not shown). All these compounds were >98% pure.

Scheme-1: Propargylation of 1,2,4-trizole thiols



Scheme 2: Allylation of 1,2,4-trizole thiols



Scheme3: Sulphones

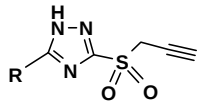


Fig. 4.6

Primary screening results. Newly synthesized triazolethiol derivatives were first screened against *M. bovis* BCG, a member of MBC group of organisms. In primary screening, all compounds were used at 100μg/ml of concentration to select the actives against *M. bovis* BCG. The cut off value was fixed at 60% for selecting the actives against aerobic stage of *M. bovis* BCG. Amongst the triazolethiol derivatives, 17 compounds showed more than 60 % inhibition on growth of aerobic bacilli (Table 4.4). Subsequently, dose response effect was monitored for all these 17 actives applying a concentration range between 100 and 1μg/ml against same aerobically growing *M. bovis* BCG. 5-(4-nitrophenyl)-3-prop-2-ynyllthio-1,2,4-triazole (**2d),** 3-allylthio-5-(4-chlorophenyl)-1,2,4-triazole **(8f)** and 5-(4-nitrophenyl)- 3-prop-2ynylsulfonyl-1,2,4-triazole **(11d**) were found to have significant dose response effect on aerobic bacilli even at 1μg/ml concentration called as hits and pursued for further characterization (Table 4.4).

Specificity of the hits against both stages of *M. bovis* BCG. These three hits were first tested against the bacilli to check their specificity against anaerobic stage also. A proper 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** on *M. bovis* BCG were found to be 2, 0.2 and 2μg/ml respectively (Table 4.5). From the above screening, it was not possible to confirm the inhibitor's action against the dormant phase. In order to know this property, 3 hits were applied on anaerobic stage bacilli in Wayne's tube model culture (20). Tube model of dormancy provided the flexibility of adding compound at any stage of culture without significantly disturbing its oxygen concentration. Hits were added (at MIC) by syringe through rubber septa to 8day old culture of *M. bovis* BCG when all the cells had reached to dormant stage and were then incubated further for 4 days. Effect of these hits on dormant stage was calculated in log value by determining the viable cell count as CFU/ml on Dubos agar plates. **2d** and **11d** reduced the viability of dormant bacilli by 0.75 and 0.95 respectively (Table 4.5). **8f** had even better effect of reducing the dormant bacilli by 1.197 log value than the aerobically growing ones.

Table 4.4 *In vitro* antimycobacterial activity of triazole derivatives against *M. bovis* BCG.

.

Compound	Antimycobacterial activity	
	% inhibition against Active stage (μg/ml)	% inhibition against Dormant stage (μg/ml)
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)
5b	10 (100)	14 (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)
Rifampin[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] 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*

Table 4.5 Secondary screening characterization of identified active molecules.

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

[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 in mycobacterial growth.

Determination of MIC against *M. tuberculosis*, *M. smegmatis* and *E. coli.* In order to verify the antitubercular specific action, these hits were applied on *M. tuberculosis* H37Ra at both the actively growing culture in flask as well as anaerobic dormant culture in Wayne's tube model. Identical MIC values were obtained in aerobic culture against *M. tuberculosis* with *M. bovis* BCG (Table 4.5). The estimated reduction of viable cell count due to the addition of 2d, 8f and 11d in dormant culture were 0.82, 1.432 and 0.95log values respectively. The data obtained indicated that all three inhibitors were almost similarly effective against both MBC organisms. In order to check their effectiveness against nonpathogenic mycobacteria, *M. smegmatis* was used. Apart from *M. smegmatis*, the hits were also tested on *E. coli* to check their specificity against other bacteria. Effect of the compounds was estimated from dose response curve obtained by applying upto 100μg/ml concentrations on cultures of *M. smegmatis* and *E. coli* at the time of inoculation. The effect on growth was calculated by measuring the absorbance of culture at 620nm after an incubation time of 6hours and 72hours for *E. coli* and *M. smegmatis* respectively. None of the hits had any significant effect on growth of either organism (Table 4.4). Hence, the result confirmed their specific action against *M. bovis* BCG and *M. tuberculosis*. Rifampicin, Isoniazid, metronidazole and itaconic anhydride used as standards in these assays gave results identical to that observed elsewhere (8, 27).

Cytotoxicity against the human cell line (THP-1). Hits should be evaluated in terms of its cytotoxicity on mammalian cells particularly the host cell line before taking them ahead into the lead stage in discovery chain. Here, cytotoxicity of the hits was estimated against THP-1

macrophage cell line by using standard cell proliferation assay. Growth inhibitory activity of the compounds was considered as their level of toxicity against mammalian cells compared to rifampicin and isoniazid (Fig 4.7A). It was observed that there was no significant effect on proliferation of THP- cell line at 10 X MIC levels of the hits. This data indicated that these series of triazolethiol derivatives could be initially considered safe from *in vitro* toxicity angle.

Efficacy of the hits against intracellular bacilli. The most serious evaluation of antituberculous hits happens from testing on *in vitro* infection model. Compounds **2d, 8f** and **11d** were applied to THP-1 macrophages infected with *M. tuberculosis* at different concentrations and the killing kinetics was followed by enumerating CFU of the bacilli (Fig 4.7B). The result clearly indicated the drop in viability of intracellular bacilli started from the very beginning of the incubation. Both compounds **2d** and **11d** could completely sterilize THP-1 macrophages from *M. tuberculosis* infection at concentration of 5μg/ml whereas **8f** could achieve sterilization at as low as 1μg/ml concentration. The MBC value of 8f was comparable with rifampicin and isoniazid under same experimental conditions. 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 monocytes.

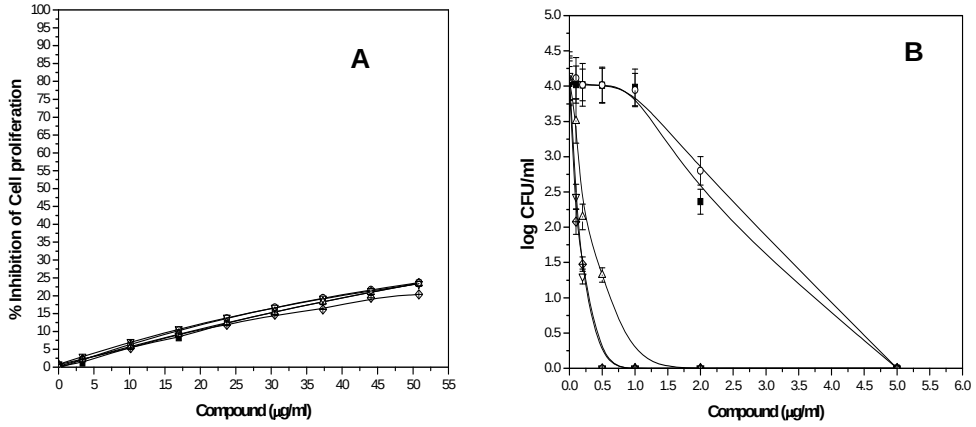
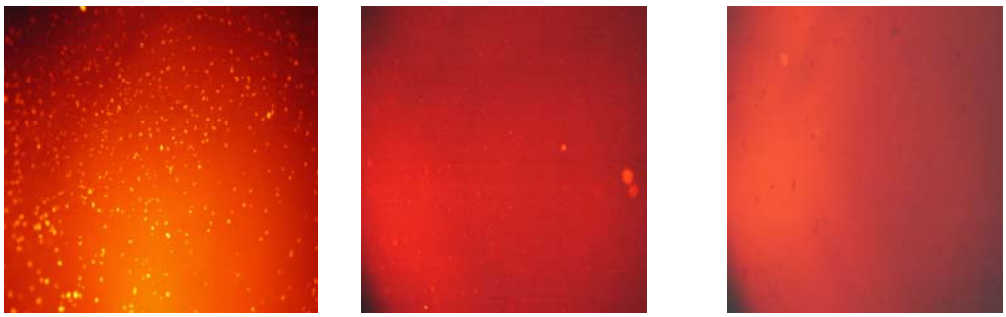


Fig. 4.7

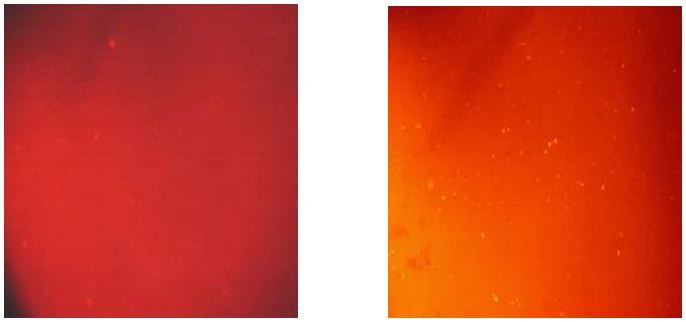
Fig. 4.7 Dose response effect of compounds on growth of (A) THP-1 cells and (B) intracellular *M. tuberculosis*. The proliferation of THP-1 cells as well as intracellular *M. tuberculosis* bacilli growing in Thp1 macrophages were monitored in presence of different concentrations of 2d(■), 8f(Δ),11d(○), rifampin(∇) and isoniazid(◊). The details of experimental procedure for cytotoxicity on Thp1 as well as

intracellular *M. tuberculosis* are described in "Materials and Methods". The results described here represent average with standard deviation of three identical experiments having triplicate values for each data set.

In order to further clarify the effect of these compounds on the viability of intracellular bacilli, microphotographs were taken by selectively staining intracellular bacilli with rodhamine-auramine (Fig. 4.8). The picture showed almost total reduction of intracellular bacilli in presence of these compounds at their respective MIC levels. Under identical condition, rifampicin was used as standard inhibitor to validate the experiment and also found to affect the viability of intracellular bacilli. Metronidazole and itaconic anhydride were also used as dormant stage inhibitors and found to interfere the viability of the intracellular bacilli (data not shown). Altogether, these results showed that the antituberculous potency of these triazolethiol derivatives was restored in intracellular environment and were comparable with best antitubercular drugs as well.



A) Control B) 11d C) 8f



D) 2d E) Rifampicin

Fig. 4.8 Photomicrographs of *M. tuberculosis* H37Ra grown in monocytes-derived macrophage. Infected macrophages were incubated with none (A), **11d** (2μg/ml) (B), **8f** (0.2μg/ml) (C), **2d** (2μg/ml) (D) and Rifampicin (0.1μg/ml) (E) for 8days. Then, the photomicrographs of *M. tuberculosis* cells in macrophages were taken after staining with auramine-rhodamine using a fluorescence microscope as described in "Materials and Methods". Bacilli are bright yellow, and macrophages are dull red.

4.2.4. Antitubercular activity of 1'acetoxychavicol acetate isolated from the rhijomes of *Alpinia galanga* (L) Swartz.

During screening of extracts, fractions and pure natural products from plant sources we also identified 1'-acetoxychavicol acetate (ACA), a major component in *Alpinia galanga* (L.) Swartz as an antimycobacterial agent. 1'-acetoxychavicol acetate, which has been used as curing/antiplasmid agent to get rid of antibiotic resistance in bacterial cells in our earlier studies. The curing mechanism of an antiplasmid agent is based on the blockage of replication of plasmid DNA. Most antiplasmid molecules form a complex with supercoiled form of plasmid DNA that ultimately leads to hindrance in its replication (28-30). The genes encoded by plasmid DNA are not essential for survival of most of the bacteria but they confer the organism extra advantages like F' factor, bacteriocin production, antibiotic resistance (31). A curing agent, who eliminates the plasmid from the daughter cells, can be advantageous in making the cells sensitive to antibiotics. In other way a curing agent makes the killing action easier for other antibiotics. This opened a new area in designing drugs against antibiotic resistant pathogen by applying curing agent in combination with the antibiotic. 1'-acetoxychavicol acetate, which has been identified as one of the most optimum-curing agent in our earlier study (32) was applied along with one antibiotic on whole cell culture of *M. tuberculosis* and *M. bovis* BCG to test the hypothesis that whether the resistance is eliminated. Interestingly, the compound itself inhibited the growth of *M. bovis* BCG and *M. tuberculosis* at very low concentration (Fig. 4.9) without applying other antibiotic. MIC of this compound was found much less (0.2μg/ml) for *M. bovis* BCG and *M. tuberculosis* when compared to other bacteria (more than 800μg/ml) (32).

These results indicated that in case of *M. tuberculosis*, the plasmid becomes essential for survival, which is not seen in other bacteria. Thus, these results add the application of 1'-acetoxychavicol acetate as an antitubercular agent and hence can be directly used as antibiotic against *M. tuberculosis*. These findings also suggest that extra-chromosomal DNA of *M. tuberculosis* could also become important from drug target point of view. Though these results indicate the importance of plasmid DNA for survival of *M. tuberculosis* the precise mechanism of action of 1'-acetoxychavicol acetate is yet to be elucidated. The specific proteins encoded by genes of plasmid DNA also needed to be elucidated along with their role in survival. Role of these proteins encoded by plasmid DNA in non-replicating latent phase could also add new dimensions in antitubercular drug research.

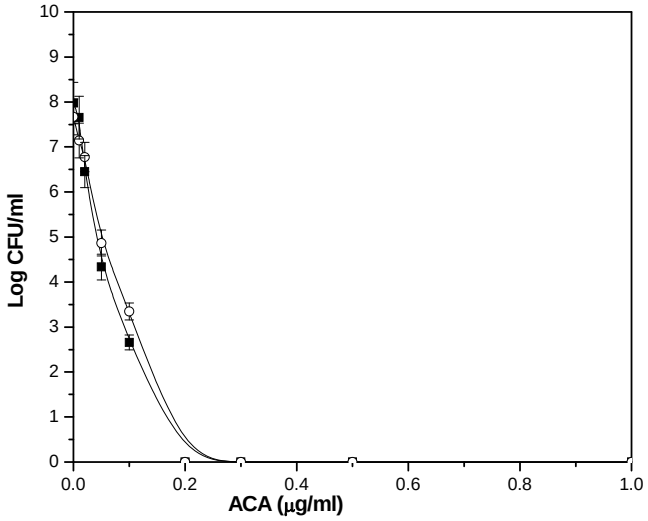


Fig. 4.9 Dose dependent effect of ACA on growth *M. bovis* BCG (■) *M. tuberculosis* H37Ra (○). Doses of ACA were dissolved in DMSO ranging from 0.01 to 10μg/ml were added at the time of inoculation and CFU/ml was measured after 8 days of incubation as described in materials and methods section of this chapter. Experiments were carried out three times with duplicate cultures and results are mean ± SD.

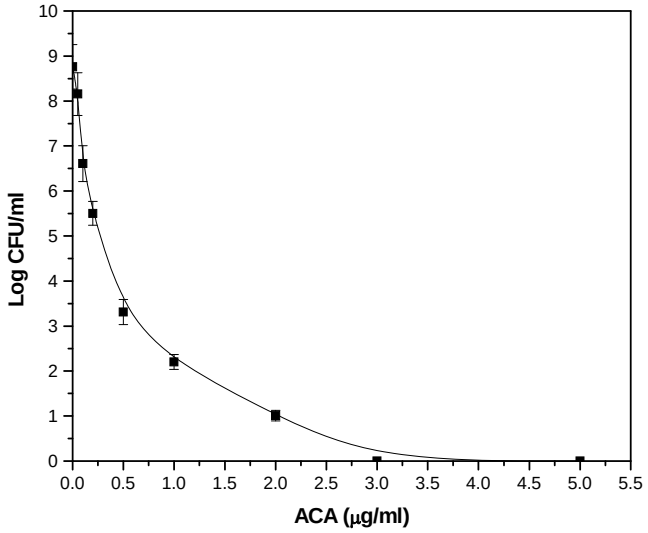


Fig. 4.10 Dose dependent effect of ACA on *M. smegmatis* mc^2155. A_{620} represented the growth of the organism which was measured after 72hours incubation in presence of the compound. Experiments were carried out three times with duplicate cultures and results are mean ± SD.

The compound was also found active against non-pathogenic strain *M. smegmatis* with MIC 2μg/ml, which also indicates the presence of similar type and properties of plasmid in this saprophytic strain (Fig. 4.10). No significant inhibition on growth of *Escherichia coli* (6.92% inhibition) at 50 X MIC values of *M. bovis* BCG and *M. tuberculosis* proved that the plasmid is dispensable for survival in case of *E. coli* (Fig.4.11).

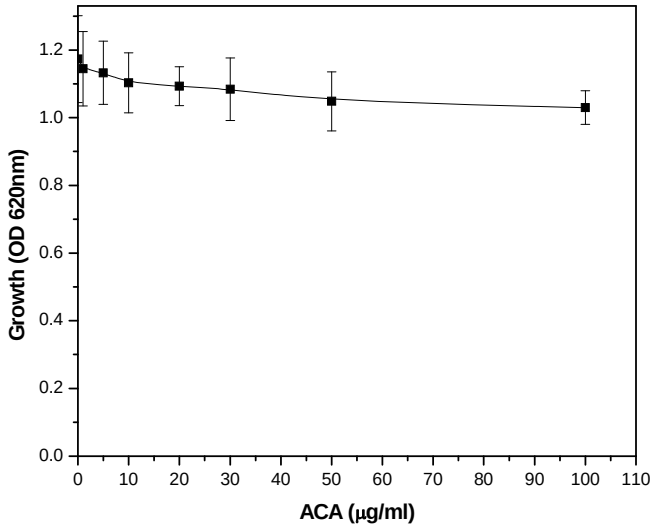


Fig. 4.11 Dose dependent effect of ACA on *E. coli*. A_{620} represented the growth of the organism which was measured after 8hours incubation in presence of the compound. Experiments were carried out three times with duplicate cultures and results are mean ± SD.

Effect of compounds against intracellular bacilli. Antimycobacterial activity of any molecule under *in vitro* axenic culture condition may lead to false conclusions unless evaluated under the condition that resembles the condition *in vivo*. Considering this factor compounds were tested against *M. tuberculosis* when residing within macrophage. Therfore ACA was also applied to THP-1 macrophage infected with *M. tuberculosis* at concentration ranging from 0.1μg/ml to 5.0μg/ml. Killing kinetics was followed by enumerating CFU of the bacilli after lysing the THP-1 cells (Fig. 4.12). The result clearly indicated the drop in viability of intracellular bacilli started from the very beginning of the incubation. The effect reached to maximum limit at concentration of 1μg/ml of the inhibitor where complete sterilization occurred. The killing kinetics of ACA clearly supported the earlier observation from *in vitro* cultures that the inhibitory action is restricted to actively growing bacilli. Furthermore, it was also observed that compound had no significant effect on the viability of macrophage THP-1 even upto 100 times of its MIC against *M. tuberculosis* and *M. bovis* BCG (data not shown). These results indicated that the reduction in

colony count was due to the bactericidal activity of the compound and not due to any direct non-specific lethal effect on monocytes THP-1. Altogether, these results showed that the antitubercular potency of ACA was restored in intracellular environment as well.

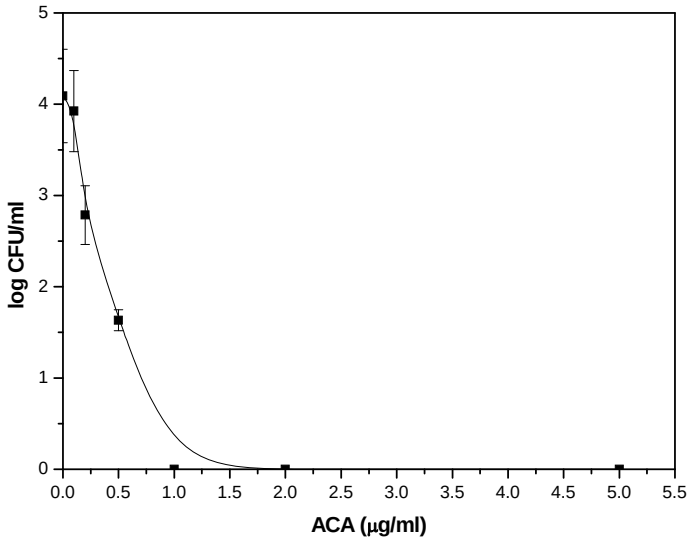


Fig. 4.12 Dose dependent effect of ACA on intracellular *M. tuberculosis* H37Ra within THP-1. Separate flask of macrophage culture was used for each concentration CFU determination keeping a vehicle control of DMSO. Experiments were carried out three times and results are mean ± SD.

Toxicity of the compound was also found insignificant upto 100μg/ml on mammalian cell line THP-1 (Fig. 4.13). This is further supported by the *in vivo* toxicological studies where the crude extract of *Alpinia galanga* did not induce any acute toxic effects in mice even at the dose as high as 3g/Kg body weight and did not show any chronic toxicity when given to mice at 100mg/Kg body weight for 90 days (33). Such activity and toxicity profiles suggest that the mode of action of ACA is rather specific than simple and non-specific interaction with any nucleophilic groups of cellular components. Altogether the invention identifies new application of 1'-acetoxychavicol acetate as a novel antitubercular molecule, targeting plasmid DNA as indispensable element for survival of *M. tuberculosis*.

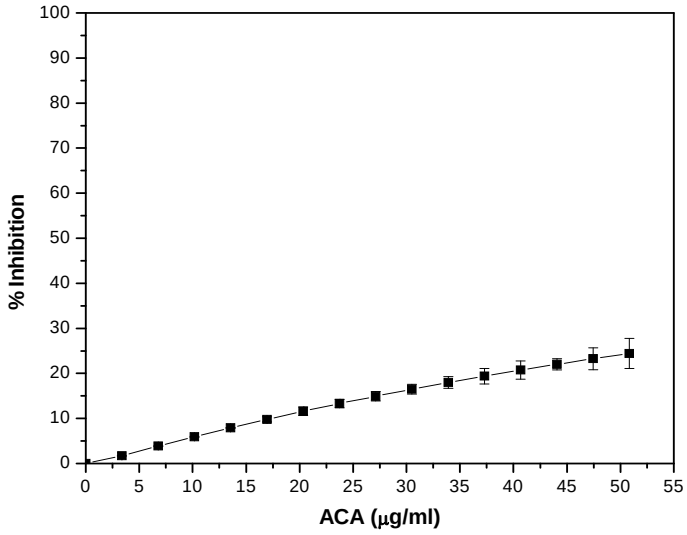


Fig. 4.13 Dose response effect of ACA on growth of THP-1 cells. The details of experimental procedure for cytotoxicity is described in details in "Materials and Methods" of the chapter. The results described here represent average with standard deviation of three identical experiments having triplicate values for each data set.

4.3 Materials and Methods

4.3.1. Bacterial strains and growth conditions

M. bovis BCG (ATCC 35745), *M. smegmatis* (ATCC 607) was obtained from AstraZeneca, India and *M. tuberculosis* H37Ra (ATCC 25177) was obtained from MTCC, India. *E. coli* strain DH5α was obtained from NCIM, India. Sub culturing of all mycobacterial strains was routinely done in Dubos albumin agar slants or plates. Liquid inocula were prepared in Dubos tween albumin broth medium, incubated in a shaker incubator rotating at a speed of 150 rpm at 37^0C. One percent of 1.0 absorbance at 620nm of the culture was used as standard inoculum size for all the experiments, yielding a final inoculum of approximately 10^5 CFU/ml.

4.3.2. Determination of antitubercular activity against active replicating bacilli

Inhibitory activity of the compounds against growing *M. bovis* BCG and *M. tuberculosis* H_{37}Ra bacilli was carried out by incubating the cells in aerobic condition in 100ml flask containing 50ml medium, shaking at 150 rpm and 37^0 C (Thermo electron Model No. 481) (27). Compounds were added at the time of inoculation and growth was measured by reading absorbance at 620nm as well as by determining CFU/ml after 8 days of incubation at which it reaches to stationary phase. The lowest concentration of drugs, yielding a differential absorbance (A_{620}) of approximately zero or less was defined as MIC. The MBC was identified as the smallest concentration of compound that prevented any growth of the test bacterium on agar plate.

4.3.3. Determination of antituberculous activity against hypoxia induced dormant bacilli

Inhibitory activity of the compounds against dormant bacilli was examined by using Wayne's 0.5 HSR (head space ratio) model (20). The Wayne's hypoxic model is based on gradual depletion of oxygen from mycobacterial cells to achieve the non- replicating dormant stage. Briefly, diluted culture of *M. bovis* BCG containing about 10^5 cells per ml 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. Attainment of cells hypoxic non replicating dormant stage was confirmed by constant CFU/ml as well as by decolorization of methylene blue (1.5µg/ml) dye in Wayne culture system. Once all the cells reached to non-replicating phase, compounds were added by using a Hamilton syringe with a 24-gauge needle and incubated for 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.

4.3.4. Determination of specificity of compounds

M. smegmatis and *Escherichia coli* were used as representative non-pathogenic mycobacterial and bacterial strain respectively to examine the specific action of molecules. Compounds were added at the time of inoculation in *M. smegmatis* and *E. coli* cultures in concentrations upto 50 time of MIC obtained against *M. tuberculosis* and *M. bovis* BCG from dose response experiments. Absorbance was measured at 620nm after 6 and 72hours for *E. coli* and *M. smegmatis* respectively to determine their effect on growth.

