Investigating the Effects of Host Factors (proteins and non-proteins) on *Mycobacteria*

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By

Muhammad Suleman Riaz

Division of Biosciences

Department of Life Sciences

College of Health and Life Sciences

Brunel University London

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Declaration

I hereby declare that the research presented in this thesis is my own work, except where otherwise specified and has not been submitted for any other degree.

Muhammad Suleman Riaz

Abstract

Mycobacterium tuberculosis (*M.tb*), the causative agent of tuberculosis, is one of the leading causes of death due to a single infectious agent and results in more than 1 million human deaths every year. *M.tb* infection of the host initiates a local inflammatory response, resulting in the migration of a number of host plasma protein and non-protein factors to the site of infection. In addition, some of these factors are also produced locally at the site of infection. It is envisaged that these host factors are likely to come in direct contact with *M.tb* and immune cells and may modulate the outcome of the infection.

In this study, a number of host factors including transferrin, lactoferrin, fibrinogen, C-reactive protein, alpha-2-macroglobulin (α2M), vitronectin, plasminogen, low-density lipoprotein (LDL), high-density lipoprotein (HDL), serotonin, L-alpha dipalmitoyl phosphatidylcholine (DPPC) and platelet activating factor C-16 (PAF C-16) were screened *in vitro* for their direct effect on the growth of *mycobacteria* using *M.smegmatis* as a model. As a result of this screening, PAF C-16, a phospholipid compound was identified that directly inhibited the growth of *M.smegmatis* and *M.bovis BCG* in a dose and time-dependent manner.

Use of a range of PAF C-16 structural analogues, including Lyso-PAF, PAF C-18, Hexanolamino PAF, 2-O-methyl PAF & Pyrrolidino PAF, revealed that small modifications in structure did not alter the direct growth inhibition property of PAF C-16 and similar levels of *M.smegmatis* and *M.bovis BCG* growth inhibition were observed as compared to PAF C-16. Structural dissection of PAF C-16 suggested that the attachment of carbon tail to the glycerol backbone via ether bond at *sn*-1 position was important for its direct growth inhibition activity against *mycobacteria*. Microscopy and flow cytometry with PAF C-16 treated *M.smegmatis* and *M.bovis BCG* showed damage to the bacterial cell membrane. The addition of membrane-stabilizing agents, α-tocopherol, tween-80 and tween-20, partially mitigated the growth inhibitory effect of PAF C-16. These results suggested that the growth inhibition activity of PAF C-16 against *mycobacteria* is most likely due to its detergent-like effect, resulting in damage to the bacterial cell membrane.

PAF C-16 and its structural analogues were also investigated for their effect on the growth of intracellular *M.smegmatis* inside THP1 cells. *In vitro*, PAF C-16, PAF C-18 and Hexanolamino PAF inhibited the growth of intracellular *M.smegmatis*, whereas, analogues such as Lyso-PAF and 2-O-methyl PAF failed to show any growth inhibitory effect, suggesting that the presence of acetyl group at *sn*-2 position was important for growth inhibition of intracellular

M.smegmatis. Use of PAF receptor antagonists partially mitigated the inhibitory effect of PAF C-16 on the growth of intracellular *M.smegmatis*, suggesting this inhibition was through receptor-mediated signalling pathways. Blocking of PAF C-16 signalling pathway components such as phospholipase C and phospholipase A_2, resulted in the increased survival of intracellular *M.smegmatis*. Arachidonic acid, a product of PAF C-16 signalling pathway directly inhibited the growth of *M.smegmatis*. Furthermore, inhibition of iNOS enzyme and antibody-mediated neutralization of TNF-α partially mitigated the inhibitory effect of PAF C-16 on intracellular *M.smegmatis* growth, suggesting that the production of NO and TNF-α were also involved in PAF C-16 induced intracellular growth inhibition.

Overall, this study has identified PAF C-16, its structural analogues such as Lyso-PAF, PAF C-18, Hexanolamino PAF and other compounds including 1-O-hexadecyl-*sn*-glycerol, miltefosine and hexadecyl lactate with novel anti-mycobacterial activity. Further investigations are needed to demonstrate their effectiveness against *M.tb* both *in vitro* and in animal models to assess their therapeutic potential as anti-TB drugs.

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List of Abbreviations

µg	Microgram
µl	Microliter
µM	Micro Molar
5-HT	5-Hydroxytryptamine
AA	Arachidonic Acid
ADC	Albumin Dextrose Catalyse
AG	Aminoguanidine hemisulfate
APC	Antigen Presenting Cell
apoA-I	Apolipoprotein A-1
apoB-100	Apolipoprotein B-100
APP	Acute Phase Proteins
ATP	Adenosine Triphosphate
BAL	Bronchoalveolar Lavage
BCG	Bacillus Calmette Guérin
BLAST	Basic Local Alignment Search Tool
BS	Benzenesulfonamide
$CaCl_2$	Calcium Chloride
CD	Cluster of Differentiation
cDNA	Complementary DNA
CDP-choline	Cytidine Diphosphate-choline
CFP10	Culture Filtrate Protein 10
CFU	Colony Forming Unit
CH_3	Methyl group
CH_3O	Acetyl group
CIITA	Class II Trans-Activator
CO_2	Carbon dioxide
CR	Complement receptor
CRP	C-Reactive Protein

DAG	Diacylglycerol
DAP	Diaminopimelic acid
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DPPC	L-Alpha Dipalmitoylphosphatidylcholine
DST	Drug Susceptibility Test
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-Linked Immunospot
ESAT-6	Early Secretory Antigenic Target of 6kDa
GSH	Glutathione
HCL	Hydrochloric Acid
HDL	High-Density Lipoprotein
HIV	Human Immunodeficiency Virus
ICAM	Intercellular Adhesion Molecule
IFN-γ	Interferon-Gamma
Ig-G	Immunoglobulin-G
IGRA	Interferon-γ release assay
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IP_3	Inositol trisphosphate
LAM	Lipoarabinomannan
LB	Luria-Bertani
LDL	Low-Density Lipoprotein
LLO	Listeriolysin O
LM	Lipomannan
LPS	Lipopolysaccharide
M. chelonei	*Mycobacterium chelonei*
M. fortuitum	*Mycobacterium fortuitum*

M.avium	*Mycobacterium avium*
M.bovis BCG	*Mycobacterium bovis*
M.smegmatis	*Mycobacterium smegmatis*
M.tb	*Mycobacterium tuberculosis*
mAb	Monoclonal antibody
MAC	Membrane Attack Complex
mAGP	Mycolylarabinogalactan-peptidoglycan
MDR-TB	Multidrug-resistant tuberculosis
mg	Milligram
MHC	Major Histocompatibility Complexes
MIC	Minimum Inhibitory Concentration
ml	Millilitre
mRNA	Messenger RNA
NAAT	Nucleic acid amplification test
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAG	N-acetylglucosamines
NAM	N-acetylmuramic acid
NCBI	National Center for Biotechnology Information
NET	Neutrophil extracellular trap
NK cell	Natural killer Cell
nm	Nanometre
NO	Nitric Oxide
O.D	Optical Density
OH	Hydroxyl group
PAF C-16	Platelet Activating Factor C-16
PAF-AH	PAF acetylhydrolase
PAFR	Platelet Activating Factor Receptor
PAMP	Pathogen Associated Molecular Pattern
PAS	Para-Aminosalicylic Acid

PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
PI	Propidium Iodide
PIM	Phosphatidylinositol mannosides
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear Leukocyte
PPD	Purified Protein Derivatives
Ppm	Parts per Million
PRR	Pattern Recognition Receptors
RD1	Region of Difference 1
RNA	Ribonucleic Acid
RNI	Reactive Nitrogen Intermediates
ROIs	Reactive Oxygen Intermediates
RRDR	Rifampicin Resistance Determining Region
rRNA	Ribosomal Ribonucleic Acid
SD	Standard Deviation
SEM	Standard Error of Mean
SIV	Simian Immunodeficiency Virus
TB	Tuberculosis
TCR	T-cell Receptor
TGF-β	Transforming growth factor-Beta
Th cells	T Helper Cells
TLR	Toll-like receptors
TNF-α	Tumor Necrosis Factor- Alpha

tPA	Tissue-type Plasminogen Activator
tRNA	Transfer Ribonucleic Acid
uPA	Urokinase-type Plasminogen Activator
WHO	World Health Organization
XDR-TB	Extensively Drug-Resistant TB
α2M	Alpha-2-Macroglobulin
γδ	Gamma Delta

Chapter 1: Literature Review

1.1 Tuberculosis

Tuberculosis (TB) is an infectious airborne disease in humans, caused by acid-fast bacillus known as *Mycobacterium tuberculosis* (*M.tb*). TB is one of the leading causes of human mortality worldwide, resulting in more than 1 million human deaths every year (WHO, Global TB report 2016). In humans, the most common type of TB is pulmonary TB, about 60-80% of the total *M.tb* cases comprises of pulmonary TB. However, *M.tb* infections are not limited to lungs and *M.tb* can spread to other organs such as lymph nodes, meninges, pleura, kidneys and gastrointestinal tract, bones and joints etc., a condition known as extra-pulmonary TB (Julceus *et al*., 2017; Gomes *et al*., 2014; Wang *et al*., 2014; Sandgren *et al*., 2013).

1.2 Epidemiology of Tuberculosis

There is an estimate that about one-third of the world's population (1.7 billion people) is latently infected with *M.tb*, a condition with no active disease symptoms due to the containment of pathogen by the host immune system. This latent infection with *M.tb* provides a huge reservoir for reactivation of active TB and its spread across the globe (Houben and Dodd, 2016).

According to World Health Organization (WHO), approximately 10.4 million new cases of TB were reported worldwide during 2015. Out of these new *M.tb* cases, 90% (9.4 million) were detected in the adult human population with a male to female ratio of 1.6:1. The remaining 10% (1.0 million) *M.tb* infections were detected in children, out of which 170,000 proved to be fatal (WHO, Global TB report 2016).

TB is more prevalent in developing world such as Asia and the Sub-Saharan African regions (Lönnroth *et al*., 2010). During 2015, 61% of new TB incidences were reported from Asian countries and 26% from Africa. About 87% of new TB cases reported were from 30 high TB burden countries including India, Indonesia, China, Nigeria, Pakistan, South Africa, Bangladesh and Philippines (WHO, Global TB report 2016). Different predisposing factors including low socioeconomic status, closed living conditions, overcrowding, malnutrition and poor health conditions contribute to the spread of TB in these countries (Lönnroth *et al*., 2009). The number of TB cases per 100,000 individuals in different countries of the world reported during 2015 is shown in Figure 1.1.

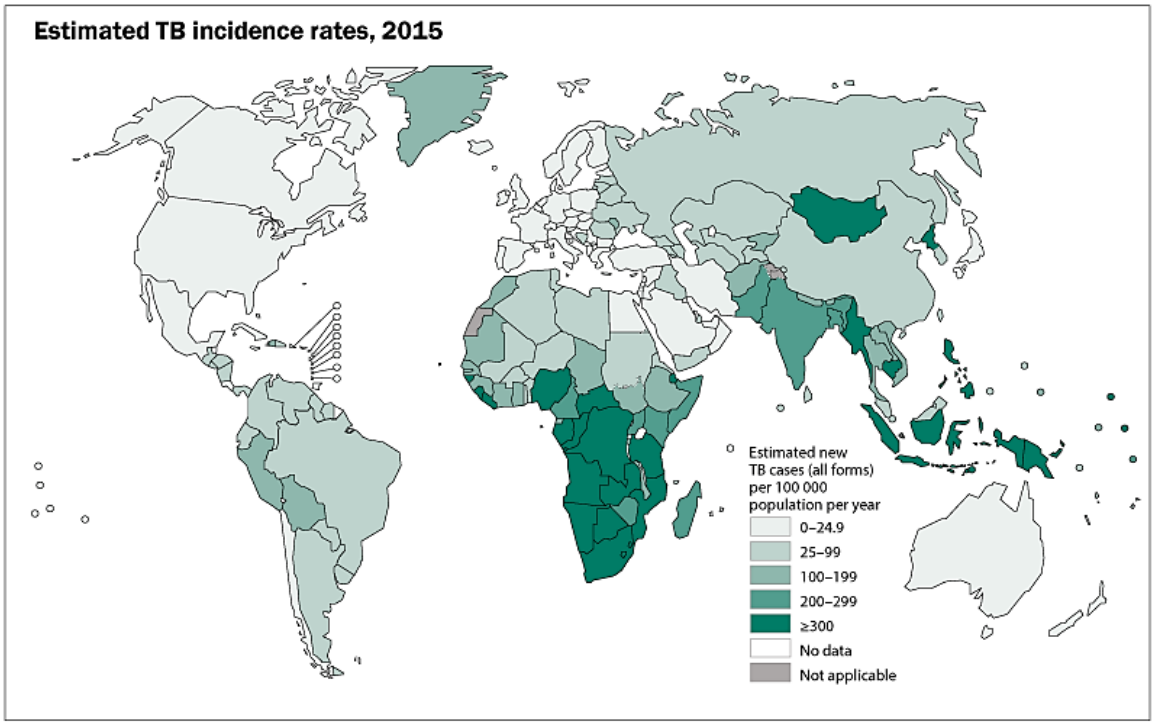


Figure1.1: Worldwide distribution of TB cases reported during 2015. Estimated TB incidences per 100,000 individuals are represented with different colours. The figure shows that TB is more common in Africa and Asia and the highest number of TB cases per 100,000 inhabitants was mostly found in African countries (WHO, Global TB report 2016).

The global menace of TB has become more severe due to Human Immunodeficiency Virus (HIV) infections (Daley *et al*., 1992). There is an increased risk of TB in HIV patients due to the killing of $CD4^+$ T cells by HIV virus, the main fighting force against *M.tb*. This co-infection of HIV and *M.tb* is a lethal combination and most patients succumb to death much earlier than individual infections on their own (Jones *et al*., 1993). HIV positive individuals are 20-30% more likely to reactivate TB as compared to HIV negative individuals (Girardi *et al*., 2000). According to WHO, the rate of TB in regions with high prevalence of HIV is more than twice (400 cases in 100,000 population per year) as compared to regions with low HIV prevalence. The incidences of TB in HIV patients are more common in Africa and account for one-fourth of the total number of TB cases in the region. An estimated 1.2 million new TB cases were reported in HIV patients worldwide in the year 2015 (WHO, Global TB report 2016).

Multidrug-resistant tuberculosis (MDR-TB) is another obstacle that threatens the control TB worldwide. In MDR-TB, *M.tb* become resistant to antibiotics and the patient fails to respond to first line anti-TB drugs (isoniazid and rifampicin). MDR-TB can develop as a result of the

inappropriate use of anti-TB drugs, inadequate treatment regimens, poor quality of anti-TB drugs and premature treatment stoppage (Gandhi *et al*., 2010; Espinal *et al*., 2003). The drug-resistant *M.tb* strains can transmit directly from person to person, contributing to the spread of the drug-resistant bacteria in a population. According to WHO Global TB report 2016, new cases of MDR/RR-TB have virtually been detected across the globe (Figure 1.2). About 480,000 new cases of MDR-TB were reported to the WHO during 2015. India, China and Russia have the highest number of patients with MDR-TB where one in eight TB patients fails to respond to the standard anti-TB drug therapy (WHO, Global TB report 2016).

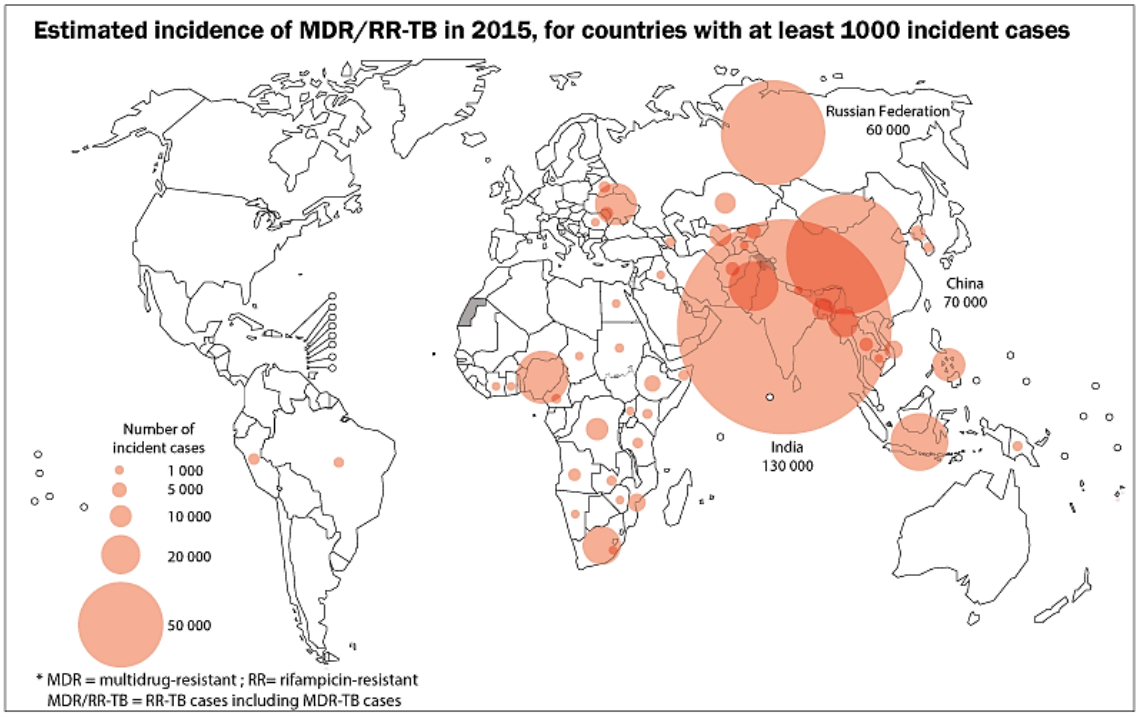


Figure1.2: Worldwide distribution of MDR/RR-TB cases reported during 2015. The size of the coloured circle represents the number of MDR/RR-TB cases reported in a particular country. The figure shows that countries like India, China and Russia have the highest number of MDR/RR-TB with 50,000 or more cases reported in the year 2015 (WHO, Global TB report 2016).

1.3 *Mycobacterium tuberculosis* (*M.tb*)

Mycobacterium tuberculosis (*M.tb*) belongs to the family Mycobacteriacae of phylum Actinobacteria. There are more than 190 different species included in the genus *Mycobacterium* (Tsukamura, 1967). Some of these species are highly pathogenic such as *Mycobacterium*

tuberculosis, *Mycobacterium africanum*, *Mycobacterium leprae*, *Mycobacterium bovis*, *Mycobacterium caprae* and *Mycobacterium microti* and cause disease in different organisms including humans.

M.tb are non-motile, rod-shaped bacteria that are about 2-4µm in length and 0.2-0.5µm in width. *M.tb* are slow growing aerobic bacteria, with a doubling time of 15-20 hours. *M.tb* have a unique cell wall with high lipid content and are poorly stained during gram staining. However, these bacteria can be identified by staining with Ziehl–Neelsen stain (acid-fast stain) and are, therefore, classified into a separate group known as the acid-fast group of bacteria (Todar, 2008).

Cole *et al* reported the complete genome sequence of *M.tb* (H37Rv strain) for the first time in 1998. *M.tb* have a circular genome that comprises of $4.4x10^6$ nucleotides and contains around 4,000 genes. The *M.tb* genome has a high GC content of 65.6% (Cole *et al.*, 1998). The *M.tb* genome was later updated with functions assigned to 2,058 bacterial proteins (Camus *et al*., 2002).

1.4 *M.tb* cell wall structure

M.tb have a thick and hydrophobic cell wall with a high content of lipids and waxes. This unique composition of the cell wall makes *M.tb* resistant to environmental stresses such as changes in pH, drought and antibiotics. The cell wall also provides protection to *M.tb* from the host organism's immune system and contributes to the virulence of *M.tb* in different ways (Hett and Rubin, 2008).

The *M.tb* cell wall has a two-layered architecture (Figure 1.3). The layer next to the cell membrane is made up of a network of peptidoglycan chains. The peptidoglycan chains in acid-fast cell wall consist of repeating units of N-acetylmuramic acid (NAM) and N-acetylglucosamines (NAG) cross-linked by short peptide chains consisting of L-alanyl-D-*iso*-glutaminyl-*meso*-diaminopimelic acid (DAP). These cell wall peptidoglycan chains are covalently attached to arabinogalactan via phosphodiester bonds. The arabinogalactan, in turn, binds to mycolic acids with varying carbon chain lengths (C70-C90). This mycolylarabinogalactan-peptidoglycan (mAGP) arrangement forms a complex, known as the "cell wall core". The mAGP core constitutes the main skeleton of the bacterial cell wall and is vital to the cell integrity. The outer surface of cell wall core in *M.tb* is infused with various

structural components including glycolipids such as lipomannan (LM), lipoarabinomannan (LAM), which are anchored by phosphatidyl-*myo*-inositol mannosides (PIMs) (Brennan, 2003; Lee *et al*., 1996).

Each LAM in *M.tb* cell wall consists of a basal PIM unit that attaches non-covalently to the cell membrane at one end. The inositol ring of PIM at the other end binds to a D-mannan polymer followed by D-arabinose chains with capping motifs at the terminal ends. A major difference in the structural composition of slow growing and fast growing *mycobacteria* is the capping of the terminal-β-Ara. In slow-growing species capping at LAM consist of mannose residues (ManLAMs), whereas, in fast-growing *mycobacteria,* LAMs are capped with phosphoinositol (PILAMs) (Alderwick *et al*., 2007). LAMs are important *M.tb* cell wall components that can bind to different receptors such as mannose receptors and TLR4 etc. on the surface of immune cells and modulate immune responses. LAMs help in inhibiting phagosomal maturation and shift the cytokine response of phagocytic cells to *M.tb* from pro-inflammatory to anti-inflammatory (Briken *et al*., 2004; Strohmeier and Fenton, 1999). *M.tb* mutants without LAMs are more sensitive to antibiotics, macrophage killing and in some cases failed to establish an effective infection in mice (Fukuda *et al*., 2013; Stoop *et al*., 2013; Kaur *et al*., 2008). Fukuda *et al*, have shown that changes in LM/LAM have a significant effect on the cell wall integrity of *mycobacteria* and structural defects in LM/LAM lead to loss of acid-fast staining, increase susceptibility to antibiotics and decrease in virulence (Fukuda *et al*., 2013).

Phosphatidyl-*myo*-inositol mannosides (PIMs) family in *mycobacteria* consists of different members with a varied number of mannose units (1-5 units) (Guerin *et al*., 2010). The PIMs have been shown to be important in the immunopathogenesis of tuberculosis. PIM_2 was shown to be important in granuloma formation in mice model by causing the recruitment of T cells during the early stages (Apostolou *et al*., 1999). PIM_2 was also shown to cause the production of pro-inflammatory cytokines such as TNF-α from mice macrophages (Rhoades *et al*., 2003).

The *M.tb* cell wall also contains other important components such as the 19kDa lipoprotein. The 19kDa lipoprotein binds to Toll-like receptor 2 (TLR2) on human monocyte-derived macrophages *in vitro* and has been shown to be involved in inducing apoptosis in these macrophages (Sánchez *et al*., 2012). However, the 19kDa lipoprotein from *M.tb* can also contribute to the virulence of the bacteria by inhibiting the processing and expression of *M.tb*

antigens via MHC class II molecules on macrophages resulting in decreased recognition by T cells (Noss *et al*., 2001).

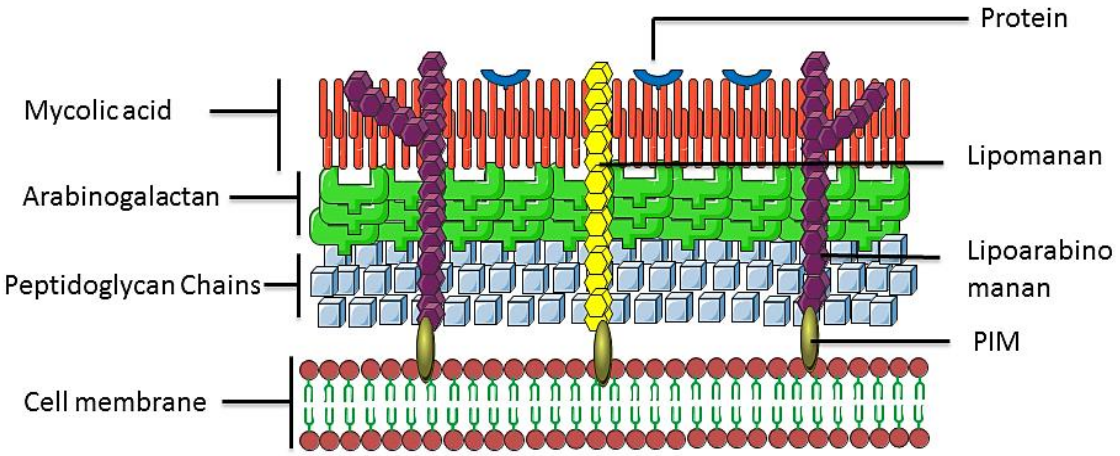


Figure 1.3: *M.tb* cell wall structure. The figure shows the arrangement of different layers of the *M.tb* cell wall. The cell wall core consists of a complex of peptidoglycan chains, arabinogalactan and mycolic acid. Structures such as Lipomannans (LMs) and lipoarabinomannans (LAMs) and different proteins are infused on the outer surface of the cell wall core, whereas the phosphatidyl-*myo*-inositol mannosides (PIMs) serve as anchors for both LMs and LAMs (Adapted from Kleinnijenhuis *et al*., 2011).