4.3.5. Determination of cytotoxicity of compounds

Toxicity of the active compounds was done by examining their dose response effect on proliferation of THP-1 cell lines. These cells were incubated with upto 50 fold higher concentration of their MIC. Approximately 100000 cells per ml were seeded in MEM medium containing 10% heat inactivated fetal bovine serum with 100µg/ml of streptomycin and ampicillin respectively. 100µl of this cell suspension was added to each well of sterile 96-well plate and compound was added in the wells at the time of inoculation in a dose dependent manner. 10µl of MTT dye solution (5mg/ml) was added after 72hours of incubation and was incubated for another 1hr. After another 4hours of incubation, 200µl isopropanol was added to the culture to read the absorbance at 490nm to examine their effect on proliferation.

4.3.6. Determination of intracellular efficacy of compounds

Monocyte cell line THP-1 (obtained from National cell repository, NCCS, India) was used to examine the inhibitory activity of the compounds against intracellular bacilli. THP-1 cells (5X 10^4 cells per ml) were treated with 100nM of phorbol myristate acetate in a culture flask for 24hours to convert them into macrophages (34). These macrophage cells were incubated for 12hours with *M. tuberculosis* $H_{37}Ra$ at MOI (multiplicity of infection) of 1:100 for infection. Extracellular mycobacteria 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. The effect of compounds was monitored by determining the bacterial load within macrophage by lysing them with hypotonic buffer (HEPES 10Mm, $MgCl_2$ 1.5mM and KCl 10mM) and spreading the samples on Dubos agar plates at different time intervals to enumerate colonies after 21 days.

4.4 Discussion

While the existence of a TB drug pipeline after decades of virtually no TB drug R&D is welcome, there are still far too few compounds that represent new chemical classes with novel mechanisms of action and a low probability of encountering pre-existing drug resistance. Out of 6 compounds being used in clinical trials, it is unlikely that a useful drug will emerge, given that only about one compound in 10 successfully emerges from these programs. Since new drugs for TB should also be used in combination, to prevent resistance, it would be a responsible act of global leadership to take whatever steps are necessary to induct more and more new lead compounds into the pipeline within least possible timeframe.

Early-stage drug discovery represents one of the key bottlenecks in the search for new anti-TB drugs. A major issue in this context is the lack of number of programs to increase the number of leads in TB drug pipeline. Combinatorial chemical libraries are likely to be of low yield, and those that are affordable may be of low quality, or may not include access to ready re-supply, which is necessary to work up hits that appear promising in early stages. The scientific rationale is that target-based approaches have been unsuccessful in the area of anti-infective agent discovery (35,36), whereas two new potential antimicrobial agents, platensimycin, and the diarylquinoline, R207910, have recently emerged from whole organism screens (37,38). However, shortening TB chemotherapy requires the discovery of novel drugs which will be able to kill dormant *M. tuberculosis* (39), justifying the search for new dormancy–related molecular targets as well. Given the urgency, both target-based screening and phenotypic screening should be pursued in parallel to increase the chances of filling the TB drug candidate pipeline. Therefore, creation of optimized compound libraries for the discovery of antituberculosis is also a realistic proposition that needs to be taken into serious consideration.

Our results of identification of 5 novel leads from a small set of compound library suggested the importance of rationale designing of library. Screening of nitroimidazoles and azoles were done based on the provided clues from previous medicinal chemistry approaches. Nitroimidazoles were selected due to their known biological activity against helminthes, protozoa fungi and bacteria (15). Based on the superior biological activity of 5-nitroimidazoles, other nitroimidazoles were evaluated initially when this drug class was being considered for its anti-tuberculous potential. Although some reports described antibacterial activity of few 2-nitroimidazole derivatives in 1970's, their anti-tuberculous activity was not appreciated or evaluated further (17). Meanwhile, major attention was created by 5-nitroimidazole derivatives and at least one of them went on to clinical trials (40). An almost 5- fold reduction in MIC value obtained in minimal

medium compared to enriched medium indicates that whole cell based anti-tubercular screening should be carried out in the former (Fig. 4.2). Otherwise protein binding due to the presence of albumin in enriched medium could mislead the assessment procedure.

MIC of 2-nitroimidazole was found to be 100-200 times lower than the MIC of reported anti-tuberculous 5-nitroimidazole compounds nitrofurantoin and furaltadone (Table 4.3). This indicated that 2-nitroimidazoles could become much more potential anti-tuberculous agents than 5-nitroimidazoles. Indeed, the MIC of 2-nitroimidazole was comparable with the lead nitroimidazole PA-824 (<1μg/ml) that has reached the clinical trial stage (40). Moreover an earlier investigation suggested a reduced probability of the development of resistance for 2-nitroimidazoles when compared to 5-nitroimidazoles (14).

The intracellular killing efficacy against *M. tuberculosis* within host macrophage proved the potential of 2-nitroimidazole. This efficacy justifies the compound being taken forward as a lead agent (Fig. 4.5). Studies in animal system are being carried out in the laboratory to assess the efficacy of compound *in vivo*.

Metronidazole, nitrofurantoin, furaltadone and nitrofurazone, which all belong to 5-nitroimidazole series, are the currently known antimycobacterial agents, effective against dormant stage of tubercle bacilli in Wayne's dormant culture system, yet their effective concentration is much higher than desired (10,11). 2-nitroimidazole on the other hand, though found ineffective against dormant bacilli, its lower MIC/MBC value indicated the possibility of finding molecules effective against dormant stage too, from this series at desired concentration (Table 4.3 and Fig. 4.4). Specific inhibitory activity of 2-nitroimidazole only against *M. tuberculosis* and *M. bovis* BCG also provides clues as to the target for this molecule, which is not present in non-pathogenic *M. smegmatis* or in other bacteria such as *E. coli* (Fig. 4.3). Revealing the mode of action and identifying the target of the compound is again anticipated before taking 2-nitroimidazoles for further developments.

The insignificant cytotoxic effect of 2-nitroimidazole on mammalian cell line THP-1 even at 100 times higher concentration than MIC strengthens the arguments for evaluating this compound further (data not shown). Synthesis of more 2-nitroimidazole derivatives based on their structure activity relationship is being carried out to find other active compounds from this class. In conclusion, our results demonstrate the potential of 2-nitroimidazole against actively growing tubercle bacilli, along with its intracellular efficacy, indicating that this compound should be considered further for optimization within an anti-tuberculous drug development program.

Selection of triazole for screening was based on known antifungal, antiviral and plant growth activity combined with information for their antimycobacterial potential in recent years (41,42). It

was earlier observed that antitubercular activity was associated with N4 substituted derivatives of 1,2,4-triazolethiols (24-26). Here, in this invention various N1,N2 and S substituted regioisomeric propargyl and allyl derivatives of 1,2,4-triazole were synthesized along with some hybrid molecules of type 5, 6 and 7 (1,2,4-triazole linked to 1,2, 3-triazole) and tested for their antituberculous activity. 17 out of 55 compounds were selected as primary screen hits based on their effect on aerobic replicating stage of *M. bovis* BCG (Table 4.4). 3 compounds (2d, 8f and 11d) were found to have significant and specific effect on both the stages even at 1.0µg/ml concentration on growing bacilli (Table 4.4 and 4.5). SAR study also indicated that neither propargylation nor allylation at N1 and N2 positions in triazole ring was effective in improving the potency of the molecules. In all these three structures, propargyl or allyl groups attached only to the thiol group in C-3 position with p-nitrophenyl/p-chlorophenyl substitutions at C-5 position pointed towards the importance of 3 and 5 positions of triazole ring in the antituberculous 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 alter its biological activity. However, both the compounds were equally effective against *M. tuberculosis* (Table 4.5). The inhibitory effect of these compounds against the organisms indicated that an inductive effect of the group attached to the *p*-position of the phenyl ring connected to C-5 position of 1,2,4 triazolethiol is very important in bringing the potency against the bacilli. An electron withdrawing group at p-position of phenyl substitute at C-5 position in the triazole ring was required to get effectiveness against tuberculosis. Replacing $-NO_2$ group by Cl- with simultaneous change of propargyl to allyl group attached with thiol at position 3 of triazole ring had increased the potency of the molecule by 10fold as anti-tubercular agent. Interestingly, in all these three leads, a C-C multiple bonds are present at the terminal position of alkyl group attached to thiol at C-3 position. Very recently, the propargyl group attached to a heterocyclic ring was proposed to tautomerise to allene, which again indicated that a double bond character at the terminal position might be important for their biological activity (43). Currently, analogues are being made to further explore the requirement of the type and position of multiple bonds with various combinations to improve the potency of the scaffold. However, specific inhibition on MBC group of organisms also raised question against their acting on cytochrome P450 carrying proteins (44). However, the nontoxic nature of these compounds (2d, 8f, 11d) on THP-1 cell line coupled with their specific action against tuberculous mycobacteria has made them most attractive inhibitors (Fig. 4.6 and 4.7). Presently, detail investigations are being carried out in our laboratory to identify their target protein/s and biochemical pathway/s in *M. tuberculosis*. The chemical structures of these three leads are simpler than the most potent antitubercular agents

being used in clinical trials, which provide rooms for accommodating further modifications, if needed, to improve the efficacy during optimization stage (37,40).

Alpinia galanga is widely cultivated in India, China and south-east Asian countries, such as Thailand, Indonesia, and Philippines. The rhizomes of this plant are extensively used as spice or ginger substitutes for flavoring foods, and also in traditional medicine for several purposes, such as stomachic, or for carminative, antiflatulent, antifungal, and anti-itching. In chemical and pharmacoloical studies of *A. galanga*, the pungent principal compound, 1'-acetoxychavicol acetate, was reported to possess antitumor, anti-inflammatory, pungency, antifungal, gastroprotective,and xanthine oxidase inhibitory activities (46). However, Very little was known about the antimycobacterial activity of the compound. Only the crude extract of *Alpinia galanga* has been demonstrated to have an activity similar to that of isoniazid (47). In Our study 1'-acetoxychavicol acetate, isolated from *Alpinia galanga* had promising antituberculous activity against both *M. bovis* BCG and *M. tuberculosis in vitro* at a very low MIC of 0.2μg/ml (Fig. 4.9). 1'-Acetoxychavicol acetate also exhibited very potent antimycobacterial activity against *M. tuberculosis* during its intracellular residence in THP-1 with an MIC value of 0.2μg/ml (Fig. 4.13). Moreover plasmid curing based activity of the compound offers new perspectives to control the replication, and the potential for this small molecule to disrupt plasmid replication and re-sensitize bacteria to antibiotics (48,49).

4.5 References

1. Cohen T, Murray M. 2004. Modeling epidemics of multidrug-resistant *M. tuberculosis* of heterogeneous fitness. *Nat Med.* **10:** 1117-1121.
2. O'Brien RJ, Spigelman M. 2005. New drugs for tuberculosis: Current status and future prospects. *Clin. Chest. Med.* **26:** 327-340.
3. Salomon JA, Lloyd-Smith JO, Getz WM, Resch S, Sánchez MS, *et al*. 2006. Prospects for advancing tuberculosis control efforts through novel therapies. *PLoS Med.* **3:** 273.
4. Arain TM, Resconi AE, Hickey MJ. 1996. Bioluminescence screening in vitro (Bio-Siv) assays for high-volume antimycobacterial drug discovery. *Antimicrob. Agents Chemother.* **40:** 1536-41.
5. Changsen C, Franzblau SG, Palittapongarnpim P. 2003. Improved Green Fluorescent Protein Reporter Gene-Based Microplate Screening for Antituberculosis Compounds by Utilizing an Acetamidase Promoter. *Antimicrob. Agents Chemother*. **47:** 3682-87.
6. Chung GAC, Aktar Z, Jackson S, Duncan K. 1995. High-throughput screening for detecting antimycobacterial agents. *Antimicrob Agents Chemother*. **39:** 2235-38.
7. Collins L, Franzblau SG. 1997. Microplate Alamar Blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. *Antimicrob. Agents Chemother*. **41:** 1004-1009.
8. Khan A, Sarkar D. 2008. A simple whole cell based high throughput screening protocol using *Mycobacterium bovis* BCG for inhibitors against dormant and active tubercle bacilli. *J. Microbiol. Methods* **73:** 62-68.
9. Barry CE, Boshoff HIM, Dowd CS. 2004. Prospects for clinical introduction of nitroimidazole antibiotics for the treatment of tuberculosis. *Curr. Pharm. Des.* **10:** 3239-
10. Wayne LG, Sramek HA. 1994. Metronidazole is bactericidal to dormant cells of *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* **38:** 2054-58.
11. Murugasu-Oei B, Dick T. 2000. Bactericidal activity of nitrofurans against growing and dormant *Mycobacterium bovis* BCG. *J. Antimicrob. Chemother.* **46:** 917-19.
12. Stover CK, Warrener P, VanDevanter DR, Sherman DR, Arain TM, Langhorne MH, *et al*. 2000. A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. *Nature* **405:** 962-66.
13. Ashtekar DR, Costa-Perira R, Nagrajan K, Vishvanathan N, Bhatt AD, Rittel W, et al. 1993. *In vitro* and *in vivo* activities of the nitroimidazole CGI 17341 against *Mycobacterium*

tuberculosis. *Antimicrob. Agents Chemother.* **37:** 183-86.

14. Smith MA, Edwards DI. 1995. Redox potential and oxygen concentration as factors in the susceptibility of *Helicobacter pylori* to nitroheterocyclic drugs. *J. Antimicrob. Chemother.* **6:** 751-764.
15. Grunberg E, Beskid G, Cleeland R, DeLorenzo WF Titsworth E, Scholer HJ, *et al*. 1967. Antiprotozoan and antibacterial activity of 2-nitroimidazole derivatives. *Antimicrob. Agents Chemother.* **7:** 513-519.
16. Roe FJ. 1985. Safety of nitroimidazoles. *Scand. J. Infect. Dis Suppl.* **46:** 72-81.
17. Sun Z, Zhang Y. 1999. Antitubercular activities of certain antifungal and antihelminthic drugs. *Tuberc. Lung Dis.* **79:** 319-320.
18. Wallace RJ Jr, Nash DR, Steele LC, Steingrube V. 1986. Susceptibility testing of slowly growing mycobacteria by a microdilution MIC method with 7H9 broth. *J. Clin. Microbiol.* **6:** 976-81.
19. Khan A, Sarkar D. 2006. Identification of a respiratory-type nitrate reductase and its role for survival of *Mycobacterium smegmatis* in Wayne model. *Microb. Pathog.* **41:** 90-95.
20. Wayne LG, Hayes LG. 1996. An *in vitro* model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of non-replicating persistence. *Infect. Immun.* **64:** 2062-2069.
21. Bellamine A, Mangla AT, Ness WD, Waterman MR. 1999. Characterization and catalytic properties of the sterol 14α- demethylase from *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. USA* **96:** 8937-8942.
22. Guardiola-Diaz HM, Foster LA, Mushrush D, Vaz ADN. 2001. Azole- antifungal binding to a novel cytochrome P450 from *Mycobacterium tuberculosis*: implications for the treatment of tuberculosis. *Biochem. Pharmacol.* **61:** 1463-470.
23. Podust LM, Poulos TL, Waterman MR. 2001. Crystal structure of cytochrome P450 14alpha-sterol demethylase (CYP51) from *Mycobacterium tuberculosis* in complex with azole inhibitors. *Proc. Natl. Acad. Sci. USA* **13:** 3068-3073.
24. Gülerman NN, Doan HN, Rollas S, Johansson C, Çelik C. 2001. Synthesis and structure elucidation of some new thioether derivatives of 1,2,4- triazoline-3-thiones and their antimicrobial activities. *Farmaco II* **56:** 953-958.
25. Kandemirli F, Shvets N, Unsalan S, Küçükgüzel I, Rollas S, Kovalishyn V, *et al*. 2006. The structure antituberculosis activity relationships study in a series of 5-(4-aminophenyl)-4-substituted-2,4-dihydro-3h-1,2,4-triazole-3-thione derivatives. A combined electronic-topological and neural networks approach. *Med. Chem.* **2:** 415-422.

26. Ozdemir A, Turan-Zitouni G, Kaplancikli ZA, Chevallet P. 2007. Synthesis of some 4-arylidenamino-4H-1,2,4-triazole-3-thiols and their antituberculosis activity. *J. Enzyme Inhib. Med. Chem.* **22:** 511-516.

27. Khan A, Sarkar S, Sarkar D. 2008. Bactericidal activity of 2-nitroimidazole against active replicating stage of *Mycobacterium bovis* BCG and *M. tuberculosis* with intracellular efficacy in THP-1 macrophage. *Int. J. Antimicrob. Agents* **32:** 40-45.

28. Spengler G, Molnár A, Schelz Z, Amaral L, Sharples D, Molnár J. 2006. The Mechanism of Plasmid Curing in Bacteria. *Current Drug Targets* **7:** 823-41.

29. Levin BR, Bergstrom CT. 2000. Bacteria are different: observations, interpretations, speculations, and opinions about the mechanisms of adaptive evolution in prokaryotes. *Proc. Natl. Acad. Sci. U. S. A.* **97:** 6981-6985

30. Hahn FE. 1979. Chemotherapy of bacterial plasmids. *Naturwissenschaften* **66:** 555-562.

31. Bouanchaud DH, Chabbert YA. 1971. Practical effectiveness of agents curing R factors and plasmids. *Ann. New York. Acad. Sci.* **182:** 305–311.

32. Lata C, Shriram VD, Jahangirdar SS, Dhakephalkar PK, Rojatkar SR. Curing of antibiotic resistance plasmid by 1'-Acetoxychavicol Acetate. Indian Patent. Application No. 297/DEL/2006 International Classification : A61K 31/05.

33. Qureshi S, Shah AH, Ageel AM. 1992. Toxicity studies on *Alpinia galanga* and *curcuma longa*. *Planta medicine.* **58:** 124-127.

34. McDonough KA, Kress Y, Bloom BR. 1993. The interaction of *Mycobacterium tuberculosis* with macrophages: a study of phagolysosome fusion. *Infect. Agents Dis.* **2:** 232-235.

35. Pasqualoto KFM, Ferreira EI. 2001. An approach for the rational design of new antituberculosis agents. *Curr. Drug Targets* ***2*****:** 427-437.

36. Payne DJ, Gwynn MN, Holmes DJ, Pompliano DL. 2007. Drugs for bad bugs: Confronting the challenges of antibacterial discovery. *Nat. Rev. Drug Discov.* **6:** 29-40.

37. Andries K, Verhasselt P, Guillemont J, Gohlmann HW, Neefs JM, *et al*. 2005. A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. *Science* **307:** 223-227.

38. Wang J, Soisson SM, Young K, Shoop W, Kodali S, *et al*. 2006. Platensimycin is a selective FabF inhibitor with potent antibiotic properties. *Nature* **441:** 358-361.

39. McKinney JD. 2000. In vivo veritas: The search for TB drug targets goes live. *Nat. Med.* **6:** 1330-1333.

40. Lenaerts AJ, Gruppo V, Marietta KS, Johnson CM, Driscoll DK, Tompkins NM, *et al*. 2005. Preclinical testing of the nitroimidazopyran PA-824 for activity against *Mycobacterium*

tuberculosis in a series of *in vitro* and *in vivo* models. *Antimicrob. Agents Chemother.* **49:** 2294-2301.

41. Elena B, Giuditta S, Daniele Z, Maria GM, Luciano V, Marco F, *et al*. 2006. Antifungal and antimycobacterial activity of new imidazole and triazole derivatives. A combined experimental and computational approach. *J. Antimicrob. Chemother.* **58:** 76-84.
42. Klimesova V, Zahajska L, Waisser K, Kaustova J, Mollmann U. 2004. Synthesis and antimycobacterial activity of 1,2,4-triazole 3-benzylsulfanyl derivatives. *Fármaco* **59:** 279-288.
43. Kumar D, David WM, Kerwin SM. 2001. N-Propargyl-2- alkynylbenzothiazolium Aza-enediynes: Role of the 2-Alkynylbenzothiazolium Functionality in DNA Cleavage. *Bioorg. Med. Chem. Letters* **11:** 2971-2974.
44. Bellamine A, Lepesheva GI, Waterman MR. 2004. Fluconazole binding and sterol demethylation in three CYP51 isoforms indicate differences in active site topology. *J. Lipid Res.* **45:** 2000-2007.
45. Hisashi M, Toshio M, Hiromi M, Masayuki Y. 2003. Antiallergic Principles from *Alpinia galanga*: Structural Requirements of Phenylpropanoids for Inhibition of Degranulation and Release of TNF-_ and IL-4 in RBL-2H3 Cells. *Bioorg. Med. Chem. Lett.* 13: 3197-3202.
46. Yang X, Eilerman RG. 1999. Pungent principle of Alpinia galanga (L.) Swartz and its applications. *J. Agricult. Food Chem.* **47:** 1657-1662.
47. Palittapongarnpim P, Kirdmanee C, Kittakoop P, Rukseree K (2002): 1'-Acetoxychavicol acetate for tuberculosis treatment. United States Patent Application 20020192262.
48. Heinemann JA. 2001. Can smart bullets penetrate magic bullet proof vests? *Drug Discov. Today* **6:** 875-878.
49. DeNap JC, Hergenrother PJ. 2005. Bacterial death comes full circle: targeting plasmid replication in drug-resistant bacteria. *Org. Biomol. Chem.* **3:** 959-966.

CHAPTER 5

Isolation, Purification and partial characterization of respiratory Nitrate Reductase (NarGHJI) from *M. tuberculosis* H37Ra.

5.1. Introduction

Nitrate reductase [NaR; EC 1.7.99.4; Nitrate: (acceptor) oxidoreductase] is a membrane-bound enzyme and is present in many anaerobic and facultative anaerobic prokaryotes (1). It plays an important role in energy production when oxygen is not readily available or completely absent in the environment. The purification and properties of Nitrate reductase (NarGHJI) has been subjected to extensive investigation and characterization in *Escherichia coli* and *Bacillus licheniformis* (2,3). The enzyme is composed of three subunits: a catalytic α subunit (NarG) of 112 to 140 kDa with MGD cofactor, a soluble β subunit (NarH) of 52 to 64 kDa with one [3Fe-4S] and three [4Fe-4S] centers, and a 19- to 25-kDa membrane biheme *b* quinol-oxidizing γ subunit (NarI) (4). Soluble α and β subunits are anchored to the cytoplasmic side of the membrane by the γ subunit and can be solubilized by detergents or heat. NarI is heat sensitive and can be lost during the purification procedure, leading to the isolation of a soluble αβ complex that can reduce nitrate with reduced viologens as electron donors. A δ polypeptide (NarJ), which is not part of the final enzyme, seems to participate in the assembly or stability of the αβ complex prior to its membrane attachment (5,6). The enzyme uses the quinol pool as the physiological electron donor and generates a PMF by a redox loop mechanism (7,8). NarI oxidizes quinols at the periplasmic side of the membrane, releasing two protons into the periplasm. Electrons are passed to NarG, via the Fe-S centers of NarH, to reduce nitrate with consumption of two cytoplasmic protons. The low- and high-potential heme *b* groups of NarI located at opposite sides of the membrane allow an effective transmembrane electron transfer (9). The enzyme has also been characterized in some other denitrifying and nitrate respiring organisms like, *Pseudomonas aeruginosa, Pseudomonas denitrificans* and *Klebsiella aerogenes* but very little is known about its counterpart in *M. tuberculosis* (10-13). Though the enzyme present in the *M. tuberculosis* was found more than 70% homologous to NarGHJI of *E. coli*, yet this genetic difference could bring a substantial divergence to the structure as well as function of the enzyme (14). It was therefore of interest to isolate, purify and characterize this membrane bound NarGHJI from *M. tuberculosis* in order to understand the basic role of this enzyme during anaerobic dormant stage. This could help in understanding the chemical and physical nature of nitrate reductase for establishing the functional structure of nitrate reductase and its relationship with the survival of the organism during latent stage. .In this chapter I have described a simple method for the preparation of pure nitrate reductase from *M. tuberculosis* H37Ra, which involves release from membranes by selective detergent and purification by a single gel filtration step. I have also described the partial

characterization of NarGHJI from *M. tuberculosis* H37Ra including its molecular weight, optimal activity conditions, substrate specificity and inhibition kinetics.

5.2. Results

5.2.1. Purification

NarGHJI from *M. tuberculosis* H37Ra was purified by modifying a method published earlier (15). Protein purification was divided into three stages. The stages were preparation of membrane fractions, extraction of enzyme from membranes and final purification. It was important that a rapid, sensitive and specific assay should be available for the detection of protein of interest during purification. A bionebulization-based method of cell lysis was found suitable to break the tough cell wall of mycobacteria and prepare membrane fractions (16). Initially, different agents such as 100mM potassium phopsphate buffer, Triton X-100 and Tween 80 were used to extract the membrane protein without losing enzyme activity. Triton X-100 based extraction of nitrate reductase from membrane fractions of *M. tuberculosis* could provide the maximum yield of the enzyme without losing any discernible activity. Ion exchange chromatography with Unosphere Q beads (anion exchanger) was found to finally purify the enzyme to almost homogeneity level with elution at 1.25M NaCl, using fast performance liquid chromatography. The final scheme of the purification after optimization of each of these steps is elaborated below.

Culture conditions for purification: *M. tuberculosis* H37Ra culture was grown in minimal medium, supplemented with 5mM of sodium nitrate at 130 rpm and 37^0C within a shaker incubator. Cultures were inoculated with 1% of 1.0 A_{620} as initial inoculums size and were incubated upto 10 days when it reached to late exponential phase of growth in 1-litre flask. These cultures were kept static for another 2 days for anaerobic induction of NR.