1.5 Pathogenesis of *M.tb*

M.tb spreads via aerosol droplets produced by a TB patient during coughing. When a healthy individual inhales these aerosol droplets, *M.tb* enters the respiratory tract and is carried to the lungs of the new host (Zumla *et al*., 2011). Inhalation of an aerosol droplet containing 1-10 bacilli is enough to initiate infection (Sundaramurthy and Pieters, 2007). Resident phagocytic cells known as alveolar macrophages along with a small number of dendritic cells serve as the first line of host defence and engulf the pathogenic *M.tb* by receptor-mediated phagocytosis in an attempt to control the infection (Details in section 1.7). Upon phagocytosis, the *M.tb* are trapped in membrane-bound vacuoles known as phagosomes. The alveolar macrophages try to eliminate the intracellular *M.tb* through various mechanisms including the formation of phagolysosome, production of reactive nitrogen species and apoptosis (Details in section 1.9).

However, there are escape mechanisms through which *M.tb* can evade intracellular killing by macrophages while residing inside the phagosomes (Details in section 1.10).

M.tb inside the alveolar macrophages cause the release different cytokines and chemokines from these cells. This results in the recruitment of different immune cells including blood-derived monocytes and neutrophils at the site of infection. The monocytes differentiate into effector macrophages and produce cytokines such as TNF-α, IL-6, IL,1 etc. which help in the elimination of intracellular *M.tb* (Giacomini *et al*., 2001; Cooper and Flynn, 1995). During initial stages of infection, a few dendritic cells with the phagocytosed *M.tb* migrate to the regional lymph nodes where they can activate the adaptive immune system by displaying *M.tb* antigens on their surfaces in association with major histocompatibility complexes (MHC) resulting in the subsequent production of antigen-specific T lymphocytes. Antigen-specific T cells from the lymph nodes then migrate to the site of infection and produce different pro-inflammatory cytokines such as IFN-γ that activates macrophages to eliminate the intracellular *M.tb* (Harding and Boom, 2010). However, the strength of host T cell response in *M.tb* infection determines whether the infection is arrested at this point or advances to the subsequent phases.

1.6 *M.tb* granuloma structure and function

Granulomas are important features associated with *M.tb* infection. Granulomas are of central importance to *M.tb* pathogenesis and determine the outcome of *M.tb* infection (Guirado and Schlesinger, 2013). A granuloma is a compact structure that consists of an organized aggregation of various immune cells at different stages of differentiation (Figure 1.4). The core region of granuloma consists of *M.tb* infected and uninfected macrophages, foamy macrophages, multinucleated giant cells (Langhans giant cells), epithelioid cells (a special form of differentiated macrophages), necrotic tissue and free *M.tb*. The outer ring surrounding the core of granuloma consist of lymphocytes, having a majority of T cells ($CD4^+$ T cells and $CD8^+$ T cells) and a small number of B cells (Gonzalez-Juarrero *et al*., 2001).

The main purpose of the granuloma is to restrict *M.tb* from spreading by creating an immune microenvironment. However, it also provides *M.tb* with a niche to survive over long periods as the bacteria can modulate the immune response. A balance between the pro-inflammatory and anti-inflammatory immune response is vital for the survival of *M.tb* inside granuloma. Pro-inflammatory cytokines such as TNF-α and IFN-γ play key roles in the development and

functioning of granulomas (Ly *et al*., 2008). TNF-α helps in maintaining the structure of granuloma by keeping sustained levels of chemokines and cellular recruitment and retention. It has been shown that in TNF-α deficient ($TNF^{-/-}$) mice there was an initial delay in CC and CXC chemokines induction due to their deregulated transcription levels. This prevented the recruitment of immune cells like macrophages and $CD4^+$ T cells in the $TNF^{-/-}$ mice when challenged with *M.tb* and caused a loose aggregation of immune cells that failed to contain the infection in the $TNF^{-/-}$ mice (Roach *et al*., 2002). TNF-α blocking therapies in humans have been shown to be associated with an increased risk of TB reactivation (Gómez-Reino *et al*., 2003). Neutralizing TNF-α by a monoclonal antibody MP6-XT22 in chronic murine tuberculosis model resulted in the reactivation of TB with a 10-fold increase in the number of *M.tb* bacilli in the lungs as compared to isotype control mice. Furthermore, histopathology of lungs tissue showed that the TNF-α neutralized mice had a loose aggregation of macrophages and the lymphoid aggregates were also less apparent as compared to the isotype control mice (Mohan *et al*., 2001). IFN-γ is also important for the stable persistence of granulomas as it causes the activation of macrophages. Challenging IFN-γ gene knockout mice ($IFN\text{-}\gamma^{-/-}$) with *M.tb* showed early mortality and 10-100 folds more *M.tb* in different organs as compared to the wild-type control. Furthermore, granulomas were formed in the absence of IFN-γ, the histopathological examination revealed that granulomas from $IFN\text{-}\gamma^{-/-}$ mice were necrotic and had a large number of extracellular *M.tb* as compared to the wild-type control (Flynn *et al*., 1993).

ICAM-1, also known as CD54 is an intercellular adhesion molecule that has been shown to be important for granuloma formation. ICAM-1 knockout mice, when challenged with *M.tb* showed higher bacterial load and lack of organized granulomas in the lungs as compared to the wild-type control. Furthermore, ICAM-1 knockout mice infected with *M.tb* died earlier as compared to wild-type control mice (Saunders *et al*., 1999).

The role of $CD4^+$ T cells in control of *M.tb* and granuloma formation is evident from the reactivation of latent TB in patients with HIV co-infection (Girardi *et al*., 2000). HIV virus kills $CD4^+$ T cells, the major fighting force against *M.tb* as they are the main producers of IFN-γ. Using CD4 knockout mice it was shown that there was a marked decrease in the number of immune cells recruitment in the lungs when challenged with *M.tb*. These CD4 knockout mice were unable to develop a normal granulomatous response and died earlier as compared to wild-type control (Saunders *et al*., 2002).

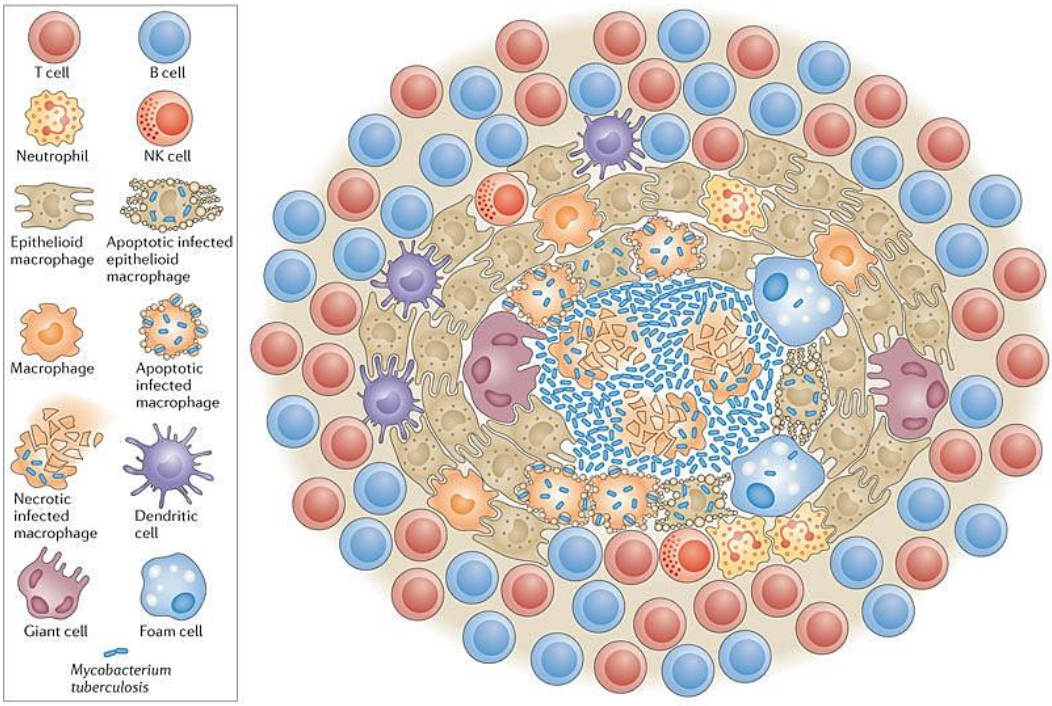


Figure 1.4: Structure of *M.tb* granuloma. The figure shows the arrangement of different immune cells involved in granuloma formation during *M.tb* infection. The inner region of granuloma mostly consists of necrotic tissue along with free *M.tb* which is surrounded by aggregates of macrophages with different phenotypes. The outer ring consists multiple layers of T cells along with a small number of B cells (Ramakrishnan, 2012).

1.7 Immune cells surface receptors involved in binding and phagocytosis of *M.tb*

Immune cells have different receptors on their surfaces that are involved in the binding and uptake of *M.tb* bacilli during infection. Binding of *M.tb* to specific receptors on the immune cells determines the kind of response that is generated by these immune cells to the pathogenic *M.tb* upon phagocytosis. The roles of some of the receptors are discussed as under:

1.7.1 Complement receptors

Complement receptors present on the surface of macrophages are responsible for the phagocytosis of *M.tb*. The complement C3 receptors like CR1, CR3 and CR4 are the major macrophage complement receptors involved in the uptake of C3b and iC3b opsonized *M.tb* (Ferguson *et al*., 2004). However, these receptors can also phagocytose un-opsonized *M.tb* by interaction with bacterial polysaccharides (Cywes *et al*., 1997), phosphatidylinositol mannosides (PIMs) and glycolipids (Villeneuve *et al*, 2005). The exact host response to *M.tb*

phagocytosed through complement receptor is still unclear. *In vitro,* macrophages from CR3 knockout mice showed reduced phagocytosis of *M.tb* as compared to macrophages from wild-type control, however, similar levels of intracellular *M.tb* survival and replication was observed in the macrophages from both CR3 knockout mice and wild-type control (Melo *et al*., 2000). *In vivo,* CR3 knockout mice when injected intravenously with *M.tb* showed similar bacterial load in organs such as lungs, liver and spleen when compared with the wild-type control. These results suggest that *M.tb* can be effectively phagocytosed even in the absence of CR3 receptors (Hu *et al*., 2000).

1.7.2 Mannose receptors

Mannose receptors are transmembrane receptors expressed on the surface of immune cells such as immature dendritic cells and mature macrophages, which interact with bacterial structures containing sugars, specifically mannose (Cochand *et al*., 1999; Taylor and Drickamer, 1993). It has been shown that mannose receptor binds virulent *M.tb* strains such as Erdman and H37Rv but not the avirulent strains such as H37Ra (Schlesinger, 1993). In case of *M.tb,* the mannose receptor chiefly binds to the mannose cap of LAM (Schlesinger *et al*., 1994). Entry of *M.tb* into the macrophage by binding to mannose receptor can result in the inhibition or delay of phagosome-lysosome fusion and hence facilitate the intracellular survival of *M.tb* (Kang *et al*., 2005). Binding of *M.tb* ManLAM to the mannose receptor is considered beneficial for the bacteria as it can inhibit the production of pro-inflammatory cytokine IL-12, important for the development of T helper type1 cells (Nigou *et al*., 2001).

1.7.3 Scavenger receptors

Cell surface scavenger receptors belong to a family of transmembrane glycoproteins and can bind to a variety of ligands. Scavenger receptors like MARCO and Class A receptors have been shown to bind to *M.tb* structures such as the cord factor and help in the phagocytosis of *M.tb* by macrophages (Bowdish *et al*., 2009).

1.7.4 Fcγ receptors

Fcγ receptors facilitate the phagocytosis of Ig-G opsonized *M.tb*. The entry of *mycobacteria* via Fcγ receptors enable the macrophages to eliminate the intracellular bacteria by causing the production of ROIs and promoting the phagosome-lysosome fusion (Schäfer *et al*., 2009; Armstrong and Hart, 1975).

1.7.5 DC specific ICAM-3 grabbing non-integrin (DC-SIGN)

DC-SIGN is a carbohydrate binding receptor majorly associated with dendritic cells (Tailleux *et al*., 2003). However, DC-SIGN may also be present on macrophage surface (Tailleux *et al*., 2005). Binding of DC-SIGN to *M.tb* cell wall structures such as ManLAMs and LMs results in the production of IL-10 and thus help in promoting anti-inflammatory response to the pathogen (Geijtenbeek *et al*., 2003).

1.7.6 Toll-like Receptors (TLRs)

Toll-like Receptors (TLRs) comprises a family of highly conserved transmembrane receptors. There are 12 different kinds of TLRs and each TLR recognizes a particular group of ligands. Each TLR consist of an extracellular amino leucine-rich repeat (LRR) domain for PAMPs recognition and intracellular carboxy-terminal tail similar to IL-1R, known as the Toll/IL-1 receptor (TIR) domain. Binding of ligands to TLRs result in activation of the transcription factor NF-κB. This NF-κB can then upregulate the production of different cytokines (Sandor *et al*., 2003; Dunne and O'Neill, 2003). TLRs such as TLR1, TLR2, TLR4, TLR6, and TLR9 can recognize a variety of structural components from *M.tb* including LAM, PIM, 19kDa lipoprotein and DNA etc. TLR2 and TLR4 are overexpressed during *M.tb* infection and have been shown to interact with 19kDa *M.tb* lipoprotein, LAM, LM and PIMs present on the surface of *M.tb* (Quesniaux *et al*., 2004; Means *et al*., 1999). Ligand binding to TLR2, TLR2/TLR1 heterodimer or TLR2/TLR6 heterodimer has been shown to be associated with a pro-inflammatory response resulting in the production of cytokines such as TNF-α and IL-1β (Kleinnijenhuis *et al*., 2009; Sandor *et al*., 2003; Takeuchi *et al*., 2002). TLR2 gene knockout ($TLR2^{-/-}$) mice when challenged with *M.tb* (100CFU/mouse) were unable to control the infection and died earlier as compared to the wild-type mice infected with the same dose of *M.tb* (Drennan *et al*., 2004).

1.8 Role of immune system during *M.tb* infection

The host's immune system is of prime importance in determining if the pathogenic *M.tb* will be eliminated, contained within granulomas or continue to spread and cause active disease. In humans, the response to pathogenic *M.tb* is multifactorial and involves immune cells from both the innate and adaptive immune system and small soluble proteins known as "cytokines" secreted by different immune cells.

1.8.1 Innate immune cells

The innate immune system comprises of various immune cells including macrophages, dendritic cells, natural killer cells and neutrophils. The role of different innate immune cells during *M.tb* infection is discussed as under:

1.8.1.1 Role of Macrophages

Macrophages play a vital role during *M.tb* infection by acting as the first line of host defence. Macrophages are phagocytic cells that can bind to and engulf pathogenic *M.tb*. These cells have an array of receptors on their surfaces such as phagocytic receptors and pattern recognition receptors (PRRs) that are important for recognition and uptake of *M.tb* (Rajaram *et al*., 2014). The PRRs are able to recognize special structures on the pathogens known as the pathogen-associated molecular patterns (PAMPs) such as LAMs, LMs, mycolic acid etc. The entry of *M.tb* to phagocytic cells is facilitated by specific binding to different cells surface receptors which result in the stimulation of different signalling pathways. The route of *M.tb* entry determines the response of macrophage and the fate of intracellular *M.tb* (Kleinnijenhuis *et al*., 2011; Akira *et al*., 2006). The macrophage cell surface receptors include complement receptors, mannose receptors, scavenger receptors, Fcγ receptors, DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) and Toll-like receptors (TLRs) (Hossain and Norazmi, 2013; Schäfer *et al*., 2009). Once the pathogenic *M.tb* is internalized by macrophages, the intracellular bacteria can be killed by different mechanisms such as formation of phagolysosomes, production of reactive nitrogen species and apoptosis (Details in section 1.9). However, *M.tb* has evolved certain mechanisms including inhibition of phagosome maturation, modulation of antigen maturation and resistance to reactive nitrogen intermediates through which it can escape killing by macrophages (Details in section 1.10).

Macrophages also process and display different antigenic proteins on their surfaces via major histocompatibility complexes (MHC), that leads to the activation of the adaptive immune system resulting in a T cells response (Ladel *et al*., 1995). These T cells secrete important cytokines like IFN-γ and TNF-α which in turn activate the macrophages to eliminate the intracellular *M.tb* (Roach *et al*., 2001).

1.8.1.2 Role of Dendritic Cells

Mature dendritic cells are powerful antigen-presenting cells (APCs) due to the expression of high levels of MHC Class I and II molecules and other co-stimulatory molecules such as CD80 and CD86 (Mellman and Steinman, 2001). These cells play a vital role in bridging the innate

and adaptive immune responses. Immature dendritic cells are specialized phagocytic cells and can engulf *M.tb* via binding to different cell surface receptors. After *M.tb* uptake, the dendritic cells migrate to the lymph nodes and mature. These mature dendritic cells in the lymph nodes can activate naïve T cell into antigen-specific $CD4^+$ T cells and $CD8^+$ T cells by presenting the processed bacterial antigen via MHC class II and MHC class I respectively (Marino *et al*., 2004). Activated $CD4^+$ T cells produce IFN-γ and TNF-α, which act on the macrophages and activates them, thus helping in eliminating the intracellular *M.tb* (Henderson *et al*., 1997). The importance of dendritic cells in *M.tb* control by activating $CD4^+$ T cells was confirmed in mice models. Depletion of dendritic cells resulted in a decreased number of activated $CD4^+$ T cells after *M.tb* challenge and higher number of *M.tb* were detected in the lungs and spleen of the test mice lacking dendritic cells as compared to the control during the early stages of infection (Tian *et al*., 2005). Dendritic cells also secrete pro-inflammatory cytokines such as IL-1β and IL-6, which help in the recruitment of other immune cells at the site of infection (Giacomini *et al*., 2001). Similarly, dendritic cells upon infection with *M.tb* produce high levels of chemokines such as CCL3, CCL4, CXCL8 and CXCL9 that play important role in the migration of NK cells and T cells to the site of infection (Slight and Khader, 2013).

In humans, the *IRF8* gene is essential for the normal development of dendritic cells and is suggested to be important in the anti-mycobacterial immunity. Mutations in *IRF8* have been shown to be associated with disseminated BCG infections in human (Hambleton *et al*., 2011).

1.8.1.3 Role of Natural Killer Cells

Natural killer (NK) cells are important cells of the innate immune systems. NK cells can bind to different bacterial pathogens such as *M.tb* and possess direct cytotoxicity by releasing different bactericidal compounds including granulysin and perforin (Lu *et al*., 2014). NK cells from a healthy donor were able to lyse *M.tb* infected monocytes and reduce the growth of intracellular *M.tb* (Vankayalapati *et al*., 2002). The cytotoxic function of NK cells seems to be associated with Glutathione (GSH), which been shown to possess direct anti-microbial activity (Guerra *et al*., 2012; Millman *et al*., 2008). Similarly, NK cells can induce apoptosis in monocytes and other immune cells by FasL/Fas-mediated pathway (Brill *et al*., 2001). HIV virus can delay FasL/Fas-mediated apoptosis, which may lead to delayed apoptosis of *M.tb* infected cells resulting in prolonged infection (Cossarizza *et al*., 2000). NK also cells express different cell surface ligands such as CD40L. Binding of CD40 expressed on the surface of infected macrophages with the CD40L on NK cells leads to the upregulation of co-stimulatory

molecules like CD80 and CD86 as well as the production of NO by macrophages (Carbone *et al*., 1997).

NK cells secrete IFN-γ during the early stages of *M.tb* infection, which activates macrophages and helps in the control of *M.tb* (Olsen *et al*., 2005; Flynn *et al*., 1993). NK cell can bind to *M.tb* cell wall components such as mycolic acid directly through natural cytotoxicity receptors known as NKp44 which causes the activation of NK cells and the production of pro-inflammatory cytokines. Cytokines such as IL-2 and IL-12 upregulate the expression of cell surface receptors such as NKp30, NKp44 and NKp46 on NK cells. These receptors interact with various stress ligands such as UL16 binding protein 1 (ULBP1) on *M.tb* infected macrophages and cause the release of INF-γ and increase macrophage activation resulting in the elimination of intracellular *M.tb* (Esin *et al*., 2013; Portevin *et al*., 2012). The protective role for NK cells during mycobacterial infections was also established in mice models. Depleting NK cells in mice using anti-NK1.1 monoclonal antibody resulted in the increased multiplication of *M.avium* as compared to control mice (Harshan and Gangadharam, 1991).

1.8.1.4 Role of Neutrophils

During *M.tb* infection, neutrophils from the blood are rapidly recruited to the site of infection. Neutrophils are phagocytic cells and have been shown to engulf *M.tb* both *in vivo* and *in vitro* (Ganbat *et al*., 2016; Eruslanov *et al*., 2005). However, there exists conflicting data about the capacity of neutrophils to eliminate intracellular *M.tb*. Several studies reported that neutrophils were unable to kill intracellular *M.tb* and activating these cell with IFN-γ showed no effect on the anti-mycobacterial ability (Eruslanov *et al*., 2005; Pedrosa *et al*., 2000). There are reports which show that neutrophils present in the sputum and BAL fluids of TB patients contain replicating *M.tb*, indicating their inability to control the bacteria (Eum *et al*., 2010). However, other groups showed that *in vitro* neutrophils and neutrophils derived products can kill *M.tb*. Kisich *et al* showed that neutrophils from a healthy individual stimulated by TNF-α possessed anti-mycobacterial activity and suggested that the poor anti-mycobacterial activity of neutrophils might be associated with improper stimulation (Kisich *et al*., 2002). Similarly, challenging rats with *M.tb* after LPS induced neutrophilia showed a significantly lower number of *M.tb* in the lungs and after 24 hours of LPS treatment and neutrophils from the bronchoalveolar lavage of these animals were able to kill *M.tb* (Sugawara *et al*., 2004). Neutrophil extracellular traps (NETs) are extracellular networks composed of DNA from neutrophils bound to special cytoplasmic proteins and are considered to play an important role

in the bactericidal activity of neutrophils. *M.tb* infected neutrophils were shown to release NETs *in vitro* however they were ineffective in killing *M.tb* (Ramos-Kichik *et al*., 2009).

An important role for neutrophils has been suggested during *M.tb* granuloma formation. Neutrophil-depleted mice showed reduction in the number, size and density of granulomas (Seiler *et al*., 2003). Early *M.tb* infection foci contain a mixture of macrophages, neutrophils and lymphocytes. Neutrophils at the site of infection secrete various chemokines such as CCL3, CCL4 CXCL2, CXCL9 and CXCL10 that attract monocytes and T lymphocytes and help in granuloma formation (Seiler *et al*., 2003).

1.8.2 Adaptive immune cells

The adaptive immune system comprises T lymphocytes/T cells and antibodies producing B cells.

The adaptive immune response in case of *M.tb* is predominantly cell-mediated involving antigen-specific T cells and plays a key role in determining the outcome of infection (Wolf *et al*., 2008). The adaptive immune response to *M.tb* is of the delayed type that becomes detectable in mice models after a minimum of 12 days post aerosol *M.tb* infection (Chackerian *et al*., 2002). T cells are produced in the thymus and are distinguished from other cell types due to the presence of T cell receptors (TCR) and CD3 molecule. T cells can be subdivided into various types including $CD4^+$ T cells, $CD8^+$ T cells and γδ T cells based on the expression of their cell surface molecules (Cooper, 2009).

1.8.2.1 Role of $CD4^+$ T Cells

$CD4^+$ T cells, also known as helper T cells are of central importance in *M.tb* infection. Based upon the cytokine secretion profile in response to antigens, $CD4^+$ T cells are subcategorized into two major groups, T helper type 1 (Th-1 cells) and T helper type 2 (Th-2 cells) (Mosmann *et al*., 1986). Th-1 cells secrete IFN-γ and IL-2 and TNF-α and provide resistance to *M.tb* infections whereas, Th-2 cells secrete IL-4, IL-5, IL-9, IL-13 and IL-10 and are associated with *M.tb* susceptibility and pathology (Al-Attiyah *et al*., 2006).

During *M.tb* infection antigen presenting cells such as dendritic cells with phagocytosed *M.tb* migrate to the mediastinal lymph nodes and present processed bacterial antigen using MHC class II molecules expressed on their cell surface to naïve T cells. This results in the activation of naïve T cells and the proliferation of *M.tb* antigen-specific $CD4^+$ T cells in the lymph nodes. These antigen-specific $CD4^+$ T cells then relocate from the lymph node to the site of *M.tb*

infection to control the infection (Boom *et al*., 2003). A Th-1 type T cell response to *M.tb* infection results in the production of cytokines such as IFN-γ at the site of infection. IFN-γ is considered to be vital for protective immunity against *M.tb* as it can activate macrophages to kill the intracellular *M.tb* (Caccamo *et al*., 2010). Furthermore, Th-1 cells also produce important cytokines such as IL-2 and TNF-β (lymphotoxin-alpha) that activates $CD8^+$ T cells (Serbina *et al*., 2001) and macrophages (Roach *et al*., 2001). Th-1 cells secreted lymphotoxin-alpha is also crucial in the control of pulmonary TB as it helps in granuloma organization (Roach *et al*., 2001).

A Th-2 T cell response to *M.tb* infection, on the other hand, results in a lack of protection mainly due to the production of anti-inflammatory cytokines like IL-10 which deactivates macrophages and causes the down-regulation of Th-1 cytokines. This cause the *M.tb* to survive inside the macrophages and leads to chronic progressive TB (Turner *et al*., 2002).