Cell lysis and preparation of membrane fractions: All the operations, except where otherwise stated, were performed at 4^0C hereafter. Culture was centrifuged at 8000 rpm for 30 minutes to harvest the cells. Cell pellet was washed twice with 50mM Tris HCL buffer of pH 7.5. Between 6 to 8gm packed wet weight of cells was obtained from 3 liters of culture. Cells were resuspended in 10 ml of 100 mM Tris/HCL buffer (pH 8.0) for e gram of pellet. This cell suspension was subjected to bionebulization based cell disruption system (BioNeb Glas-Col, USA) for 10 cycles of 3 minutes each at 200psi (16). Clumps were broken completely after 10 cycles of bionebulization and a homogenized cell suspension was obtained. Lost volume of

suspension during bionebulization was adjusted with same Tris/HCL buffer. 5mM EDTA, 0.5mg/ml lysozyme and 50µg/ml DNAse + RNAse was added to this lysate and slowly stirred for 30 minutes to lyse the remaining intact cells. Further efficiency of lysis was increased with fridge thawing of the suspension for 5 times. Lysate was then centrifuged at 3000 rpm for 5 minutes at room temperature to pellet down the cell debris. Supernatant was centrifuged at 25000 g for 2 hours to collect the membrane fractions at bottom. Enzyme activity was examined in these membrane fractions to ensure that these membrane fractions contain the enzyme in sufficient quantity (Table 5.1).

Extraction of enzyme from membrane fractions: Resulting pellet of membrane fractions was washed twice with same Tris/HCL buffer and resuspended in 50mM sod phosphate buffer (pH 7.2) containing 0.1mM of 2-mercaptoethanol and 0.1 mM phenyl methanesulfonylfluoride (PMSF). This suspension was added with Triton X-100 to a final concentration of 2% triton. The mixture was stirred at 100 rpm on a magnetic stirrer for 30 minutes and centrifuged at 25000 g for 2 hours. This supernatant found to contain significant amount of nitrate reductase activity while pellet fraction obtained after this centrifugation did not show any discernible nitrate reductase activity, hence confirmed the effective extraction of the enzyme (Table 5.1).

Heat treatment: A heat treatment step commonly used to remove unwanted proteins during the purification of this nitrate reductase was also attempted here (17). After treating the extracted protein sample at 65^0C for 7 minutes, sample was kept overnight at 4^0C and centrifuged at 15,000 rpm for 60 minutes to remove heat precipitated proteins. This could remove many unwanted proteins and increased the purity of the enzyme. However, inclusion of this also resulted in the loss of one of the subunit from the enzyme confirmed by SDS PAGE done after FPLC (Figure 5.1). Though loss of one of the subunit did not cause any loss of enzyme activity, this heat treatment step was excluded in this protocol to retain all the three subunits of nitrate reductase (NarGHI). Samples were therefore subjected to ion exchange chromatography in the next step itself.

Table 5.1 Purification scheme of nitrate reductase from *Mycobacterium tuberculosis* at different steps.

Fraction	Total protein (mg)	%	Activity units[a]	(%)	Specific activity (units/mg protein)	Purification (fold)
Cell suspension	467	100	1184	100	2.53	1
Cell free lysate	351	75.16	872	73.6	2.48	0.98
Crude membrane fraction	98	20.98	790	66.72	8.06	3.18
Triton X 100 extract	32	6.85	722	60.97	22.56	8.91
Pooled Unosphere Q eluted	3.4	0.72	602	50.76	177.1	69.96
Dialyzed and concentrated by filtration	3.1	0.66	589	49.74	190	75.09
After Gel filtration and concentration	2.7	0.57	440	37.16	162.97	64.41

[a] 1 Unit = 1 μM NO_2 produced per minute at 37^0C.

[b] Protein concentration were determined by using the BioRad protein assay kit with bovine serum albumin as standards (BioRad Laboratories, Hercules, CA, USA).

Ion exchange chromatography: Ion exchange chromatography was used for purification of this enzyme from *M. tuberculosis*. Unosphere Q anion exchanger column (size 26mm X 10 cm) was attached with the basic system (BioRad, Biological Duoflow) for fast performance liquid chromatography (FPLC). Extracted protein solution was injected into the column already, equilibrated with 50mM of sodium phosphate buffer (pH 7.2). The column was washed sequentially with 50 ml of the above buffer, with a linear gradient of 0-2.0M of NaCl in the above buffer at a flow rate of 1ml/min. The A_{280}, conductivity, pH and nitrate reductase activity were measured on all fractions and SDS-PAGE analysis was performed for fractions of interest. Active fractions from the column were pooled, dialyzed for 12 hours against the above phosphate buffer and concentrated by filtration with 3Kd filter (AMIKON, Millipore). The profile of protein in active fractions confirmed the preparation of an almost homogenous nitrate reductase in SDS-PAGE (Fig. 5.1). The purification achieved by this fast flow column was more than 60 fold and with a yield of about 50% (Table 5.1). The peak nitrate reductase activity eluted at salt

concentration of 1.25M NaCl in the gradient (Fig. 5.2). The fractions with the activity were eluted in the salt concentration range between 1.35 to 1.15 M NaCl concentrations. The pooled fractions which contained more than 20% of the peak of activity yielded a total volume of 3.6ml. The specific activity of enzyme was increased from 22.55 to 177.1 units/mg of protein after this chromatography. There is a clear protein band seen at the expected molecular weight range in lane 7 of the SDS PAGE which corresponds with the elution of nitrate reductase activity at peak (Fig. 5.1).

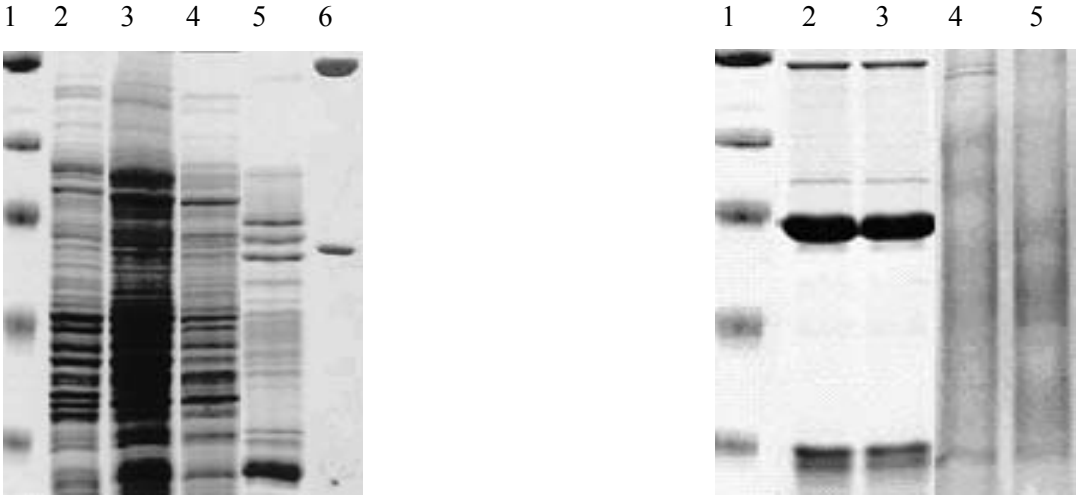


Fig. 5.1 SDS-PAGE analysis during different steps of purification of nitrate reductase. A and B shows the purification of enzyme including and excluding the heat treatment step respectively. Molecular weight markers used in the lane 1 of A and B were: β- galactosidase (Mr 117 Kd), phosphorylase b (Mr 97 Kd), bovine serum albumin (Mr 66 Kd), ovalbumin (Mr 45 Kd) and soybean trypsin inhibitor (Mr 21). Lane 2,3,4,5 and 6 of A were loaded respectively with crude cell lysate, membrane extract, heat treated extract at 65^0C, heat treated extract at 70^0C, FPLC peak activity fraction of heat treated sample. Lane 2,3,4 and 5 of B were loaded respectively with concentrated FPLC peak activity fraction, duplicate of lane 2, membrane extract, crude cell lysate. 20µl of protein sample having concentration of at least 200µg/ml was loaded and stained with comassie blue.

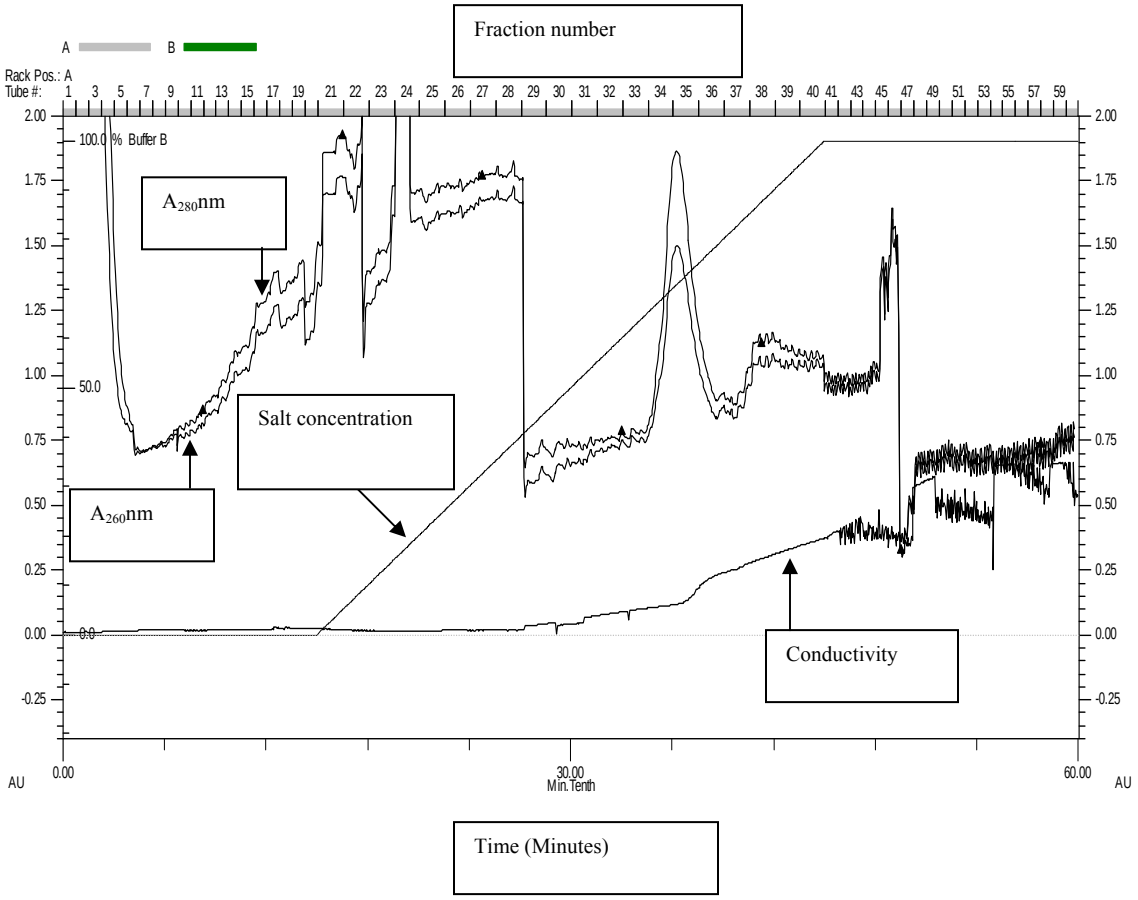


Fig. 5.2 Profile of nitrate reductase elution from the Unosphere Q fast flow anion exchange chromatography column. The peak of nitrate reductase activity was eluted at salt concentration of 1.25mM NaCl in 50mM sodium phosphate buffer of pH 7.2. Salt concentration, conductivity, A_{280}nm and A_{260}nm monitored along with time with corresponding number of fraction as denoted in the figure.

5.2.2. Determination of Molecular Weight

MW of nitrate reductase was determined by gel filtration chromatography using a Sephacryl S-300 beads column, pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.2), containing 0.1mM 2-mercaptoethanol and 0.1mM PMSF. Purified protein at 1mg/ml concentration was applied to the column and buffer was pumped through the column at 0.25ml per minute and fractions each of 0.5 ml were collected. In order to find out the molecular weight of the purified protein, first molecular weight markers were passed through the column and a standard curve was generated with the elution volume. Based on this standard curve, the molecular weight of nitrate reductase was found to be 214Kd (Fig. 5.3). This data was also in confirmation with the

denatured SDS-PAGE data, which indicated to have three subunits of about 125, 60 and 25 in the enzyme preparation respectively for NarG, H and I (Fig. 5.1) (18,19).

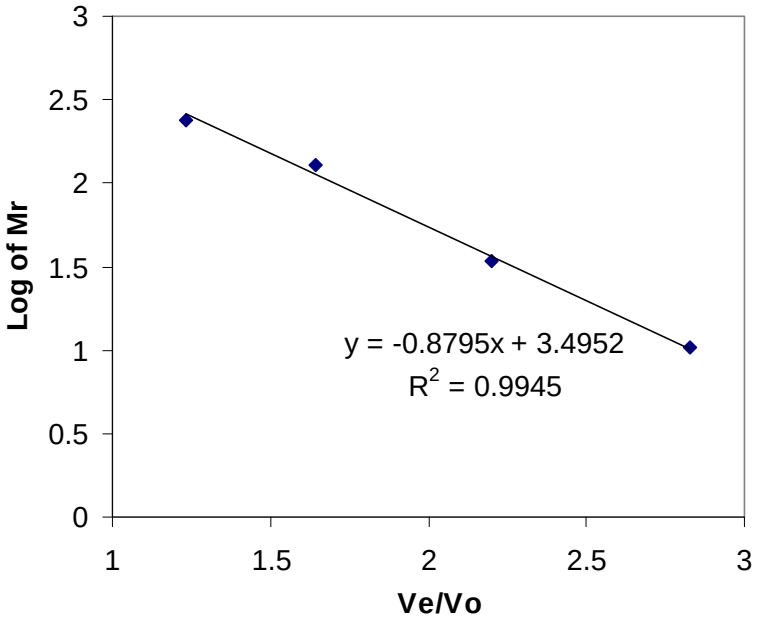


Fig. 5.3 Standard curve of the elution of molecular weight markers from non denaturing sephacryl 300 size exclusion chromatography column. The markers include; thyroglobulin (670Kd), bovine gamma globulin (158Kd), ovalbumin (44Kd), and myoglobulin (17Kd). Nitrate reductase activity peak was eluted at Ve/Vo of 1.3243 corresponding to a molecular weight of 214 Kd.

5.2.3. Stability, Effect of pH, temperature and metal ions on activity of enzyme

Stability of the enzyme was first examined in various buffers, and other stabilizing agents to determine its native environment and optimum preservation conditions. Among different buffers, it was found to be most stable in 50mM sodium phosphate buffer (pH 7.2). Addition of glycerol in the buffer increased the longevity of the enzyme's activity compared to other stabilizers such as PMSF, β-MSH, DTT, threonine, cysteine, leupeptin and dithionite (Fig. 5.4) (20). Without addition of glycerol there was a consistent loss of activity of pure enzyme with time and the loss was minimized with addition of 2% glycerol (Fig. 5.5A and B). PMSF (10 μM) and β-MSH (10 μM) were also added in the enzyme preparation to avoid the proteolytic degradation.

Nitrate reductase activity was optimal at pH 7.0 (Fig. 5.6). It maintained more than 50% of the optimal activity from pH 5 to 9 and still detectable activity in the range of 4-12. This

indicated the enzyme has ability to adapt to a wide range of pH and functioning in environment at different physiological pHs. The optimal temperature of the enzyme was found to be 45^0C and more than 50% of it was retained in the range of 20-60^0C (Fig. 5.7). Activity was almost completely lost at below 10^0C and above 70^0C. In case of *E. coli* nitrate reductase, optimal temperature was reported to be 23^0C (21). This indicated that the extreme temperature adaptability and thermo tolerant nature of *M. tuberculosis* nitrate reductase.

Different metal ions were examined thereafter to determine their effect on nitrate reductase activity. There was no significant effect seen of any of the metal ions on nitrate reductase activity. Some favorable effect by Mo^{5+} and Wo^{5+} could be seen by only 11-20% at concentration of (10nM) (Fig. 5.8).

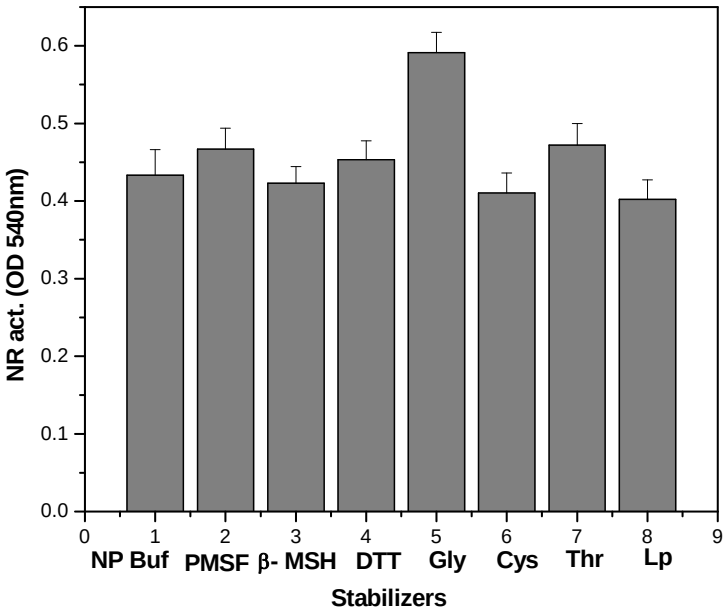


Fig. 5.4 Effect of different agents on stability of nitrate reductase. The effect on nitrate reductase activity was measured after 24 hours of addition of these agents to enzyme solution. The concentration used for gycerol was 2% and 0.1mM for other agents. Where NP Buf = sodium phosphate buffer, PMSF= phenyl methane sulfonyl fluoride, β-MSH = 2-mercaptoethanol, DTT = dithiothreiotol, Gly = glycerol, Cys = cysteine, Thr = threonine, Lp = Leupeptin. Results are mean +/- SD of three identical experiments.

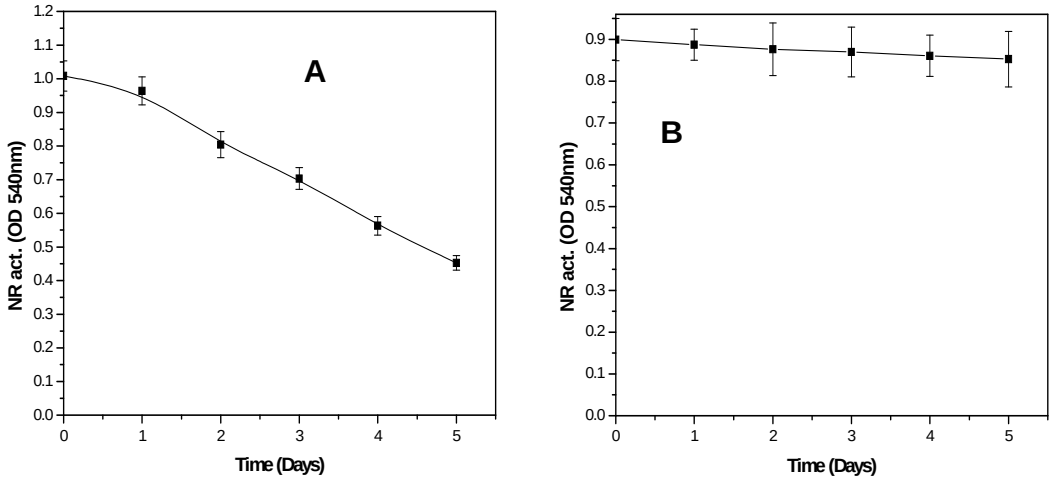


Fig. 5.5 Stability of nitrate reductase activity of purified enzyme without addition of glycerol (A) as compared to with addition of 2% glycerol (B). The reaction was carried out at pH 6.8, 37^0C and 60minutes incubation time in a final assay volume of 2ml containing 20mM sodium phosphate buffer, 5mM sodium nitrate, 100µM Methyl Viologen, 10µM Clealand reagent, 20µg/ml of enzyme and 0.08% sodium dithionate solution. Results are mean +/- SD of three identical experiments.

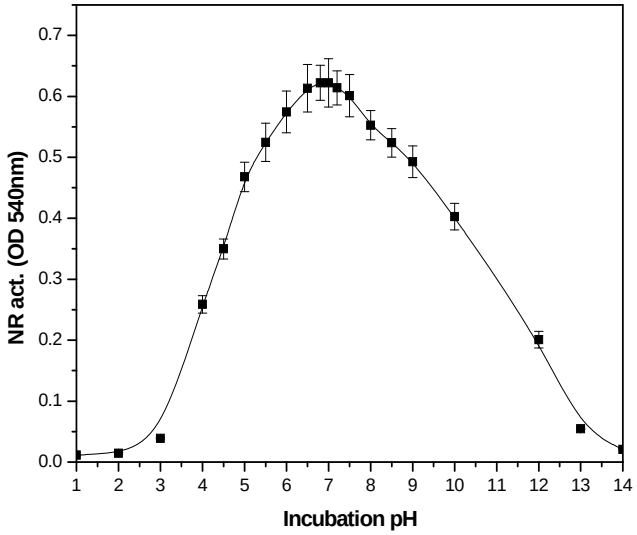


Fig. 5.6 Effect of pH on nitrate reductase activity of purified enzyme. The activity was assayed in different pH phosphate buffers. The reaction was carried out 37^0C and 60minutes incubation time in a final assay volume of 2ml containing 20mM sodium phosphate buffer, 5mM sodium nitrate, 100µM Methyl Viologen, 10µM Clealand reagent, 20µg/ml of enzyme and 0.08% sodium dithionate solution. Results are mean +/- SD of three identical experiments.

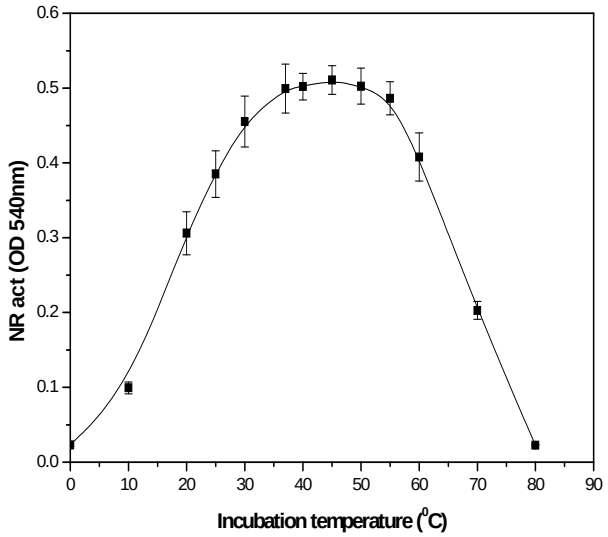


Fig. 5.7 Effect of different temperature on nitrate reductase activity of purified enzyme. The reaction was carried out at pH 6.8, 60minutes incubation time in a final assay volume of 2ml containing 20mM sodium phosphate buffer, 5mM sodium nitrate, 100µM Methyl Viologen, 10µM Clealand reagent, 20µg/ml of enzyme and 0.08% sodium dithionate solution. Results are mean +/- SD of three identical experiments.

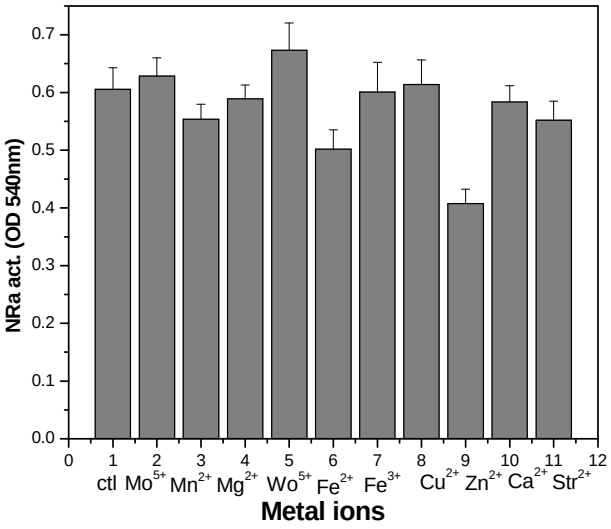


Fig. 5.8 Effect of different metal ions on nitrate reductase activity of purified enzyme. 10µl of 10µM solution of each metal ion solution was added in the assay reaction just before addition of enzyme. The reaction was carried out at pH 6.8, 37^{0}C and 60minutes incubation time in a final assay volume of 2ml containing 20mM sodium phosphate buffer, 5mM sodium nitrate, 100µM Methyl Viologen, 10µM Clealand reagent, 20µg/ml of enzyme and 0.08% sodium dithionate solution. Results are mean +/- SD of three identical experiments.