The importance of $CD4^+$ T cell-mediated immunity during *M.tb* infection was shown in mice. MHC class II-deficient (MHC $II^{-/-}$) mice due to a lack of functional $CD4^+$ T cells showed a higher number of *M.tb* in their lungs and succumbed to the infection earlier as compared to the control mice (Mogues *et al*., 2001). Similarly, mice models deficient in the CD4 molecule ($CD4^{-/-}$) were also shown to be highly susceptible to *M.tb* infection and died earlier as compared to the control (Caruso *et al*., 1999). Both MHC $II^{-/-}$ and $CD4^{-/-}$ mice showed lower levels of INF-γ in the lungs as compared to wild-type mice after infection with *M.tb*.

The loss of $CD4^+$ T cells in humans during HIV infection can trigger the reactivation of latent TB to an active form and also increase the susceptibility of HIV patients to new *M.tb* infection as well (Alimonti *et al*., 2003). The protective role of $CD4^+$ T cells was also confirmed in latent TB macaque models. It was shown that SIV infection in these animals led to a loss in the number of $CD4^+$ T cells in the lungs as compared to the SIV negative control animals that resulted in the reactivation of latent TB (Diedrich *et al*., 2010).

1.8.2.2 Role of $CD8^+$ T cells

$CD8^+$ T cells also play an important role during *M.tb* infection as these cells are able to recognize a number of *M.tb* antigens. Naïve T cells develop into antigen-specific $CD8^+$ T cells in response to bacterial antigen presented via MHC class 1 molecule expressed on the surface of antigen-presenting cells (Weerdenburg *et al*., 2010). *M.tb* specific $CD8^+$ T cells have been detected in the lungs of mice infected with *M.tb* (Lewinsohn *et al*., 2003). The protective role of $CD8^+$ T cells during *M.tb* infections can be attributed to their ability to produce cytokines

such as INF-γ and TNF-α along with cytotoxic molecules (granzymes) that causes apoptosis (Jeong *et al*., 2014; Serbina and Flynn, 1999). Another important characteristic of $CD8^+$ T cells is their cytolytic activity. $CD8^+$ T cells have been shown to kill intracellular *M.tb* via perforin and granulysin. Stenger *et al* showed *in vitro* that granulysin produced by $CD8^+$ T cells possess direct *M.tb* growth inhibition ability in cultures and proposed that $CD8^+$ T were able to inhibit intracellular *M.tb* growth in granule-mediated fashion (Stenger *et al*., 1998). $CD8^+$ T cells recognize *M.tb* antigen on infected macrophage surface via MHC class I molecules. Proteins such as β2-microglobulin and TAP-1 play important roles in the packaging and trafficking of MHC class I heavy chain. Mice models with a disruption in β2-microglobulin gene or TAP-1 gene upon infection with *M.tb* showed a higher number of *M.tb* bacilli in lungs and succumbed to infection earlier as compared to control mice due to lack of functional $CD8^+$ T cells (Behar *et al*., 1999). $CD8^+$ T cells are particularly important during latent *M.tb* infection and cytokine-producing $CD8^+$ T cells are present in *M.tb* granulomas. Depletion of $CD8^+$ T cells in mice model with chronic *M.tb* infection caused an increase in *M.tb* burden indicating their protective role (van Pinxteren *et al*., 2000). The importance of $CD8^+$ T cells in controlling *M.tb* infection was also shown by depleting $CD8^+$ T cells in BCG vaccinated rhesus macaques using Anti-CD8 antibody. $CD8^+$ T cells depletion resulted in a significant decrease in vaccine-induced immunity to pulmonary *M.tb* infection as compared to isotype antibody treated control animals (Chen *et al*., 2009).

1.8.2.3 Role of γδ T cells

γδ T cells are a special type of T cells, having a T cell receptor (TCR) that can be distinguished from αβ T cells due to its physical properties and ligand binding sites. These cells are generally present in a low percentage (5%) in humans (Carding and Egan, 2002). Janis *et al* first reported the detection of γδ T cells in *M.tb* infected mice and showed that these cells do not require antigen presentation through MHC and can interact directly with soluble *M.tb* antigens *in vitro* (Janis *et al*., 1989). *In vitro,* human monocytes/macrophages infected with live *M.tb* were shown to be potent inducers of human γδ T cells expansion. γδ T cells from healthy donors were shown to produce high levels of IFN-γ in response to *M.tb* infected monocytes suggesting their protective role during *M.tb* infections (Gioia *et al*., 2003). A special subset of γδ T cells known as Vγ9Vδ2 T cells was shown to recognize and directly bind mycobacterial antigens such as phosphoantigens (Tanaka *et al*., 1994; Havlir *et al*., 1991). Furthermore, Vγ9Vδ2 T cells were shown to kill macrophage phagocytosed *M.tb* by producing cytolytic enzymes granulysin and perforin indicating their direct role in host protection against *M.tb* (Dieli *et al*.,

2001). γδ T cells are considered to be involved in connecting the innate and adaptive immune responses during *M.tb* infection. Vγ9Vδ2 T cells were shown to induce maturation in *M.tb* infected immature dendritic cells by upregulating the expression of CD80 and CD40 in these cells (Meraviglia *et al*., 2010), which are powerful antigen-presenting cells and can strengthen the cellular immune response against *M.tb* infection.

1.8.2.4 Role of B cells and antibodies

Until early 20th century the protective role of B cells and antibody-mediated immunity during *M.tb* infections was generally considered to be insignificant. A number of serum transfer studies using antibodies as protective agents failed to show consistent results (Glatman-Freedman and Casadevall, 1998). However, several recent studies suggest that B cells and antibodies can help in protection against *M.tb* by participating in the formation of granuloma, T cells activation through antigen presentation and the production of cytokine (Kozakiewic *et al*., 2013; Almeida *et al*., 2011; Tsai *et al*., 2006).

The importance of B cells during *M.tb* infection was established using mice models. Maglione *et al* showed that B cells deficient (B cell$^{-/-}$) mice were more susceptible to *M.tb* infection and showed a higher number of *M.tb* in lungs as compared to wild-type control. Furthermore, increased recruitment of neutrophils and higher levels of IL-10 were detected in lungs of these B cell$^{-/-}$ mice as compared to wild-type mice (Maglione *et al*., 2007). Another study by Vordermeier *et al* showed that 3-6 weeks after *M.tb* infection, B cell-deficient mice showed up to 8 times higher bacterial load in different organs such as lung, liver, spleen etc. as compared to wild-type control (Vordermeier *et al*., 1996).

Serum analysis from children with tuberculosis suggested that reduction in serum IgG is associated with disseminated *M.tb* infections, as the levels of serum IgG to *M.tb* antigens such as LAM were significantly lower in children with disseminated *M.tb* infections as compared to those having localised *M.tb* infections (Costello *et al*., 1992). The protective role of antibodies against *M.tb* was shown in mice models where administration of specific monoclonal antibodies (mAb) against *M.tb* surface antigens such as arabinomannan, LAM etc. increased the survival of *M.tb* infected mice by inhibiting the growth of *M.tb*, reducing *M.tb* dissemination and helping in granuloma formation. (Hamasur *et al*., 2004; Williams *et al*., 2004; Pethe *et al*., 2001; Teitelbaum *et al*., 1998). Antibody opsonisation of *M.tb* helps in the uptake of the bacteria by macrophages through FcγR and has been shown to promote phagosome maturation and enhance the killing of intracellular *M.tb* (Kumar *et al*., 2015).

1.8.3 Role of cytokines during *M.tb* infection

Cytokines are small soluble proteins produced by a variety of cells in the immune system and can affect the activity of other cells by acting as messengers, regulating their functions. The role of different cytokines during *M.tb* infection is discussed as under:

1.8.3.1 Interferon- γ (IFN-γ)

During *M.tb* infections IFN-γ is mainly produced by T cells ($CD4^+$ T cells, $CD8^+$ T cells) (Jeong *et al*., 2014) and NK cells (Olsen *et al*., 2005). IFN-γ has been shown to perform multiple roles during *M.tb* infection and is important for host protection. IFN-γ activates macrophages by upregulating the transcription of different genes including genes coding for antimicrobial molecules such as reactive nitrogen species and helps them in eliminating the intracellular *M.tb* (Cavalcanti *et al*., 2012). Mutations in the IFN-γ receptor 1 encoding genes in humans have been shown to be associated with a loss of protective immunity against *mycobacteria* (*M. fortuitum*, *M. chelonei*, and two strains of *M. avium*) resulting in disseminated infections that were fatal (Newport *et al*., 1996). Individuals with IFN-γ receptor mutations are vulnerable to *M.bovis BCG* infections which are otherwise poorly pathogenic in normal humans (Dorman *et al*., 2004; Jouanguy *et al*., 1996). Cooper *et al* and Flynn *et al* have shown the importance of IFN-γ during *M.tb* infection using mice model. They showed that mice with a disruption in IFN-γ gene upon infection with sublethal doses of *M.tb* showed a higher number of *M.tb* bacilli in the lungs (100-1000 folds increase) as compared to control which was reduced by administration of exogenous IFN-γ. The IFN-$\gamma^{-/-}$ mice were unable to contain the infection and large number of *M.tb* was detected in organs like kidneys and bone marrow indicating dissemination of *M.tb* infection (Cooper *et al*., 1993). Although granulomas were formed in the absence of IFN-γ, the granulomas in IFN-$\gamma^{-/-}$ mice were shown to be highly necrotic and contain a large number of *M.tb* (Flynn *et al*., 1993).

1.8.3.2 Tumor necrosis factor- α (TNF-α)

TNF-α is a pro-inflammatory cytokine, produced by monocytes, macrophages (Valone *et al*., 1988), dendritic cells (Henderson *et al*., 1997) and T cells (Allie *et al*., 2013) in response to *M.tb*. During TB, TNF-α is produced at the site of infection and this increase production of TNF-α is associated with undesirable inflammatory effects and fever (Tramontana *et al*, 1995). TNF-α is one of the most important cytokines produced during *M.tb* infection and has vital roles in macrophage activation and granuloma formation (Flesch and Kaufmann, 1990). In humans, the use of the TNF-α neutralizing antibody for conditions such as Crohn's disease and rheumatoid arthritis has been shown to be associated with an increase in reactivation of latent

TB (Keane *et al*., 2001). In mice model, TNF-α has been shown to help in the containment of *M.tb* during latent infection. Neutralizing TNF-α in these organisms resulted in the reactivation of latent TB that proved to be fatal (Mohan *et al*., 2001, Flynn *et al*., 1995). Mice with disruption in TNF gene ($TNF^{-/-}$), when challenged with *M.tb*, succumbed to infection much earlier as compared to control and had a greater number of *M.tb* in their lungs and poorly formed granulomas (Bean *et al*., 1999).

1.8.3.3 Interleukin-12 (IL-12)

IL-12 is another important host defence cytokine produced during *M.tb* infection. IL-12 acts as a bridge between the innate and adaptive immune responses and enhances the production of IFN-γ (Zhang *et al*., 1994). IL-12 is produced upon the phagocytosis of *M.tb* by immune cells such as monocytes, macrophages and dendritic cells (Ladel *et al*., 1997; Fulton *et al*., 1996). Administration of exogenous IL-12 in BALB/c mice at the time of infection with virulent *M.tb* was shown to be associated with a protective effect as indicated by lower bacterial counts in lungs and increased survival time when compared with the untreated control mice (Flynn *et al*., 1995). Similarly, mice model with a disruption in IL-12 gene ($IL\text{-}12p40^{-/-}$) when challenged with *M.tb* were unable to control *M.tb* infection and died earlier as compared to wild-type control mice. Cytokine analysis from these $IL\text{-}12p40^{-/-}$ mice showed reduced expression of IFN-γ mRNA while the expression of TNF-α was delayed (Cooper *et al*., 1997). In humans, deletion mutations in IL-12p40 and IL-12R genes have been shown to be associated with a reduction in IFN-γ production and recurrent mycobacterial infection (Altare *et al*., 2001; de Jong *et al*., 1998).

1.8.3.4 Interleukin-1 (IL-1)

Pro-inflammatory cytokine IL-1α and IL-1β (collectively referred as IL-1 here) are produced by host immune cells such as monocytes, macrophages and dendritic cells in response to *M.tb* (Giacomini *et al*., 2001). IL-1α and IL-1β double knockout mice when challenged with *M.tb* showed larger granulomatous lesions without Langhans like giant cells and higher number of *M.tb* in lungs as compared to the control mice 3 weeks post infection (Yamada *et al*., 2000). Furthermore, cultured alveolar macrophages from the IL-1 knockout mice showed low level of NO production as compared to those from control mice (Yamada *et al*., 2000). In addition, IL-1R deficient ($IL\text{-}1R^{-/-}$) mice were more susceptible to *M.tb* infection, had a high mortality rate, showed greater dissemination of bacteria to different organs and defective granuloma formation (Juffermans *et al*., 2000).

1.8.3.5 Interleukin-6 (IL-6)

IL-6 is also produced at the site of infection during TB (Hoheisel *et al*., 1998). IL-6 deficient mice showed increased susceptibility to *M.tb* (Saunders *et al*., 2000) and succumbed to *M.tb* doses that were otherwise non-lethal in control mice (Ladel *et al*., 1997) indicating a protective role for this cytokine in *M.tb* infection. However, other studies have shown that *in vitro* IL-6 promotes the growth of *M.avium* (Shiratsuchi *et al*., 1991) and also inhibits the production of IL-1 and TNF-α (Schindler *et al*., 1990) suggesting a negative role for this cytokine in controlling TB.

1.8.3.6 Interleukin-18 (IL-18)

IL-18 is a pro-inflammatory cytokine produced mainly by macrophages. IL-18 has been shown to activate Th-1 cells and induce the production of IFN-γ from these cells and thus has a protective role during *M.tb* infection (Giacomini *et al*., 2001). It was shown *in vitro* that neutralizing IL-18 in human PBMC infected with *M.tb* resulted in a marked decrease in IFN-γ production (Vankayalapati *et al*., 2000). IL-18 gene knockout mice were shown to be highly susceptible to *M.tb*. In these knockout mice greater number of *M.tb* was detected in the lungs and had larger granulomas as compared to wild-type control (Sugawara *et al*., 1999). Administration of exogenous IL-18 lowered the number of *M.tb* in the lungs of IL-18 knockout mice and reduced the sizes of granulomatous lesions. Furthermore, decreased expression of IFN-γ was observed in the IL-18 knockout mice as compared to the wild-type control (Sugawara *et al*., 1999).

1.8.3.7 Interleukin-17 (IL-17)

IL-17 is produced by specific $CD4^+$ T cells and γδ T cells in the lungs during the early phase of mycobacterial infections in mice models (Lockhart *et al*., 2006; Aggarwal *et al*., 2003). IL-17 gene knockout mice ($IL\text{-}17^{-/-}$) showed delayed-type hypersensitivity responses with a lower number of IFN-γ secreting T cells during BCG infection and developed impaired granulomas (Yoshida *et al*., 2010; Umemura *et al*., 2007). IL-17 has been suggested to play a protective role during chronic *M.tb* infection in mice. $IL\text{-}17^{-/-}$ mice when challenged with virulent *M.tb* died earlier as compared to control mice and the histological examination of lungs from these $IL\text{-}17^{-/-}$ mice showed impaired granulomas (Umemura *et al*., 2016)

1.8.3.8 Interleukin-23 (IL-23)

IL-23 is a pro-inflammatory cytokine produced by monocytes, macrophages and dendritic cells (McKenzie *et al*., 2006). During early pulmonary *M.tb* infection, IL-23 is required for the

production of IL-17 (Khader *et al*., 2011). IL-23 knockout mice showed effective control of *M.tb* suggesting IL-23 has no direct role during early *M.tb* infection (Khader *et al*., 2005). However, IL-23 has been shown to be involved in the long-term protection against *M.tb* in mice model. IL-23a deficient (IL-23a$^{-/-}$) mice, lacking the p19 component of IL-23 showed significantly higher number of *M.tb* in the lungs during late stages of infection (150-250 days post infection) as compared to wild type control. IL-23 causes the expression of important chemokine CXCL13 in the lungs of *M.tb* infected mice. This CXCL13 has an important role in T cell accumulation and granuloma formation during *M.tb* (Khader *et al*., 2011).

1.8.3.9 Interleukin-4 (IL-4)

IL-4 is an anti-inflammatory cytokine that has a negative impact on INF-γ production and macrophage activation (Lucey *et al*., 1996) and is mainly associated with a Th-2 type CD4^{+} T cell response (Killar *et al*., 1987). Increased production of IL-4 by circulating T lymphocytes has been detected in human TB patients (van Crevel *et al*., 2000). Similarly, mice infected with *M.tb* showed increased production of IL-4 which was associated with disease progression and reactivation of latent infection (Hernandez-Pando *et al*., 1996). Buccheri *et al* showed that neutralizing IL-4 in BALB/c mice by anti-IL-4 antibody treatment significantly reduced the number of *M.tb* in the lungs during the early stage of infection as compared to untreated control (Buccheri *et al*., 2007). Similar results were obtained using IL-4 gene knockout (IL-4$^{-/-}$) BALB/c mice that showed a lower number of *M.tb* in the lung and spleen tissue as compared to wild-type control. Administration of exogenous IL-4 in these IL-4$^{-/-}$ mice resulted in an increase in the number of *M.tb*, suggesting a protective effect of IL-4 for the pathogenic *M.tb* (Buccheri *et al*., 2007). Overexpression of IL-4 in mice model was shown to affect *M.tb* induced granulomas by increasing the overall size of the granuloma and greater accumulation of eosinophils (Lukacs *et al*., 1997).

1.8.3.10 Interleukin-10 (IL-10)

IL-10 is an anti-inflammatory cytokine that can be found in the serum and alveolar lavage of TB patients (Olobo *et al*., 2001; Verbon *et al*., 1999). IL-10 is produced as a result of phagocytosis of *M.tb* by macrophages (Shaw *et al*., 2000) and binding of bacterial structures such as LAM play an important role in the production of IL-10 from these cells (Dahl *et al*., 1996). IL-10 is also produced by other types of cells such as T- lymphocytes (Barnes *et al*., 1993). IL-10 production during *M.tb* infection interferes with the host defence and helps in the bacterial survival by downregulating the production of different pro-inflammatory cytokines like TNF-α, IL-12 and INF-γ. The role of IL-10 in TB progression was investigated *in vivo*

using CBA/J mouse model and it was observed that blocking the IL-10 receptor with anti-IL-10R1 improved the control of *M.tb* inside the lungs due to an increase in the number of $CD4^+$ and $CD8^+$ T cells capable of producing INF-γ and increased the survival duration as compared to the mice treated with control antibody (Beamer *et al*., 2008). Similarly, IL-10 deficient (IL-$10^{-/-}$) mice models when challenged with virulent *M.tb* were able to control the infection and showed a significant decrease in the number of *M.tb* in the lungs and spleen as compared to control. Furthermore, in IL-$10^{-/-}$ mice increased percentage of IFN-γ producing $CD4^+$ T cells were present in the lungs indicating the early control of *M.tb* (Redford *et al*., 2010).

1.8.3.11 Transforming growth factor- β (TGF-β)

TGF-β is an anti-inflammatory cytokine produced by monocytes, macrophages and dendritic cells in response to *M.tb* (Toossi *et al*., 1995). TGF-β is produced in excess at the site of infection during TB (Toossi *et al*., 1995) and *M.tb* virulence factors such as LAM can induce the selective production of TGF-β (Dahl *et al*., 1996). TGF-β protects the pathogenic *M.tb* from the host's immune system by suppressing the immune response. TGF-β has a negative impact on cell-mediated immunity as it inhibits T cell proliferation and the production of INF-γ, whereas in macrophages TGF-β affects cellular activation by downregulating the production of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α (Toossi and Ellner, 1998). *In vitro,* exogenous TGF-β was shown to increase the intracellular growth of *M.tb* in human monocytes while neutralizing TGF-β by a monoclonal antibody resulted in decreased replication of intracellular *M.tb* as compared to control treated with isotype antibody (Hirsch *et al*., 1994). Naturally occurring TGF-β inhibitors, Decorin and LAP *in vitro* restored T cells blastogenesis and improved the production of INF-γ in PBMCs isolated from TB patients (Hirsch *et al*., 1997).

1.9 Mechanism involved in killing of intracellular *M.tb*

The phagocytosis of *M.tb* can result in the activation of macrophages. Activated macrophages eliminate the intracellular *M.tb* through various mechanisms that are discussed as under:

1.9.1 Acidification of phagosome

Macrophages can eliminate intracellular *M.tb* contained within membrane-bound phagosomes by making the environment inside the phagosomes highly acidic. This acidification is vital for the fusion of phagosomes with lysosomes and helps in the activation of hydrolytic enzymes involved in the degradation of pathogens. In addition, the acidic environment inside the

phagosome provides a hostile environment to the bacteria where metabolism is difficult and can lead to the elimination of intracellular *M.tb* (Flannagan *et al*., 2009). Macrophages achieve this acidification of phagosome through a large number of vacuolar H^+-ATPases or proton pumps present on the phagosomal membrane. The H^+-ATPase consists of a cytoplasmic V_1 domain, responsible for ATP hydrolysis resulting in energy production and a membrane-embedded V_0 domain that uses the energy from ATP hydrolysis to translocate protons across the membrane (Kissing *et al*., 2015).

1.9.2 Reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs)

An effective host defence mechanism against intracellular pathogens involves the production of toxic compounds such as reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) by phagocytic immune cells such as macrophages (Nathan and Shiloh, 2000). Macrophages can produce ROIs through the activity of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase complex assembled on the phagosomal membrane. Activated NADPH-oxidase transfer cytosolic electron to oxygen (O_2) inside the phagosome resulting in the production of superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•) and other ROIs for killing the phagosome bound pathogen (Slauch, 2011). However, ROIs have been suggested to play a non-significant role in the elimination of intracellular *M.tb* (Howell Wescott *et al*., 2017). It was suggested that different *M.tb* products such as sulfatides and LAM are able to scavenge ROIs (Chan *et al*., 1991), hence limiting the role of ROIs in the control of *M.tb* infection. *In vitro* both ROI deficient and normal mice macrophages with intracellular *M.tb* showed a comparable level of anti-mycobacterial activity. In addition, the use of oxygen radical scavengers such as catalase and superoxide dismutase showed no effect on the anti-mycobacterial activity of macrophages (Chan *et al*., 1992). However, there are other studies that show the involvement of ROIs in the control of *M.tb* infection in model organisms. The gene knockout mice lacking $p47^{(phox)}$ gene required for NADPH-oxidase activity showed significantly increased numbers of *M.tb* during the early stages of infection, indicating a protective role of ROIs during *M.tb* infection (Cooper *et al*., 2000).

In addition to ROIs, macrophages are also able to produce increased quantities of nitric oxide (NO) upon stimulation with cytokines such as IFN-γ TNF-α and IL-1 and different pathogen products such as LPS (Mastroeni *et al*., 2000; Flynn *et al*., 1993). NO is produced as a result of oxidation of L-arginine by an enzyme known as inducible NO synthases (iNOS). This NO can be further converted into reactive nitrogen intermediates (RNIs) such as peroxynitrite ($ONOO^-$) upon reaction with oxygen radicals (Fang, 2004). *In vitro,* NO has been shown to be

highly toxic to *M.tb* even at low concentrations. *M.tb* exposure to <100 parts per million (ppm) NO resulted in more than 99% *M.tb* killing in cultures (Long *et al*., 1999). There are different mechanisms through which NO and other RNI can kill *M.tb*. These include damage to the bacterial DNA by causing strand breaks as well as modification of bacterial cell surface and intracellular proteins and lipids (Bogdan, 2001). The importance of NO in control of intracellular *M.tb* was shown using mice models. It was shown that iNOS gene knockout mice failed to produce NO and other RNI and were more susceptible to *M.tb* infection with a higher mortality rate as compared to the wild type control (Adams *et al*., 1997). Similarly, iNOS inhibitors treatment in mice model with latent TB caused the reactivation of disease and resulted in early mortality as compared to the untreated control (Flynn *et al*., 1998). *In vitro,* NO and RNIs were shown to be involved in the intracellular killing of *M.tb* (Bose *et al*., 1999) and *M.bovis BCG* (Nozaki *et al*., 1997) in human macrophages suggesting their important role in the host defence.

1.9.3 Apoptosis

Apoptosis is the host cell defence response to intracellular pathogens such as viruses and bacteria. Apoptosis of *M.tb* infected macrophages involves programmed cell death that results in the killing of intracellular *M.tb* (Behar *et al*., 2011).

A family of serine proteases known as caspases is vital for the initiation and execution of apoptosis. Apoptotic pathways can be divided into three distinct types depending on the type of caspase initially activated. The extrinsic apoptotic pathway involves the initial activation of procaspase 8 or procaspase 10 by TNF-α or FasL binding to the cell surface receptors such as TNFR1 and Fas (Brunner *et al*., 2003). The intrinsic apoptotic pathway involves the initial activation of procaspase 9 by cytochrome c released from the mitochondria in response to intracellular stress signals such as DNA damage, nutrient deficiency or oxidative stress (Saelens *et al*., 2004). The third apoptotic pathway involves caspase activation through granzymes B released by $CD8^+$ T cells or NK cells along with perforin (Trapani and Smyth, 2002). All the three apoptotic pathways ultimately converge at the same target and activate effector caspases including caspase 3, caspase 6 and caspase 7 that can induce different processes such as condensation of nuclear chromatin, DNA breakage and the formation of apoptotic vesicles leading to cell death.