5.2.4. Substrate specificity of nitrate reductase

Nitrate reductase activity was assayed against a concentration range of 0 to 100mM of nitrate in the reaction to determine the affinity of enzyme for substrate. Maximum activity of enzyme could be achieved at a concentration of 1.28mM (Fig. 5.9). K_m for nitrate was found to be 380µM as determined from Hans-Wolf plot. These low Km values indicate high affinity of the enzyme towards nitrate (Fig. 5.10). Along with methyl viologen, which is commonly used as electron donor in the reaction, some other substrates were also examined for their ability to be used as artificial electron donor in the reaction catalyzed by nitrate reductase. It was found that benzyl viologen could also effectively support the purified enzyme activity (data not shown). Other electron donors such as NADH, NADPH, reduced glutathione, succinate, fumarate and formate could not support the reaction. The K_m values of methyl viologen and benzyl viologen were calculated to be 60 and 80µM respectively (Fig 5.11 and 5.12). As compared to the K_m values obtained in nitrate reductases of other prokaryotes, the K_m values for nitrate, methyl viologen and benzyl viologen were very similar and indicated the high affinity and efficiency of *M. tuberculosis* nitrate reductase (Table 5.2) (22).

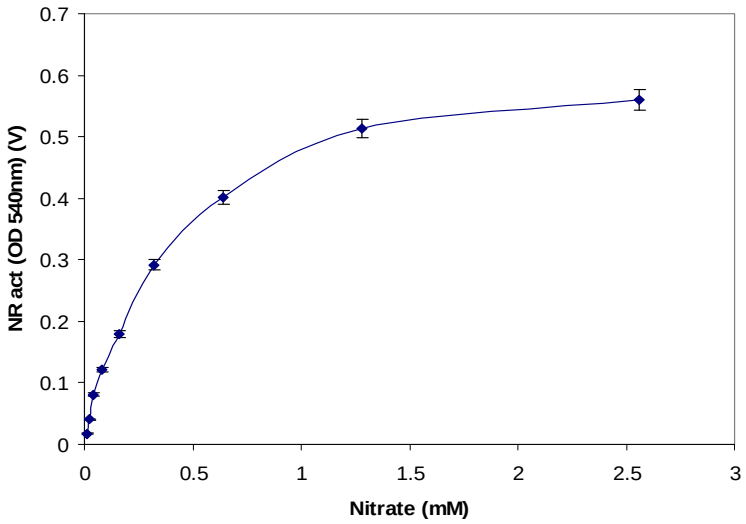


Fig. 5.9 Nitrate reductase activity of purified enzyme at different nitrate concentrations. The reaction was carried out at pH 6.8, 37^0C and 60minutes incubation time in a final assay volume of 2ml containing 20mM Sodium Phosphate buffer, 100µM Methyl Viologen, 10µM Clealand reagent, 20µg/ml of enzyme and 0.08% sodium dithionate solution. Results are mean +/- SD of three identical experiments.

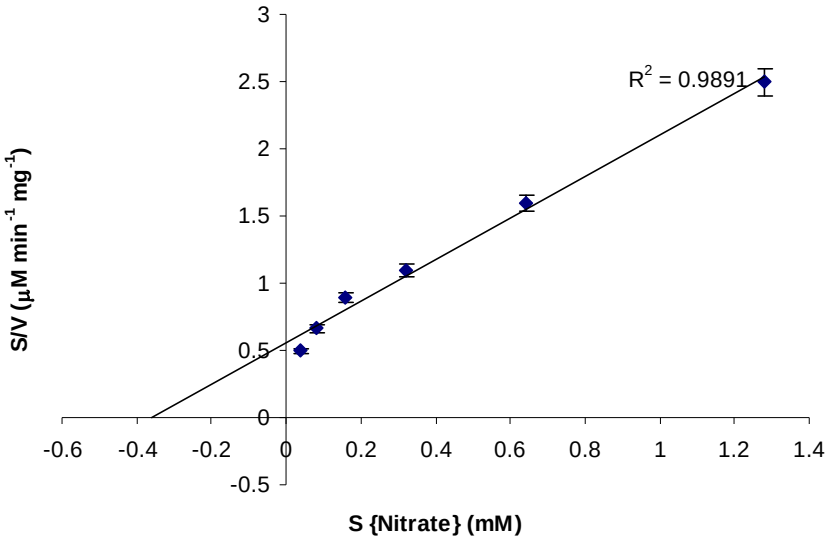


Fig. 5.10 Hans-Wolf plot for determination of the K_m of nitrate for nitrate reductase. Where V = Activity of nitrate reductase (OD 540nm). Experiments was carried out at 20µg/ml of enzyme concentration in the final assay volume of 2ml. Enzyme reaction was carried out at pH 6.8 , 37^0C and with incubation time of 60 minutes. Results are mean +/- SD of three identical experiments. Other details of the experiment are described in materials and method section.

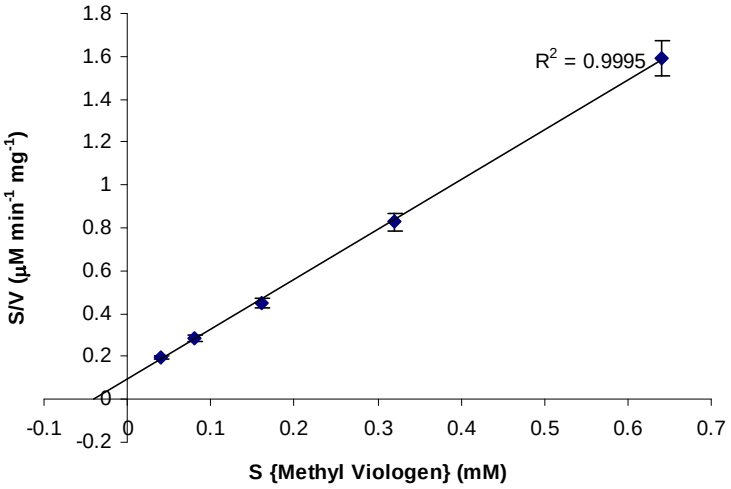


Fig. 5.10 Hans-Wolf plot for determination of the K_m of Methyl Viologen for nitrate reductase. Where V = Activity of nitrate reductase (OD 540nm). Experiments was carried out at 20µg/ml of enzyme concentration in the final assay volume of 2ml. Enzyme reaction was carried out at pH 6.8 , 37^0C and with

incubation time of 60 minutes. Results are mean +/- SD of three identical experiments. Other details of the experiment are described in materials and method section.

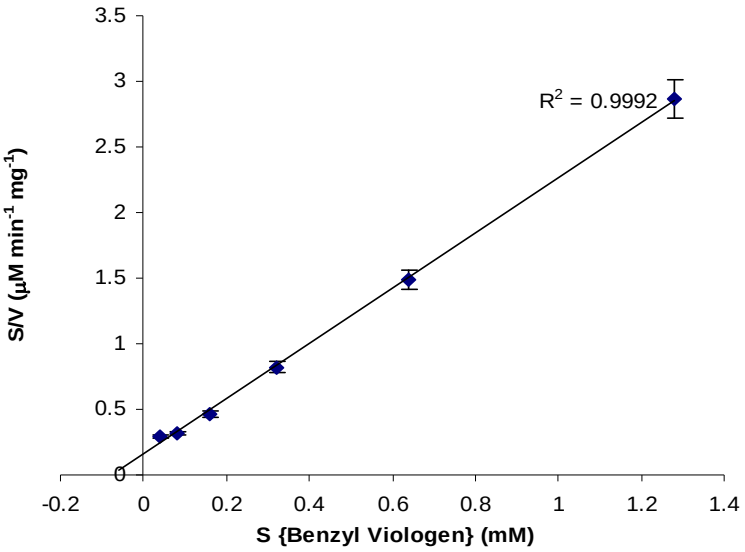


Fig. 5.10 Hans-Wolf plot for determination of the K_m of Benzyl Viologen for nitrate reductase. Where S = Concentration of Benzyl Viologen in mM, V = Activity of nitrate reductase (OD 540nm). Experiments was carried out at 20µg/ml of enzyme concentration in the final assay volume of 2ml. Enzyme reaction was carried out at pH 6.8 , 37^0C and with incubation time of 60 minutes. Results are mean +/- SD of three identical experiments. Other details of the experiment are described in materials and method section.

Table 5.2 Km of nitrate, MV, BV determined for NR purified from *M. tuberculosis* H37Ra compared with previously reported values for *E. coli*.

Substrate	K_m for *M. tuberculosis* NR	K_m for *E. coli* NR
Nitrate	380µM	330uM
Methyl viologen	60µM	29uM
Benzyle viologen	80µM	34uM

5.2.5. Inhibition kinetics of Nitrate reductase

Inhibition characteristics of the purified enzyme were determined by applying the known inhibitors of nitrate reductase to the reaction in dose dependent manner. Azide which was used for the inhibition of nitrate reductase in the whole cell culture showed a similar pattern of inhibition on isolated enzyme (23). Lineweaver-Burk plot drawn from the results obtained from different concentration of azide at varied concentrated of nitrate indicated the typical competitive inhibition of the enzyme (Fig 5.13). Thiocyanate on the other hand showed an un-competitive type of inhibition of nitrate reduction without being affected by nitrate concentration in the reaction (Fig. 5.14).

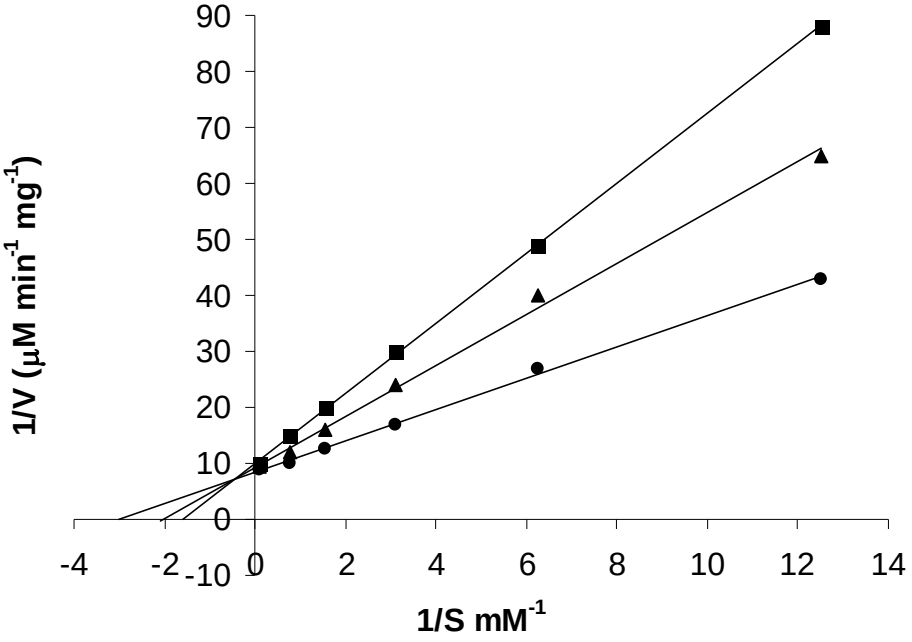


Fig. 5.13 Lineweaver-Burk plot showing competitive inhibition of purified nitrate reductase by azide. The concentration of 5μM (■), 20μM (▲) and 50μM (●) of azide resulted in subsequent decrease in the inhibition constant of 3.1, 2.2 and 1.7mM respectively. Experiments was carried out at 20μg/ml of enzyme concentration in the final assay volume of 2ml. Enzyme reaction was carried out at pH 6.8 , 37^0C and with incubation time of 60 minutes. Different doses of inhibitor were added just after addition of enzyme in the reaction mixture. Results are mean +/- SD of three identical experiments.

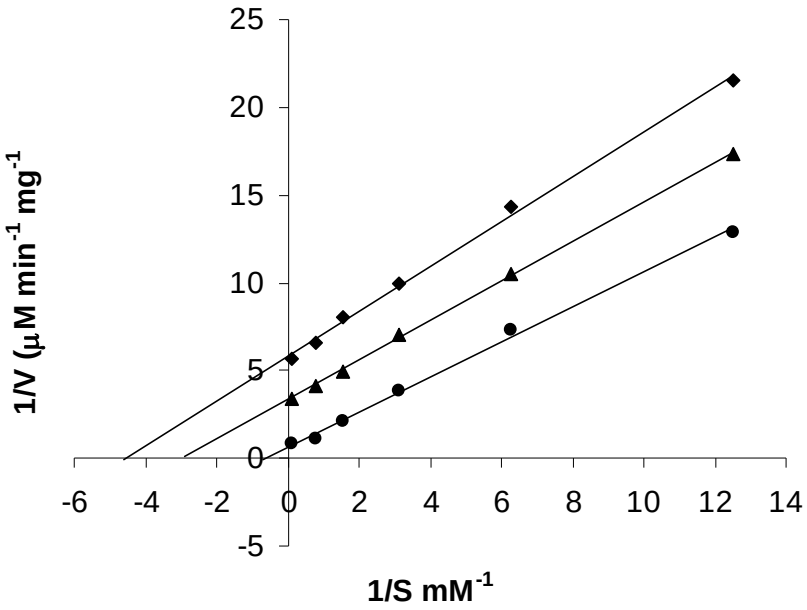


Fig. 5.14 Lineweaver-Burk plot showing un-competitive inhibition of purified nitrate reductase by thiocyanate. The concentration of 5mM (■), 20mM (▲) and 50Mm (●) of thiocyanate resulted in subsequent increase in the inhibition constant of 0.8, 3.0 and 4.7mM respectively. Experiments was carried out at 20μg/ml of enzyme concentration in the final assay volume of 2ml. Enzyme reaction was carried out at pH 6.8 , 37^0C and with incubation time of 60 minutes. Different doses of inhibitor were added just after addition of enzyme in the reaction mixture. Results are mean +/- SD of three identical experiments.

5.2.6. UV-VIS absorption spectroscopy of Nitrate reductase

Absorbance spectra of the purified nitrate reductase in UV-Visible region are shown in figure 5.15. The position of maximum absorbance is seen in the range of 240-290nm having three different peaks in this region (Fig. 5.15A). After addition of dithionite, there was a new peak seen with λ_{max} at 340nm which is generally seen with the reduced form of an enzyme (5.15B). Dithionite which provides a reducing environment may cause a different rearrangement of iron and sulfur clusters within the enzyme subunits (24,25). There could also be seen a change of absorbance pattern showing decrease in the peak at 250nm and increased peak at 275nm after addition of dithionate which is being under characterization process in the future laboratory work.

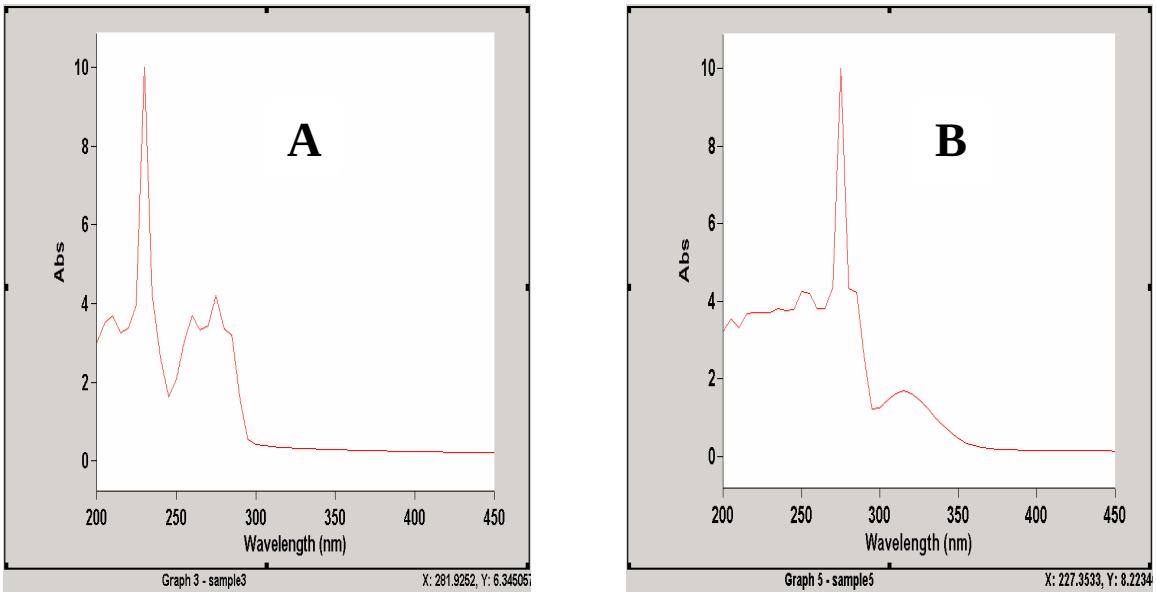


Fig. 5.15 UV-Visible spectra of purified nitrate reductase before (A) and after addition of dithionite (B). A baseline correction was done for the buffer solution in which the enzyme was kept before taking the spectra. Enzyme concentration used for spectra was 200µg/ml and 10µM of dithionite was added in the enzyme solution.

5.3. Materials and Methods

5.3.1. Bacterial strains, media, culture conditions, buffers and solutions

M. tuberculosis H37Ra (ATCC 25177), an avirulent strain, was used as source of nitrate reductase (NarGHJI). Recipies for media, buffers, solutions used in the production, purification and characterization of nitrate reductase were as per described previously (21,22).

5.3.2. *In vitro* Nitrate reductase assay

Nitrate reductase activity was assayed by measuring the reduction of nitrate to nitrite with methyl viologen as the electron donor (15). The assay mixture contained 0.5ml of 100mM sodium phosphate buffer (pH 7.1); 0.1ml of 100mM sodium nitrate; 0.2ml of 0.05% methyl viologen; 10µl of 10mM Cleland's reagent and 1.1ml of water plus enzyme. To start the reaction, 0.1ml of a solution containing 0.8% sodium bicarbonate and 0.8% sodium dithionite was added to the assay mixture and it was gently swirled until uniformly blue. After incubation for 60 minutes at 37^0C, the reaction was stopped by mixing rapidly in a Vortex mixer until the blue color had disappeared. Nitrite was then determined by the following the diazo coupling procedure (26). Two milliliters of a 1% solution of sulfanilic acid in 20% HCI was added to the assay mixture and it was mixed thoroughly. Then, 2 ml of a 0.129% solution of N- naphthylethylenediamine diHC1 was added and, after 10 minutes, the absorbance at 540 nm was measured. Of the above reagents, the dithionite- bicarbonate solution and the methyl viologen were prepared fresh daily and the Cleland's reagent was kept frozen. All other reagents were stable indefinitely. A unit of activity is defined as the production of 1µmole of NO_2 per min at 37^0C. Specific activity is expressed in units per mg of protein.

5.3.3. SDS-PAGE

SDS-PAGE was performed as described by Laemmli, under reducing condition using a miniprep protein gel apparatus (Amersham Biosciences) (27). Gels were 0.5mm thick and contained 12.5% polyacrylamide in the separating gel. Protein samples were mixed 1:1 with 2X sample buffer and heated to 95^0C for 5 minutes prior to loading. Standards used were BioRad's high molecular weight markers diluted 1:50 in the buffer. Standards include: b-galactosidase (119000) phosphorylase b (Mr 97500) bovine serum albumin (Mr 66200), ovalbumin (Mr 45000) and soybean trypsin inhibitor (Mr 21500). Gels were electrophoresed at constant current of 40 miliampere at room temperature and stained with BioRad's Commassie blue stain kit. Native

PAGE was performed with similar running conditions except the addition of SDS and heat treatment of samples.

5.3.4. Unosphere Q anion exchange chromatography

Protein sample was loaded at 1ml per minute flow rate on the Unosphere Q fast flow column which had been pre-equilibrated with 50mM sodium phosphate buffer (pH 7.2) and fractions of 0.3 ml each were collected. After washing 3 column volumes of sodium phosphate buffer (pH 7.2) at 10ml per minute flow rate, baseline absorbance 280 nm was returned to zero and protein was eluted with a 100ml linear gradient of 0 to 2.0 M NaCl in 50mM sodium phosphate buffer (pH 7.2) at a flow rate of 1 ml per minute. Column was washed with 3 column volumes of 50mM sodium phosphate (pH 7.2) buffer with 2M NaCl at a flow rate of 10ml per minute.

5.3.5. Determination of molecular weight of native nitrate reductase

Estimation of the molecular weight of the nitrate reductase was made by gel filteration chromatography on the sephacryl 300 column. A standard curve of the log of the molecular weight versus elution volume was produced using 5 molecular weight standards. Molecular weight standards used were: blue dextran (Mr 2000000), beta amylase (Mr 200000), alcohol dehydrogenase (Mr 150000), bovine serum albumin (Mr 66000) and carbonic anhydrase (29000). Standards (500µg of each protein in a total volume of 0.5ml) were loaded on the column which had been pre-equilibrated with 50mM sodium phosphate buffer.

5.3.6. Enzyme Kinetics

For all the experiments related to kinetics studies of enzyme such as effect of temperature, pH, metal ions, inhibitors, substrate specificity, etc, 10µl of purified enzyme having a concentration of 200 µg/ml enzyme was added in the reaction. Reaction was carried out at pH 6.8, temperature 37^0C and incubation time of 60 minutes, once this was standardized. Substrates, inhibitors and other modulators were added after the addition of enzyme in the assay. Frozen enzyme kept in aliquots, added with 2% glycerol, 10µM PMSF and 10µM β-MSH, was thawed and dilute to 200µg/ml in sodium phosphate buffer (50mM pH 7.2) before use.

5.4. Discussion

Nitrate reductase (NarGHJI), belongs to an important family of reductases and plays a key role of energy generation in a wide variety of bacteria (28). These organisms which contain this enzyme are either facultative anaerobes or obligatory anaerobes and generally reside in environments where nitrate is readily available. There were certain differences seen among these organisms for the structural as well as chemical properties in nitrate reductase. It was surprising initially that *M. tuberculosis*, which was known to be an obligate aerobe, contained this enzyme (29). However, once the concept of hypoxic dormant stage in mycobacteria was proved, it was accepted as a facultative anaerobe which can use nitrate in either limited or complete absence of oxygen (30). Although it is almost a decade since the availability of genome draft revealing that *M. tuberculosis* contains this respiratory nitrate reductase, yet the attention towards the study of this enzyme in isolation could not be attracted so far (14). As our results and earlier findings strengthened this enzyme becoming a possible drug target against dormant tubercle bacilli, an elaborated study on the enzyme in isolation became imminent to better understand the role of this enzyme in pathogenicity before taking it up as drug target studies (23,29). It was reasonably more complex to purify nitrate reductase from *M. tuberculosis* due to its slow growing nature, tough wall to break and the membrane bound nature of the enzyme. A successful purification of nitrate reductase from *M. tuberculosis* H37Ra in this study provided a major step forward isolating and purifying enzymes from wild type strains of mycobacteria. In this purification we could also retain all three subunits of enzyme which was not shown in many of the organisms (3,10,11). There were some noticeable differences seen as well in the characteristics of enzyme compared to nitrate reductase of other organisms. Although *M. tuberculosis* is a slow growing organism in nature, nitrate reductase was found to have much higher specific activity of 190 units/mg protein compared to the fast growing *E. coli*, where it is f only 76units/mg protein in the purified enzyme preparation (Table 5.1) (11).

The molecular size of the enzyme was nearly similar to what was reported in other organisms (Fig. 5.1 and 5.3) (12). However, the native form of the enzyme was found here to remain in monomer form showing 220 Kd band in the gel exclusion chromatogram, which was seen to be tetramer form with MW ~880Kd in the native gel exclusion chromatogram of protein other organisms (17). The subunits of enzyme were also found to have a more or less similar molecular weight to what was reported for other bacterial nitrate reductase subunits. It is not very clear about the association of NarI in the purified preparation in preventing them to form the tetramer.

Comparative molecular weight of the enzyme as determined by gel exclusion chromatography subunit indicated the existence of monomer form in both preparation of enzyme with and without containing NarI (Fig 5.3). This data point out towards a different subunit association of *M. tuberculosis* nitrate reductase as compared to the nitrate reductases of other organisms (13,17).

Addition of glycerol in the enzyme solution resulted in increased stability of enzyme, hence enzyme's activity could be preserved for longer time, which also suggested the possible hydrophobic nature of the enzyme (Fig 5.4 and 5.5). The pH and temperature studies of nitrate reductase also indicated the wide range of adaptability to pH and temperature which indicated its ability to adapt according to different physiological conditions as the pathogen is supposed to face changing environment during the course of disease and particularly during the latent stage (Fig. 5.6.and 5.7). The K_m values of Nitrate, MV and BV determined for *M. tuberculosis* nitrate reductase were quite similar to that reported for *E. coli* and other organisms (5.9, 5.11 and 5.12) (10). The competitive and non-competitive nature of inhibition of this enzyme by azide and thiocyanate respectively, was expected based on their well known mode of action and indicated similar binding sites available in this enzyme as well (Fig 5.13 and 5.14). Presence of an oxidized and reduced form similar to other nitrate reductases was seen by UV-Visible spectra (Fig. 5.15) (24,25).

Electron paramagnetic resonance, differential light scattering, small angle X-ray scattering, chorionic dichroism and X-ray crystallography in the subsequent studies are being carried out in our laboratory to understand the physical and chemical nature of the enzyme in detail. An enzyme based microplate nitrate reductase assay is also being developed in the laboratory which could be effectively used to search novel anti- tuberculosis molecules targeting nitrate reductase of *M. tuberculosis*.

Altogether, this work and the subsequent characterization studies should contribute to the understanding of the bioenergentics mechanism mediated by NarGHJI and allow us to explore the development of new anti-mycobacterial agents that target key stage of latency in *M. tuberculosis*.

5.5. References

1. Berks BC, Ferguson SJ, Moir JWB, Richardson DJ. 1995. Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. *Biochim. Biophys. Acta* **1232:** 97-173.
2. Bonnefoy V, DeMoss JA. 1994. Nitrate reductases in *Escherichia coli*. *Antonie Leeuwenhoek* **66:** 47-56.
3. Van't Riet J, Wientjes FB, Van Doom J, Planta RJ. 1979. Purification and characterization of the respiratory nitrate reductase of *Bacillus licheniformis*. *Biochim. Biophys. Acta* **576:** 347-360.
4. Moreno-Vivián C, Ferguson SJ. 1998. Definition and distinction between assimilatory, dissimilatory and respiratory pathways. *Mol. Microbiol.* **29:** 664-666.
5. Dubourdieu M, DeMoss JA. 1992. The *narJ* gene product is required for biogenesis of respiratory nitrate reductase in *Escherichia coli*. *J. Bacteriol.* **174:** 867-872.
6. Blasco F, Pommier J, Augier V, Chippaux M, Giordano G. 1992. Involvement of the *narJ* or *narW* gene product in the formation of active nitrate reductase in *Escherichia coli*. *Mol. Microbiol.* **6:** 221-230.
7. Merrick MJ, Edwards RA. 1995. Nitrogen control in bacteria. *Microbiol. Rev.* **59:** 604-622.
8. Richardson DJ, Watmough NJ. 1999. Inorganic nitrogen metabolism in bacteria. *Curr. Opin. Chem. Biol.* **3:** 207-219.
9. Denis K, Dias FM, Rowe JJ. 1990. Oxygen regulation of nitrate transport by diversion of electron flow in *Escherichia coli*. *J. Biol. Chem.* **265:** 18095-18097.
10. Fewson, C. A., and D. J. D. Nicholas. 1961. Nitrate reductase from *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta* **49:** 335-349.
11. Radcliffe BC, Nicholas DJD. 1970. Some properties of a nitrate reductase from *Pseudomonas denitrificans*. *Biochim. Biophys. Acta* **205:** 273-287.
12. Van't Riet J, Planta RJ. 1975. Purification, structure, and properties of the respiratory nitrate reductase of *Klebsiella aerogenes*. *Biochim. Biophys. Acta* **379:** 81-94.
13. Van't Riet J, Van Ee JH, Wever R, Van Gelder BF, Planta RJ. 1975. Characterization of the respiratory nitrate reductase of *Klebsiella aerogenes* as a molybdenum-containing iron-sulfur enzyme. *Biochim. Biophys. Acta* **405:** 306-317.
14. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, *et al*. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393:** 537-544.

15. MacGregor CH, Schnaitman CA, Normansell D, Hodgins MG. 1974. Purification and properties of nitrate reductase from *Escherichia coli* K-12. *J. Biol. Chem.* **249:** 5321-5327.
16. Dick LR, Cruikshank AA, Grenier L, Melandri FD, Nunes SL, Stein RL. 1996. Mechanistic studies on the inactivation of the proteasome by lactacystin: a central role for clasto lactacystin beta-lactone. *J. Biol. Chem.* **271:** 7273-7276
17. Forget P. 1974. The bacterial nitrate reductases. Solubilization, purification, and properties of the enzyme A of *Escherichia coli* K12. *Eur. J. Biochem.* **42:** 325-332.
18. Graham A, Boxer DH. 1980. Arrangement of respiratory nitrate reductase in the cytoplasmic membrane of Escherichia coli. *FEBS Lett.* **113:** 15-20.
19. Graham A, Boxer DH. 1980. Implication of a subunit of *Escherichia coli* nitrate reductase in catalytic activity. *Biochem. Soc. Trans.* **8:** 329-330.
20. DeMoss JA. 1977. Limited proteolysis of nitrate reductase purified from membranes of *Escherichia coli*. *J. Biol. Chem.* **252:** 1696-1701.
21. Clegg RA. 1976. Purification and some properties of nitrate reductase from *Escherichia coli*. *Biochem. J.* **153:** 533-541.
22. Morpeth FF, Boxer DH. 1985. Kinetic analysis of respiratory nitrate reductase from *Escherichia coli* K12. *Biochem.* **24:** 40-46.
23. Khan A, Sarkar D. 2006. Identification of a respiratory-type nitrate reductase and its role for survival of *Mycobacterium smegmatis* in Wayne model. *Microb. Pathog.* **41:** 90-95.
24. Magalon A, Asso M, Guigliarelli B, Rothery RA, Bertrand P, Giordano G, Blasco F. 1998. Molybdenum cofactor properties and [Fe-S] cluster coordination in *Escherichia coli* nitrate reductase A: investigation by site-directed mutagenesis of the conserved His-50 residue in the NarG subunit. *Biochemistry* **37:** 7363-7370.
25. Breton J, Berks BC, Reilly A, Thomson AJ, Ferguson SJ, Richardson DJ. 1994. Characterization of the paramagnetic iron-containing redox centers of *Thiosphaera pantotropha* periplasmic nitrate reductase. *FEBS Lett.* **345:** 76-80.
26. Nicholas DJD, Nason A. 1957. Determination of nitrate and nitrite. *Methods Enzymol.* 3**:** 981-984.
27. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227**:** 680-685.
28. Wayne LG, Sohaskey CD. 2001. Non-replicating persistence of *Mycobacterium tuberculosis* *Annu. Rev. Microbiol.* **55:** 139-163.
29. Weber I, Fritz C, Ruttkowski S, Dreft A, Bange FC. 2000. Anaerobic nitrate reductase (NarGHJI) activity of *Mycobacterium bovis* BCG *in vitro* and its contribution to virulence in

immunodeficient mice. *Mol. Microbiol.* **35:** 1017-1025.

30. Honer K, Bentrup Z, Russel GD. 2001. Mycobacterial persistence: adaptation to a changing environment. *Trends Microbiol.* **9:** 597-605.

APPENDIX 1

Reprints of Publications from thesis

Available online at www.sciencedirect.com
ScienceDirect
Microbial Pathogenesis 41 (2006) 90–95
MICROBIAL PATHOGENESIS
www.elsevier.com/locate/micpath

Identification of a respiratory-type nitrate reductase and its role for survival of *Mycobacterium smegmatis* in Wayne model

Arshad Khan, Dhiman Sarkar*

CombiChem Bio Resource Center, National Chemical Laboratory, Dr. Homi Bhabha Road, Pune 411008, India

Received 18 October 2005; received in revised form 11 April 2006; accepted 28 April 2006
Available online 27 June 2006

Abstract

Nitrate reductase (NR) is found to be expressed in certain mycobacterium sp. whose link with the development of persistence is yet to be resolved. The present study demonstrates the action of selective inhibitors on NR as well as in the survival of *Mycobacterium smegmatis* using Wayne's model. During gradual shift down to anaerobic stage in Wayne's model, conversion of nitrate to nitrite became apparent in *M. smegmatis*. More than 97 percent inhibition was observed for the conversion of nitrate to nitrite by azide (0.05 mM) and thiocyanate (20 mM) in both whole-cell as well as its cell-free lysate, respectively. Under identical condition, chlorate (20 mM) inhibited nitrate reduction by 67 and 10 percent, respectively. At these concentrations, neither of azide, thiocyanate nor chlorate had any significant effect on cell growth under aerobic condition. In Wayne's culture model, thiocyanate and chlorate inhibited the growth of *M. smegmatis* by almost 2 logs at the same concentrations whereas azide inhibited by almost 1.75 log when added at the time of inoculation. Exposure of same culture at 96 h after inoculation in Wayne's model to these inhibitors showed 1.74, 1.95 and 2.37 log inhibition of viable cells with respect to azide, thiocyanate and chlorate. These findings further indicated that NR inhibitors kill the bacilli at anaerobic stage under the experimental condition mentioned. Metronidazole (MTZ) (2 mM) and Nitrofurantoin (NIT) (0.3 mM) reduced the cell number at both stages by <0.7 log. They did not have any effect on NR. Altogether, the results clearly indicate that NR-specific inhibitors could become more promising in killing the bacilli at anaerobic stage than the available conventional drugs.

Keywords: Nitrate reductase; *Mycobacterium smegmatis*; Wayne model

1. Introduction

Mycobacterium tuberculosis is the causative agent for tuberculosis, a widespread disease that is responsible for 2–3 million deaths annually [1,2]. One-third of the world's population is infected with the latent form of the organism [3]. The organism resides in two different phases, one in replicating aerobic and the other in non-replicating persistent phase [4–7]. During survival in persistent phase, metabolic activity of the bacilli decreases to a minimum level, which makes most of the drugs ineffective [8]. Very recently it has been postulated that nitrate; through nitrate respiration, could provide energy for bacterial metabolism in an anaerobic environment, because anaerobic nitrate reductase (NR) (*narGHJI*) couples the reduction of nitrate to the generation of ATP in absence of oxygen as terminal electron acceptor in *Mycobacterium bovis* BCG [9–12]. A gene cluster homologous to *narGHJI* of *Bacillus subtilis* was first identified during sequencing of *M. tuberculosis* genome [13], which codes for a respiratory type of NR. Apart from identifying tissue-specific infection and reduced virulence in SCID mice by the *narG* mutant of *M. bovis* BCG, no major attention has been paid so far on NR as drug target [14,15]. Major difficulty in working on *M. tuberculosis* is its very high generation time and pathogenicity. There is still no concrete evidence supporting the presence of NR in *Mycobacterium smegmatis*. Earlier studies indicated that *M. smegmatis* follows similar pattern of dormancy in Wayne model as *M. tuberculosis* [16]. In this model culture is gradually shifted to anaerobic condition in a sealed tube and when conditions become

*Corresponding author. Tel.: +91 20 25902400; fax: +91 20 25893355/25902601.
E-mail addresses: arshu_ali2002@yahoo.co.in (A. Khan), d.sarkar@ncl.res.in (D. Sarkar).

0882-4010/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.micpath.2006.04.006

anaerobic, organism stops multiplying but remains viable. Alanine dehydrogenase activity increased during dormancy in *M. megmatis* [17]. In fact, Wayne model has broadened the scope of developing a thorough understanding about the metabolic pathways operating at anaerobic stage in all Mycobacterial species including *M. smegmatis*. This is essential before accepting NR as target for identifying novel anti-tubercular molecules. Its role in nitrogen metabolism as well as persistence could only be revealed through extensive biochemical studies on anaerobic stage using Wayne model. *M. Smegmatis* could provide an advantage in developing target-based whole-cell assay for a simple high throughput screening of chemical library.

In this study, for the first time the authors have reported the presence of respiratory NR activity in *M. smegmatis*. Abrogation of this activity by applying specific inhibitors at anaerobic stage could yield far better result in killing the bacilli than Metronidazole (MTZ) and Nitrofurantoin (NIT) which favors the selection of NR as potential target for persistence-specific anti-tubercular drug development.

2. Results

2.1. Growth and nitrate to nitrite conversion in aerobic and Wayne's model by M. smegmatis

The essential requirement for these kinds of studies is the availability of a defined medium where comparable growth of the organism/s under study could be seen with respect to changes in composition of the medium. Most of the media used for growth of *Mycobacterium* sp. are always complex in nature. Here, whole study was carried out using a minimal medium called *M. phlei* medium. The growth of *M. smegmatis* in this medium under aerobic condition was comparable with other complex media (data not shown) [16].

The patterns of growth in aerobic and Wayne's 0.5 HSR model were also comparable with that obtained in complex medium (Fig. 1A). Nitrate was added in the medium that the organism is supposed to utilize as alternate electron acceptor. The extent of conversion of nitrate to nitrite was an indication of the level of dependence of the cell on the alternate electron transport chain during that stage. The result showed that there is no significant conversion of nitrate to nitrite in aerobic culture of *M. smegmatis* (Fig. 1B). Significant NR activity was seen in Wayne culture in spite of having less viable cells in it. The pattern indicated that reduction of nitrate starts at the onset of microaerophilic phase of cell growth. This indicates that nitrate reduction could be due to the newly synthesized anaerobic stage-specific enzyme. Increased NR activity could also be due to increased transport of nitrate inside the cell and/or due to an increase in electron flow to NR under hypoxic condition.

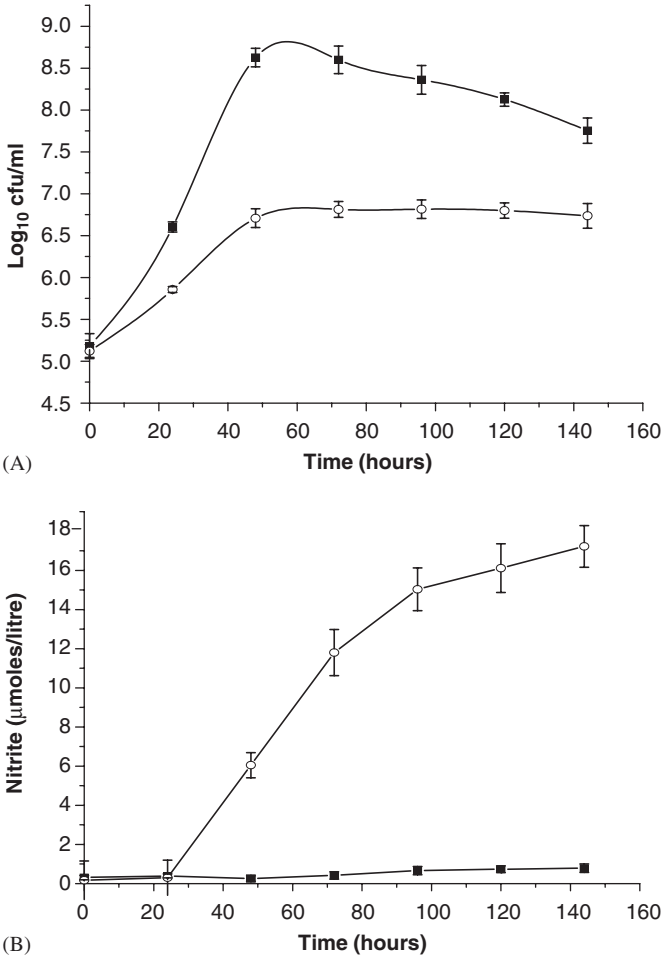


Fig. 1. Growth of *M. smegmatis* and its nitrate reduction in aerobic and Wayne dormancy culture system. (A) Growth of *M. smegmatis* was monitored in aerobic (■) and Wayne's model (○) in minimal medium in presence of 10 mM nitrate. (B) Nitrate reduction by *M. smegmatis* was monitored in aerobic (■) and in Wayne's model (○) under identical conditions as described in Sections 4.3 and 4.4. The result was an average of duplicate readings of three identical experiments.

2.2. Effect of inhibitors on the viability of M. smegmatis and its nitrate reduction in aerobic and Wayne's model

Earlier reports indicated about using sodium chlorate, sodium thiocyanate and sodium azide as inhibitors of anaerobic NR in prokaryotes [18,19]. Azide is a competitive inhibitor of NR with respect to nitrate [20]. By chelating free molybdenum ion from the medium, thiocyanate inhibits NR activity [21]. On the other hand, chlorate inhibits nitrate reduction by competing with nitrate and gets reduced preferentially by the enzyme [22]. End product chlorite leads to the death of bacterial cells through non-specific manner [23]. Apart from using above inhibitors, MTZ and NIT were used in this study as standard anaerobic stage-specific inhibitors to compare the results. In order to validate the same in *M. smegmatis*, these inhibitors were tested under aerobic condition, at concentrations where no significant effect on growth was observed

(Fig. 2). Under similar condition, the viability of cells in Wayne's 0.5 HSR model was also checked in presence of the inhibitors. The inhibitors were added in the culture at the time of inoculation and experiment was terminated at 120 h of incubation. It was observed that the cell number was reduced by 1.75, 2.0 and 1.98 log when azide, thiocyanate and chlorate were used at concentrations of 0.05, 20 and 20 mM, respectively. The result indicated that the growth was inhibited possibly because of very specific effect of inhibitors on anaerobic NR. In parallel with the culture, NR activity was also monitored in whole-cell lysate (Table 1). The results clearly indicated that the conversion of nitrate to nitrite was reduced by almost 100 percent in presence of azide and thiocyanate whereas chlorate inhibited 67 percent in whole cell and 10 percent in lysate with respect to the control value. This result further strengthened the assumption that the conversion of nitrate to nitrite is due to anaerobic NR. To confirm that reduction in viable cell count is due to specific inhibition of NR, these inhibitors were applied in absence of nitrate in Wayne culture. Azide and thiocyanate did not affect the viability at all whereas chlorate affected the viability because of its use as a substrate by NR and reduction into toxic chlorite (data not shown). This proves that inhibition of NR by these compounds is very specific in nature. This could also be explained by the fact that presence of nitrate will control the electron flow through NR. Other alternate respiratory chain will become non-functional even in conditions when NR is completely inhibited by application of inhibitors when nitrate is added in the culture. Along with those specific inhibitors of NR, anaerobic stage-specific inhibitors like MTZ [24–26] and NIT [27,28] were also used to understand if they have any effect on the same. MTZ reduced the number of cells in aerobic and Wayne's model by 0.2 and 0.75 log, respectively. Under similar condition, NIT reduced the cell number by 0.65 and 0.8 log, respectively. The result indicated that the effect of MTZ and NIT was to some extent independent of either aerobic or anaerobic stage of the bacterium. None of MTZ and NIT had any inhibitory effect on the conversion of nitrate to nitrite observed. Both MTZ and NIT did not have any effect on the enzyme activity in cell lysate also (Table 1).

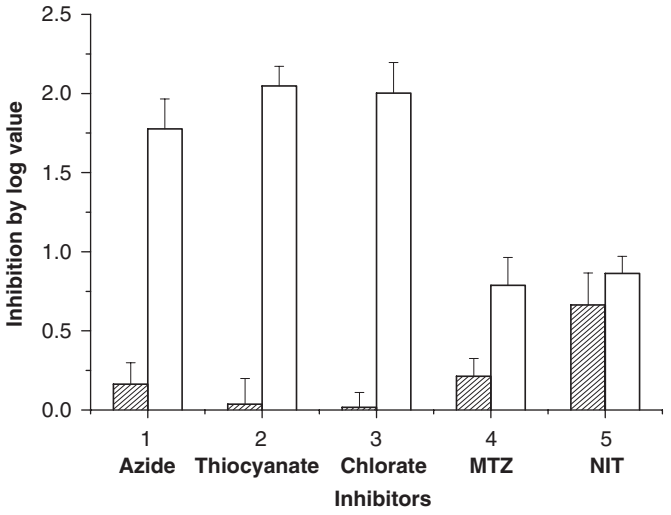


Fig. 2. Effect of inhibitors on viability of *M. smegmatis*. 0.5 mM azide, 20 mM thiocyanate, 20 mM chlorate, 2 mM MTZ and 0.3 mM NIT were added during inoculation of aerobic (dark bars) and Wayne's model culture (light bars). The viable counts were taken at 120 h of incubation. Inhibitions were calculated by subtracting log values with control. The results were an average of three identical experiments.

Table 1
Effect of inhibitors on nitrate reduction by whole cell and lysate of *M. smegmatis*

Inhibitors	% Inhibition	
	Whole cell[a]	Membrane fractions[b]
Azide (0.05 mM)	99.34±1.89	98.45±3.18
Thiocyanate (20 mM)	97.86±2.67	96.74±1.98
Chlorate (20 mM)	67.25±4.16	10.22±1.2
MTZ (2 mM)	6.67±0.51	Nil
NIT (0.3 mM)	19.47±1.11	Nil

[a]Whole cell, inhibitors were added at the time of inoculation.
[b]Membrane fractions, 96 h Wayne culture was used to prepare cell lysate.

2.3. Inhibition kinetics on nitrate reduction and viability during anaerobic shift down in Wayne's model

In order to get an idea about the phase where azide, thiocyanate and chlorate were most active in Wayne's model, the growth and viability was monitored at every 24 h interval. Initially, growth was seen in all the cultures but the viability reduced with time more drastically after ~48 h of inoculation in those cultures where NR-specific inhibitors were applied (Fig. 3A). Initial growth seen was due to the availability of dissolved O_2 in the medium and the inhibitors were not affecting their growth at that stage. In order to remain viable at anaerobic stage, the dependence of culture increased on NR, it failed to grow in presence of the inhibitors. This was clearly observed when NR activity was also monitored in same culture samples (Fig. 3B). In presence of azide and thiocyanate, NR activity remained at insignificant level. Chlorate inhibition was increased upto 65 percent at the termination of the experiment. This clearly indicated that NR activity plays an important role for the viability *of M. smegmatis* during anaerobic shift down in Wayne's model.

2.4. Effect of inhibitors on the viability of M. smegmatis at anaerobic stage

In order to find out more precisely the phase in Wayne's model where inhibition of NR has maximum impact on the viability of *M. smegmatis*. Inhibitors were added after every 24 h of inoculation. The experiment was terminated

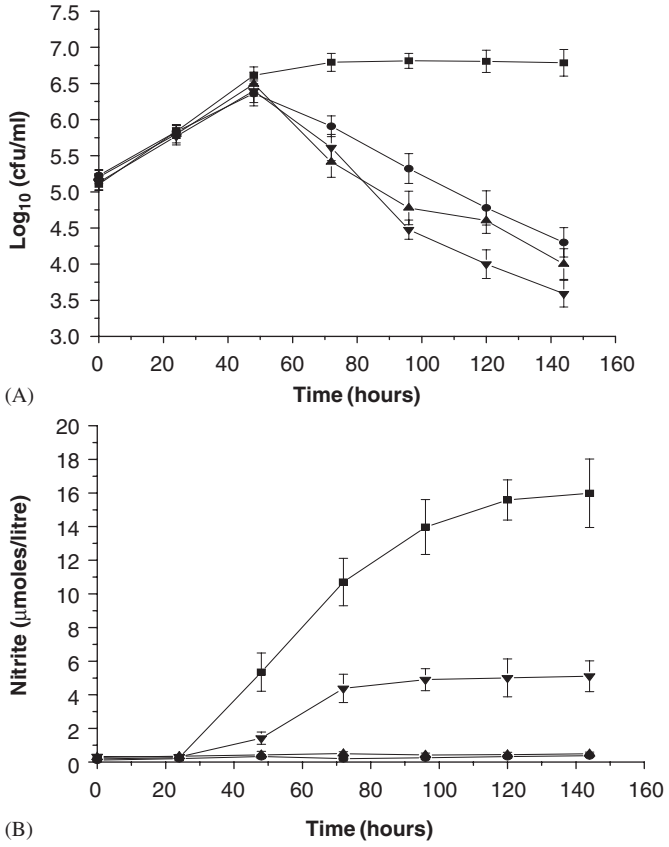


Fig. 3. Kinetics of inhibition on nitrate reduction and viability of *M. smegmatis* in Wayne's model by NR inhibitors. 0.05 mM of azide (●), 20 mM thiocyanate (▲), 20 mM chlorate (▼), none (■) were added during inoculation. (A) Viable cell count and (B) NR activity was monitored in presence of the inhibitors. The details of the experiment are described in Sections 4.3 and 4.4. The result is an average of three identical experiments done in duplicates.

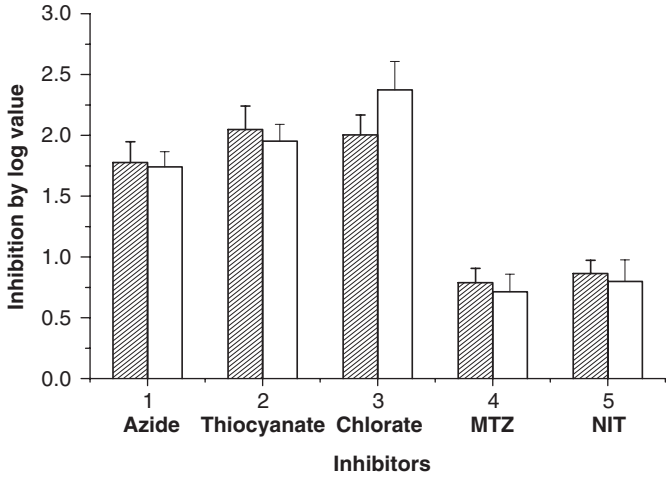


Fig. 4. Effects of different inhibitors on the viability of *M. smegmatis* when added at different time stages in Wayne's model. Inhibitors were added in the culture at 0 h (dark bars) and 96 h (light bars) through rubber septa used in sealing the tubes by a syringe. The experiment was terminated simultaneously at 120 h after inoculation and viability was checked as described in Section 4.3. The result is an average of three identical experiments.

at 120 h and the viable counts were taken. Almost similar effect on the viability of *M. smegmatis* was observed when the inhibitors were added at 0 and 96 h (Fig. 4). Viable cell count was reduced by ~2–2.4 log value in presence of thiocyanate and chlorate compared to the control. Azide reduced the viable count by ~1.75 log value compared to the control. This clearly indicated that the effect of azide, thiocyanate and chlorate on the viability of *M. smegmatis* in Wayne's model was due to the inhibition of NR at anaerobic stage. Interference of NR led to drastic killing of the bacilli. MTZ reduced the viable count only by 0.7 log value at the same time period. NIT has more effect on the viability of aerobic cells than anaerobic cells at the concentrations used. So, the results clearly indicated that NR is essential for the viability of anaerobic stage of *M. smegmatis* culture. The results also indicated that NR inhibitors had superior inhibitory impact on the viability of anaerobic bacilli compared to MTZ and NIT under experimental condition mentioned.