The extrinsic apoptotic pathway was shown to be involved in the killing of intracellular *M.tb*. Keane *et al* showed for the first time that *M.tb* was able to induce apoptosis in human alveolar

macrophages through TNF-α production (Keane *et al*., 1997). They also showed that attenuated *M.tb* strain such as H37Ra were more potent inducers of apoptosis as compared to virulent strain H37Rv, although both induced similar level of TNF-α production by macrophages (Keane *et al*., 1997). This reduced apoptotic potential of virulent *M.tb* strains was later shown to be associated with the increased expression of the anti-apoptotic Mcl-1 gene of macrophages by virulent *M.tb* (Riendeau and Kornfeld, 2003; Oddo *et al*., 1998).

Apoptotic cell death leads to the production of membrane-bound vesicles containing the cytoplasmic content of dying cells along with bacterial components called apoptotic bodies. These apoptotic bodies are eliminated as they attract phagocytic cells predominantly macrophages by releasing signal such as ATP and expressing phosphatidylserine (Martin *et al*., 2012).

1.10 *M.tb* survival strategies inside host cells

M.tb has evolved several unique strategies through which it can evade the hostile environment inside phagocytic macrophages. Some of these survival mechanisms are discussed as under:

1.10.1 Inhibition of Phagosome maturation

In order to eliminate the pathogenic *M.tb*, macrophages engulf the bacteria in membrane-bound structures called phagosomes. These phagosomes then undergo a maturation process involving sequential fusion with early endosomes, late endosomes and finally lysosomes (Aderem and Underhill, 1999). However, in case of *M.tb*, the phagosome maturation process is blocked beyond the fusion with early endosome. This early fusion provides the phagocytosed *M.tb* to access nutrient and protection. There are several mechanisms through which *M.tb* halt phagosome maturation.

First, *M.tb* produced enzymes such as urease resulting in the production of ammonia. This causes an increase in the pH of the phagosome making it more alkaline which hampers the transport of material from early to late endosome leading to disruption in phagosome maturation (Harth and Horwitz, 1999; Clemens *et al*., 1995). In addition, intracellular *M.tb* are able to exclude the vacuolar H^+-ATPase from the phagosome membranes, resulting in lack of acidification and higher pH in the phagosomal lumen (Wong *et al*., 2011).

The phagosome membrane contains various proteins that are involved in the interaction of phagosome with early and late endosome as well as the lysosome. One such protein is a small

GTPase protein known as Rab7 that controls the maturation of early endosome to late endosome. *M.tb* avoids phagosome maturation by blocking the acquisition of Rab7 (Fratti *et al*., 2003; Via *et al*., 1997). Rab14 is another protein that *M.tb* utilizes to avoid phagosome maturation. Rab14 is not needed during the later stages of phagosome maturation and is disassociated during phagosome maturation. However, *M.tb* blocks the disassociation of Rab14 and hence halts the phagosome maturation (Kyei *et al*., 2006). *M.tb* also produce an acid phosphatase called SapM, which causes the release of Phosphatidylinositol-3-phosphate (PI3P) from the phagosome. This PI3P is essential for phagosome maturation as it provides a site for docking several proteins involved in the maturation of phagosome into phagolysosome (Vergne *et al*., 2005; Saleh and Belisle, 2000; Wurmser *et al*., 1999).

Another possible mechanism used by *M.tb* for inhibiting phagosome maturation is through the accumulation of host cell coronin I protein around the phagosome (Ferrari *et al*., 1999). It is suggested that this envelope of coronin I around phagosome prevent phagosome-lysosome fusion by regulating calcium-dependent signalling processes (Jayachandran *et al*., 2007).

1.10.2 Modulation of antigen presentation

Macrophages can initiate adaptive immune response by processing the *M.tb* antigens and presenting them to T cells via MHC molecules expressed on their cell surfaces. However, *M.tb* has evolved strategies through which it can interfere with antigen processing and its presentation by macrophages. *M.tb* cell surface molecule such as ManLAM can inhibit the production of IFN-γ resulting in reduced expression of MHC class II molecules (Baena and Porcelli, 2009). Although the exact mechanism is not clear it has been suggested that attenuation of MHC class II molecules could be due to the inhibition of IFN-γ gene expression and/or downregulation of different molecules such as MHC Class II trans-activator (CIITA). CIITA is a major transcriptional regulator of MHC class II expression (Chang *et al*., 1994). The interaction of 19kDa lipoprotein molecule from *M.tb* has also been shown to cause inhibition of antigen processing and downregulation of MHC class II molecules *in vitro* in mouse macrophages as compared to untreated control (Noss *et al*., 2001). TLR 2 receptors on macrophages were shown to play an important role in this downregulation of MHC Class II molecules. It was shown that macrophages isolated from TLR2 knockout ($TLR2^{-/-}$) mice when treated with 19kDa lipoprotein showed greater expression of MHC class II molecules as compared control (Noss *et al*., 2001).

1.10.3 Resistance to reactive oxygen and nitrogen intermediates

Activated macrophages can eliminate intracellular *mycobacteria* by producing highly toxic compounds such as reactive nitrogen intermediates (RNIs) and reactive oxygen intermediates (ROIs) (Fang, 2004). *M.tb*, however, as a part of its resistance mechanism produces different enzymes that can inactivate some of these toxic compounds. For example, the production of KatG, a catalase-peroxidase can inactivate reactive oxygen inside the phagosomes and help in the growth of *M.tb* in animal models such as mice and guinea pigs (Li *et al*., 1998). Similarly, another protective mechanism involves *M.tb* proteasome, which helps in the removal or refolding of proteins damaged by reactive nitrogen intermediates (Darwin *et al*., 2003).

1.11 Diagnosis of TB

Some of the diagnostic tests currently used for TB are discussed as under:

1.11.1 Chest X-ray

Chest X-rays are performed to detect pulmonary TB in patients with abnormal respiratory symptoms. Chest X-rays showing cavitation or calcified shadows in the upper and middle lobes of the lungs are positive indications for pulmonary TB (van Cleeff *et al*., 2005). However, a chest radiograph is not by itself diagnostic for TB and only provide clues for the diagnosis.

1.11.2 Sputum Microscopy

Sputum smear microscopy using a light microscope is the primary method for diagnosing pulmonary TB in most of the low income and middle income countries with a high number of TB cases. This method of diagnosis is advantageous because it is inexpensive, rapid and relatively simple (Dye *et al*., 2005). For *M.tb* detection via microscopy, sputum samples are collected from the patient on 3 consecutive days. The sputum is smeared on a glass side and acid-fast staining (Ziehl–Neelsen staining) is performed to detect red *M.tb* bacilli using a light microscope. This technique has been reported to have a sensitivity ranging from 50-80% in detecting pulmonary TB in the adult population (Levy *et al*., 1989; Aber *et al*., 1980). Sputum microscopy has a poor track record in detecting pulmonary TB in children (20%) and in HIV positive patients (Perkins *et al*., 2006; Shingadia and Novelli, 2003). Other limitation of this method including non-specificity for *M.tb* as the presence of other mycobacterial species may produce false positive results.

An important new innovation to this diagnostic technique in high income countries is the introduction of fluorescence microscopy using acid-fast fluorescence dyes such as auramine-O or auramine-rhodamine. Comparison of fluorescence and conventional microscopy showed similar specificity and 10% higher sensitivity for fluorescence microscopy than that of conventional microscopy (Steingart *et al*., 2006).

1.11.3 *M.tb* cultures

For the definitive diagnosis of TB, cultures are grown from samples such as sputum, bronchoalveolar lavage etc. of suspected patients for the detection of *M.tb*. A culture test is considered to be the gold-standard for *M.tb* detection and has a higher sensitivity of around 82%. *M.tb* number as low as 10 bacilli per ml of the specimen can be detected using this method (Yeager, Jr. *et al*., 1967). Conventionally 3 sputum samples are used for culture diagnosis however, the use of 2 specimens has been shown to be equally sensitive (Leonard *et al*., 2005). One of the major drawback associated with this technique is the longer duration of diagnosis since *M.tb* are slow growing bacteria and may require 3-6 weeks for detectable growth.

Automated culture detection systems such as BACTEC MGIT 960 and MB/BacT system have been introduced for diagnosis of TB. These systems utilize modified Middlebrook 7H9 broth and have improved sensitivity combined with a shorter *M.tb* detection duration of 8-10 days as compared to conventional culturing methods. These systems have the additional advantage of performing drug susceptibility test (DST) simultaneous to the detection of *M.tb* in the diagnostic samples (Muyoyeta *et al*., 2009; Palomino *et al*., 2008). Hasan *et al* showed that BACTEC MGIT-960 was 100% sensitive and 93.3% specific in detecting *mycobacteria* when compared to Löwenstein–Jensen (LJ) culture method in a study conducted in Bangladeshi population (Hasan *et al*., 2013). Whereas, in case of detecting *M.tb* susceptibility to first-line TB drugs, MB/BacT system showed 95-100% sensitivity and 100% specificity when compared to LJ culture method (Barreto *et al*., 2002).

1.11.4 Tuberculin skin test

Tuberculin skin test is one of the oldest diagnostic test mostly used for the detection of latent TB due to its lower cost. This test is also known as intradermal Mantoux test. During tuberculin skin test, intermediate strength purified protein derivatives (PPD) from *M.tb* are injected intradermally. An indurate develops in the skin at the site of injection due to cell-mediated immunity against the injected PPD. The size of this indurate is measured after 48-72 hours and conclusion is drawn about latent *M.tb* infection based on the size (Huebner *et al*., 1993). The

sensitivity of Tuberculin skin test in detecting pulmonary *M.tb* infections in the first time TB patients is approximately 80% (Nash and Douglass, 1980). However, there are limitations to this diagnostic method. The PPD which is a culture filtrate of tubercle bacilli shares almost 200 antigens with *M.bovis BCG* and other non-pathogenic *mycobacteria*. Therefore, previous BCG vaccination or exposure to environmental *mycobacteria* can hamper the results from this test (Wang *et al*., 2002).

1.11.5 Interferon-γ release assay (IGRA)

Interferon-γ (IFN-γ) release assay (IGRA) is a blood-based diagnostic test for TB. It relies on the release of IFN-γ by T-lymphocytes upon exposure to specific *M.tb* antigens, ESAT-6 and CFP-10 which are encoded by the genes in the region of difference 1 (RD1) of *M.tb* genome.

Two commercially available immunological tests, QuantiFERON-TB Gold and T-SPOT.TB tests are used *ex vivo* for diagnosing *M.tb* infections. QuantiFERON-TB Gold test uses enzyme-linked immunosorbent assay (ELISA) to measures IFN-γ released by sensitized T lymphocytes after incubation *M.tb* specific antigens (Mazurek *et al*., 2005). The second IGRA test known as T-SPOT.TB test. This test identifies *M.tb* specific T lymphocytes from the blood sample secreting INF-γ using enzyme-linked immunospot (ELISpot) assay technique (Lalvani *et al*., 2001).

The main advantage of these tests over Tuberculin skin test is that the ESAT-6 and CFP-10 are specific to *M.tb*, hence excluding false positive results from BCG vaccinated individuals or people exposed to environmental *mycobacteria.* A comparative study conducted for detection of active pulmonary tuberculosis in patients with clinical and radiological symptoms in Rawalpindi, Pakistan showed QuantiFERON-TB Gold test to be 80% sensitive while the sensitivity for Tuberculin skin test was 28% (Khalil *et al*., 2013). Similarly, the T-SPOT.TB test was shown to have a higher sensitivity of 83% in the diagnosis of active pulmonary TB as compared to Tuberculin skin test with a sensitivity of 38.3% (Simsek *et al*., 2010). The T-SPOT.TB test was shown to be 10% more sensitive that QuantiFERON-TB Gold test in detecting latent TB infections in health care workers in South Africa (van Zyl-Smit *et al*., 2016).

The major limitation of both QuantiFERON-TB Gold test and T-SPOT.TB test is that these tests are unable to distinguish between active and latent tuberculosis.

1.11.6 Nucleic acid amplification tests (NAATs)

Amplification of specific *M.tb* DNA sequences from the patient specimen can be used for the diagnosis of TB. These tests are fast and can give results in 3-6 hours (Catanzaro *et al*., 1997). Cobas TaqMan MTB assay is a commercially available NAAT that consists of a real-time PCR (qPCR) kit for rapid detection of *M.tb* from clinical specimens. Using Culture test as a reference Cobas TaqMan MTB assay has shown a sensitivity of 83% and a specificity of 97% in patients with clinical symptoms of TB (Lee *et al*., 2013). Similarly, another commercially available NAAT known as Gene Xpert MTB/RIF assay not only allows rapid *M.tb* detection through DNA amplification but can simultaneously detect rifampicin resistance causing mutations by using *rpoB* gene as a target (Zeka *et al*., 2011). GeneXpert MTB/RIF assay has shown a sensitivity of around 87-93% and a specificity of 98% when compared to culture test as a gold standard during different studies (Moussa *et al*., 2016; Bunsow *et al*., 2014). The Gene Xpert MTB/RIF assay has a sensitivity of 100% and a specificity of 96% in detecting RIF resistance strains of *M.tb* (Bunsow *et al*., 2014).

1.12 Treatment of TB

Currently, there are more than 20 drugs used in different combinations for treatment of TB. The use of particular combinations of drugs depends upon the circumstances during which it is used. The anti-TB drug can be divided into 2 major groups known as first-line drugs and second-line drugs.

1.12.1 First Line Drugs

The first line drugs consist of four anti-TB drugs including isoniazid, rifampicin, pyrazinamide and ethambutol. First line drugs are used for the treatment of TB in new patients as these patients have the least chances of showing resistance to any of these drugs. During standardized short-course therapy for TB, all the four antibiotics are administered to the patient once every day for the first 2 months. The purpose of taking all 4 drugs simultaneously is to eliminate *M.tb* mutants and avoid drug resistance. Isoniazid and rifampicin are continued for another 4 months while pyrazinamide and ethambutol are discontinued (van Deun *et al*., 2010). There is a 5% relapse chance for TB in patients who have undergone the standardized short-course therapy with first line drugs (Gegia *et al*., 2017).

The first line anti-TB drug are discussed as under:

1.12.1.1 Isoniazid

Isoniazid is an important anti-TB drug that has been used since 1952. Isoniazid inhibits the production of mycolic acid in *mycobacteria* and hence prevents the synthesis of bacterial cell wall. It also causes the production of oxygen derived free radicals (superoxide, peroxide and hydroxyl radicals) and organic free radicals (carbon-containing compounds with a free electron such as $^{\bullet}CH_3$) that damage DNA (Timmins and Deretic, 2006). The minimum inhibitory concentration (MIC) of isoniazid ranges from 0.02-0.20 µg/mL for *M.tb* (Arbex *et al*., 2010). However, isoniazid is a prodrug and requires *M.tb* catalase-peroxidase enzyme *KatG* for its conversion to active form isonicotinic acid (Zhang *et al*., 1992). Resistance to isoniazid can arise due to missense mutations causing amino acid substitutions, or small deletions or insertions in the *KatG* genes. This results in the lower efficacy of isoniazid and inhibits the prodrug from being converted into its functional metabolite (Slayden and Barry, 2000). Furthermore, isoniazid can efficiently kill actively growing *M.tb* however, it has limited effect against slow-growing intracellular *M.tb* (Zhang, 2005).

1.12.1.2 Rifampicin

Rifampicin is the most important drug included in the first line drugs for TB treatment and has been in use since 1966. Rifampicin can kill metabolically active *M.tb* as well as bacteria in stationary phase. Rifampicin blocks the transcription of mycobacterial genes by binding to the β-subunit of DNA-dependent RNA polymerase. This stops the synthesis of mRNA resulting in a lack of important protein for *M.tb* and finally cell death (Blanchard, 1996). The MIC of rifampicin is 0.05-0.50 µg/mL for *M.tb* (Arbex *et al*., 2010). Point mutations or deletions in the RNA polymerase B gene (*rpoB*) result in resistance to rifampicin (Telenti *et al*., 1993). About 95% of this resistance causing mutations in bacteria are restricted to an 81bp region of *rpoB* called RRDR (rifampicin resistance determining region) (Caws *et al*., 2006).

1.12.1.3 Pyrazinamide

Pyrazinamide has been used as an anti-TB drug since 1952. Chemically pyrazinamide is a nicotinic acid derivative and is similar to isoniazid in structure. The antibacterial activity of pyrazinamide is pH dependent and the minimum inhibitory concentration (MIC) of pyrazinamide is 6.25-50.0 µg/mL for *M.tb* at pH 5.5 (Arbex *et al*., 2010). Pyrazinamide is a prodrug and requires *M.tb* enzyme nicotinamidase/pyrazinamidase for its conversion to active form pyrazinoic acid (Konno *et al*., 1967). The mechanism of action for pyrazinamide is still not fully clear. It is suggested that pyrazinamide kills *M.tb* by passively entering the bacteria and converting into pyrazinoic acid. The bacteria lack an efficient system efflux system causing

accumulation of pyrazinoic acid in the cytoplasm leading to a decrease in intracellular pH (Zhang *et al*., 2003). It is also suggested that this change in pH leads to the inactivation of important bacterial enzymes such as fatty acid synthase I, which plays important role in mycolic acid synthesis (Zimhony *et al*., 2007). Pyrazinamide has sterilizing effect by eliminating non-replicating persistent *M.tb* present in acidic environments such as phagolysosome that is responsible for bacteriological relapse (Mitchison, 1985). Pyrazinamide is used along with other first-line TB drugs due to its sterilizing effect and can shorten the treatment time to 6 months. Resistance to pyrazinamide can arise due to missense mutations, or small deletions or insertions in the *pncA* gene leading to the production of non-functional nicotinamidase/pyrazinamidase enzyme (Scorpio and Zhang, 1996).

1.12.1.4 Ethambutol

Ethambutol is a bacteriostatic drug that has been used for treating TB since 1966. Ethambutol can inhibit the growth of *M.tb* and is effective against intracellular as well as extracellular *M.tb*. The minimum inhibitory concentration (MIC) of ethambutol is 1-5 µg/mL for *M.tb* (Arbex *et al*., 2010). Ethambutol inhibits the formation of *M.tb* cell wall by interfering with the biosynthesis of an important polysaccharide arabinogalactan. Ethambutol inactivates the enzyme arabinosyl transferase, which mediates polymerization arabinose into arabinan and then arabinogalactan (Mikusova *et al*., 1995). Resistance to ethambutol develops due to missense mutations in the *embB* gene cluster coding for arabinosyl transferase (Telenti *et al*., 1997).

1.12.2 Second Line Drugs

Multidrug-resistant TB (MDR-TB) is defined as a condition in which patient does not respond to both isoniazid and rifampicin. The second line drugs are only used during MDR-TB when *M.tb* is resistant to the first line drugs. Treatment of MDR-TB with second-line drugs requires longer durations and the treatment is continued for 24 months. MDR-TB patients are treated by giving them different anti-TB drugs in combination with fluoroquinolones (Gegia *et al*., 2017). Proper treatment is of utmost importance in case of MDR-TB for recovery of patients and to stop further acquisition of resistance mutation by the pathogenic *M.tb*. If MDR-TB is not treated properly it can develop into extensively drug-resistant TB (XDR-TB). During XDR-TB the patients become resistant to a fluoroquinolone and one drug from aminoglycosides or cyclic peptides groups (CDC, 2006).

Some of the second line drugs used for treating MDR-TB are described as under:

1.12.2.1 Fluoroquinolones

Fluoroquinolones including fluorine-containing nalidixic acid derivatives are used for treating pulmonary, extrapulmonary and disseminated TB (Rustomjee *et al*., 2008). Fluoroquinolones include a number of drugs such as sparfloxacin, gatifloxacin, moxifloxacin, levofloxacin, ciprofloxacin, and ofloxacin. Among all fluoroquinolones, gatifloxacin and moxifloxacin were shown to be the most effective in treating rifampicin-tolerant persistent *M.tb in vitro* and had MIC of 0.20-0.25µg/mL (Arbex *et al*., 2010). Fluoroquinolones are also bactericidal against intracellular *M.tb*. In mice, moxifloxacin was the most effective in killing *M.tb* followed by sparfloxacin, levofloxacin, and ofloxacin respectively (Shandil *et al*., 2007). In humans, fluoroquinolone such as ciprofloxacin or ofloxacin is commonly used for treating MDR-TB (Bass *et al*., 1994).

Fluoroquinolones kill *M.tb* by targeting the bacterial topoisomerases II (DNA gyrase) that are essential for DNA replication, repair and recombination. Fluoroquinolones directly inhibit bacterial DNA synthesis by binding to the enzyme-DNA complex and cause a blockade of DNA replication and strand breakage (Aubry *et al*., 2004). Resistance to fluoroquinolones develops due to mutations in the *gyrA* and *gyrB* genes of *M.tb* coding for A and B subunits of topoisomerase II. Majority cases of *M.tb* with fluoroquinolones resistance have missense mutations in a small portion of *gyrA* gene called quinolone resistance-determining region (QRDR) (Sun *et al*., 2008; Cheng *et al*., 2004).

1.12.2.2 Streptomycin

Streptomycin was the first antibiotic used for treating TB, however, *M.tb* became resistant to this antibiotic due to its use as monotherapy. Streptomycin binds to the 16S rRNA and inhibits the translation of bacterial proteins (Moazed and Noller, 1987). Resistance to streptomycin is due to mutations in the *rrs* or *rpsL* gene that alter the streptomycin binding site on 16S rRNA (Ramón-García *et al*., 2006; Finken *et al*., 1993).

1.12.2.3 Aminoglycosides and Cyclic peptides

Aminoglycosides and cyclic peptides used for TB treatment share the same mechanism of action for killing *M.tb*. These drugs kill *M.tb* by binding irreversibly to the ribosomal subunits and prevent the initiation of translation during protein synthesis (Maus *et al*., 2005; Kotra *et al*., 2000).

Kanamycin and amikacin are aminoglycosides used for treating TB. These drugs inhibit protein synthesis by binding to the 16S rRNA specifically in regions that interact with tRNA and lead

to misreading of tRNA (Alangaden *et al*., 1998). Resistance to these drugs is due to mutations at position 400 and 1401 of the *rrs* gene coding for 16S rRNA result in mutants with high-level resistance to kanamycin and amikacin (Suzuki *et al*., 1998).

Capreomycin and viomycin are structurally related cyclic peptides used for TB treatment. These drugs share the same mechanism and inhibit protein synthesis in *M.tb* by binding at the interface of the 30S small and 50S large ribosomal subunits (Stanley *et al*., 2010). Resistance to these drugs is due to mutations in *tlyA* gene that codes for rRNA methyltransferase (Johansen *et al*., 2006)

1.12.2.4 Ethionamide

Ethionamide is a pro-drug, structurally similar to isoniazid and is used for treating TB. The pro-drug is activated by ethA-encoded enzyme mono-oxygenase, resulting in the formation of the functionally active ethionamide-NAD adduct. This active ethionamide-NAD adduct interferes with mycolic acid synthesis by inhibiting the enoyl-ACP reductase enzyme (Wang *et al*., 2007; Baulard *et al*., 2000). Resistance to ethionamide occurs due to mutations in *ethA* gene encoding mono-oxygenase (Brossier *et al*., 2011) or the transcriptional repressor *ethR* gene (Carette *et al*., 2011).

1.12.2.5 Para-Aminosalicylic Acid

Para-Aminosalicylic Acid (PAS) is bacteriostatic in nature and was the second drug found effective for treating TB (Dooneief *et al*., 1950). Earlier PAS was used in combination with isoniazid and streptomycin for treating TB, however, PAS is now used as a second line drug for treating MDR-TB. The mechanism of action for PAS is not completely understood. It is suggested that PAS interferes with folic acid synthesis by limiting the supply of dihydropteroate synthase enzyme needed by para-aminobenzoic acid (Rengarajan *et al*., 2004). Mutations in the *thyA* gene encoding thymidylate synthase A enzyme which is important for activation of PAS and *folC* gene have been shown to be associated with resistance to PAS (Mathys *et al*., 2009; Rengarajan *et al*., 2004).

1.12.2.6 D-Cycloserine

D-cycloserines are bacteriostatic drugs that are used for treating MDR-TB. D-cycloserines are analogues of D-alanine and interfere with peptidoglycan synthesis by blocking the activity of D-alanine : D-alanine ligase (Feng and Barletta, 2003). This drug also prevents the conversion of L-alanine to D-alanine by inhibiting the enzyme D-alanine racemase (Strych *et al*., 2001).

The actual target for this antibiotic is unknown in *M.tb* however, recombinant *M.smegmatis* with mutations in *alrA* were shown to be resistant to cycloserine (Caceres *et al*., 1997).

1.12.2.7 Linezolid

Linezolid used for treating MDR-TB belongs to the oxazolidinones family of drugs. Linezolid binds to the 50S ribosomal units and inhibit the early steps in protein synthesis (Thompson *et al*., 2002). Resistance to linezolid is rare in *M.tb* and only 1.9% of the MDR strains don't respond to linezolid (Richter *et al*., 2007). *In vitro* analysis of resistant strains showed mutations in 23S rRNA and *rplC* gene encoding 50S ribosomal subunit (Beckert *et al*., 2012). There are also reports showing the involvement of *M.tb* efflux pumps in resistance to linezolid (Escribano *et al*., 2007).

1.12.3 New Anti-TB Drugs

Because of the emergence of resistant *M.tb* strains to the currently available anti-TB drugs, a number of new anti-TB drugs are being developed with the aim of improved efficacy and shorter treatment duration. Some of these new drugs are discussed as under:

1.12.3.1 Bedaquiline

Bedaquiline is a new antibiotic used specifically for treating MDR-TB (Diacon *et al*., 2009). This drug inhibits the ATP-synthase of *M.tb* by blocking the proton pumps (Hards *et al*., 2015). Bedaquiline is currently in phase III clinical trials.