3. Discussion

Nowadays, persistence has gained a lot of attention with respect to developing novel anti-tubercular drug, which could drastically reduce the duration of tuberculosis therapy. A persistence-specific drug should ideally work at anaerobic stage like NRP2 shown in *M. tuberculosis* [29,30]. Identification of a target specific for this stage as well as having an easy assay system for screening a chemical library could provide a novel anti-TB inhibitor. From earlier studies, it was very difficult to predict any target worth pursuing for drug development against this persistent bacillus. Looking at different options, working on the anaerobic *M. smegmatis* as well as its NR could help in building a thorough understanding on the potentiality of NR as drug target. Very recently, two genes have been identified in *M. smegmatis* having ~80 percent sequence homology with *M. tuberculosis narG* and *H* genes from genome sequence analysis [31]. The results very clearly indicated that NR activity appears only under anaerobic condition (Fig. 1A and B). The possibility of this anaerobic NR becoming *narGHJI* was further strengthened by its almost complete inhibition in presence of azide, thiocyanate and to major extent by chlorate under anaerobic condition (Fig. 2). The inhibition of nitrate reduction by whole-cell lysate in presence of azide, thiocyanate and to a negligible extent by chlorate further strengthened earlier prediction (Table 1). Presently, the work is in progress in our laboratory to thoroughly characterize this anaerobic stage-specific NR.

As the stages were not clearly defined from growth curve of *M. smegmatis* in Wayne's model, it could be divided into aerobic and anaerobic stages based on the rapid change observed in our studies also at ~48 h after inoculation (Fig. 1A). Therefore, the period between 96 and 120 h in Wayne's model could be assumed as clearly an anaerobic stage (Fig. 1) [16]. The inhibitory action of azide,

thiocyanate and chlorate is exhibited at later stage (after 48 h) (Figs. 3 and 4). The presence of nitrate in the medium is an obligatory requirement for the functioning of NR during anaerobic respiration [19,32]. In presence of nitrate in the medium, whole respiratory mechanism becomes completely dependent on NR. The presence of an alternate respiratory mechanism and fermentation pathway also cannot be ruled out in Mycobacterial system because it could survive at anaerobic stage in absence of nitrate (data not shown). The presence of nitrate in host system is quite evident because of the release of NO by the immune cells and its subsequent conversion into nitrate [33]. The relevance of NR in in vivo survival also could be predicted from tissue-specific growth of a *narG* mutant of *M. bovis* BCG [14]. Applying specific inhibitors in the host system could only make possible to understand the exact role of NR in the maintenance of persistence of the bacilli.

The results further indicated that NR-specific inhibitors have reduced cell count by ~2 log compared to only ~0.8 log by MTZ (Figs. 2 and 4). These data clearly indicated that NR inhibitors have much deeper impact on the survival of bacilli at late anaerobic stage compared to MTZ and NIT (Figs. 3 and 4). MTZ and NIT were identified as anaerobic stage-specific anti-TB molecules and the former is used as medicine also for TB treatment. Our studies indicated that these two molecules are not very specific in either of the stages [26,28]. As the bacilli could survive with or without nitrate during persistent stage, to have an impact on NR-specific inhibitors, nitrate should be present as alternate electron acceptor in the vicinity of its residence. It is not very clear so far how the development and spreading of persistent bacilli occurs in in vivo condition. If the in vivo development occurs in the same way as predicted by Wayne's model, then complete sterilization is possible only through the inclusion of NR inhibitors in the combination therapy. It remained to be clear about the fate of nitrite in the culture. Altogether the results indicated that induction of anaerobic stage-specific NR activity is observed in *M. smegmatis* and abrogation of the activity dramatically kills dormant bacilli. Therefore, NR could be adopted as a better target than others for the development of persistent stage-specific inhibitor.

4. Materials and methods

4.1. Chemicals

All the chemicals were purchased from Sigma-Aldrich, USA. Dubos medium was purchased from DIFCO, USA.

4.2. Strain and media

M. smegmatis strain ATCC 607 was used throughout the present study which was grown in a defined medium containing 0.5 g KH_2PO_4, 0.2 g sodium citrate, 60 mg $MgSO_4$, 0.5 g asparagine and 2 mL glycerol in 100 mL of distilled water at pH 6.6. 10 mM sodium nitrate was always added in the medium unless otherwise it is mentioned. The stock culture was maintained at −70 °C and subcultured once in liquid medium before inoculation to an experimental culture.

4.3. Cultivation of the aerobic and dormant bacilli

For aerobic cultivation, cultures were grown in 20 mL medium in 100 mL flask at 37 °C and incubated on an orbital shaker at 190 rpm (Thermo electron Model No. 481). One percent of 1 O.D. culture was used as inoculums for routine experiments.

To cultivate the anaerobic bacilli, Wayne's 0.5 HSR tube model was followed, using 20 mm/125 mm tube with a total volume of 25.5 mL [29]. Inoculums size used here was about 10^5 cells per ml by diluting the culture upto 0.008 A_{580}. After putting 8 mm Magnetic spin bar, the tube was made airtight using rubber septa. The cultures were gently stirred at 100 rpm on a magnetic stirring platform. Viable cells were counted by the following method described earlier [29].

4.4. Estimation of conversion of nitrate to nitrite in liquid culture

Nitrate to nitrite conversion in the whole-cell culture was measured by estimating the end product nitrite by using NEDD and sulphanilic acid reagents to generate a pink diazonium dye which could be monitored after 15 min of adding the reagent at 540 nm [34]. To estimate the nitrite in molar concentration, the data were compared with a standard curve generated by using serially diluted solutions of sodium nitrite of known concentrations.

4.5. Preparation of cell extracts and cell-free NR assay

To make the cell-free mycobacterial extract, the culture was washed twice with 5 × volume of cell pellet using Tris buffer (0.1 M pH 7.5) and resuspended in 10 × volume of same buffer. The cell suspension was sonicated and the sonic extract was washed once again in the same buffer [35]. The pallet containing the membrane fraction was resuspended in sodium phosphate buffer (20 mM pH 7.2). After determining the protein concentration, suspension was used for NR assay by following an earlier described method [36].

Acknowledgments

The authors are thankful to Director, National Chemical Laboratory, Pune India for providing financial assistance through an In-house Project. We are also thankful to AstraZeneca R&D Bangalore, India for providing *M. smegmatis* strain ATCC 607.

References

[1] Dye C, Cheele S, Olin P, Ikram P, Aviglioen MC. Global burden of tuberculosis. JAMA 1999;282:677–86.

[2] Honer K, Bentrup Z, Russel GD. Mycobacterial persistence: adaptation to a changing environment. Trends Microbiol 2001;9: 597–605.

[3] Kochi A. The global tuberculosis situation and the new control strategy of the World Health Organization. Tubercle 1991;72: 1–6.

[4] McKinney JD, Jacobs WR, Bloom BR. Persisting problems in tuberculosis. In: Krause RM, editor. Emerging infections. London: Academic Press; 1998. p. 51–139.

[5] Parrish NM, Dick JD, Bishai WR. Mechanisms of latency in *Mycobacterium tuberculosis*. Trends Microbiol 1998;6:107–12.

[6] Wayne LG. Dormancy of *Mycobacterium tuberculosis* and latency of disease. Eur J Clin Microbiol Infect Dis 1994;13:908–14.

[7] Wayne LG, Sohaskey CD. Non-replicating persistence of *Mycobacterium tuberculosis*. Annu Rev Microbiol 2001;55:139–63.

[8] Manabe YC, Bishai WR. Latent *Mycobacterium tuberculosis*—persistence, patience, and winning by waiting. Nat Med 2000;6: 1327–9.

[9] Berks BC, Ferguson SJ, Moir JW, Richardson DJ. Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. Biochim Biophys Acta 1995;1232:97–173.

[10] Hutter B, Dick T. Analysis of the dormancy-inducible *narK2* promoter in *Mycobacterium bovis* BCG. FEMS Microbiol Lett 2000;188:141–6.

[11] Hutter B, Dick T. Upregulation of *narX*, encoding a putative "fused nitrate reductase" in anaerobic dormant *Mycobacterium bovis* BCG. FEMS Microbiol Lett 1999;178:63–9.

[12] Philippot L, Hojberg O. Dissimilatory nitrate reductases in bacteria. Biochim Biophys Acta 1999;1446:1–23.

[13] 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;393:537–44.

[14] Fritz C, Maass S, Kreft A, Bange FC. Dependence of *Mycobacterium bovis* BCG on anaerobic nitrate reductase for persistence is tissue specific. Infect Immun 2002;70:286–91.

[15] Weber I, Fritz C, Ruttkowski S, Dreft A, Bange FC. Anaerobic nitrate reductase (*narGHJI*) activity of *Mycobacterium bovis* BCG in vitro and its contribution to virulence in immunodeficient mice. Mol Microbiol 2000;35:1017–25.

[16] Dick T, Lee BH, Murugasu-Oei B. Oxygen depletion induced dormancy in *Mycobacterium smegmatis*. FEMS Microbiol Lett 1998;163:159–64.

[17] Hutter B, Dick T. Increased alanine dehydrogenase activity during dormancy in *Mycobacterium tuberculosis*. FEMS Microbiol Lett 1998;167:7–11.

[18] Hochstein LI, Tomlinson GA. The enzymes associated with denitrification. Annu Rev Microbiol 1988;42:231–61.

[19] Moreno-Vivián C, Cabello P, Martínez-Luque M, Blasco R, Castillo F. Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. J Bacteriol 1999;181: 6573–84.

[20] Forget P. The bacterial nitrate reductases. Solubilization, purification and properties of the enzyme A *of Escherichia coli* K 12. Eur J Biochem 1974;42:325–32.

[21] Van't Riet J, Van Ee JH, Wever R, Van Gelder BF, Planta RJ. Characterization of the respiratory nitrate reductase of *Klebsiella aerogenes* as a molybdenum- containing iron-sulfur enzyme. Biochim Biophys Acta 1975;405:306–17.

[22] Siddiqi MY, King BJ, Glass ADM. Effects of nitrite, chlorate and chlorite on nitrate uptake and nitrate reductase activity. Plant Physiol 1992;100:644–50.

[23] Callaway TR, Anderson RC, Genovese KJ, Poole TL, Anderson TJ, Byrd JA, et al. Sodium chlorate supplementation reduce *E. coli* 0157:H7 population in cattle. J Anim Sci 2002;80:1683–9.

[24] Brooks JV, Furney SK, Orme IM. Metronidazole therapy in mice infected with tuberculosis. Antimicrob Agents Chemother 1999;43: 1285–8.

[25] Edwards DI. Mechanism of antimicrobial action of metronidazole. J Antimicrob Chemother 1979;5:499–502.

[26] Wayne LG, Sramek HA. Metronidazole is bactericidal to dormant cells of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 1994;38:2054–8.

[27] McOsker CC, Fitzpatrick PM. Nitrofurantoin: mechanism of action and implications for resistance development in common uropathogens. J Antimicrob Chemother 1994;33:23–30.

[28] Murugasu-Oei B, Dick T. Bactericidal activity of nitrofurans against growing and dormant *Mycobacterium bovis* BCG. J Antimicrob Chemother 2000;46:917–9.

[29] Wayne LG, Hayes LG. An in vitro model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of non-replicating persistence. Infect Immun 1996;64:2062–9.

[30] Wayne LG. In vitro model of hypoxically induced non-replicating persistence of *Mycobacterium tuberculosis*. Methods Mol Med 2001;54:247–69.

[31] Tyagi JS, Sharma D. *Mycobacterium smegmatis* and tuberculosis. Trends Microbiol 2002;10:68–9.

[32] Rowe JJ, Ubbink-Kok T, Molenaar D, Konings WN, Driessen AJM. NarK is a nitrite-extrusion system involved in anaerobic nitrate respiration by *E. coli*. Mol Microbiol 1994;12:579–86.

[33] Kelm M. Nitric oxide metabolism and breakdown. Biochim Biophys Acta 1999;1411:273–89.

[34] Nicholas DJD, Nason A. Determination of nitrate and nitrite. Methods Enzymol 1957;3:983.

[35] Sohaskey CD, Wayne LG. Role of *narK2X* and *narGHJI* in hypoxic upregulation of nitrate reduction by *Mycobacterium tuberculosis*. J Bacteriol 2003;185:7247–56.

[36] Macgregor CH, Schnitman CA, Normansell DE. Purification and properties of nitrate reductase from *E. coli* K12. J Biol Chem 1974;249:5321–7.

Available online at www.sciencedirect.com
ScienceDirect
Microbial Pathogenesis 44 (2008) 71–77
MICROBIAL PATHOGENESIS
www.elsevier.com/locate/micpath

Presence of a functional nitrate assimilation pathway in *Mycobacterium smegmatis*

Arshad Khan, Shamim Akhtar, Jawid N Ahmad, Dhiman Sarkar*

Combichem Bio Resource Center, National Chemical Laboratory, Dr. Homi Bhabha Road, Pune 411008, India

Accepted 1 August 2007
Available online 14 August 2007

Abstract

Ability of *Mycobacterium smegmatis* to assimilate nitrate was evaluated in its active and dormant phase. Nitrate (10 mM), nitrite (0.5 mM) and ammonia (10 mM) allowed growth of *M. smegmatis* concomitant with their complete depletion from the culture in 144, 120 and 96 h, respectively, when used as sole nitrogen source. Azide (50 μM) stopped the growth of *M. smegmatis* when nitrate was used as sole nitrogen source. L-methionine-*S*-sulfoximine (L-MSO), which is a well-known inhibitor of glutamine synthetase, an enzyme also involved in nitrogen metabolic pathway, when applied at 10 μg/ml concentration, completely inhibited the growth of the organism when nitrate or nitrite was used as sole nitrogen source. There was no effect of either azide or L-MSO at above concentrations on the growth of the organism when asparagine or ammonia was used as sole nitrogen source. More significantly, utilization of nitrate, nitrite and ammonia continued even in oxygen depletion induced dormant culture at the rates of 289, 25 and 354 μM/day, respectively. These rates were 5–8 times slower than the rates of 1966, 127 and 2890 μM/day, respectively, in active replicating phase. In the presence of azide (50 μM) and L-MSO (10 μg/ml), 2.1 and 1.51 logs reduction in viability of dormant *M. smegmatis* was observed using nitrate and nitrite, respectively, as sole nitrogen source. Altogether, the results indicated the presence of nitrate assimilation pathway operating in both active and dormant stage of *M. smegmatis*.

Keywords: Nitrate assimilation; *Mycobacterium smegmatis*; Dormant bacilli; Glutamine synthetase; L-methionine-*S*-sulfoximine

1. Introduction

Mycobacterium tuberculosis, the etiological agent of tuberculosis (TB) causes upto 2 million deaths per year and latently infects approximately one-third of the world's population [1]. In the last 40 years, no new medicine has been discovered for the treatment of this deadly pathogen [2]. A worldwide rise in TB cases in the last decade and the emergence of multidrug resistant strains led the World Health Organization to declare TB a global emergency [3]. One of the major reasons behind the failure of modern therapy is the existence of the bacilli in persistent stage [4,5]. The survival strategy of *M. tuberculosis* is precisely tuned with its ability to adapt in changing environment, understanding of which is being considered as the major bottleneck for the development of an effective therapy against this disease.

M. tuberculosis is a strong reducer of nitrate [6]. A respiratory type nitrate reductase coded by *narGHJI* was found to be responsible for this reduction of nitrate to nitrite [7]. The evidence of a point mutation in the promoter region of *narGHJI* was responsible for the increased reduction of nitrate compared to other mycobacteria [8]. Although the enzyme was constitutively expressed in *M. tuberculosis*, eight-fold induction of its activity observed during hypoxic shiftdown to dormancy suggested that nitrate could serve as terminal electron acceptor in the absence of oxygen to provide energy through nitrate respiration [9]. 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 membrane [10]. Very recently, it was shown that inhibition of respiratory nitrate reductase kills

*Corresponding author. Tel.: +91 20 25902400; fax: +91 20 25893355.
E-mail addresses: aa.khan@ncl.res.in (A. Khan), s.akhtar@ncl.res.in (S. Akhtar), jna_research@yahoo.co.in (J. N Ahmad), d.sarkar@ncl.res.in (D. Sarkar).

0882-4010/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.micpath.2007.08.006

dormant *Mycobacterium smegmatis* more efficiently than metronidazole [11]. In contrast to all these information, except few preliminary reports published long back, little was known about the assimilation of nitrate by any mycobacterium [12]. This was essential in developing a better understanding about the nitrogen metabolism of the organism as well as in the evaluation of enzymes involved in this pathway for their potential as drug targets in active and dormant stage.

The authors have reported here the presence of a functional nitrate assimilation pathway in *M. smegmatis* by using different substrates as sole nitrogen source and specific inhibitors of the enzymes involved in the pathway. Nitrate-assimilation capability of this bacillus has also been evaluated in the dormant stage where it is unable to multiply.

2. Results

2.1. Growth of *M. smegmatis* in aerobic culture in the presence of nitrate/nitrite or ammonia as sole nitrogen source

Assimilation of nitrate in bacteria involves a multistep pathway, which requires a series of enzymes functioning in combination (Fig. 1) [13]. Nitrate is first converted into nitrite with the help of a nitrate reductase (NR), which subsequently is converted into ammonia by nitrite reductase (Nir). Ammonia is normally taken up into glutamine via glutamine synthetase (GS). In bacterial system, this nitrogen could then be distributed for various cellular requirements of nitrogen through glutamine 2-oxoglutarate amidotransferase (GOGAT) [14].

In this study, all the experiments were carried out using a minimal medium called as *Mycobacterium phlei* medium, which normally contained asparagine as the sole nitrogen source [11]. The growth of *M. smegmatis* in this medium was comparable with other complex media (data not shown). In order to assess nitrate assimilation pathway in *M. smegmatis*, the growth in presence of nitrate, nitrite and ammonia and their utilization were monitored using them as sole nitrogen source (Fig. 2). In presence of nitrate (10 mM) and ammonia (10 mM) as sole nitrogen source, *M. smegmatis* grew as good as it grew in a medium with asparagine as sole nitrogen source (Fig. 2A). The growth of *M. smegmatis* in presence of nitrite (0.5 mM) was proportional to its availability in the medium. Higher concentration of nitrite exerts toxic effect on the bacilli (data not shown). The rate of depletion of nitrate, nitrite and ammonia were almost comparable with the growth of the bacilli, which indicates that they were utilized for the growth of the organism (Fig. 2B). Ten millimolar of nitrate and 0.5 mM of nitrite was completely used up in 144 h whereas 10 mM ammonia was depleted in 96 h. These results indicated that this saprophytic bacillus must contain all the enzymes required for assimilation of nitrate. It was also observed that the traces of nitrite and ammonia were not detected in the culture when nitrate was used as sole nitrogen source and similarly ammonia was absent in culture when nitrite was used as nitrogen source (data not shown). It thus indicated that all these three nitrogen sources of the pathway when used separately would be channelized for assimilation purpose in tandem with growth requirements. All these evidences well indicated the presence of functional nitrate assimilation pathway in *M. smegmatis*.

2.2. Effect of azide and L-methionine-sulfoxamine (L-MSO) on growth and nitrate/nitrite/ammonia utilization

It was earlier known that azide inhibits prokaryotic assimilatory nitrate reductase [13]. In order to check the assimilation of nitrate catalyzed by a respective nitrate reductase in the bacilli, sodium azide was applied in the

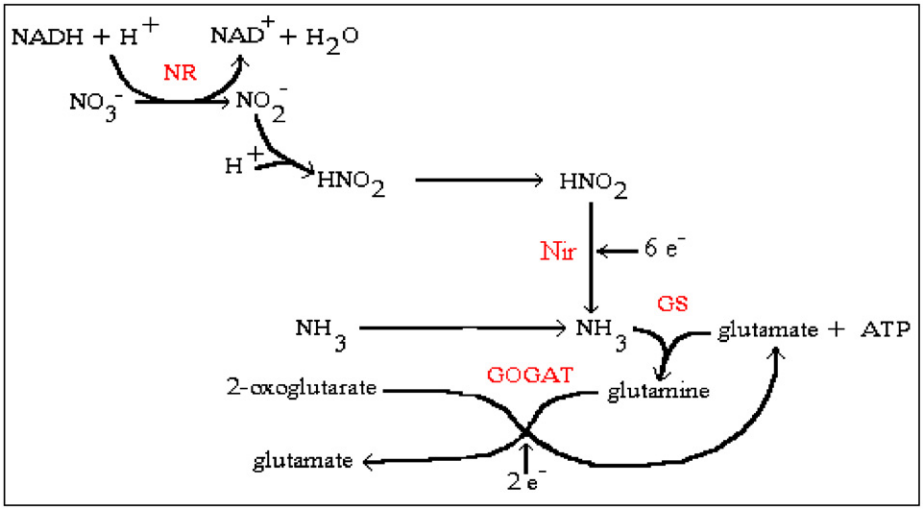


Fig. 1. Biochemical pathway of nitrate assimilation through NR (nitrate reductase), Nir (nitrite reductase) and GS (glutamine synthetase) in bacterial system.

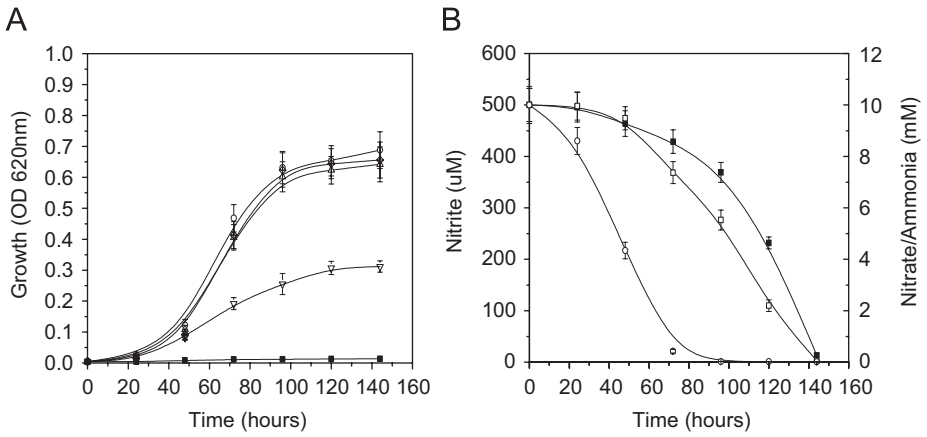


Fig. 2. (A) Growth curve of *M. smegmatis* in presence of 34 mM asparagine (○), 10 mM sodium nitrate (△), 500 μM sodium nitrite (▽) and 10 mM ammonium chloride (◇) and none (■) during aerobic culture condition and (B) utilization of nitrate (■), nitrite (□) and ammonia (○) by *M. smegmatis* with time during aerobic culture condition. The experiments were carried out more than three times and results are mean ± S.D.

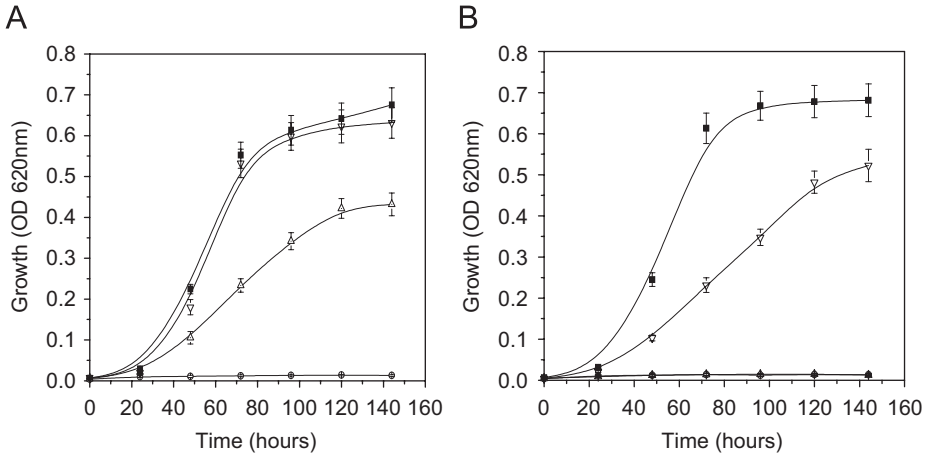


Fig. 3. Effect of (A) azide (50 μM) and (B) L-MSO (10 μg/ml) on growth of *M. smegmatis* in aerobic culture condition with 34 mM asparagine (■), 10 mM sodium nitrate (○), 500 μM sodium nitrite (△) and 10 mM ammonium chloride (▽) as sole nitrogen source. The results are the mean ± S.D. of three identical experiments.

culture (Fig. 3A). At 50 μM concentration, sodium azide did not affect the growth of *M. smegmatis* in presence of asparagine as nitrogen source. At same concentration of azide, there was no growth observed in *M. smegmatis* culture in presence of nitrate as sole source of nitrogen. The effect of azide on growth was also not seen when nitrite and ammonia were used as sole nitrogen source. As expected, azide stopped the depletion of only nitrate and not of nitrite and ammonia in the culture (4A). This evidence proved that conversion of nitrate to nitrite occurred specifically by a nitrate reductase. Since a respiratory nitrate reductase is also present in *M. smegmatis*, it was necessary to identify the type of nitrate reductase involved in the conversion of nitrate to nitrite. In order to find this, a more specific inhibitor thiocyanate (40 mM), which specifically inhibits the respiratory nitrate reductase under such condition, was found to remain ineffective on growth as well as on nitrate reduction (data not shown). This result confirmed that conversion of nitrate to nitrite was catalyzed here by an assimilatory type nitrate reductase. The genome database of *M. smegmatis* was also found to have *narB* (Gene Accession No. YP_887157) gene, which might function as assimilatory nitrate reductase.

In step 3 of nitrate assimilation pathway, ammonia reacts with glutamate in presence of ATP and metal ion Mg^{2+} to produce glutamine, ADP and inorganic phosphate in the course of its assimilation. This biosynthetic reaction is catalyzed by glutamine synthetase [15]. L-methionine sulfoximine (L-MSO), which is a well-known inhibitor of glutamine synthetase, was used here to more precisely elucidate this assimilation pathway [16]. There was no effect of L-MSO on growth of *M. smegmatis* observed in medium with asparagine as sole nitrogen source whereas 10 μg/ml of L-MSO completely inhibited the growth of *M. smegmatis* when grown in medium with nitrate/nitrite as sole nitrogen source (Fig. 3B). Utilization of nitrate/nitrite from the medium was not seen in the

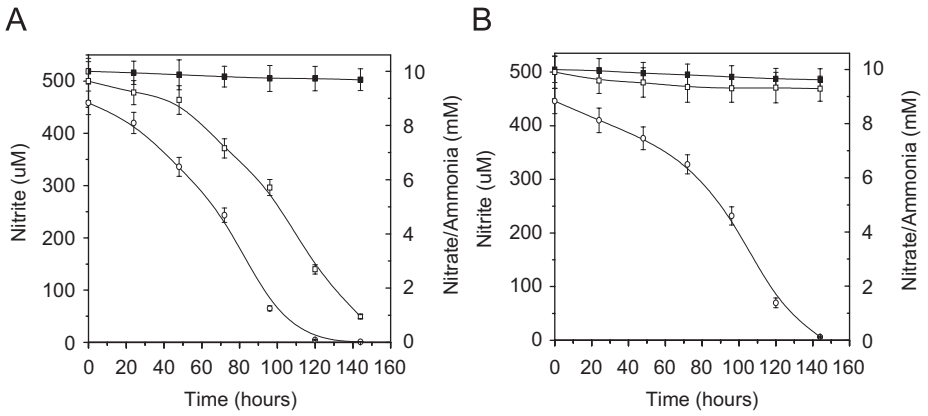


Fig. 4. Effect of (A) azide (50 μM) and (B) L-MSO (10 μg/ml) on utilization of sodium nitrate (■), sodium nitrite (□) and ammonium chloride (○) as sole nitrogen source by *M. smegmatis* in aerobic culture condition. The results are the means ± S.D. of three identical experiments.

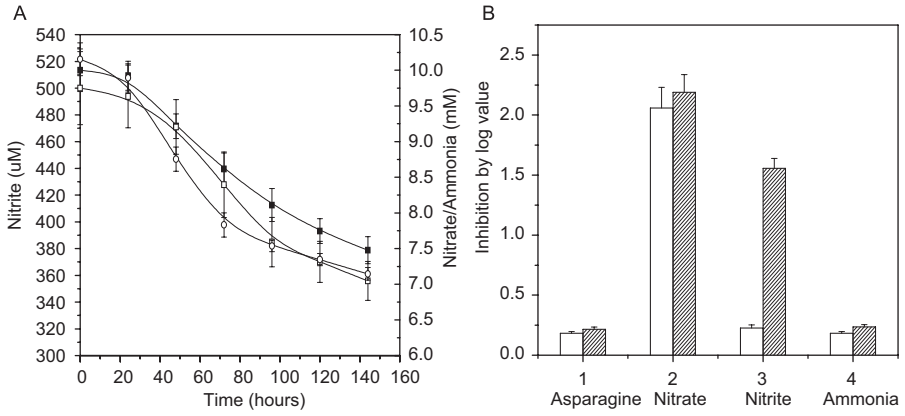


Fig. 5. (A) Utilization of sodium nitrate (■), sodium nitrite (□) and ammonium chloride (○) by *M. smegmatis* with time when cultured in Wayne's dormancy system and (B) effect of azide (light bars) and L-MSO (dark bars) on viability of *M. smegmatis* during its survival in Wayne's dormancy model when sodium asparagines, sodium nitrate, sodium nitrite and ammonium chloride were used as sole nitrogen source. Azide (50 μM) and L-MSO (10 μg/ml) were added at 4-day-old Wayne culture by syringe when all the cells had reached to non-replicating phase and viability was determined after 96 h of addition of these inhibitors. Experiments were carried out more than three times and results are mean ± S.D.

culture as well in presence of L-MSO, which indicated direct involvement of glutamine synthetase in nitrate assimilation process (Fig. 4B). Unexpectedly, L-MSO did not show any effect on growth of *M. smegmatis* grown in medium with ammonia as sole nitrogen source and depletion of ammonia was also seen to occur with time, indicating that ammonia could be utilized by other enzymes in the pathway. Glutamate dehydrogenase (GDH) has already been reported to bypass the function of glutamine synthetase when higher concentration of ammonia is available in the medium [17]. Possibility of the conversion of ammonia into glutamine by a thermolabile glutaminase (EC 3.5.1.2) again could not be denied, as the gene MSMEG_3818 encoding for this enzyme (Gene Accession No. YP_888111) is also present in *M. smegmatis* genome. Altogether, these results confirmed that nitrate assimilation pathway is functional in *M. smegmatis* and inhibition of glutamine synthetase could affect the growth of the bacilli during the assimilation of nitrate/nitrite.

2.3. Utilization of nitrate, nitrite and ammonia by *M. smegmatis* in Wayne's in vitro dormancy model

The evidences discussed above indicated the presence of nitrate assimilation pathway in this saprophytic mycobacterium during its growth in aerobic culture. The role of this pathway in dormant culture could provide a better insight to understand the nitrogen metabolism operative in latent phase. In this course, organism's growth with nitrate, nitrite and ammonia utilization was monitored in Wayne's dormancy model. Interestingly, it was noticed that the depletion of nitrate, nitrite and ammonia did not stop in non-replicating phase (Fig. 5). Rate of utilization of nitrate, nitrite and ammonia were found to be 289, 25 and 354 μM per day, respectively, in this non-replicating stage of the bacilli which was almost 5–8 times less than the rate of utilization seen in actively replicating stage (Table 1). These results indicated that assimilation of nitrate/nitrite/

Table 1
Rate of nitrate, nitrite and ammonia utilization by *M. smegmatis* in aerobic and dormant stage

Substrates[a]	Rate in active replicating stage (μM/day)	Rate in dormant non-replicating stage (μM/day)
Nitrate	1966.67±43.57	289.64±11.26
Nitrite	127±3.41	25.48±1.35
Ammonia	2890.64±51.24	354.79±17.77

[a]These substrates were added with initial concentration of 10, 0.5 and 10 mM, respectively, for nitrate, nitrite and ammonia.

ammonia, even though at a slow rate, continued in dormant phase.

2.4. Effect of azide and L-MSO on viability of dormant bacilli of M. smegmatis

Dependence of the organism for survival in dormant stage on nitrate assimilation pathway was further evaluated by applying azide and L-MSO to the Wayne culture after 96 h of inoculation. It was incubated for another 96 h to examine the effect on viability of the bacilli. A significant reduction of 2.05 and 2.18 logs, respectively, by azide (50 μM) and L-MSO (10 μg/ml) was observed on viability of dormant bacilli in medium where nitrate was used as sole nitrogen source (Fig. 5B). This reduction in viability of the bacilli might have occurred due to the inhibition of either respiratory or assimilatory type of nitrate reductase because inhibition of respiratory nitrate reductase using thiocyanate also reduced the viability of dormant bacilli by ~2 logs [11]. Interestingly, L-MSO reduced the viability of bacilli by 1.51 logs in the presence of nitrite as sole nitrogen source which indirectly proved that whatever may be the nature of nitrate reduction in dormant stage, it will be channelized for assimilation (Fig. 5B). In contrast, negligible effect of these inhibitors was seen on viability of the dormant bacilli when asparagine and ammonia were used as sole nitrogen source. This observation indicated that assimilation of ammonia and asparagine does not take a separate route in this dormant phase of *M. smegmatis*. Thus, it could be concluded that nitrate assimilation pathway remains active even in dormant stage of *M. smegmatis* and also plays an important role in the survival of this non-replicating stage in case when nitrate or nitrite is available as sole nitrogen source.

3. Discussion

Nitrate reductase activity has long been used as a diagnostic tool to identify different mycobacterial species [6,18,19]. This activity has also been explored in few drug susceptibility assays [20,21]. Contribution to virulence as well as dependence on this nitrate reductase (NarGHJI) to maintain persistent infection in SCID mice was then proved this enzyme as a potential drug target particularly against the latent phase of the pathogen [22,23]. Interestingly, in our previous studies it was observed that even though nitrate reduction reaches a plateau after a certain period of time in Wayne's dormancy model, the bacilli were still dependent on NarGHJI for their survival. This indicated that nitrite could be subsequently reduced either for assimilation/respiration purpose. Following of *in vitro* dormancy system with added advantage of its non-pathogenic and fast-growing nature allowed us to use *M. smegmatis* as a model organism for this kind of study [24]. Use of a defined minimal medium where a single nitrogen source could be added at a time according to the experimental requirement again provided advantage to study the complete nitrate metabolism [11]. Earlier reports though suggested the capabilities of *M. smegmatis* to assimilate nitrate but could not reveal the mechanism of complete pathway [12]. In this study, the capability of nitrate assimilation of *M. smegmatis* has been investigated by using three substrates mentioned as sole nitrogen source along with the use of respective inhibitor of the enzymes involved in the pathway. Growth of *M. smegmatis* in presence of nitrate, nitrite and ammonia as sole nitrogen source along with its depletion from the culture provided a strong evidence in favor of an active nitrate assimilation pathway present in the bacilli (Figs. 2 and 3A). Total inhibition on growth and utilization of nitrate by *M. smegmatis* in presence of azide as well as mention of *narB* gene (Gene Accession No. YP_887157) in genome database confirmed the existence of an active nitrate assimilatory pathway (Fig. 3). Another characteristic of the pathway noticed was that the intermediates of nitrate metabolism did not appear in the medium (data not shown). L-MSO blocked the utilization of both nitrate and nitrite as sole nitrogen source for growth, further confirmed the presence and utility of this pathway in *M. smegmatis* (Figs. 3 and 4). The lack of inhibition observed by L-MSO on the assimilation of ammonia as well as growth of the bacilli could be justified by the presence of genes for glutaminase (Gene Accession No. YP_888111) and glutamate dehydrogenase (Gene Accession No. YP_889681) making a bypass of glutamine synthetase for the survival of the organism [17]. Further studies are being carried out in our laboratory to investigate the specific role played by these three enzymes in pathogenic as well as saprophytic mycobacteria. Most significantly, this assimilation pathway remained active even when the organism was shifted to oxygen depletion induced dormant stage (Fig. 5). Inhibitor of the enzymes involved in the pathway also had significant effect on the viability of the dormant bacilli, hence, indicated the importance of this pathway during persistent stage. These results also provided an indication, in contrary to an earlier report, that L-MSO inhibits intracellular glutamine synthetase [25]. Analysis of this nitrate assimilation pathway could also help in finding new drug targets, which could be equally useful against active and persistent bacilli of the pathogen.

Though the results clearly indicated about the utility and dependence on this pathway under certain conditions when

nitrate/nitrite is available as sole nitrogen source, presence of nitrate/nitrite in nutrient-deprived host cells harboring these mycobacteria may not be at all surprising [26]. Presence of similar type of nitrate assimilation pathway in *Mycobacterium bovis* BCG and *M. tuberculosis* was also observed during initial experiments in our laboratory (unpublished). Studies regarding the availability and utilization capabilities of other nitrogen sources by these mycobacterial species is needed further to provide a deeper insight in relating a possible link between the different enzymes involved in the pathway with the manifestations of the disease by different mycobacterial species to identify a futuristic drug target.

4. Materials and methods

4.1. Bacterial strains, chemicals and media

M. smegmatis (ATCC607) was obtained from AstraZeneca, India. All the chemicals were purchased from Sigma, USA, unless mentioned. A defined medium containing 0.5 gm KH_2PO_4, 0.2 gm sodium citrate, 60 mg $MgSO_4$, 0.5 gm asparagine and 2 ml glycerol in 100 ml of distilled water at pH 6.6 used throughout the whole study called as *M. phlei* medium [11]. Asparagine was replaced with other nitrogen sources according to the experimental requirement.

4.2. Cultivation of aerobic and dormant bacilli

For aerobic cultivation of the bacilli, 5 ml of the culture was added to a 25 ml capacity tube containing 8 mm magnetic bar and was then incubated at 37 °C on a magnetic stirrer rotating at 100 rpm. Inoculum was prepared by first growing the organism upto stationary phase in minimal medium with asparagine as nitrogen source and then aseptically washed the cells by centrifuging at 10,000 rpm for 10 min. Cell pallet was then resuspended to equal volume of the minimal medium without any nitrogen source. 0.1% of this suspension was used as inoculum size for each experiment yielding approximately 10^5 cells/ml.

Dormant bacilli of *M. smegmatis* were cultivated by following Wayne's 0.5 headspace ratio model using 20×125 mm tubes with a total volume of 25.5 ml [11]. Inoculum size used here was about 10^5 cells/ml by appropriately diluting the culture to 0.008 A_{620}. After putting 8 mm magnetic spin bar, the tube was made airtight using rubber septa. The cultures were gently stirred at 100 rpm on a magnetic stirrer.

4.3. Measurement of growth

Growth of the organism was measured by reading absorbance of culture at 620 nm following an earlier described method [27]. Viability of dormant bacilli was determined by spreading different dilutions of the culture on agar plates made of *M. phlei* medium and counting the colonies appeared.

4.4. Estimation of nitrate, nitrite and ammonia

Concentration of the nitrate in the whole cell culture was estimated by a method based on salicylic acid nitration [28]. Briefly, 50 µl of the culture was added with 200 µl of 5% salicylic acid solution prepared in conc. H_2SO_4. The solution was incubated for 20 min and 4.75 ml of 2 N NaOH was added to develop a yellow color. Absorbance of the sample was read at 410 nm and nitrate concentration was calculated by comparing with a standard nitrate curve. Cell culture and nitrite did not interfere in the estimation of nitrate.

Nitrite concentration in the culture was determined by using Griess reaction [29]. Briefly, 1 ml of the culture was added with 1 ml of 1% sulphanilic acid solution (prepared in 20% HCL) and 1 ml of 0.1% NEDD solution (prepared in distilled water). The tubes were incubated for 15 min to develop pink color. Absorbance of the samples was read at 540 nm and nitrite concentration was calculated by using nitrite standard curve.

Ammonia concentration in the culture was determined by following salicylate–hypochlorite method [30]. Briefly, 3.5 ml of sample were added with 0.5 ml of salicylate solution (40% in D/W), 0.5 ml of potassium ferrocyanide solution (2% $K_4Fe(CN)_6 \cdot 2H_2O$ and 10% Na_3 citrate $\cdot 2H_2O$ (in 0.1 N NaOH) and 0.2 ml of hypochlorite solution (1.93% in 0.1 N NaOH). After covering the tubes with parafilm, the sample was vortexed and placed in water bath at 37 °C for 45 min. Samples were then cooled for 15 min, vortexed and absorbance at 650 nm was measured. Standard curve made of ammonia was used to estimate the ammonia level in culture samples.

Acknowledgments

Authors are thankful to Director, National Chemical Laboratory, Pune, India, for providing financial assistance through an in-house project. We are also thankful to AstraZeneca R&D, Bangalore, India, for providing *M. smegmatis* strain ATCC 607.

References

[1] Dye C, Cheele S, Olin P, Ikram P, Aviglioen MC. Global burden of tuberculosis. JAMA 1999;282:677–86.
[2] Helen P. Drug giant to offer new, cut-price tuberculosis treatment. Lancet Infect Dis 2005;5:744.
[3] Bloom BR, Murray CJ. Tuberculosis: commentary on a reemergent killer. Science 1992;257:1055–64.
[4] Wayne LG. Dormancy of *Mycobacterium tuberculosis* and latency of disease. Eur J Clin Microbiol Infect Dis 1994;13:908–14.
[5] Manabe YC, Bishai WR. Latent *Mycobacterium tuberculosis*—persistence, patience, and winning by waiting. Nat Med 2000;6:1327–9.
[6] Virtanen S. A study of nitrate reduction by mycobacteria. Acta Tuberc Scand 1960;47:1–116.

[7] Wayne LG, Sohaskey CD. Non-replicating persistence of *Mycobacterium tuberculosis*. Annu Rev Microbiol 2001;55:139–63.
[8] Stermann M, Sedlacek L, Maass S, et al. A promoter mutation causes differential nitrate reductase activity of *Mycobacterium tuberculosis* and *Mycobacterium bovis*. J Bacteriol 2004;186:2856–61.
[9] Wayne LG, Hayes LG. Nitrate reduction as a marker for hypoxic shift down of *Mycobacterium tuberculosis*. Tuberc Lung Dis 1998; 79:127–32.
[10] Sohaskey CD, Wayne LG. Role of *narK2X* and *narGHJI* in hypoxic upregulation of nitrate reduction by *Mycobacterium tuberculosis*. J Bacteriol 2003;185:7247–56.
[11] Khan A, Sarkar D. Identification of a respiratory-type nitrate reductase and its role for survival of *Mycobacterium smegmatis* in Wayne model. Microb Pathog 2006;41:90–5.
[12] Hedgecock LW, Costello RL. Utilization of nitrate by pathogenic and saprophytic mycobacteria. J Bacteriol 1962;84:195–205.
[13] Moreno-Vivián C, Cabello P, Martínez-Luque M, Blasco R, Castillo F. Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. J Bacteriol 1999;181: 6573–84.
[14] Merrick MJ, Edwards RA. Nitrogen control in bacteria. Microbiol Rev 1995;59:604–22.
[15] Harth G, Clemens DL, Horwitz MA. Glutamine synthetase of *Mycobacterium tuberculosis*: extracellular release and characterization of its enzymatic activity. Proc Natl Acad Sci USA 1994;91:9342–6.
[16] Manning JM, Moore S, Rowe WB, Meister A. Identification of L-methionine-*S*-sulfoximine as the diastereomer of L-methionine-*S, R*-sulfoximine that inhibits glutamine synthetase. Biochemistry 1969;8:2681–5.
[17] Kanamori K, Weiss RL, Roberts JD. Role of glutamate dehydrogenase in ammonia assimilation pathways in nitrogen-fixing *Bacillus macerans*. J Bacteriol 1987;169:4692–5.
[18] Master RN. Mycobacteriology. In: Isenberg HD, editor. Clinical microbiology procedures handbook. Washington, DC: ASM Press; 1992. p. 3.0.1–3.16.4.
[19] Metchock BG, Nolte FS, Wallace RJ. Mycobacterium. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, editors. Manual of clinical microbiology. Washington. DC: ASM Press; 1999. p. 399–437.
[20] Angeby KA, Klintz L, Hoffner SE. Rapid and inexpensive drug susceptibility testing of *Mycobacteriiim tuberculosis* with a nitrate reductase assay. J Clin Microbiol 2002;40:1553–5.
[21] Palomino JC, Portaels F. Simple procedure for drug susceptibility testing of *Mycobacterium tuberculosis* using a commercial colorimetric assay. Eur J Clin Microbiol Infect Dis 1999;18:380–3.
[22] Weber I, Fritz C, Ruttkowski S, Dreft A, Bange FC. Anaerobic nitrate reductase (*narGHJI*) activity of *Mycobacterium bovis* BCG *in vitro* and its contribution to virulence in immunodeficient mice. Mol Microbiol 2000;35:1017–25.
[23] Fritz C, Maass S, Kreft A, Bange FC. Dependence of *Mycobacterium bovis* BCG on anaerobic nitrate reductase for persistence is tissue specific. Infect Immun 2002;70:286–91.
[24] Dick T, Lee BH, Murugasu-Oei B. Oxygen depletion induced dormancy in *Mycobacterium smegmatis*. FEMS Microbiol Lett 1998;163:159–64.
[25] Harth G, Horwitz MA. An inhibitor of exported *Mycobacterium tuberculosis* glutamine synthetase selectively blocks the growth of pathogenic mycobacteria in axenic culture and in human monocytes: extracellular proteins as potential novel drug targets. J Exp Med 1999;189:1425–36.
[26] Kelm M. Nitric oxide metabolism and breakdown. Biochim Biophys Acta 1999;1411:273–89.
[27] Wayne LG, Hayes LG. An in vitro model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of non-replicating persistence. Infect Immun 1996;64: 2062–9.
[28] Caltado DA, Haroon M, Schrader LE, Youngs VL. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun Soil Sci Plant Anal 1975;6:71–80.
[29] Nicholas DJD, Nason A. Determination of nitrate and nitrite. Methods Enzymol 1957;3:981–4.
[30] Bower CE, Holm-Hansen T. A salicylate-hypochlorite method determining ammonia in sea water. Can J Fisheries Aquat Sci 1980;37:794–8.

ELSEVIER

International Journal of Antimicrobial Agents 32 (2008) 40–45

INTERNATIONAL JOURNAL OF Antimicrobial Agents

www.ischemo.org

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*

Combi Chem-Bio Resource Center, National Chemical Laboratory, Dr Homi Bhabha Road, Pune 411008, India

Received 13 October 2007; accepted 29 February 2008

Abstract

This study evaluated the antituberculous potential of 2-nitroimidazole under in vitro conditions. Minimal bactericidal concentrations of the compound against actively replicating *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* H37Ra were found to be 0.226 μg/mL and 0.556 μg/mL in enriched and minimal medium, respectively. Minimal inhibitory concentrations were >100 times lower than reported antituberculous nitroimidazoles such as nitrofurantoin and furaltadone, indicating the greater potential of 2-nitroimidazole. No discernible effect of 2-nitroimidazole was seen on saprophytic *Mycobacterium smegmatis* and the representative bacterial strain *Escherichia coli* DH5α, indicating the specificity of the molecule against tuberculous mycobacteria. The compound was also found to be effective against *M. tuberculosis* in the intracellular environment of the human monocytic cell line THP-1, with a reduction in viability of bacilli by 2.5 log after 144 h of incubation at a concentration of 0.113 μg/mL. A five-fold higher concentration (0.565 μg/mL) of 2-nitroimidazole sterilised the macrophages of intracellular pathogens within 192 h, without affecting the host. However, 2-nitroimidazole was unable to affect significantly the viability of dormant non-replicating bacilli of *M. bovis* BCG and *M. tuberculosis* in Wayne's in vitro model. Overall, the results indicate that 2-nitroimidazole is a potent antituberculous agent active against the organism's active replicating stage, with promising intracellular efficacy as well.

Keywords: Tuberculosis; Antimycobacterial agents; 2-Nitroimidazole; Nitroimidazoles; THP-1 macrophage

1. Introduction

Tuberculosis (TB) is the leading cause of death of ca. 2 million of the world's population every year [1,2]. In the last 40 years, no new drug has been developed for the treatment of TB [3]. The length and complexity of antibiotic therapy for TB and the emergence of multidrug-resistant strains make a compelling case for the development of new efficacious antituberculous drugs [4,5]. There is currently a great deal of interest in developing new drugs that are not only active against drug-resistant TB but that can also shorten the duration of therapy. New drug development for TB has been directed towards the use of the nitroimidazole class of compounds because of their novel mechanism of action and lower chances of developing resistance [6]. Metronidazole, nitrofurantoin, furaltadone, nitrofurazone, nitroimidazopyran PA-824 and CGI-17341 are the active molecules identified in the recent past against TB, which all belong to the 5-nitroimidazole series [7–10]. A recent finding in *Helicobacter pylori* has clearly shown that 2-nitroimidazole-derived molecules are highly effective against strains resistant to even 5-nitroimidazole derivatives [11]. So far, attention has not been paid to evaluation of the effectiveness of 2-nitroimidazole series compounds for their antituberculous potential despite their known antibacterial activity [12]. Nitroimidazoles also remain a major attraction to medicinal chemists to work with because of their very low

* Corresponding author. Tel.: +91 20 2590 2400; fax: +91 20 2590 2624.
E-mail address: dhimansarkar77@gmail.com (D. Sarkar).

0924-8579/$ – see front matter © 2008 Elsevier B.V. and the International Society of Chemotherapy. All rights reserved.
doi:10.1016/j.ijantimicag.2008.02.022

level of in vivo toxicity [13]. These observations prompted us to evaluate more precisely the antituberculous potential of 2-nitroimidazoles.

The present study evaluated the effectiveness and specificity of 2-nitroimidazole against in vitro actively replicating and dormant non-replicating tubercle bacilli as well as its efficacy in an intracellular environment.

2. Materials and methods

2.1. Bacterial strains and inocula preparation

Mycobacterium bovis BCG (ATCC 35745) and *Mycobacterium smegmatis* (ATCC 607) were obtained from AstraZeneca (Bangalore, India) and *Mycobacterium tuberculosis* H37Ra (ATCC 25177) was obtained from MTCC (Chandigarh, India). *Escherichia coli* strain DH5α was obtained from NCIM (Pune, India). Subculturing of all mycobacterial strains was routinely done on Dubos albumin agar slants or plates. Liquid inocula were prepared in Dubos Tween–albumin broth medium and incubated in a shaking incubator rotating at a speed of 150 rpm at 37 °C. One percent of 1.0 absorbance at 620 nm of the culture was used as the standard inoculum size for all the experiments, yielding a final inoculum of ca. 10^5 colony-forming units (CFU)/mL.