1.12.3.2 Delamanid

Delamanid is a nitro-dihydro-imidazooxazole derivative that inhibits the synthesis of mycolic acid and is highly effective against both drug-susceptible and drug-resistant *M.tb* (Diacon *et al*., 2011; Matsumoto *et al*., 2006). Delamanid is currently in phase III clinical trials.

1.12.3.3 Pretomanid (PA-824)

Pretomanid (PA-824) is a nitroimidazole derivative that kills *M.tb* by inhibiting the synthesis of cell wall lipids (Manjunatha *et al*., 2009). PA-824 is effective against both actively dividing and non-replicating persistent *M.tb* (Diacon *et al*., 2012; Lenaerts *et al*., 2005). PA-824 is currently in phase III clinical trials.

1.12.3.4 SQ-109

SQ-109 is a synthetic ethambutol analogue that is effective against extensively drug-resistant *M.tb* strains in addition to actively dividing and persistent non-replicating *M.tb*. The minimum inhibitory concentration of SQ-109 is 0.16-0.63 mg/L for *M.tb* (Protopopova *et al*., 2005). This

drug inhibits *M.tb* growth by interfering with the assembly of mycolic acid into the mycobacterial cell wall core (Tahlan *et al*., 2012). It is currently in phase II clinical trials. When used in combination with isoniazid and rifampicin in mice models, SQ-109 enhanced the anti-*M.tb* activity and reduced the time required to cure TB (Nikonenko *et al*., 2007).

1.12.3.5 Benzothiazinone

Benzothiazinone derivatives such as BTZ043 are a new class of anti-TB drug that interferes with the bacterial cell wall synthesis by blocking the synthesis of arabinans. BTZ043 targets the bacterial enzyme decaprenylphosphoryl-beta-d-ribose 2'-epimerase (Makarov *et al*., 2009). This enzyme is essential for the synthesis of decaprenylphosphoryl arabinose, which serves as a precursor for cell wall arabinans (Mikusová *et al*., 2005). These drugs have been shown to be effective in against drug-susceptible and MDR *M.tb* strains (Pasca *et al*., 2010).

1.13 Anti-TB vaccines

1.13.1 *M.bovis* Bacillus Calmette–Guérin (BCG) vaccine

BCG is the only licenced vaccine currently available against TB and is used worldwide for TB prevention. This vaccine consists of an attenuated *M.bovis* strain that was derived from a virulent strain after passaging it *in vitro* for over a decade (Calmette *et al*, 1927; Calmette and Plotz, 1929). *M.bovis* BCG is closely related to *M.tb* and its genome has almost 99.95% similarity with *M.tb*. Genome analysis showed that the *M.bovis* BCG genome has undergone a large number of mutations during it's *in vitro* propagation including a number of deletions in the RD1 region (Behr *et al*., 1999). These deletions resulted in the loss of several important T cell stimulating antigens such as the ESAT-6 (early secretory antigenic target of 6kDa) and CFP10 (culture filtrate protein 10) (Pym *et al*., 2002).

The BCG vaccine was first introduced in France and Belgium from 1921-1927 and showed high efficacy in protecting children against TB. Initial use of BCG vaccine was limited to tuberculin negative children as this vaccine was not expected to have a positive effect in *M.tb* infected individuals. Later studies indicated that it was safe to use BCG vaccine in tuberculin positive people which led to the mass vaccination programs in TB-endemic areas. Currently, BCG vaccine is administered to infants soon after birth in the form of a single intradermal dose in countries with high TB risk. It is estimated that >3 billion people have been vaccinated against TB using BCG vaccines.

There have been a number of efficacy trials and epidemiological studies that have established the protective role of BCG vaccines in children against different forms of TB. These studies showed that BCG vaccines have 60-80% protective efficacy in children against various forms of TB such as meningitis and miliary TB, while the protection against pulmonary TB varies from 20-80% geographically (Michelsen *et al*., 2014; Trunz *et al*., 2006; Wünsch Filho *et al*., 1990; Romanus, 1987; Padungchan *et al*., 1986). In the adult population, BCG vaccine has shown limited protection against pulmonary TB. The largest community-based trail for BCG vaccine efficacy was conducted in Madras, South India from 1968-1971. Individuals who were vaccinated as adults during this trail on re-evaluation after 5 and 15 years respectively showed no protective effect against the development of active TB. This trial also showed much lower levels of protection by BCG vaccine against pulmonary TB in children (17%) as compared to other studies (Tripathy, 1987). Other controlled-field trials performed showed variable efficacy (0-80%) for BCG vaccine in controlling pulmonary TB in adults, with the lowest levels of protection in countries of high burden (Aronson *et al*., 2004; Colditz *et al*., 1994; Palmer and Long, 1966).

This variability in BCG vaccine efficacy can be attributed to a number of factors including strain variation in BCG vaccine preparations. The original attenuated *M.bovis* culture produced in France was distributed to different laboratories without cloning. These laboratories then propagated the vaccine *M.bovis* BCG culture under different conditions using different protocols, which led to variations in the bacteria. Other factors such as genetic differences between population, nutritional condition of individuals and environmental factors may also lead to variability in vaccine efficacy (Behr, 2002).

Pre-exposure to environmental *mycobacteria* can have a deleterious effect on BCG vaccination. Two hypothesis are put forward in this regard. The first hypothesis suggests that some level of protective immunity is generated in individuals exposed to environmental *mycobacteria* which mask the protective effect from a subsequent BCG vaccine (Fine and Vynnycky, 1998). The second hypothesis suggests that pre-exposure to environmental *mycobacteria* results in the generation of an immune response to antigens shared by different mycobacterial species. Subsequent BCG vaccination of pre-exposed individual prevents BCG from replication and affect the efficacy of the vaccine (Brandt *et al*., 2002).

1.13.2 New candidate anti-TB vaccines

Due to the variability in efficacy of BCG vaccine and its limited effectiveness in regions with higher TB burden, novel anti-TB vaccines are required. Research is being done to develop new TB vaccines that can either be used to boost the immune response produced by prior BCG vaccination or to develop novel vaccines that can replace BCG altogether. Some of these new candidate anti-TB vaccines are discussed as under:

1.13.2.1 MVA85A

MVA85A was a subunit vaccine used in BCG vaccinated individual to boost the number of antigen-specific T cells. MVA85A consists of a recombinant modified vaccinia virus Ankara expressing *M.tb* antigen 85A (McShane *et al*., 2004). In BCG vaccinated mice models MVA85A was shown to be effective in further enhancing the protective immunity against *M.tb*. MVA85A was shown to increase the number of IFN-γ secreting $CD4^+$ T cells (Goonetilleke *et al*., 2003). Phase I clinical trial for MVA85A showed the vaccine to be safe in humans and capable of inducing high levels of antigen-specific IFN- γ secreting T cells (McShane *et al*., 2004). However, unfortunately, the phase IIb clinical trial conducted in infants in South Africa showed no significant enhancement in protection against TB in BCG prime MVA85A boosted individuals (Tameris *et al*., 2013). Further analysis revealed that MVA85A vaccine was unable to elicit the same levels of T cell response as the phase I clinical trials conducted in the UK. The T cell response generated by MVA85A in African population during phase IIb trials was 10-fold less than the UK population observed in phase I trials (Wilkie and McShane, 2015).

1.13.2.2 M72/AS01$_E$

M72/AS01$_E$ is another subunit vaccine that is currently in phase IIb clinical trials. This vaccine consists of a recombination fusion antigenic protein, M72, derived from two mycobacterial proteins Mtb32a and Mtb39a. The fusion protein M72 is delivered in the adjuvant AS01. The M72/AS01$_E$ exerts its protective effect by causing the production of high levels polyfunctional M72 specific $CD4^+$ T cells and $CD8^+$ T cells (Leroux-Roels *et al*., 2013). This vaccine has shown good safety profile in multiple phase I and phase IIa clinical trials carried out in adults (Montoya *et al*., 2013) and BCG vaccinated infants (Idoko *et al*., 2014).

1.13.2.3 H1/IC31 and H56/IC31

H1/IC31 and H56/IC31 are fusion protein vaccines currently in phase IIa clinical trials. H1/IC31 consist of *M.tb* secreted Ag85B protein fused with another antigenic *M.tb* protein ESAT-6. This fusion protein is combined with adjuvant IC31 composed of the antibacterial

peptide (KLK) and a synthetic oligodeoxynucleotide (ODN1a), for the vaccine delivery (Olsen *et al*., 2001). This vaccine can elicit a potent T cell response and has shown good results during phase I and some phase II clinical trials in naïve (van Dissel *et al*., 2010), BCG vaccinated (van Dissel *et al*.., 2011) and HIV^+ individuals (Reither *et al*., 2014).

H56/IC31 is a modified version of the H1/C31 vaccine. This vaccine consists of an additional protein Rv2660c fused with the Ag85B and ESAT-6. The advantage of H56/IC31is its ability to provide protection against latent TB by eliciting a strong cellular and humoral immune response as was shown during phase I trial conducted in South Africa (Luabeya *et al*., 2015).

1.13.2.4 VPM1002

The VPM1002 vaccine consists of recombinant BCG that produces enzyme listeriolysin O (LLO) due to the incorporation of genes from *Listeria monocytogenes*. The recombinant BCG also has a deletion of the *ureC* gene in order to improve the activity of LLO enzyme as it requires a pH of 5.5 (Farinacci *et al*., 2012). The LLO enzyme makes the phagosome membrane porous resulting in the leakage different enzymes and bacterial antigens into the cytoplasm leading to increased apoptosis and enhanced $CD4^+$T cells and $CD8^+$T cells responses (Grode *et al*., 2013). This vaccine is currently in phase IIa clinical trials.

1.13.2.5 MTBVAC

MTBVAC vaccines contain live attenuated *M.tb*. The *M.tb* used in these vaccines have two independent stable deletion mutations in the virulence genes *fadD26* and *phoP* (Arbues *et al*., 2013). This vaccine has shown a greater level of protective immunity as compared to BCG vaccines in pre-clinical models such as mice by enhancing T cell-based immunity (Nambiar *et al*., 2012). Similarly, these vaccines have shown good results in phase I clinical trials in humans and resulted in the production of high levels polyfunctional $CD4^+$ T cells without any adverse effects (Spertini *et al*., 2015).

Table1.1: Candidate anti-TB vaccines.

Category	Vaccine	Sponsor	Clinical trial stage	Vaccine Description
Subunit-protein in adjuvant	M72/AS01	GlaxoSmithKline (GSK), Aeras	Phase IIb	Fusion protein (Mtb39a, Mtb32a) in AS01 adjuvant
	H1/IC31	Statens Serum Institut (SSI), TBVI, EDCTP, Valneva	Phase IIa	Fusion protein (Ag85B, ESAT-6) in IC31 adjuvant
	H4/IC31	SSI, Sanofi, Institut Pasteur, Aeras	Phase II	Fusion protein (Ag85B,TB10.4) in IC31 adjuvant
	H56/IC31	SSI, Valneva, Aeras	Phase IIa	Fusion protein (Ag85B, ESAT-6, Rv26660c) in IC31 adjuvant
Subunit- viral vectors	MVA85A	Oxford University, Aeras, EDCTP	Phase IIb	Replication deficient vaccinia Ankara virus expressing Ag85A
	Ad5Ag85A	McMaster University, CanSino	Phase I	Replication-deficient adenovirus-5 expressing Ag85A
	AD35.TB-S	Aeras	Phase I	Replication deficient adeno virus-35 expressing Ag85A, Ag85B, TB10.4
Recombinant BCG	rBCG30	Aeras	Phase I	Recombinant BCG overexpressing Ag85B
	VPM1002	Vakzine Projekt Management GmbH, Max Planck Institute, TBVI, Serum Institute of India	Phase IIa	Recombinant BCG with deleted *ureC* gene and expressing *L.monocytogenes* LLO
Attenuated *M.tuberculosis*	MTBVAC	University of Zaragoza, Biofabri, TBVI	Phase IIa	Live attenuated *M.tb* with deleted *phoP* and *fadD26* genes
Inactivated Mycobacteria	Dar-901	Dartmouth University, Aeras	Phase I	Heat-inactivated *Mycobacterium obuense*

1.14 *Mycobacterium smegmatis* and *Mycobacterium bovis BCG* as models for *M.tb*

Studying pathogenic bacteria of the genus *mycobacteria* such as *M.tb* is challenging due to a number of reasons. These bacteria are highly virulent due to which working with them require special protective measures including a biosafety level-3 laboratory and there is a persistent risk of accidental exposure to these pathogens which can result in serious diseases. In addition, the pathogenic *M.tb* are slow growing with a generation time of almost 22-24 hours in broth media. It takes about 3-4 weeks for the colonies to appear on agar plates and the colonies

generation requires special media. Therefore, other similar *mycobacteria* are used as models instead of using *M.tb* where feasible (Shiloh and Champion, 2010). Two non-pathogenic mycobacterial species, used as models for *M.tb* in the current study are discussed as under:

1.14.1 *Mycobacterium smegmatis* (*M.smegmatis*)

M.smegmatis are saprophytic bacteria mostly found in soil, water and plants. The genome size of *M.smegmatis* is 6.98 x 10^6 nucleotides, with high GC content of 67%. The genome mostly consists of the coding region (90% genome) and a total of 6716 proteins are encoded by *M.smegmatis* genome. *M.smegmatis* shares 95% gene homology with *M.tb* (Mohan *et al*., 2015).

M.smegmatis is used as a model organism for *M.tb* due similarities in basic structures, metabolism and cellular processes. *M.smegmatis* and *M.tb* have the same mechanism for cell wall synthesis (Takayama and Kilburn, 1989). This feature of *M.smegmatis* can be translated into treatments for *M.tb* by using *M.smegmatis* as a model to find ways to inhibit the synthesis of different cell wall components. Different previous studies have used *M.smegmatis* as a model during the screening process to select potentially active compounds against *M.tb* (Andries *et al*., 2005; Lu and Drlica, 2003; Murdock *et al*., 1978).

In addition, *M.smegmatis* are non-pathogenic, fast-growing bacteria with a generation time of 2-3 hours and the colonies appear after 72 hours. These bacteria do not have any special media requirements and can grow in commonly available synthetic or complex laboratory media which makes it much easier to work with them.

1.14.2 *Mycobacterium bovis BCG* (*M.bovis BCG*)

M.bovis BCG is an attenuated strain of *Mycobacterium bovis* that bears a very high level of similarity to *M.tb* showing 99.95% identity at the genomic level (Garnier *et al*., 2003). Because of this high level of genomic similarity, *M.bovis BCG* is used as a model to understand and investigate different aspects of *M.tb* such as physiology, pathogenesis and immunity (Altaf *et al*., 2010; Alli *et al*., 2009). However, the disadvantage of using *M.bovis BCG* is that it is slow growing bacteria and *M.bovis BCG* cultures require about 2-3 weeks for growth. In addition, *M.bovis BCG* cannot be grown in the commonly available culture media and requires a specially formulated growth medium to support its growth.

1.15 Host protein and non-protein factors used during screening

Different host protein and non-protein factors used in the current study during the screening are briefly described as under:

1.15.1 Transferrin

Transferrin is an important plasma protein that consists of 679 amino acids and has a molecular weight of ~70kDa (Parkkinen *et al*., 2002). Transferrin is mainly produced by hepatocytes in the liver with two high affinity iron-binding domains at N-terminal and C-terminal (Beutler *et al*., 2000). Other cells such as sertoli (Lecureuil *et al*., 2004), oligodendroglial cells (Bloch *et al*., 1985), melanoma cells (Nicolson *et al*., 1990) and human breast cancer cells (Inoue *et al*., 1993) can also synthesize transferrin. Transferrin is present in various body fluids such as blood plasma, lymph, bile, cerebrospinal, and breast milk. The plasma concentration of transferrin ranges from 2g-3g/L and the half-life is about 8 days (Cazzola *et al*., 1985). Transferrin facilitates the transport of iron in the body in a soluble form to different cells. Transferrin binds free iron with high affinity and hence prevents iron mediated free radical toxicity (Gomme *et al*., 2005). Lower levels of plasma transferrin (0.1g/L) are associated with anaemia, growth retardation and increased risk of infection (Hayashi *et al*., 1993). Transferrin also possess direct antimicrobial properties and can inhibit the growth of bacteria as well as fungi by causing damage to the cell membrane (Lin *et al*., 2014; Ellison *et al*., 1988).

1.1.5.2 Lactoferrin

Lactoferrin is an iron-binding glycoprotein that was first discovered in human milk and was named Lactoferrin due to its functional similarity to transferrin protein. Lactoferrin is produced by epithelial cells in apo-form and is present in most of the body fluids including plasma, milk, saliva, tears, bile and gastric fluids (Montreuil *et al*., 1960). Lactoferrin consists of 690 amino acids and bears almost 60% amino acid similarity with human transferrin (Lönnerdal and Iyer, 1995). The normal plasma concentration of lactoferrin ranges from 0.4–2 mg/L, however, its concentration can increase up to 200 mg/L during inflammation and infections (Legrand *et al*., 2008). The biological role of lactoferrin is not limited to its role in iron transportation. Lactoferrin also plays an important role in the innate immune system. Increased Lactoferrin levels at the site of infection due to pathogen induced inflammation results in binding more free iron, an important substrate for the growth bacterial pathogens and thus creates an environment difficult for bacterial survival. Lactoferrin can bind to the cell membrane of the pathogenic microorganisms such as bacteria, fungi and different parasites and can directly inhibit their growth (Rodriguez-Franco *et al*., 2005; Ellison *et al*., 1988).

1.15.3 Fibrinogen

Fibrinogen is a precursor blood plasma protein that is converted enzymatically by thrombin into the active form fibrin (Mosesson, 2005). Fibrinogen is synthesized in the liver by hepatic cells and is secreted into the blood. Fibrinogen is present in the blood plasma at a concentration ranging from 150-400mg/dL with a half-life of about 4 days (Oswald *et al*., 1983). Fibrinogen has a molecular weight of 340kDa and structurally consists of two trimers held together by disulphide bonds. Each trimer is further composed of three polypeptide chains namely fibrinogen alpha, beta and gamma chains (Henschen *et al*., 1983). The major function of this protein is to help in blood clotting during tissue or vascular injury (Mosesson, 2005). Fibrinogen is a positive acute phase protein and its level increases during inflammation (Davalos and Akassoglou, 2012). High levels of fibrinogen can be dangerous as it cause damage to the blood vessels and facilitate in the formation of blood clots leading to stroke and heart attack (Wilhelmsen *et al*., 1984). Fibrinogen can bind to different bacteria and was shown to possess antibacterial properties against group A and group B streptococci, however, it was unable to inhibit the growth of gram negative bacteria such as *E.coli* (Påhlman *et al*., 2013).

1.15.4 C-reactive protein

C-reactive protein (CRP) is a ring shaped plasma protein that belongs to the 'pentraxin' family of proteins. Structurally CRP consists of five non-covalently associated protomers arranged around a central axis. Each protomer consists of 206 amino acid forming two anti-parallel β sheets and a single α helix (Thompson *et al*., 1999; Shrive *et al*., 1996). CRP an acute phase protein, synthesized by hepatic cells in the liver and is released in to the blood. The standard concentration of CRP in human plasma is between 5-10mg/L (Albert *et al*., 2003), however, it is increased by several folds during tissue injury, infection or other causes of inflammation (McIntyre *et al*., 1997). CRP hence serves as an important marker for inflammation and is useful in determining disease progression and effectiveness of treatment. CRP also plays an important role in the immune system by acting as a pattern recognition receptor (PRR). CRP binds phosphocholine moieties from the membranes of bacteria, fungi or dead cells in a Ca^{+2} dependent manner. This CRP-phosphocholine complex leads to the activation of complement system through C1q protein and also promotes macrophage phagocytosis (Kaplan and Volanakis, 1974).

1.1.5.5 Alpha-2-macroglobulin

Alpha-2-macroglobulin (α2M) is a large blood plasma protein with a molecular size of 720kDa. Structurally α2M is a tetrameric protein having 4 identical subunits held together by disulphide

bonds (Feldman *et al*., 1985). α2M is mainly synthesized in the liver (Petersen *et al*., 1988) however, other cells such as macrophages (White *et al*., 1980) and fibroblasts (Mosher and Wing, 1976) can also make some α2M. The plasma levels of α2M in normal humans ranges from 200-300mg/dL (Housley, 1968). The major functions of α2M includes its ability to inactivate a variety of proteinases and act as a carrier protein for different cytokines (TGFβ, IL-1β) and growth factors (platelet-derived growth factor) (Broth, 1992). Administration of exogenous α2M was shown have a protective effect against *Pseudomonas aeruginosa* infections in guinea pigs (Khan *et al*., 1994).

1.15.6 Vitronectin

Vitronectin is a glycoprotein found in body fluids such as blood plasma, amniotic fluid and urine (Shaffer *et al*., 1984). The plasma concertation of vitronectin ranges from 0.25-0.45mg/mL (Boyd *et al*., 1993). Majority of the vitronectin in human body is synthesized in the liver by hepatic cells and is secreted in to the blood. Vitronectin has also been detected in smooth and skeletal muscle, renal tissue, skin and vascular walls (Hayman *et al*, 1983). Structurally vitronectin is made up of 459 amino acids with a molecular weight of 75kDa. Vitronectin can exist as a single chain molecule or in the form of double chains held together by a disulfide bonds (Preissner, 1991). In humans, vitronectin is mainly involved in cellular adhesion, cell migration and angiogenesis (Leavesley *et al.*, 1992). Vitronectin has also been shown to bind to bacteria and augment in the attachment of these bacteria to the host cells and possibly their uptake (Chhatwal *et al*., 1987). Vitronectin deposition on the surface of bacteria prevents the assembly of membrane attack complex (MAC) and protects the bacteria from MAC mediated lysis inside the host (Singh *et al*., 2010).

1.15.7 Plasminogen

Plasminogen is a precursor protein consisting of 810 amino acids that is produced in the liver and released in the blood circulation (Forsgren *et al*., 1987). Circulatory plasminogen upon binding clots or cell surfaces is converted into the active form by enzymes such as tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), kallikrein, and factor XII (Hageman factor). The active form of plasminogen is known as plasmin and consist 791 amino acids. The major function of plasminogen/plasmin system to dissolve fibrin clots (fibrinolysis) (Castellino and Ploplis, 2005) and also plays important roles in wound healing, embryogenesis and angiogenesis (Law *et al*., 2013). Host plasminogen form complexes by interacting with Streptokinase (SK) and Staphylokinase (SAK) enzymes from *Streptococci* and *Staphylococci*,

resulting in the formation of proteolytic surfaces that can help in the spread of bacteria (Bergmann *et al.,* 2013).

1.15.8 Plasma Lipoproteins

Plasma lipoproteins are spherical particles that consist of a single layer of phospholipids, cholesterol and triglycerides with embedded apolipoproteins. Their main function is the transportation lipids to different parts of the body in a water soluble form. The plasma lipoproteins are categorized into high density lipoproteins (HDL), low density lipoproteins (HDL), intermediate density lipoproteins (IDL) and very low density lipoproteins (VLDL) based on their density and size (Morrisett *et al*., 1975).

LDL particle ranges from 220-275Å in diameter and consists of 3000-6000 fat molecules (Fisher *et al*., 1972). Each LDL particle contains a 514 kDa apolipoprotein B-100 molecule along with other small proteins (Knott *et al*., 1986). The major function of LDL in the blood is to deliver cholesterol to different cells by binding to LDL receptors on their surfaces (Goldstein *et al*., 1983). Oxidatively modified forms of LDL (ox-LDL) play an important role the blood vessel biology and are involved in atherosclerosis. These ox-LDL causes injury to the endothelial cells of vascular vessels due to the increased recruitment of monocytes and activation of different pro-inflammatory signalling pathways (Steinberg and Witztum, 2010).

High Density Lipoproteins (HDL) are small lipoprotein with a particle size of about 100 Å. HDL particles are rich in proteins and apoA-I is the most abundant protein present in the HDL (Brewer and Rader, 1991). The major function of HDL is to carry cholesterol from cells and tissue back to liver (Fisher *et al*., 2012).

Bacterial infection accompanied by inflammation results in the production of different cytokines such as IL-6, TNF-α, and IL-1 etc, these cytokines cause profound changes in the concentration, composition and function of the plasma lipid and lipoproteins such as LDL and HDL (Nassaji and Ghorbanni, 2012).

1.15.9 Serotonin

Serotonin, chemically known as 5-Hydroxytryptamine or 3-(β-Aminoethyl)-5-hydroxyindole is an important chemical with diverse functions found in humans and other animals (Erspamer, 1986). Serotonin is mostly present in the gastrointestinal tract (GI) and the central nervous system (CNS) performing important function like regulating intestinal movements and working as a neurotransmitter (Berger *et al*., 2009). It is also present in the serum and helps in the

constriction of smooth muscles in respiratory and cardiovascular systems (Saxena and Villaló, 1990). During the process of inflammation serotonin is released by degranulation of mast cells and platelets leading to increased vascular permeability and vasodilation (Mössner and Lesch, 1998). Serotonin has been shown to enhance phagocytosis in murine macrophages (Freire-Garaba *et al*., 2003) and was also able to directly inhibit the growth of fungi such as candida species (Lass-Flörl *et al*., 2003).