2.2. Antimycobacterial agents and media

2-Nitroimidazole, furaltadone, nitrofurantoin, 4-nitroimidazole, 1,2-dimethyl 5-nitroimidazole and metronidazole were purchased from Sigma (St Louis, MO) and their stock solutions were prepared in dimethyl sulfoxide (DMSO) stored at −20 °C. Dubos broth base with Dubos albumin supplements was purchased from Difco (Sparks, MD) and was used as enriched media for the study. A defined medium containing 0.5 g of KH_2PO_4, 0.2 g of sodium citrate, 60 mg of $MgSO_4$, 0.5 g of asparagine and 2 mL of glycerol in 100 mL of distilled water at pH 6.6 was used as minimal medium [14].

2.3. In vitro activity testing against the active replicating stage

The inhibitory activity of the compounds against growing *M. bovis* BCG and *M. tuberculosis* H37Ra bacilli was tested by incubating the cells in aerobic condition in a 100 mL flask containing 50 mL of medium with shaking at 150 rpm at 37 °C (Thermo Electron Model No. 481; Thermo Electron Corp., Marietta, OH) [14]. Compounds were added at the time of inoculation at doses ranging from 0.1 μg/mL to 10 μg/mL and growth was measured by reading the absorbance at 620 nm as well as by determining CFU/mL after 8 days of incubation, at which time the bacilli reaches stationary phase [15]. The lowest concentration of drug yielding a differential absorbance (A_{620}) of approximately zero or less was defined as the minimal inhibitory concentration (MIC). The minimal bactericidal concentration (MBC) was defined as the lowest concentration of compound that prevented any growth of the test bacterium on an agar plate.

2.4. Activity testing against the dormant non-replicating stage

The inhibitory activity of the compounds against dormant bacilli was examined using Wayne's 0.5 head space ratio in vitro model [15]. Wayne's hypoxic model is based on gradual depletion of oxygen from mycobacterial cells to achieve the non-replicating dormant stage. Briefly, diluted culture containing ca. 10^5 cells/mL was transferred to 20 mm × 125 mm tubes. Culture tubes were then sealed with rubber septa and gently stirred with the help of 8 mm magnetic beads rotating at 100 rpm on a magnetic stirring platform. Attainment of cells of the hypoxic non-replicating dormant stage was confirmed by constant CFU/mL as well as by decolourisation of methylene blue (1.5 μg/mL) dye in the Wayne culture system [15]. Once all the cells reached the non-replicating phase, compounds were added using a Hamilton syringe with a 24-gauge needle followed by incubation for 4 days. Doses of the compounds were kept in the range of their determined MIC/MBC (0.226 μg/mL) up to 20 times the MIC/MBC (4.52 μg/mL). Culture samples were then spread on Dubos agar plates and colonies were enumerated on Day 21 to examine the effect of the compound on the dormant stage.

2.5. Intracellular activity in THP-1 macrophages

The monocytic cell line THP-1 (obtained from the national cell repository, National Centre for Cell Science, Pune, India) was used to examine the inhibitory activity of the compounds against intracellular bacilli. THP-1 cells (5×10^4 cells/mL) were treated with 100 nM of phorbol myristate acetate in a culture flask for 24 h to convert them into macrophages. These macrophage cells were incubated for 12 h with *M. tuberculosis* H37Ra at a multiplicity of infection of 1:100 for

Table 1
Minimal inhibitory concentration (MIC) of 2-nitroimidazole compared with other antimycobacterial nitroimidazole compounds

Compound[a]	MIC (μg/mL)[b]	
	Mycobacterium bovis BCG	*Mycobacterium tuberculosis*
2-Nitroimidazole	0.226	0.226
4-Nitroimidazole	>50	>50
1,2-Dimethyl 5-nitroimidazole	>50	>50
Nitrofurantoin	48	32
Furaltadone	32	32
Rifampicin	0.08	0.05
Isoniazid	0.075	0.05
Streptomycin	0.3	0.2
Ethambutol	3	2

[a] All these compounds were dissolved in dimethyl sulfoxide (DMSO).
[b] Determined in minimal medium.

infection. Extracellular mycobacteria were removed by washing twice with phosphate-buffered saline and then adding fresh medium to adhered cells. Compounds were then added to these infected macrophages at different concentrations of 0.113–1.13 μg/mL. The effect of the compound was monitored by determining the bacterial load within macrophages by lysing them with hypotonic buffer (10 mM HEPES, 1.5 mM $MgCl_2$ and 10 mM KCl) and spreading the samples on Dubos agar plates at different time intervals to enumerate colonies after 21 days.

3. Results

3.1. Effect of 2-nitroimidazole on the active replicating stage of M. tuberculosis and M. bovis BCG

Most of the nitroimidazole compounds are poorly soluble in aqueous solution. The hydrophobic character of these compounds results in a general tendency for protein binding in solution, which ultimately could affect their bactericidal efficacy even under in vitro conditions. To obtain a better understanding of the inhibitor, MIC and MBC values were determined both in minimal medium and albumin–dextrose–saline-enriched Dubos medium. Different doses of 2-nitroimidazole (0.1–10 μg/mL) were added at the time of inoculation of the culture flasks and absorbance at 620 nm and CFU/mL were measured after 8 days incubation to determine the MICs and MBCs for the compounds, respectively. For both *M. tuberculosis* and *M. bovis* BCG, identical MBC values of 0.226 μg/mL and 0.556 μg/mL were found in enriched and minimal medium, respectively (Fig. 1). The sharp decrease in the MBC of approximately five times in minimal medium could be attributed to the availability of limited nutrients to the cells compared with enriched medium. These results also indicated that medium with minimal nutrient support could be more useful in evaluating the antituberculous activity of a compound. A higher MIC of 2-nitroimidazole described in an earlier report could have been observed due to the use of enriched medium in the study [16]. This MIC value of 2-nitroimidazole was almost on par with other frontline antituberculous drugs such as rifampicin, isoniazid, streptomycin and ethambutol (Table 1) [17]. MIC values for some of the known antituberculous nitroimidazoles (nitrofurantoin, furaltadone) as well as the representative 4- and 5-nitroimidazole compounds were also determined to compare the data with 2-nitroimidazole (Table 1) [8]. It was interesting to note that the MIC value of 2-nitroimidazole was ≥100 times lower than other nitroimidazoles used in this study.

Specificity of the molecule for its antimycobacterial activity was also examined by applying it to a saprophytic mycobacterial strain *M. smegmatis* and a representative bacterial strain *E. coli* DH5α (Fig. 2). 2-Nitroimidazole showed a negligible effect on the growth of these organisms even in up to 50-fold higher concentrations than its determined MIC and MBC against *M. bovis* BCG and *M. tuberculosis*. These results indicated that 2-nitroimidazole is specifically active against tuberculous bacilli.

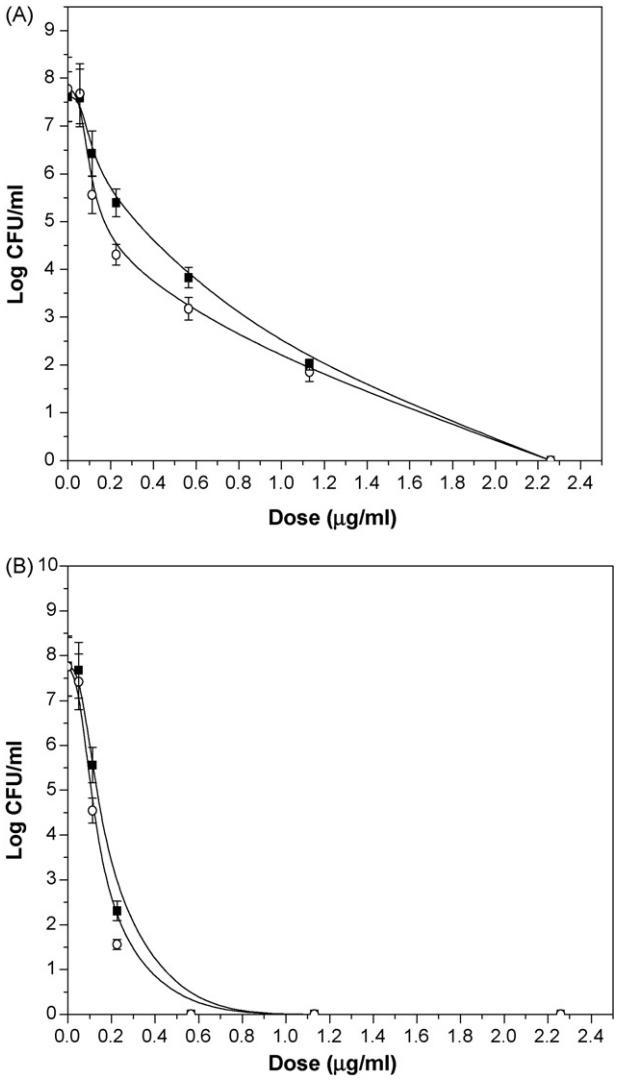


Fig. 1. Dose-dependent effect of 2-nitroimidazole on growth in (A) enriched medium and (B) minimal medium against *Mycobacterium bovis* BCG (■) and *Mycobacterium tuberculosis* (◯). Doses of 2-nitroimidazole (0.1–10 μg/mL) dissolved in dimethyl sulfoxide (DMSO) were added at the time of inoculation and colony-forming units (CFU)/mL were counted after 8 days of incubation, as described in Section 2.3. Experiments were carried out three times with duplicate cultures and results are mean ± standard deviation.

3.2. Effect of 2-nitroimidazole against dormant non-replicating bacilli

2-Nitroimidazole was also tested against *M. tuberculosis* and *M. bovis* BCG in an in vitro dormancy model to evaluate its activity against persistent bacilli. Wayne's model, which is the most accepted model to mimic dormancy in vitro, was selected to assess the effect of 2-nitroimidazole on persistent

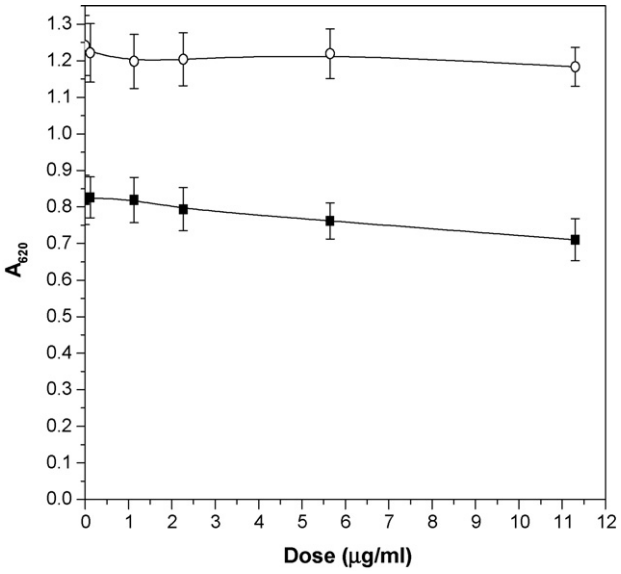


Fig. 2. Dose-dependent effect of 2-nitroimidazole on *Mycobacterium smegmatis* (■) and *Escherichia coli* (◯). A_{620} represents the growth of the organism and was measured after 72 h and 15 h of incubation in the presence of the compound for *M. smegmatis* and *E. coli*, respectively. Experiments were carried out three times with duplicate cultures and results are mean ± standard deviation.

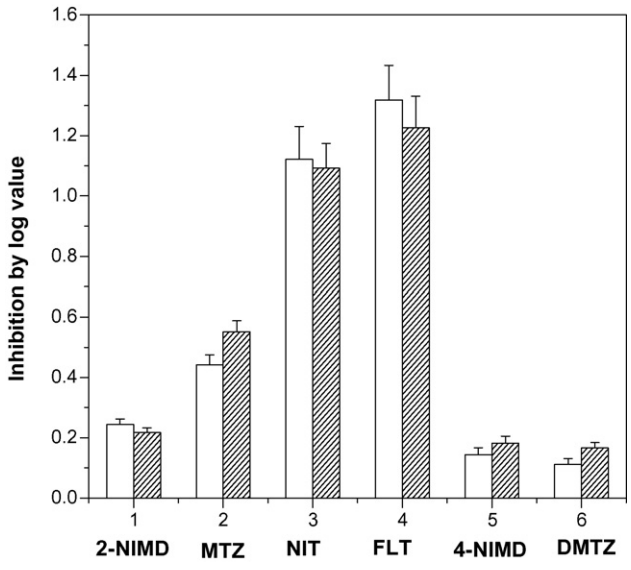


Fig. 3. Effect of 2-nitroimidazole (2-NIMD), metronidazole (MTZ), nitrofurantoin (NIT), furaltadone (FLT), 4-nitroimidazole (4-NIMD) and dimetridazole (DMTZ) on the viability of dormant bacilli in minimal medium at doses of 4.52, 85.5, 48, 32, 50 and 50 μg/mL, respectively. Dark bars represent *Mycobacterium bovis* BCG and light bars represent *Mycobacterium tuberculosis*. Inhibitors were added to an 8-day-old Wayne culture through a syringe and the effect was observed after 96 h of incubation by measuring the viable cell count. Inhibition was calculated by subtracting log values of inhibitors from control. Experiments were carried out three times with duplicate culture and results are mean ± standard deviation.

bacilli [15]. Compound was added to an 8-day-old Wayne culture without disturbing the oxygenic environment inside the container. Doses of the compounds were kept in the range of their determined MIC/MBC (0.226 μg/mL) up to 20 times the MIC/MBC (4.52 μg/mL). The effect of the compound on the viability of these dormant bacilli was then examined by CFU enumeration after 96 h of incubation in the presence of the compound. Interestingly, the effect of 2-nitroimidazole was found to be very different on these hypoxic dormant bacilli. The compound did not affect the viability of dormant bacilli of either *M. tuberculosis* or *M. bovis* BCG even up to 20× MIC (4.52 μg/mL) (Fig. 3). On the other hand, nitrofurantoin and furaltadone had a comparatively better effect (1.1 log and 1.3 log reduction) on the viability of these dormant bacilli under such conditions [8]. Consistent with previous results, metronidazole showed only moderate bactericidal activity against these hypoxic dormant bacilli [7]. These results indicated the inefficacy of 2-nitroimidazole against non-replicating dormant bacilli. Differential antituberculous activity of 2-nitroimidazole (only against the replicating stage), metronidazole (only against dormant bacilli) and nitrofurans (both against active and dormant stages) suggest a thorough evaluation of the proposed mechanism of action of reactive nitrogen intermediate-based killing of bacilli through DNA damage by nitroheterocyclic compounds [7,8].

3.3. Effect of 2-nitroimidazole against intracellular bacilli

The antimycobacterial activity of any molecule under in vitro axenic culture conditions may lead to false conclusions unless evaluated under conditions that resemble the condition in vivo. Considering this factor, 2-nitroimidazole was tested against *M. tuberculosis* when residing within macrophages. 2-Nitroimidazole was applied to THP-1 macrophages infected with *M. tuberculosis* at a concentration of 0.113–1.13 μg/mL. Killing kinetics was followed by enumerating CFU/mL of the bacilli after lysing the THP-1 cells (Fig. 4). The result clearly indicated a drop in viability of intracellular bacilli from the very beginning of the incubation. At 0.5× MIC (0.113 μg/mL) of 2-nitrimidazole, 2.5 log reduction in the viability of *M. tuberculosis* residing in THP-1 macrophages occurred within 144 h. The inhibitory effect reached its maximum at 2.5× MIC (0.565 μg/mL) of the inhibitor, where complete sterilisation occurred within 192 h. The killing kinetics of 2-nitroimidazole clearly supported the earlier observation from axenic cultures that the inhibitory action is restricted to actively growing bacilli.

Furthermore, it was also observed that 2-nitroimidazole had no significant effect on the viability of THP-1 macrophages even up to 100 times its MIC against *M. tuberculosis* and *M. bovis* BCG (data not shown). These results indicated that the reduction in colony count was due to the bactericidal activity of 2-nitroimidazole and not due to any direct non-specific lethal effect on THP-1 monocytes. Altogether, these results showed that the antituberculous potency of 2-nitroimidazole was also effective in an intracellular environment.

4. Discussion

Nitroimidazoles have long been used for their biological activity against helminths, protozoa, fungi and bacteria [12]. Based on the superior biological activity of 5-nitroimidazoles, other nitroimidazoles were evaluated initially when this drug class was being considered for its antituberculous potential. Although some reports described the antibacterial activity of a few 2-nitroimidazole derivatives in the 1970s, their antituberculous activity was not appreciated or evaluated further [18,19]. Meanwhile, major attention was created by 5-nitroimidazole derivatives and at least one of them went on to clinical trials [20]. Incidentally, 2-nitroimidazole was identified as one of the hits from our in-house screening programme developed to discover novel antitubercular agents against actively growing and/or dormant bacilli (results unpublished). An almost five-fold reduction in the MIC obtained in minimal medium compared with enriched medium indicates that whole cell-based antitubercular screening should be carried out in the former medium (Fig. 1). Otherwise, protein binding due to the presence of albumin in enriched medium could mislead the assessment procedure.

The MIC of 2-nitroimidazole was found to be ca. 100–200 times lower than the MIC of the reported antituberculous 5-nitroimidazole compounds nitrofurantoin and furaltadone (Table 1) [8]. This indicated that 2-nitroimidazoles could become much greater potential antituberculous agents than 5-nitroimidazoles. Indeed, the MIC of 2-nitroimidazole was comparable with the lead nitroimidazole PA-824 (<1 μg/mL) that has reached the clinical trial stage [21]. Moreover, an earlier investigation suggested a reduced probability of the development of resistance for 2-nitroimidazoles compared with 5-nitroimidazoles [11].

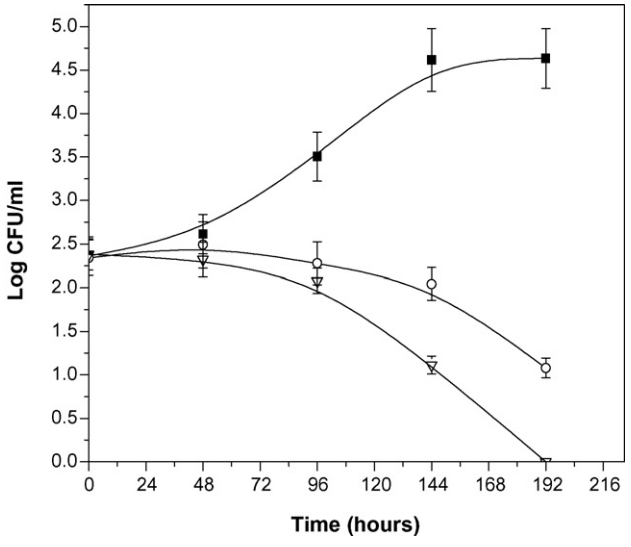


Fig. 4. Killing kinetics of 2-nitroimidazole against intracellular *Mycobacterium tuberculosis* H37Ra. Dimethyl sulfoxide (DMSO) control (■), 0.113 μg/mL 2-nitroimidazole (◯) and 0.565 μg/mL 2-nitroimidazole (▽) was added just after infection of THP-1 macrophages with *M. tuberculosis*. A separate flask of macrophage culture was used for each colony-forming unit's (CFU) determination at each time point. Experiments were carried out three times and results are mean ± standard deviation.

The intracellular killing efficacy against *M. tuberculosis* within host macrophages proved the potential of 2-nitroimidazole (Fig. 4). This efficacy justifies the compound being taken forward as a lead agent. Studies in animal systems are being carried out in the laboratory to assess the efficacy of the compound in vivo.

Metronidazole, nitrofurantoin, furaltadone and nitrofurazone, which all belong to the 5-nitroimidazole series, are the currently known antimycobacterial agents, effective against the dormant stage of the tubercle bacilli in Wayne's dormant culture system, yet their effective concentration is much higher than desired [7,8]. The inhibition pattern of these nitroimidazoles indicates that there is the possibility of finding a 2-nitroimidazole derivative effective against the dormant stage as well (Table 1; Fig. 3). Specific inhibitory activity of 2-nitroimidazole only against *M. tuberculosis* and *M. bovis* BCG also provides clues as to the target for this molecule, which is not present in non-pathogenic *M. smegmatis* or in other bacteria such as *E. coli* (Fig. 2). Revealing the mode of action and identifying the target of the compound is again anticipated before taking 2-nitroimidazoles for further development.

The insignificant cytotoxic effect of 2-nitroimidazole on the mammalian cell line THP-1 even at 100 times higher concentration than the MIC strengthens the arguments for evaluating this compound further (data not shown). Synthesis of more 2-nitroimidazole derivatives based on their structure–activity relationship is being carried out to find other active compounds from this class. In conclusion, our results demonstrate the potential of 2-nitroimidazole against actively growing tubercle bacilli, along with its intracellular efficacy, indicating that this compound should be considered further for optimisation within an antituberculous drug development programme.

Funding: This work was supported by grants provided by an in-house project of the National Chemical Laboratory, Pune, India.

Competing interests: None declared.

Ethical approval: Not required.

References

[1] World Health, Organization, Global tuberculosis control: surveillance, planning, financing. WHO report 2007. WHO/HTM/TB/2007.376 Geneva, Switzerland: WHO; 2007.

[2] Raviglione MC, Snider DE, Kochi A. Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic. JAMA 1995;273:220–6.

[3] Helen P. Drug giant to offer new, cut-price tuberculosis treatment. Lancet Infect Dis 2005;5:744.

[4] World Health Organization. Anti-tuberculosis drug resistance in the world. Report No. 2. Prevalence and trends. Geneva, Switzerland:

WHO; 2000. http://www.who.int/bookorders/anglais/detart1.jsp?sesslan=1&codlan=1&codcol=93&codcch=2117 [accessed 20 March 2008].
[5] Schluger NW. The impact of drug resistance on the global tuberculosis epidemic. Int J Tuberc Lung Dis 2000;4(Suppl. 1):S71–5.
[6] Barry CE, Boshoff HIM, Dowd CS. Prospects for clinical introduction of nitroimidazole antibiotics for the treatment of tuberculosis. Curr Pharm Des 2004;10:3239–62.
[7] Wayne LG, Sramek HA. Metronidazole is bactericidal to dormant cells of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 1994;38:2054–8.
[8] Murugasu-Oei B, Dick T. Bactericidal activity of nitrofurans against growing and dormant *Mycobacterium bovis* BCG. J Antimicrob Chemother 2000;46:917–9.
[9] Stover CK, Warrener P, VanDevanter DR, Sherman DR, Arain TM, Langhorne MH, et al. A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. Nature 2000;405:962–6.
[10] Ashtekar DR, Costa-Perira R, Nagrajan K, Vishvanathan N, Bhatt AD, Rittel W. In vitro and in vivo activities of the nitroimidazole CGI 17341 against *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 1993;37:183–6.
[11] Smith MA, Edwards DI. Redox potential and oxygen concentration as factors in the susceptibility of *Helicobacter pylori* to nitroheterocyclic drugs. J Antimicrob Chemother 1995;6:751–64.
[12] Grunberg E, Beskid G, Cleeland R, DeLorenzo WF, Titsworth E, Scholer HJ, et al. Antiprotozoan and antibacterial activity of 2-nitroimidazole derivatives. Antimicrob Agents Chemother 1967;7:513–9.
[13] Roe FJ. Safety of nitroimidazoles. Scand J Infect Dis Suppl 1985;46:72–81.
[14] Khan A, Sarkar D. Identification of a respiratory-type nitrate reductase and its role for survival of *Mycobacterium smegmatis* in Wayne model. Microb Pathog 2006;41:90–5.
[15] Wayne LG, Hayes LG. An in vitro model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of non-replicating persistence. Infect Immun 1996;64:2062–9.
[16] Sun Z, Zhang Y. Antituberculosis activities of certain antifungal and antihelmintic drugs. Tuber Lung Dis 1999;79:319–20.
[17] Wallace Jr RJ, Nash DR, Steele LC, Steingrube V. Susceptibility testing of slowly growing mycobacteria by a microdilution MIC method with 7H9 broth. J Clin Microbiol 1986;6:976–81.
[18] Cavalleri B, Ballotta R, Arioli V, Lancini G. New 5-substituted 1-alkyl-2-nitroimidazoles. J Med Chem 1973;16:557–60.
[19] Cavalleri B, Volpe G, Arioli V. Synthesis and biological activity of some vinyl-substituted 2-nitroimidazoles. J Med Chem 1977;20:656–60.
[20] Lenaerts AJ, Gruppo V, Marietta KS, Johnson CM, Driscoll DK, Tompkins NM, et al. Preclinical testing of the nitroimidazopyran PA-824 for activity against *Mycobacterium tuberculosis* in a series of in vitro and in vivo models. Antimicrob Agents Chemother 2005;49:2294–301.
[21] Tyagi S, Nuermberger E, Yoshimatsu T, Williams K, Rosenthal I, Lounis N, et al. Bactericidal activity of the nitroimidazopyran PA-824 in a murine model of tuberculosis. Antimicrob Agents Chemother 2005;49:2289–93.