1.16 Platelet Activating Factor (PAF)

Platelet activating factor (PAF) or PAF-acether is a membrane-derived phospholipid with diverse physiological effects in mammals. Benveniste *et al* assigned the name "platelet activating factor" to a compound that was released from rabbit basophils upon stimulation with Ig-E and was able to activate platelets (Benveniste *et al*., 1972). Another group of researchers isolated a polar lipid similar to PAF in properties from the renal medulla and named it "antihypertensive polar renal lipid" due to its ability to reduce blood pressure in mice model (Muirhead *et al*., 1981). Other laboratories were able to synthesize a semisynthetic phospholipid compound, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, and observed that this compound had identical physiochemical and biological properties to the naturally produced PAF and antihypertensive polar renal lipid (Benveniste *et al*., 1979; Blank *et al*., 1979; Demopoulos *et al*., 1979). Hanahan *et al* described the chemical structure for PAF produced by stimulated rabbit basophils by using techniques such as gas-liquid chromatography and mass spectral analysis and showed it as a 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (Hanahan *et al*., 1980). Although the term PAF is still commonly used for this compound, it can be sometimes misleading suggesting that its activity is limited to platelets. On the contrary, this lipid mediator possesses a variety of physiological effects due to its role as intercellular and intracellular messenger.

A variety of cell types including platelets (Alam *et al*., 1983), neutrophils (Biffl *et al*., 1996), monocytes/macrophages (Yagnik, 2014; Leaver *et al*., 1990), endothelial cells (Bussolino *et al*., 1986(a)) and mast cells (Schleimer *et al*., 1986) are able to produce PAF upon proper stimulation. Under normal physiological conditions, the concentration of PAF is very low in tissues and blood. PAF can be produced in higher amounts during inflammation and anaphylactic reactions by cells such as leukocytes (Vadas *et al*., 2013).

1.16.1 Structure of PAF

Chemically PAF is 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (Hanahan *et al*., 1980). The glycerol backbone of PAF has an alkyl group attached to the *sn*-1 position via ether linkage that makes the tail of the compound. The most common form of naturally produced PAF in the body contains a 16-carbon alkyl chain and is known as PAF C-16 (Clay *et al*., 1984). The length of carbon tail can vary and there are naturally produced PAF analogues with 18-carbon chain (Oda *et al*., 1985). A short chain acyl group in the form of acetyl is attached at the *sn*-2 position. This acetyl group increases the solubility of the molecule and helps PAF to function as a soluble signalling molecule. At the *sn*-3 position, a phosphocholine group is attached that constitutes the polar head region of the compound (Figure 1.5). All the structural elements of PAF can influence the biological potency of this phospholipid. Substitution of the acetyl group at the *sn*-2 position of PAF has been shown to affect the activity of the PAF significantly and even lead to the biological inactivation of the compound (McManus *et al*., 1993).

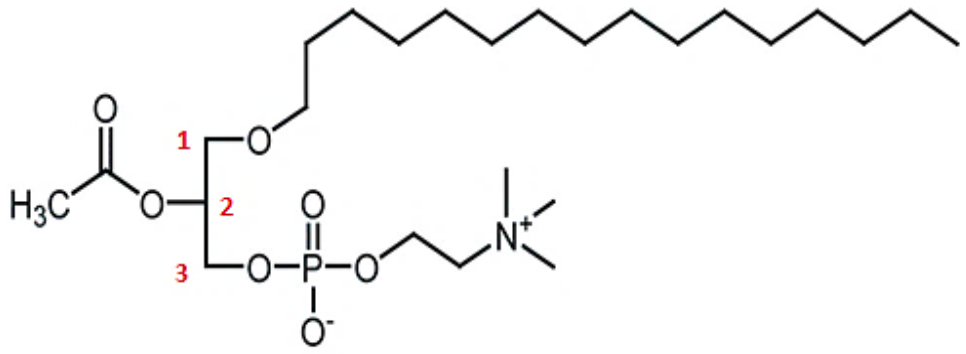


Figure1.5: Chemical structure of Platelet Activating Factor C-16. At position *sn*-1, the aliphatic carbon tail consisting of 16 carbon atoms is attached. Acetyl group is present at position *sn*-2 and phosphocholine at *sn*-3.

1.16.2 PAF C-16 biosynthesis

There are two distinct pathways for the production of PAF C-16 *in vivo*. These pathways include the remodelling pathway and the *de novo* pathway for synthesis of PAF C-16. These pathways are described in detail as under:

1.16.2.1 The remodelling pathway for PAF C-16 synthesis

The remodelling pathway was the first pathway described for the synthesis of PAF C-16 and involves structural modifications to pre-existing, membrane ether-linked phospholipids

(Wykle *et al*., 1980). The remodelling pathway of PAF C-16 synthesis is mostly used by a number of activated cells involved in inflammatory and allergic responses (Lee *et al*., 1984; Alonso *et al*., 1982).

The synthesis of PAF C-16 is initiated by the enzyme phospholipase A_2 (PLA_2) (Ninio *et al*., 1982) and carried out in two steps as shown in figure 1.6. The PLA_2 enzyme first hydrolyzes arachidonate from the membrane ether-linked phospholipids such as 1-O-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine. This enzymatic action results in the formation of 1-O-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (Lyso-PAF), a precursor form of PAF C-16 and the release of free arachidonic acid. In the next step, the enzyme acetyl coenzyme A (Lyso-PAF acetyltransferase) adds an acetyl group at the position *sn*-2 of the 1-O-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (Lyso-PAF) resulting in the formation of PAF C-16 (Balestrieri *et al*., 1997; Uemura *et al*., 1991).

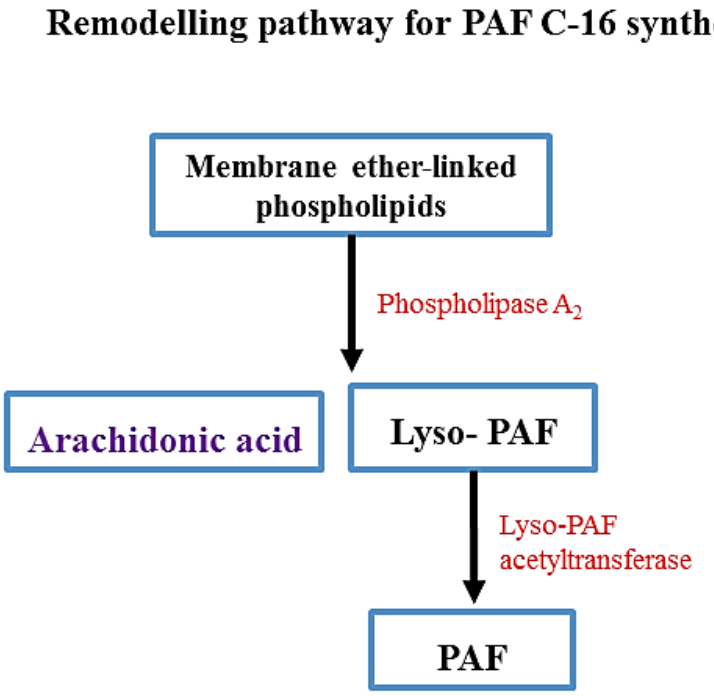


Figure1.6: Remodelling pathway for PAFC-16 synthesis. During the first step, PLA_2 enzyme hydrolyzes the membrane phospholipids and produces the inactive precursor from Lyso-PAF and arachidonic acid. In the second step, Lyso-PAF is converted to PAF by the enzyme acetyl transferase, which adds an acetyl group at position *sn*-2 replacing the hydroxyl group.

1.16.2.2 The *de novo* pathway for PAF C-16 synthesis

PAF is synthesized continuously in small amounts in kidneys and central nervous system through the *de novo* pathway (Snyder, 1995; Bussolino *et al*., 1986(b)). This pathway consists of three steps including acetylation/acylation, dephosphorylation and transfer of phosphocholine from cytidine diphosphate-choline (CDP-choline) moiety to 1-O alky-*sn*-glycero-3-phosphate inside the cells (Figure 1.7).

During the first step, the enzyme acetyl coenzyme A acetyltransferase adds an acetyl group at the *sn*-2 position of 1-O alky-*sn*-glycero-3-phosphate resulting in the formation of 1-O alky-2-acetyl-*sn*-glycero-3-phosphate. The phosphohydrolase enzyme then removes the phosphate group from position *sn*-3, forming 1-O alky-2-acetyl-*sn*-glycerol. Finally, the enzyme CDP-choline phosphotransferase catalyses the transfer of a phosphocholine group resulting in the formation of PAF C-16 (Lee *et al*., 1988; Renooij and Snyder, 1981).

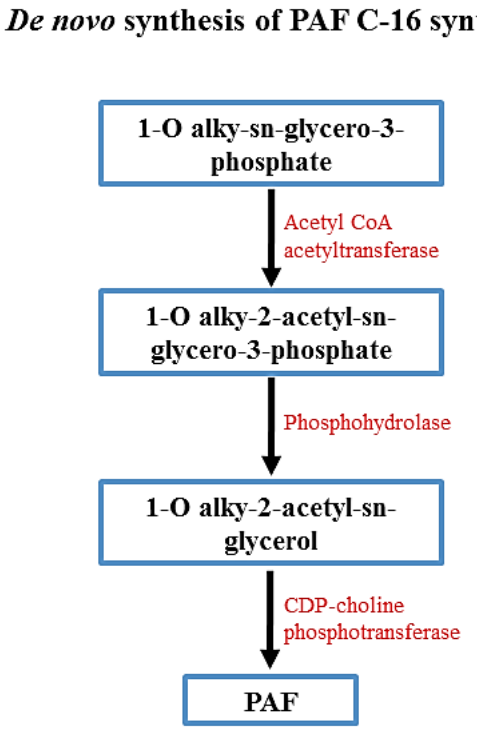


Figure 1.7: *De novo* pathway for PAF C-16 synthesis. PAF is synthesized from 1-O-alkyl-*sn*-glycero-3-phosphate in three steps including acetylation, dephosphorylation and transfer of phosphocholine during the last step.

1.16.3 PAF acetylhydrolase (PAF-AH)

PAF C-16 concentration and activity is tightly regulated by specific calcium-independent subfamilies of acetylhydrolase enzymes known as PAF acetylhydrolase (PAF-AH). PAF-AH limit the availability of active PAF C-16 by removing the short chain acyl group from the *sn*-2 position resulting in the formation of Lyso-PAF (Farr *et al*., 1980). PAF-AH is present both in the blood plasma (extracellular form) (Farr *et al*., 1980) and in different tissues (intracellular form) (Nijssen *et al*., 1986). The plasma form of PAF-AH is normally secreted by macrophages and circulates in the plasma, primarily in association with HDL and LDL particles (Elstad *et al*., 1989). The intracellular forms of PAF-AH can be exclusively cytosolic as in case of brain cells and erythrocytes or both cytosolic and membrane-bound as in case of liver and kidney cells (Stafforini *et al*., 1991).

1.16.4 PAF receptor and signalling

PAF C-16 can communicate signals to target cells by binding to specific receptors known as the platelet activating factor receptor (PAFR) on the surface of the target cells (Honda *et al*., 1991; Nakamura *et al*., 1991). A wide variety of cells including platelets (Valone *et al*., 1982), monocytes/macrophages (Liu *et al*., 1992), neutrophils (O'Flaherty et al., 1986), eosinophils (Ukena *et al*., 1989), endothelial cells (Korth *et al*., 1989), cardiomyocytes (Sugimoto *et al*., 1992), smooth muscle cells (Hwang *et al*., 1983) and cells of the central nervous system (Domingo *et al*., 1988) were shown to possess PAFR on their plasma membrane.

PAFR belongs to "serpentine receptors" which are a family of transmembrane receptors. Structurally PAFR consist of seven α-helices that passes through the plasma membrane seven times (Figure 1.8). PAFR has a molecular weight of 39.2kDa and comprises of 342 amino acids. G proteins are attached to the third intracellular loop and to the carboxyl tail of the PAFR (Montrucchio *et al*., 2000; Shimizu *et al*., 1996).

Binding of PAF C-16 to PAFR on target cells results in the activation of phosphatidylinositol-specific phospholipase C (PLC) through the associated G proteins. This activated membrane-bound PLC causes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) from the plasma membrane causing the transient production of second messengers including inositol trisphosphate (IP_3) and diacylglycerol (DG). The IP_3 produced then causes the mobilization of intracellular Ca^{+2} along with the influx of extracellular Ca^{+2} via cell membrane-associated channels, leading to elevated levels of Ca^{+2} in the cytoplasm (Ishii and Shimizu, 2000). This Ca^{+2} together with DAG can activate protein kinase C (PKC) enzyme that results in the

phosphorylation of different intracellular proteins and leads to different cellular responses including leukocyte activation, platelet aggregation, chemotaxis, generation of reactive oxygen and nitrogen species and upregulation of different cytokines such as TNF-α, IL-6, IL-4 etc. (Chao and Olson, 1993). PAF C-16 binding to its receptor PAFR on target cells also causes the activation of cytosolic phospholipase A_2 ($cPLA_2$) mediated by intracellular Ca^{+2} levels. The activation of $cPLA_2$ leads to the hydrolysis of arachidonic acid from intracellular phospholipids resulting in the production of lysophosphatides. The arachidonic acid is further utilized in the synthesis of bioactive lipid mediator eicosanoid (Nakashima *et al*., 1989).

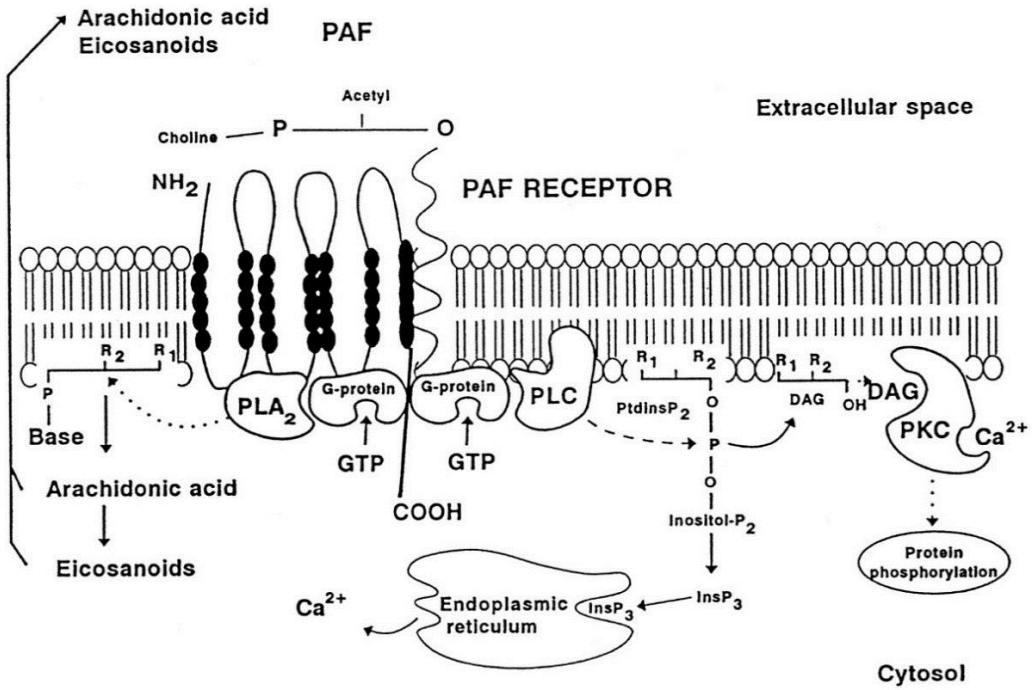


Figure 1.8: PAF receptor (PAFR) and its signal transduction mechanisms. The figure shows the structure of trans-membrane PAFR with its associated G proteins. Binding of PAF C-16 to PAFR results in the activation of PLC, PKC and PLA_2 along with the production of different intracellular second messengers including DAG, IP_3 and Ca^{+2} (Montrucchio *et al*., 2000).

1.16.5 Physiological and pathophysiological roles of PAF C-16

PAF C-16 is a phospholipid with diverse biological activities that works as a signalling molecule by binding to specific receptors known as PAF receptors (PAFR) on the target cells (Nakamura *et al*., 1991). The diverse effects of PAF C-16 can be attributed to the presence of PAFR on a variety of cells and the ability of PAF C-16 to activate different intracellular signalling pathways resulting in the production of downstream effector molecules specific for

cell type (Ishii and Shimizu, 2000). PAF C-16 can work as an autocrine molecule by acting on the cells producing it and can also mediate intercellular communication by acting over short distances as a paracrine signalling molecule or as an endocrine molecule where it is circulated in the blood (Prescott *et al*., 2000). Some of the important physiological and pathophysiological roles of PAF C-16 are discussed as under:

1.16.5.1 Role of PAF C-16 in platelets aggregation

One of the most well-known function of PAF C-16 is its ability to cause platelet aggregation. PAF C-16 is the most potent platelet aggregating agent and can cause complete aggregation of platelets at very low concentrations approaching nanomolar. PAFC-16 treatment causes the degranulation of platelets, resulting in the release of different molecules such as serotonin, ADP, ATP from their intracellular granules (Chesney *et al*., 1982; Vargaftig *et al*., 1981).

1.16.5.2 Role of PAF C-16 in reproduction

PAF C-16 has been suggested to plays an important role in the reproductive system in humans and other mammals. PAF C-16 improves sperm motility and enhances fertilization by acting as a capacitation factor (Ricker *et al*., 1989; Davis, 1981). PAF C-16 is produced by mammalian embryos from the 2-cell zygote till the blastocyst stage (Ripps *et al*., 1993). A number of studies indicated that PAF C-16 is involved in the implantation, development and parturition of mammalian embryos (Nishi *et al*., 1995; O'Neill *et al*., 1989). It was shown that preimplantation exposure of murine embryos to exogenous PAF C-16 resulted in improved birth rate and also improved size and weight at birth (Roudebush *et al*., 2004).

1.16.5.3 Role of PAF C-16 in inflammation

Inflammation is considered to have evolved as a protective mechanism contributing to the host innate immune system. However, it is also the key mediator mechanism that generates different complications in the body. These inflammation related complications can ultimately lead to different types of diseases (Medzhitov, 2008).

Most of the current research focuses on the role of PAF C-16 as a mediator of inflammation. *In vitro,* acute inflammation models comprising of human endothelial cells stimulated with thrombin or histamine showed the production of PAF C-16 along with the expression of cell adhesion molecule P-selectin. This PAF C-16 form endothelial cells was able to bind to PAFR on the surface of polymorphonuclear leukocyte (PMNs) and activated these key effectors of acute inflammatory response, indicating an active role for PAF C-16 during inflammation (Lorant *et al*., 1999).

Nagase *et al* showed the association of PAF C-16 and inflammation in disease conditions by examining acute lung injury in mice models. It was observed that PAFR knockout mice showed reduced lung injury due to acid aspiration, whereas mice overexpressing PAFR showed more severe lung injury that proved to be fatal when compared with control mice (Nagase *et al*., 1999). In humans, excess of PAF C-16 due to deficiency in PAF acetylhydrolase enzyme results in unregulated pro-inflammatory signalling that has been suggested to be associated with different disease conditions such as acute pancreatitis (Chen *et al*., 2008), Crohn's disease (Sobhani *et al*., 1992), ulcerative colitis (Ferraris *et al*., 1993) and neonatal necrotizing enterocolitis (Caplan *et al*., 1990).

1.16.5.4 Role of PAF C-16 in asthma

Another important pathological effect of PAF C-16 due to its pro-inflammatory nature is its role in asthma. Asthma is a respiratory system disorder characterized by activation of inflammatory cells, microvascular leakage, bronchoconstriction, and airway hyperresponsiveness. Asthma patients were shown to have increased level of PAF C-16 in their blood (Kurosawa *et al*., 1994) and higher expression of PAFR mRNA was detected in the lung tissue (Shirasaki *et al*., 1994) as compared to that of normal individuals. Treating antigen-induced asthma in humans and guinea pigs with PAF antagonists that block PAFR, improved the disease symptoms (Kagoshima *et al*., 1997, Hozawa *et al*., 1995). Furthermore, mutations in the genes coding for PAF acetylhydrolase leading to loss of functional activity were found to be prevalent in asthma patients, suggesting the association of functional PAF acetylhydrolase deficiency and elevated PAF C-16 levels with increased risk of asthma (Stafforini *et al*., 1999).

1.16.5.5 Role of PAF C-16 in type 1 diabetes

PAF C-16 is also involved in the development of type 1 diabetes in humans due to its pro-inflammatory nature. Type 1 diabetes results from the destruction of pancreatic β cells present in the islets of Langerhans (insulitis). About 50-fold more PAF C-16 was detected in the blood of type 1 diabetes individuals as compared to healthy controls (Nathan *et al*., 1992). A study in diabetes prone BB-rat models using PAF antagonist BN52021 showed that administration of PAF antagonist either protected these animals from diabetes or delayed the onset of diabetes as compared to the untreated control (Jobe *et al*., 1993). During another study in BB-rat models, it was shown that the use of recombinant PAF acetylhydrolase (rPAF-AH) reduced the frequency of diabetes by lowering the concentration of active PAF C-16 as compared to the untreated control. The immunohistochemistry of the islets showed decreased insulitis and

higher numbers of insulin-positive cells in rPAF-AH treated rats as compared to untreated control (Lee *et al*., 1999).

1.16.5.6 Role of PAF C-16 in renal diseases

PAF C-16 is produced in the kidneys (Schlondorff et al., 1986; Pirotzky *et al*., 1984) and elevated levels of PAF C-16 have been suggested to be involved in renal diseases such as acute renal failure (Lopez-Farré *et al*., 1988). It was shown that administration of exogenous PAF C-16 in dogs and mice had negative effect on the kidney function and resulted in a dose-dependent decrease in glomerular filtration rate (Badr *et al*., 1989; Santos *et al*., 1988). This damaging effect of PAF C-16 at higher concentration was shown to be due to the vasoconstriction of the arterioles (Juncos *et al*., 1993). The damaging effect of exogenous PAF C-16 on the kidneys function was prevented by the use of PAF receptor antagonists (Yoo *et al*., 1990).

1.16.5.7 Role of PAF C-16 in cancer

There are reports which suggest a possible role for PAF C-16 in some cancers by promoting cells growth and tumours formation. PAF C-16 causes angiogenesis (Camussi *et al*., 1995), which contributes to the growth and metastasis of tumours. PAF C-16 was shown to trigger the formation of new blood vessels in breast cell-induced cancer in mice by stimulating the recruitment of endothelial cells along with the production of angiogenic factors such as FGF, placental growth factor, VEGF, HGF etc. (Bussolati *et al*., 2000). Human tumour-derived endothelial cells (TEC) were shown to produce greater quantities of PAF C-16 as compared to normal endothelial cells, which contributes to cell motility, adhesion and vessel formation. Overexpression of PAF-AH in these cells resulted in the inactivation of PAF C-16 resulting in reduced cell motility, cell adhesion and restored the sensitivity of these cells to apoptosis (Doublier *et al*., 2007). In mouse models, exogenous PAF C-16 was shown to have growth enhancing effects on tumour cells and augmented the metastasis of melanoma to lungs (Im *et al*., 1996). Disrupting PAF C-16 mediated signalling by using PAF receptor antagonists inhibited the cell proliferation and metastatic potential of different cancers such as human breast adenocarcinoma, colon adenocarcinoma, fibrosarcoma, hepatocarcinoma and neuroblastoma *in vitro* (Cellia *et al*., 2006).

1.16.5.8 Role of PAF C-16 in infection

The role of PAF C-16 during infection is not completely understood. Elevated levels of PAF C-16 have been detected in the blood during HCV and dengue virus infections (Jeewandara *et al*., 2015; Caini *et al*., 2007). *In vitro* intestinal epithelial cells were shown to produce increased

quantities of PAF C-16 when challenged *Salmonella enteritidis* (Egea *et al*., 2008). A protective role for endogenously produced PAF C-16 on the host organism was suggested during *Candida albicans* infection in mice. PAF C-16 was shown to cause the activation of NF-κB and upregulation of TNF-α in these infected mice (Choi *et al*., 2001). The protective role of endogenous PAF C-16 acting through its receptor was shown by using PAFR deficient mice during *K. pneumoniae* infection. The PAFR deficient mice showed higher bacterial load in the lungs and died earlier as compared to the wild-type control (Soares *et al*., 2002). A number of studies have shown that exogenous PAF C-16 while acting through PAFR can help in the elimination of intracellular pathogenic microorganisms such as Leishmania (Borges *et al*., 2017; Lonardoni *et al*., 2000), Trypanosoma (Aliberti *et al*., 1999) and *Candida albicans* (Kim *et al*., 2008) both *in vivo* and *in vitro*. These studies showed that PAF C-16 treatment resulted in enhanced production of ROIs, RNIs and cytokines such as TNF-α. Furthermore, exogenous PAF C-16 was also shown *in vitro* to have direct inhibitory effect on the growth gram-positive bacteria (Steel *et al*., 2002) and lower eukaryotes such as yeast (Nigam *et al*., 2013).

Hypothesis

M.tb infection elicits an inflammatory response in the host which causes leakage of a number of plasma proteins and non-protein factors at the site of infection. A number of such factors including PAF C-16 and serotonin are either present or produced locally at the site of infection, which we hypothesized are likely to have protective functions during microbial infections. We, therefore, investigated a number of such endogenously produced factors *in vitro* for their effect on the growth of *mycobacteria*.

Aim and Objectives of the study

Aim: To investigate the *in vitro* inhibitory effect of different endogenously produced host factors (proteins and non-proteins) on the growth of *mycobacteria* using non-pathogenic *M.smegmatis* and *M.bovis BCG* as models for *M.tb*.

Objective 1: *In vitro* screening of different host factors for their direct growth inhibitory effect against *mycobacteria* including proteins such as Transferrin, Lactoferrin, Fibrinogen, C-Reactive Protein (CRP), Alpha-2-Macroglobulin (α2M), Vitronectin, Plasminogen, Low-Density Lipoprotein (LDL), High-Density Lipoprotein (HDL) and non-protein factors such as Serotonin, L-Alpha Dipalmitoylphosphatidylcholine (DPPC) and Platelet Activating Factor C-16 (PAF C-16).

Objective 2: Characterization of the effect of small structural changes to PAF C-16 on mycobacterial growth inhibition by using PAF C-16 analogues, identification of the active portion of PAF C-16 and elucidation of the molecular mechanism of PAF C-16 induced mycobacterial growth inhibition.

Objective 3: Investigation of the effect of PAF C-16 and its analogues on the growth of intracellular *mycobacteria* using phagocytic THP-1 cells and understanding the mechanism of PAF C-16 induced intracellular *M.smegmatis* growth inhibition.

Chapter2: General Methods and Materials

2.1 Growing and Storing *mycobacteria*

Non-pathogenic mycobacterial species, *M.smegmatis* (mc^2 155) and *M. bovis BCG* (Pasteur 1173P2) were used in the current study. The bacteria were grown in broth cultures and stored at -80°C for use in future experiments.

2.1.1 Growing *M.smegmatis* cultures in LB-broth

Liquid cultures of *M.smegmatis* (mc^2 155) were grown in Luria-Bertani (LB) broth. The culture media was prepared by dissolving 10grams of tryptone (Fisher Scientific, UK), 5grams of yeast extract (Fisher Scientific, UK) and 0.5gram of sodium chloride (Fisher Scientific, UK) in 1 liter distilled water and autoclaved at 121°C for 15 minutes. Using a sterile inoculating loop three isolated *M.smegmatis* colonies streaked on LB-agar plate were transferred to 200ml LB broth supplemented with 0.1% (v/v) glycerol (Fisher Scientific, UK) and 0.05% (v/v) tween-80 (Fisher Scientific, UK). A selective antibiotic, carbenicillin (Fisher Scientific, UK) was also added at a final concentration of 50µg/ml to the culture media. The bacterial culture was kept in a shaker at 37°C for the *M.smegmatis* to grow. Optical density ($O.D_{600}$) for the bacterial culture was checked at regular intervals using a spectrophotometer (Ultrospec 10 Cell Density Meter, Amershem Biosciences). On reaching 0.70-0.90 $O.D_{(600nm)}$ *M.smegmatis* culture was removed from the shaker, checked for purity using acid-fast staining and stored at -80°C.

2.1.2 Growing *M.bovis BCG* cultures in 7H9 media

Liquid cultures of *M. bovis BCG* (Pasteur 1173P2) were grown in Middlebrook 7H9 broth (Sigma Aldrich, UK). The 7H9 broth media was prepared by dissolving 2.5grams of 7H9 powder and 2.5ml of glycerol in 450ml distilled water (Fisher Scientific, UK). The media was sterilized by autoclaving at 121°C for 15 minutes. Using a sterile inoculating loop, three isolated *M. bovis BCG* colonies streaked on a 7H10 agar-plate (BD Biosciences, UK) were transferred to 200ml of 7H9 supplemented with 10% Albumin Dextrose Catalyse (ADC) and 0.05% (v/v) tween-80 (Fisher Scientific, UK). The bacterial culture was kept in a shaker at 37°C to allow *M.bovis BCG* to grow and the optical density ($O.D_{600}$) was checked at regular intervals after the first week using a spectrophotometer (Ultrospec 10 Cell Density Meter, Amershem Biosciences). On reaching 0.70-0.90 $O.D_{(600nm)}$ *M.bovis BCG* culture was removed from shaker, acid-fast staining was performed to ensure that there was no contamination and stored at -80°C for future experiments.

2.1.3 Acid-fast staining for *M.smegmatis* and *M.bovis BCG*

Acid-fast staining, a special type of staining technique was performed for every batch of mycobacterial culture grown in order to identify *M.smegmatis* and *M.bovis BCG* and determine the purity of the culture.

To perform acid-fast staining, 1ml aliquots of *M.smegmatis* or *M. bovis BCG* from their respective cultures were centrifuged at 5000rpm for 10 minutes (Eppendorf 5424 Microcentrifuge). After centrifugation the supernatant was discarded and the bacterial pellet was resuspended in 50µl of culture media. About 10-15µl of the bacterial suspension was smeared on a clean glass slide and left to air dry for 10-15 minutes at room temperature. The bacterial smear was heat fixed by passing the glass slide 3-4 times through a Bunsen burner flame. The heat fixed smear was covered with an absorbent paper, flooded with carbol-fuschin stain (Pro Lab Diagnostics, UK) and heated for 5-10 minutes until it started steaming. During the entire heating process carbol-fuschin was continuously added to keep the smear flooded with the dye. After heating, excess carbol-fuschin was removed by gently washing the glass slide with tap water. A few drops of decolorizing solution (Pro Lab Diagnostics, UK) were added to the smear, followed by gentle washing with tap water. The smear was then flooded with methylene blue (Pro Lab Diagnostics, UK) for 45 seconds and washed with tap water. After air drying the glass slides with stained *M.smegmatis* or *M. bovis BCG,* they were observed under a light microscope (Brunel SP100) at 1000X magnification. A pure culture consisted of entirely red rods due to the retention of carbol-fuschin stain by the *mycobacteria* (Figure 2.1.3).

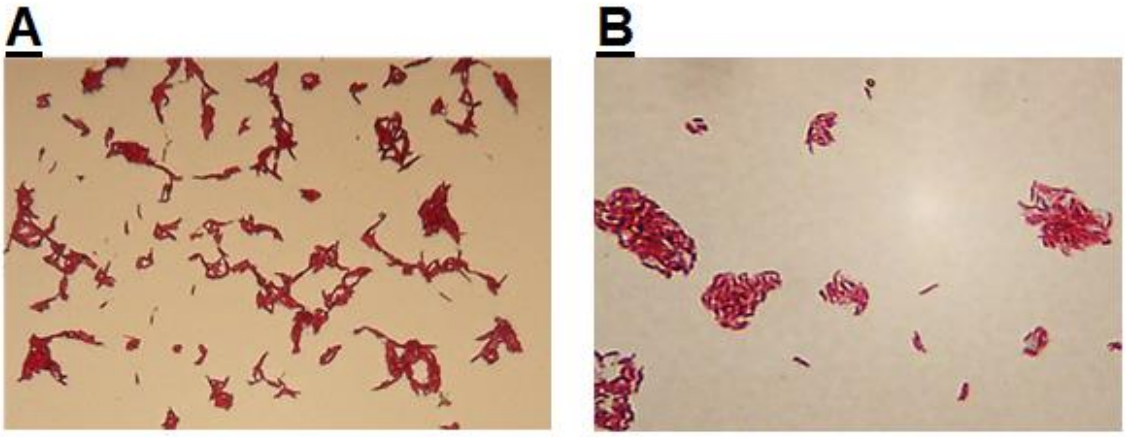


Figure 2.1.3: Acid fast staining of *mycobacteria*. Microscopic images of *M.smegmatis* (Slide A) and *M.bovis* BCG (Slide B) grown in cultures at 1000X after performing acid fast staining.

2.1.4 Storing *M.smegmatis* and *M.bovis BCG*

After establishing the purity of mycobacterial cultures by acid-fast staining, both *M.smegmatis* and *M. bovis BCG* were stored at -80°C for further use. *M.smegmatis* and *M. Bovis BCG* grown in broth cultures were transferred to 50ml falcon tubes and pelleted by centrifugation at 5000rpm for 30 minutes. The supernatant was discarded and the bacterial pellet was resuspended in 3ml freezing solution. The freezing solution was prepared by mixing sterile LB broth or 7H9 and glycerol in equal volumes (1:1). About 200µl of *M.smegmatis* or *M.bovis BCG* suspension in freezing solution was transferred to sterile 1.2 ml cryotubes and stored at -80°C.

2.1.5 Preparation of agar plates for *M.smegmatis* and *M.bovis BCG*

M.smegmatis colonies were grown on LB-agar plates. The LB-agar media was prepared by dissolving 10grams of tryptone (Fisher Scientific, UK), 5grams of yeast extract (Fisher Scientific, UK), 0.5grams of sodium chloride (Fisher Scientific, UK) and 15grams of nutrient agar (Fisher Scientific, UK) in 1 liter distilled water and autoclaved at 121°C for 15 minutes. About 15-20 ml of melted LB-agar was poured in petri plates under sterile conditions using laminar flow cabinets and petri plates were allowed to solidify and air dry for 90-120 minutes before plating *M.smegmatis*.

M.bovis BCG colonies were grown on commercially available 7H10 plates purchased from BD Biosciences, UK. The plates were stored at 4°C and were air dried at room temperature in a laminar flow hood using sterile conditions for 30-45 minutes before using them for plating *M.bovis BCG*.

2.1.6 Calculating the number of bacteria for stock *M.smegmatis* and *M.bovis BCG*

In order to achieve consistency and uniformity in experiments, the number of bacteria per microliter for *M.smegmatis* and *M.bovis BCG* stocks was calculated. The bacterial stocks kept at -80^{o}C were thawed at room temperature and sonicated using Transsonic T480, CAMLAB for 15 seconds to break bacterial clumps. The bacteria were mixed uniformly by vortexing for 5-10 seconds and serial 10-fold dilutions of stock *M.smegmatis* and *M.bovis BCG* were prepared until $1x10^{-8}$ dilution in LB broth or 7H9 respectively. Finally, 200µl of bacterial suspension was plated from dilution 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} on agar plates (LB-agar for *M.smegmatis* and 7H10 for *M.bovis BCG*) in triplicates. The plates were incubated at 37°C for 72 hours for *M.smegmatis* and 2-3 weeks for *M.bovis BCG*. Dilution plates with countable

number of bacterial colony forming units (CFUs) were enumerated with naked eye and the number of bacteria per ml was calculated for the stock by using the following equation

$$\frac{\textbf{\textit{Average number of CFUs from triplicate plates}}}{\textbf{\textit{Volume plated (ml) x Dilution factor used for plating}}}$$

2.2 Direct mycobacterial growth inhibition assays using different test compounds

To investigate *in vitro* the direct effect of different test compounds (proteins and non-proteins) on the growth of *M.smegmatis* and *M.bovis BCG*, direct growth inhibition assays were performed *in vitro*. Stock *M.smegmatis* or *M.bovis BCG* stored at -80°C with known number of bacteria per µl was thawed at room temperature, sonicated for 15 seconds to break any bacterial clumps and mixed uniformly by vortexing for 5 seconds. Stock bacteria were serially diluted, starting with an initial 1:100 (10^{-2}) dilution prepared by adding 10µl of stock bacteria to 990µl broth media (LB or 7H9) in an eppendorf tube. After the initial 1:100 (10^{-2}) dilution, 10-fold serial dilution was performed by transferring 100µl of bacterial suspension from 10^{-2} dilution to 900µl of broth media (LB or 7H9) for making10^{-3} dilution and so on. The process of 10-fold serial dilution was repeated until the dilution that gave countable number of CFUs on agar plates was achieved as shown in figure 2.2a

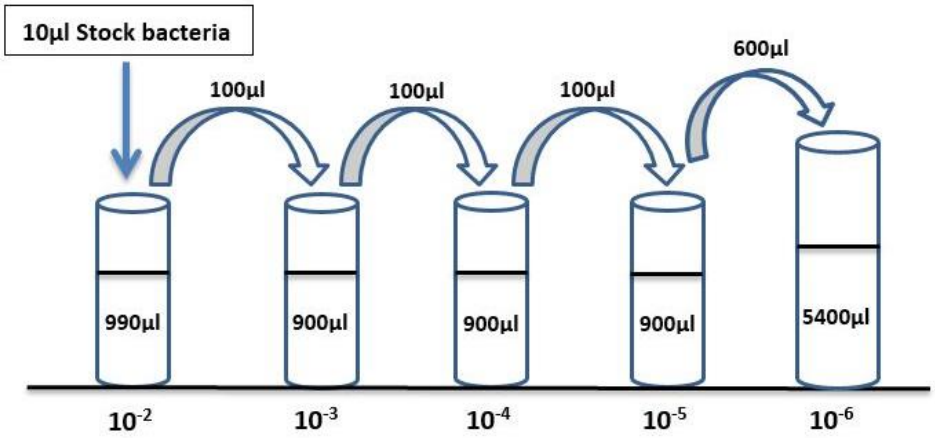


Figure 2.2a: Serial dilution of stock bacteria (*M.smegmatis* and *M.bovis BCG*). Stock bacteria was serially diluted starting from an initial 1:100 (10^{-2}) dilution followed 10-fold serial dilution afterwards till 10^{-6} dilution was achieved.

The bacteria at 10^{-6} dilution were used in the direct *in vitro* assays to find the effect of test compounds on mycobacterial growth. A set of properly labelled eppendorf tubes, each containing 1ml of bacterial suspension at dilution 10^{-6} were prepared. Different concentrations of test compound were added to the tubes. A suitable solvent control (solvent for test compound) was also included in the experiment as shown in figure 2.2b. The eppendorf tubes were then incubated at 37°C for 2 hours and the contents were mixed after every 15 minutes by inverting each tube 5 times. After incubation, the eppendorf tubes were vortexed for 5 seconds and bacteria from each condition were plated on agar plates (LB-agar plates for *M.smegmatis* or 7H10 plates for *M.bovis BCG*) in triplicates by using 200µl of bacterial suspension. The agar plates were incubated at 37°C for 72 hours for *M.smegmatis* and 2-3 weeks for *M.bovis BCG* after which the number of bacterial CFUs was counted with the naked eye. A comparison of CFUs number between different test conditions and the solvent control was done to determine the effect of the test compound on the growth of bacteria *in vitro*.

Additional experiments to determine the effect of increase in incubation time on the growth inhibitory concentration of PAF C-16 against *M.bovis BCG* and *M.smegmatis* were performed in a similar manner as described above, however the treatment times were increased to 6, 12 and 24 hours.

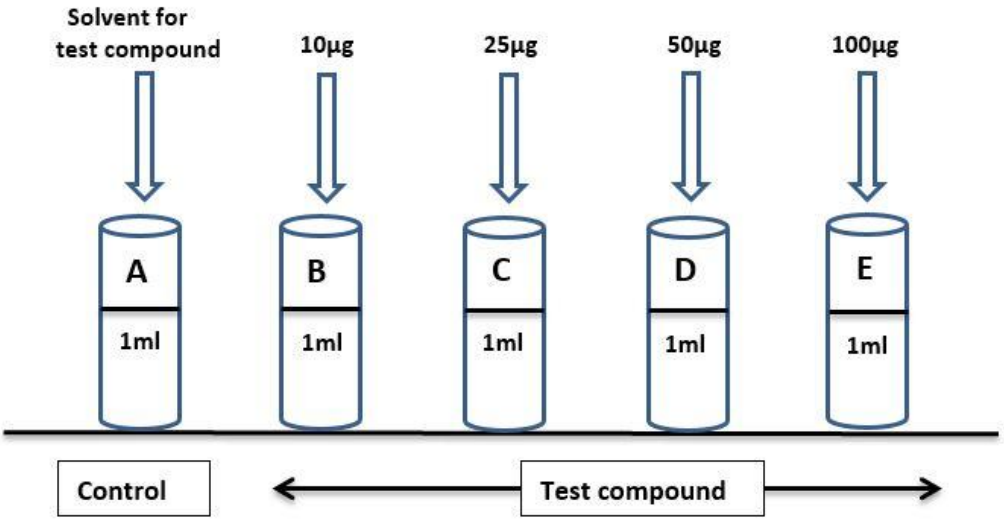


Figure 2.2b: Diagrammatic representation of experimental setup for one of the direct growth inhibition assays. All the tubes contain 1ml of bacterial suspension in broth media (LB or 7H9)

at 10^{-6} dilution. 10µl ethanol (solvent for test compound) was added to tube A, whereas, test compound at concentrations of 10µg, 25µg, 50µg and 100µg was added to tube B, C, D and E respectively.

2.3 Microscopy with PAF C-16 treated *mycobacteria*

In order to investigate the effect of PAF C-16 treatment on *M.smegmatis* and *M.bovis BCG* microscopy was performed. The protocols are given as under:

2.3.1 Light Microscopy with PAF C-16 treated *M.smegmatis* and *M.bovis BCG*

The effect of PAF C-16 treatment on *M.bovis BCG* and *M.smegmatis* cell membrane was examined under a light microscope using cell viability dye, trypan blue. Approximately $1x10^8$ *M.smegmatis* or *M.bovis BCG* were transferred to appropriately labelled 1.5 ml eppendorf tubes and washed once with phosphate buffer saline (PBS) by centrifugation at 5000rpm for 10 minutes. The bacteria were then resuspended in 1ml of broth media (LB or 7H9). 100µg/ml PAF C-16 was added to the bacterial suspension in "Test" eppendorf tube and 10µl/ml ethanol (solvent control) was added to the bacteria in the "Control" eppendorf tube. The tubes were incubated for 4 hours at 37°C with mixing after every 15 minutes. After incubation, the bacterial samples were washed twice with PBS at 5000rpm for 10 minutes. The supernatant was discarded and the pellets were resuspended in 20µl PBS and 20µl of 0.4% trypan blue (Gibco, UK). Slides were prepared by transferring 10µl of the mycobacterial suspension from each condition onto clean glass slides. The glass slides with stained bacteria were covered with glass coverslips, sealed and observed under a light microscope (Brunel SP100) at 400X magnification and images were taken for PAF C-16 treated and solvent control *mycobacteria*.

2.3.2 Fluorescence Microscopy with PAF C-16 treated *M.smegmatis* and *M.bovis BCG*

Propidium Iodide (PI), a nucleic acid binding fluorescent dye was used to detect damage to the mycobacterial cell membrane. Mid-logarithmic cultures for both *M.bovis BCG* and *M.smegmatis* at 0.90-1.00 $O.D_{(600nm)}$ were used for fluorescence microscopy. 1ml bacteria from fresh grown cultures was transferred to properly labelled eppendorf tubes. The bacteria were pelleted by centrifugation at 5000rpm for 10 minutes. The supernatant was removed and the bacterial pellets were washed by resuspending in PBS and centrifugation at 5000rpm for 10 minutes. After washing the bacterial pellets were resuspended in 1ml broth media (LB or 7H9) and "Test" and "Solvent control" bacteria were treated with 100µg PAF C-16 and 10µl ethanol per ml bacterial suspension respectively for 2 hours at 37°C with mixing after every 15 minutes.

A positive control comprising of heat treated bacteria (100ºC for 10 minutes) was also included in the experimental design. After incubation for 2 hours, the mycobacterial samples were washed twice with PBS at 5000rpm for 10 minutes to remove the residual PAF C-16 and ethanol. This was followed by staining with PI (BioLegend, USA) at a concentration of 1µg/ml for 20 minutes at room temperature in dark. Excess dye was removed by washing the bacterial samples twice with PBS by centrifugation at 5000rpm for 10 minutes. The bacterial pellet was finally resuspended in 100µl of PBS, and slides were prepared by smearing 5-10µl of the bacterial suspension onto a glass slide overlay with a coverslip and sealed. Images of the PAF C-16 treated and solvent control *mycobacteria* were acquired at 400X magnification with a Leica DM4000® fluorescence microscope using bright field channel to focus living and dead/injured bacteria and CY3 channel to detect the red fluorescence from the PI indicating bacteria with damaged cell membranes.

2.4 Flow cytometry analysis of PAF C-16 treated *M.smegmatis and M.bovis BCG*

For flow cytometry, 1ml bacteria was transferred from fresh grown cultures of *M.bovis BCG* and *M.smegmatis* at 0.90-1.00 $O.D_{(600nm)}$ into properly labelled eppendorf tubes. The bacteria was washed once with PBS by centrifugation at 5000rpm for 10 minutes and resuspended in 1ml broth media (LB or 7H9). The test *mycobacteria* was treated with PAF C-16 at a concentration of 100µg/ml and to the solvent control 10µl ethanol per ml bacterial suspension (solvent for PAF C-16) was added. The eppendorf tubes were incubated for 2 hours at 37°C with mixing after every 15 minutes. A positive control comprising of heat treated *mycobacteria* at 100ºC for 10 minutes was also included in the experimental design. After incubation, the mycobacterial samples were washed twice with PBS by centrifugation at 5000rpm for 10 minutes to remove residual chemicals. Test and control bacteria were then stained with 1µg/ml PI (BioLegend, USA) for 20 minutes at room temperature and excess dye was removed by washing the bacteria twice with PBS at 5000rpm for 10 minutes. The bacterial pellets were finally resuspended in 250µl of PBS. The damage to the bacterial cell membrane in test and control bacteria was quantified by performing flow cytometry using ACEA NovoCyte® Flow Cytometer. First, 20,000-25,000 untreated and unstained bacteria were acquired as a compensation. In the second step 20,000-25,000 bacteria were acquired for each solvent control, heat treated positive control and 100µg/ml PAF C-16 treated conditions. The data was plotted using NovoExpress® software and analyzed for membrane damage.

2.5 Assays to neutralize PAF C-16 induced direct growth inhibition of *M.smegmatis*

Assays were performed to detect the neutralizing effect of a number of compounds including PAF receptor antagonists (ABT0491 and WEB-2086), dexamethasone, benzenesulfonamide, alpha-tocopherol, ascorbic acid, tween-80 and tween-20 on PAF C-16 induced mycobacterial growth inhibition *in vitro* using a similar protocol. In this section, the protocol for experiments to determine the effect of PAF receptor antagonists (ABT0491) on PAF C-16 induced *M.smegmatis* growth inhibition is described.

M.smegmatis stock at -80°C was thawed, sonicated for 15 seconds to break any clumps and mixed uniformly by vortexing for 5 seconds. Serial dilution of stock *M.smegmatis* was performed according to the protocol mentioned in section 2.2., 1ml of *M.smegmatis* suspensions at 10^{-6} dilution were transferred to properly labelled eppendorf tubes. *M.smegmatis* in eppendorf tubes labelled "E" and "F" was first incubated with 100µg/ml PAF receptor antagonist (ABT-491) for 1 hour at 37°C with mixing at 15 minutes intervals. After 1 hour PAF C-16 at a concentration of 50µg/ml and 100µg/ml was added to the eppendorf tubes E and F respectively, and the tubes were incubated at 37°C for another 2 hours with mixing after every 15 minutes. Individual controls comprising of *M.smegmatis* treated with 20µl/ml ethanol (solvent of test chemicals) (eppendorf tube A), 50µg/ml PAF C-16 (eppendorf tube B), 100µg/ml PAF C-16 (eppendorf tube C) and 100µg/ml PAF receptor antagonist (ABT-491) (eppendorf tube D) were also included in the experiment (Figure 2.5). After incubation, 200µl/ml of *M.smegmatis* suspension from all the tubes was plated on LB agar plates in triplicates. The plates were incubated for 72 hours at 37°C for the colonies to appear and the number of CFUs was counted by naked eye and test were compared to controls.

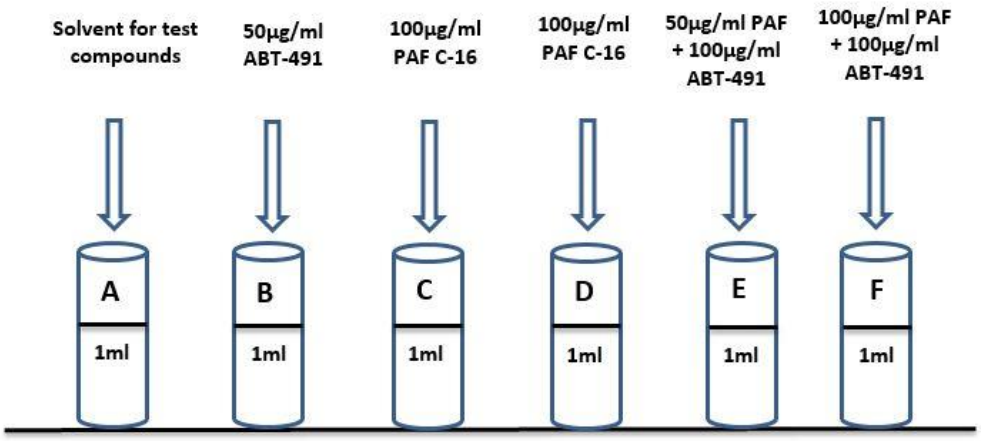


Figure 2.5: Diagrammatic representation of experimental setup for effect of PAF receptor antagonist ABT-491 on PAF C-16 induced growth inhibition. All the tubes contain 1ml of *M.smegmatis* suspended in LB media. 20µl/ml ethanol (solvent for test compounds) was added to tube A, 50µg/ml PAF C-16 was added tube B, 100µg/ml PAF C-16 was added to tube C and 100µg/ml PAF receptor antagonist (ABT-491) was added to tube D. The M.smegmatis in tube E and F were first treated with 100µg/ml PAF receptor antagonist (ABT-491) for 1 hour followed by treatment with 50µg/ml and 100µg/ml PAF C-16 respectively for another 2 hours.

2.6 Growing and Storing human monocytes derived THP-1 cells

Human monocytes derived THP-1 cells (ATCC® TIB-202™) were used as model phagocytic cells. THP-1 cells were grown in complete RPMI media at 37°C using an incubator with 5% CO_2 supply. The complete RPMI was prepared by adding 10% fetal bovine serum (FBS) (HyClone, UK), 1% sodium pyruvate (Sigma Aldrich, UK), 1% L-glutamine (Sigma Aldrich, UK) and 1% penicillin/streptomycin (Sigma Aldrich, UK) to RPMI-1640 cell culture medium (Sigma Aldrich, UK).

2.6.1 Thawing and culturing THP-1 cells

THP-1 cells were grown from frozen stocks kept in liquid nitrogen. THP-1 cryotubes containing $5x10^6$ cells/ml were thawed on ice. The defrosted THP-1 cells were quickly transferred to 10ml of RPMI media and washed twice by centrifugation at 1500rpm for 10 minutes each to remove the DMSO from the freezing mixture. The cell pellet was finally resuspended in 10ml complete RPMI and the number of viable THP-1 cells was counted using

trypan blue staining. For trypan blue staining 20µl of THP-1 cell suspension was mixed with 20µl of 0.4% trypan blue stain (Gibco, UK). From this mixture, 10µl was loaded on a haemocytometer and the viable THP-1 cells were counted by viewing them at 400X using an inverted microscope (Leica,DI ML). After determining the number of viable THP-1 cells, the cells were transferred to a cell culture flask and incubated at 37°C in a CO_2 incubator (MCO-18AC-PE IncuSafe, Panasonic) at 5% CO_2 supply.

The viability of cultured THP-1 cells was checked the following day by viewing them under a microscope. THP-1 cells that appeared to be round and shiny were considered alive. The THP-1 cells were fed every 3 days by removing half of the culture media and replacing it with fresh complete RPMI. When necessary the THP-1 cells were split to keep the cell density at $0.5x10^6$/ml.

2.6.2 Storing THP-1 cells

To store THP-1 cells for future experiments, about 25ml of THP-1 cells were grown to a density of $1\text{-}1.5x10^6$/ml. The THP-1 cell suspension was then centrifuged at 1500rpm for 10 minutes. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in 5 ml freezing solution (10% DMSO in FBS) such that the final number of THP-1 cells per ml of freezing solution was $5x10^6$. 1ml of THP-1 cell suspension in freezing solution was transferred to cryotubes and the cryotubes were quickly moved to -80^{o}C freezer (New Brunswick, U41085). After 24 hours at -80°C, the frozen THP-1 cells cryotubes were transferred to liquid nitrogen for long-term storage.

2.7 Intracellular growth inhibition assays for *mycobacteria* inside THP-1 cells using different test compounds

Intracellular mycobacterial growth inhibition assays were performed to investigate the effect of test compounds such as PAF C-16 and different PAF C-16 structure analogues on the growth of phagocytosed *mycobacteria* (*M.smegmatis* and *M.bovis BCG*) inside THP-1 cells.

2.7.1 Infecting THP-1 cells with *mycobacteria*

About 20ml of THP-1 cells grown to a density of $0.5\text{-}0.75x10^6$/ml in culture flasks were transferred to a 50ml falcon tube and centrifuged at 1500rpm for 10 minutes. The supernatant was discarded and the cell pellet was washed twice by resuspending in plain RPMI media and centrifugation at 1500rpm for 10 minutes. The cell pellet was finally resuspended in 15ml

complete RPMI without antibiotics and the number of cells per ml was counted using a haemocytometer. From the THP-1 cells suspension, $0.25x10^6$ were transferred to properly labelled eppendorf tubes and the final volume in each tube was made 1ml by adding complete RPMI without antibiotics.

Stock *M.smegmatis* or *M.bovis BCG* was sonicated for 15 seconds to break any clumps and mixed uniformly by vortexing. The stock bacteria was diluted by 10-fold in plain RPMI and $1.25x10^6$ bacteria were incubated with THP-1 cells in a ratio 5:1 (bacteria : THP-1 cell) at 37°C in a CO_2 incubator for 2 hours to allow THP-1 cells to phagocytose the bacteria (Figure 2.7.1).

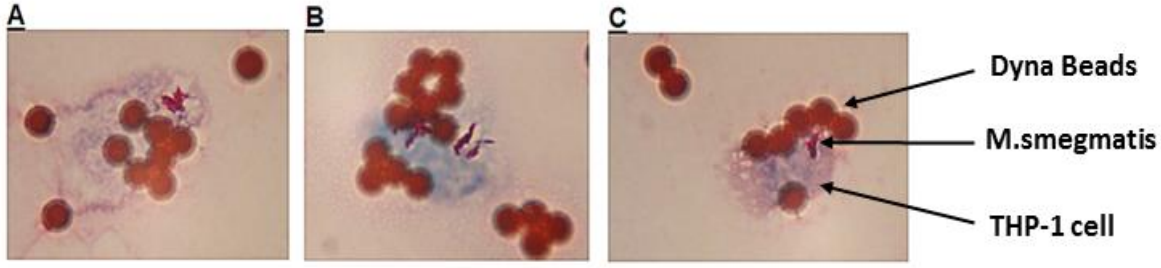


Figure 2.7.1: Acid fast staining for phagocytosed *mycobacteria* inside THP-1. The microscopic images show *M.smegmatis* (red rods) inside the THP-1 cells after 2 hours incubation. The magnetic beads used for isolation of THP-1 cells can be seen attached to the THP-1 cells as well.

2.7.2 Dyna beads™ preparation

Dyna beads™ Pan Mouse IgG (Thermo Fisher Scientific, UK) attached to anti-human MHC Class I antibody were used to remove non-phagocytosed extracellular *mycobacteria* from THP-1 cells after incubation. The beads were prepared by taking 12µl ($5x10^6$ beads) anti-mouse IgG coated magnetic beads from stock ($4x10^8$ beads/ml) and incubating with 6µl (1mg/ml stock concentration) mouse anti-human MHC Class I antibody (W6/32 against HLA A, B& C) (BioLegend, USA) for 90 minutes on ice to allow binding of W6/32 to the beads. Finally the beads were washed with plain RPMI twice by applying a Dynal® magnet (Thermo Fisher Scientific, UK), resuspended in 210µl plain RPMI and stored on ice.

2.7.3 Binding of W6/32 coated Dyna beads with THP-1 cells

After incubation of bacteria with THP-1 cells (section 2.7.1) for 2 hours to allow phagocytosis, 50µl ($1x10^6$) of W6/32 coated Dyna beads were added to each eppendorf tube in a ratio of 4:1 (Beads : THP-1 cells). The eppendorf tubes were then buried horizontally in ice and kept in a

shaker for 45 minutes to allow the attachment of Dyna beads to the THP-1 cells. After incubation, extracellular *mycobacteria* was removed by applying a Dynal® magnet. Dyna beads attached to THP-1 cells with intracellular *mycobacteria* migrated to a side and the supernatant along with extracellular bacteria was removed as illustrated in figure 2.7.2. The washing of extracellular *mycobacteria* was repeated three times with plain RPM by applying a Dynal® magnet and finally the THP-1 cell with intracellular *mycobacteria* were resuspended in 1ml complete RPMI without antibiotic.

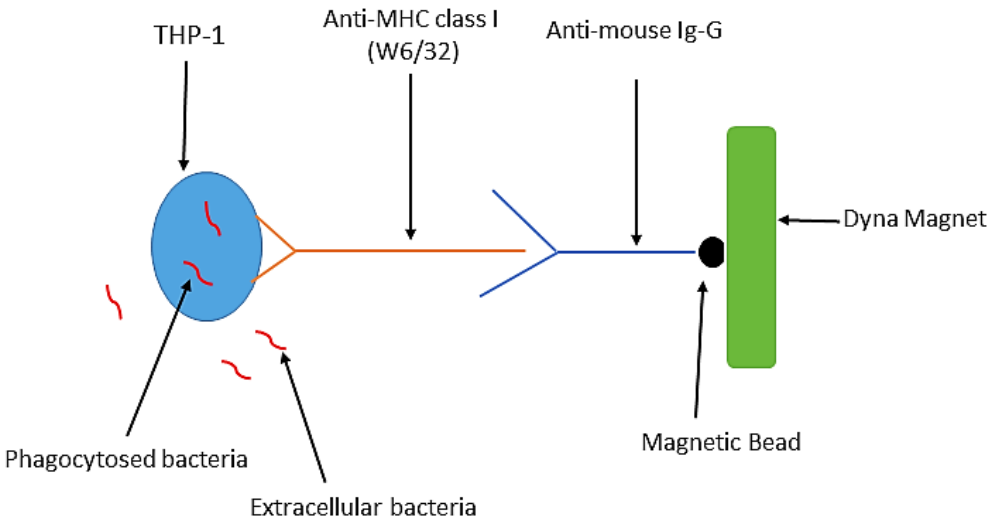


Figure 2.7.2: Separation of THP-1 cells by magnetic beads. W6/32 coated Dyna beads were used to isolated THP-1 cells having intracellular *mycobacteria* by binding to MHC class I on the THP-1 surface and applying a magnet.

2.7.4 Treating *mycobacteria* infected THP-1 cells with test compound

Test compounds such as PAF C-16 or PAF structure analogues were added to the eppendorf tubes containing THP-1 cells with intracellular *mycobacteria* in different concentrations. A solvent control for test compound was also included in the experiment and all the eppendorf tubes were incubated at 37°C in a CO_2 incubator for 24 hours.

2.7.5 Lysing THP-1 to release intracellular *mycobacteria*

After 24 hours incubation, Dynal® magnet was applied eppendorf tubes containing THP-1 cells with intracellular *mycobacteria*. The Dyna beads attached to THP-1 cells migrated to the side of eppendorf tube. The supernatant was transferred to appropriately labelled 15ml falcon tubes

to collect any extracellular bacteria. The THP-1 cells attached Dyna bead in the eppendorf tubes were lysed by adding 1ml of 1% saponin solution (Fisher Scientific, UK) and vortexing the mixture for 10 minutes. After vortexing the cell lysate for each condition was transferred to the respective 15ml falcons already containing 1ml of bacterial supernatant previously collected for each condition. The contents of each falcon tube were mixed by vortexing for 5 seconds and serial dilutions (10^{-1}, 10^{-2} and 10^{-3}) were prepared in sterile water.

2.7.6 Plating bacteria from cell lysates

Bacterial suspensions at dilutions of 10^{-2} and 10^{-3} were used for platting. 200µl of bacterial suspension was plated for each experimental condition in triplicates using LB-agar or 7H10 plates. Plates were incubated at 37°C for 72 hours in case of *M.smegmatis* and 2-3 weeks for *M.bovis BCG* after which the number of bacterial CFUs were enumerated. A comparison of CFUs number between different test plates and the solvent control was done to determine the effect of the test compound on the growth of intracellular *mycobacteria* inside THP-1 cells.

2.8 Flow cytometry to detect apoptosis in PAF C-16 treated THP-1 cells

About 20ml of THP-1 cells grown to a density of 0.5-0.75x10^6/ml in culture flasks were transferred to a 50ml falcon tube and pelleted by centrifugation at 1500rpm for 10 minutes. The supernatant was discarded and the cells were washed twice by resuspending them in plain RPMI media and centrifugation at 1500rpm for 10 minutes. Finally the cells pellet was resuspended in complete RPMI media and the number of THP-1 cells per ml was counted using a haemocytometer.

Approximately 0.5x10^6 THP-1 cells were transferred to properly labelled eppendorf tubes and the final volume in each tube was made 1ml by adding complete RPMI. PAF C-16 at a concentration of 1µg/ml was added to eppendorf tube labelled "Test", 2µl/ml ethanol (solvent control for PAF C-16) was added to the THP-1 cells in the eppendorf tube labelled "Solvent control" and incubated at 37°C for 24 hours in a CO_2 incubator. THP-1 cells treated with 1µM staurosporine for 4 hours were included as positive control for apoptosis.

FITC Annexin V Apoptosis Detection Kit with PI (BioLegend, USA) was used to detect apoptosis in 1µg/ml PAF C-16, 2µl/ml ethanol and 1µM staurosporine treated THP-1 cells according to the manufacturer's protocol.

After incubation with test compounds, THP-1 cells were washed twice with PBS by centrifugation at 1500rpm for 10 minutes to remove any residual PAF C-16, ethanol or staurosporine. After washing the cells were resuspended in 1ml of Annexin V binding buffer. About 100µl of cell suspension was transferred to new eppendorf tubes and incubated with 5µl of Annexin V conjugated FITC and 2µl Propidium Iodide at room temperature for 15 minutes. Finally 400µl of Annexin V binding buffer was added to each tube and analyzed for apoptosis by performing flow cytometry. 20,000 THP-1 cell were acquired for test and control conditions using ACEA NovoCyte® Flow Cytometer. The data was plotted using NovoExpress® software and PAF C-16 treated THP-1 cells were compared with controls to detect PAF C-16 induced apoptosis.

2.9 Flow cytometry to detect apoptosis in PAF C-16 treated THP-1 cells having intracellular *M.smegmatis*

M.smegmatis infected THP-1 cells treated with PAF C-16 were analyzed for apoptosis using flow cytometry according to the following protocol:

2.9.1 Infection of THP-1 cells with *M.smegmatis*

About 20ml of THP-1 cells grown to a density of 0.5-0.75x10^6/ml in culture flasks were transferred to a 50ml falcon tube and pelleted by centrifugation at 1500rpm for 10 minutes. The supernatant was discarded and the cell pellet was washed twice by resuspending in plain RPMI media and centrifugation at 1500rpm for 10 minutes. Finally the THP-1 cells were resuspended in complete RPMI without antibiotics and the number of cells per ml was counted using a haemocytometer. About 0.5x10^6 THP-1 cells were transferred to properly labelled eppendorf tubes and the final volume in each tube was made 1ml by adding complete RPMI without antibiotics.

Stock *M.smegmatis* with known number of bacteria per µl was used for infecting THP-1 cells. Approximately 2.5x10^6 *M.smegmatis* were added to THP-1 cells in a ratio 5:1 (bacteria : THP-1 cell) and incubated at 37°C in a CO_2 incubator for 2 hours to allow phagocytosis of *M.smegmatis* by THP-1 cells. After 2 hours the non-phagocytosed *M.smegmatis* were removed by washing the THP-1 cells three times with plain RPMI at 1000rpm for 5 minutes each.

2.9.2 Treatment of *M.smegmatis* infected THP-1 cells with PAF C-16

M.smegmatis infected THP-1 cells were then treated with 1µg/ml PAF C-16 for 24 hours in a CO_2 incubator at 37°C. Different controls comprising of THP-1 cells only, *M.smegmatis* infected THP-1 cells treated with 2µl/ml (solvent control for PAF C-16) and THP-1 cells treated with 1µM staurosporine for 4 hours were also included in the experiment.

2.9.3 Staining of THP-1 cells

Apoptosis was detected in PAF C-16 treated test and control THP-1 cells by using FITC Annexin V Apoptosis Detection Kit with PI (BioLegend, USA). THP-1 cells were washed twice with PBS by centrifugation at 1500rpm for 10 minutes to remove any residual PAF C-16, ethanol or staurosporine and resuspended in 1ml of Annexin V binding buffer. About 100µl of cell suspension was transferred to new eppendorf tubes and incubated with 5µl of Annexin V conjugated FITC and 2µl PI for 15 minutes at room temperature. 400µl of Annexin V binding buffer was added to each tube and analyzed for apoptosis by performing flow cytometry.

2.9.4 Flow cytometry

20,000 THP-1 cell were acquired for test and control conditions using ACEA NovoCyte® Flow Cytometer. The data was plotted using NovoExpress® software and PAF C-16 treated THP-1 cells were compared with controls to detect PAF C-16 induced apoptosis.

2.10 Investigating the effects of PAFR antagonists, iNOS inhibitors, phospholipases inhibitors and cytokine neutralizing antibodies on PAF C-16 induced intracellular *M.smegmatis* growth inhibition

A number of compounds including PAF C-16 receptor antagonists (ABT-491 and WEB-2086), iNOS inhibitor (Aminoguanidine hemisulfate), phospholipase C inhibitor (U-73122), phospholipase A_2 inhibitor (Benzenesulfonamide) and neutralizing antibodies for IL-6, IL-10 and TNF-α were used to investigated their effect on PAF C-16 induced intracellular *M.smegmatis* growth inhibition.

Assays with these compounds were carried out according to the protocol mentioned in section 2.7 however, the only difference was in the treatment step. In the current assays *M.smegmatis* infected THP-1 cells were treated with test compounds mentioned above for 1 hour prior to treatment with PAF C-16. After one 1 hour, PAF C-16 (1µg/ml) was added to the cells and were incubated for 24 more hours at 37°C in a CO_2 incubator. An additional control comprising of *M.smegmatis* infected THP-1 cells treated with test compound only was also included in the experimental design.

2.11 Studying the effect of PAF C-16 treatment on THP-1 cells phagocytosis

Phagocytic assays were performed to check the effect of PAF C-16 on the uptake of *M.smegmatis* by THP-1 cells according to the following protocol:

2.11.1 Treating THP-1 cells with PAF C-16

About 20-25ml THP-1 cells grown in culture flasks to a cell density of 0.5-0.75x10^6/ml were transferred to a 50ml falcon tube and centrifuged at 1500rpm for 10 minutes. The supernatant was discarded and the cell pellet was washed twice by resuspending in plain RPMI and centrifugation at 1500rpm for 10 minutes. The cell pellet was finally resuspended in 15ml complete RPMI without antibiotics and the number of THP-1 cells per ml was counted using a haemocytometer. From the THP-1 cells suspension, 1x10^6 cells were transferred to properly labelled eppendorf tubes and the final volume in each tube was made 1ml by adding complete RPMI without antibiotics.

PAF C-16 in different concentrations (0.01µg, 0.1µg, 1µg and 5µg/ml) was added to the eppendorf tubes containing 1x10^6 THP-1 cells. A solvent control (1µl ethanol/ml) was also included in the experimental design. All the eppendorf tubes were incubated at 37°C in a CO_2 incubator for 2 hours with gentle mixing after every 20 minutes. After incubation PAF C-16 was removed by washing THP-1 cells with plain RPMI three times at 1500rpm for 10 minutes each. The THP-1 cells were then resuspended in complete RPMI without antibiotics and the number of THP-1 cells for all PAF C-16 treated conditions and solvent control was counted using a haemocytometer. Finally, 0.25x10^6 THP-1 cells were transferred into new labelled eppendorf tubes and the final volume in each tube was made 1ml by adding complete RPMI without antibiotics.

2.11.2 Infecting THP-1 cells with *M.smegmatis*

Stock *M.smegmatis* was sonicated for 15 seconds to break any clumps and mixed uniformly by vortexing. The bacteria was then diluted by 10-fold in plain RPMI and 1.25x10^6 *M.smegmatis* was added to THP-1 cells in each eppendorf tube in a ratio of 5:1 (*M.smegmatis* : THP-1 cell). The eppendorf tubes with THP-1 cell and *M.smegmatis* were incubated at 37°C in a CO_2 incubator for 2 hours to allow phagocytosis of *M.smegmatis* by THP-1 cells.

2.11.3 Dyna beads™ preparation

Dyna beads™ Pan Mouse IgG (Thermo Fisher Scientific, UK) attached to anti-human MHC Class I antibody were used to remove non-phagocytosed extracellular *M.smegmatis* from THP-1 cells after incubation. The beads were prepared by taking 16µl (5x10^6 beads) anti-mouse IgG

coated magnetic beads from stock ($4x10^8$ beads/ml) and incubating with 8µl (1mg/ml stock concentration) mouse anti-human MHC Class I antibody (W6/32 against HLA A, B& C) (BioLegend, USA) for 90 minutes on ice to allow binding of W6/32 to the beads. Finally the beads were washed with plain RPMI twice by applying a Dynal® magnet (Thermo Fisher Scientific, UK), resuspended in 270µl plain RPMI and stored on ice.

2.11.4 Binding of W6/32 coated Dyna beads with THP-1 cells

After incubating *M.smegmatis* with THP-1 cells for 2 hours to allow phagocytosis (section 2.11.2), 50µl ($1x10^6$) of W6/32 coated Dyna beads were added to each eppendorf tube in a ratio of 4:1 (Beads : THP-1 cells). The eppendorf tubes were then buried horizontally in ice and kept in a shaker for 45 minutes to allow the binding of Dyna beads to THP-1 cells. After incubation, extracellular bacteria was removed by applying a Dynal® magnet. Dyna beads attached to THP-1 cells with intracellular *M.smegmatis* migrated to a side and the supernatant along with extracellular bacteria was removed. The washing of extracellular bacteria was repeated three times with plain RPM by applying a Dynal® magnet.

2.11.5 Lysing THP-1 with phagocytosed *M.smegmatis*

THP-1 cells with phagocytosed *M.smegmatis* were finally resuspended in 1% saponin solution (Fisher Scientific, UK) and lysed by vortexing for 10 minutes. After vortexing the cell lysate for different PAF C-16 treated and solvent control conditions were serially diluted (10^{-1} and 10^{-2} dilutions) in sterile water.

2.11.6 Plating *M.smegmatis* from cell lysates

M.smegmatis suspensions at dilutions 10^{-1} and 10^{-2} were used for platting. 200µl of bacterial suspension PAF C-16 treated and solvent control conditions was plated in triplicates using LB-agar plates. Plates were incubated at 37°C for 72 hours after which the number of *M.smegmatis* CFUs were enumerated. A comparison of CFUs number between different PAF C-16 treated and the solvent control plates was done to determine the effect of PAF C-16 treatment on the phagocytosis of *M.smegmatis* by THP-1 cells.

2.12 Determining the Minimum Inhibitory Concentration (MIC) for Platelet Activating Factor (PAF C-16) against *M.smegmatis*

The minimum inhibitory concentration is a qualitative experiment to determine lowest concentration of PAF C-16 required to inhibit the growth of *M.smegmatis.*

First sterile glass culture tubes were labelled properly. 4 ml of LB broth was added to the first tube labelled "100µg" and 2ml LB broth each was added to the rest of the tubes labelled "50µg, 25µg, 12.5µg, 6.25µg, 3.12µg, 1.5µg and 0.78µg" respectively. To the first tube labelled "100µg", PAF C-16 was added at a final concentration of 100µg/ml and mixed by vortexing. 2-fold serial dilution was performed for PAF C-16 by transferring 2ml media from the 100µg tube to the next tube labelled "50µg". This process was repeated until PAF C-16 concentration of 0.78µg/ml was achieved. From the last tube 2ml LB was discarded so that at the end each tube had a final volume of 2ml. A solvent control tube containing 2ml of LB and ethanol (10µg/ml solvent for PAF C-16) was also included in the experiment.

Stock *M.smegmatis* was sonicated for 15 seconds to break any clumps and mixed uniformly by vortexing. About $1.0x10^6$ *M.smegmatis* was added to each tube and the tubes were kept in a shaker incubator at 37°C for 48 hours. After 48 hours the culture tubes were taken out and examined with naked eye. Cloudiness of the growth media in the tubes indicated bacterial growth were as the clear tubes indicated effective bacterial control. The minimum inhibitory concentration was found to lie between last tube with clear media and the first tube with cloudiness. After visual inspection, optical density $O.D_{(600)}$ was also calculated for each tube.

2.13 Statistical Analysis

For bacterial growth inhibition assays, all the experiments were performed in triplicates and repeated 3-6 times. The data were expressed as mean ± SEM where "solvent control" was considered 100% bacterial survival and different test condition were compared to it. GraphPad Prism® software (Version 5.01) was used to determine the level of significance. Non-parametric multiple comparison Kruskal-Wallis test was applied on ranks and individual datasets were compared by performing post hoc Dunn's multiple comparison test. For comparison of two particular data sets non-parametric Mann Whitney test was used.

p-value of less than or equal to 0.05 ($p \leq 0.05$) was considered to be significant. The *p*-values were represented on the graphs with an asterisk (*) and different denotations are shown in table 1.2:

Table 1.2: Denotations used for *p*-value on graphs.

***p*-value**	**Denotation**
0.01 to 0.05	*
0.001 to 0.01	**
< 0.001	***

Chapter 3: *In vitro* screening of different endogenous factors (proteins and non-proteins) for their inhibitory effect on mycobacterial growth

3.1 Introduction

M.tb infection of the host elicits localized inflammation in the lungs, resulting in the migration of different immune cells and leakage of plasma protein and non-protein factors at the site of infection (Sherwood and Toliver-Kinsky, 2004; Toossi, 2001). In addition, a number of host factors including phospholipids such as PAF C-16 (Camussi *et al*., 1987) and proteins such as C1q (Loos *et al*., 1989) can also be synthesized by immune cells such as macrophages, which are present at the site of infection. These host factors are likely to come in direct contact with the bacterial pathogen and immune cells and thus, may modulate the outcome of the infection. The direct effect of most these factors on *M.tb* growth is either poorly understood or completely unknown and therefore, needs further investigation.

In this chapter, a number of endogenously produced host factors (proteins and non-proteins) were screened *in vitro* for their ability to directly inhibit the growth of *mycobacteria* using *M.smegmatis* (mc^2 155) and *M. Bovis BCG* (Pasteur 1173P2) as model organisms for *M.tb*. All the compounds were first screened against the fast-growing *M.smegmatis*. Compounds that directly inhibited the growth of *M.smegmatis* were then tested against a slow-growing more similar *M.tb* model, *M.bovis BCG* during the second phase of screening.

Different protein and non-protein factors investigated for their direct growth inhibitory effect against *mycobacteria* in this study included:

a) Transferrin
b) Lactoferrin
c) Fibrinogen
d) C-Reactive Protein (CRP)
e) Alpha-2-Macroglobulin (α2M)
f) Vitronectin
g) Plasminogen
h) Low-Density Lipoprotein (LDL)
i) High-Density Lipoprotein (HDL)
j) Serotonin
k) L-Alpha Dipalmitoylphosphatidylcholine (DPPC)
l) Platelet Activating Factor C-16 (PAF C-16)

Aim:

1) Screening a number of endogenously produced host protein and non-protein factors *in vitro* for their direct growth inhibitory effect against *mycobacteria*.

3.2 Direct effect of Transferrin on *M .smegmatis* growth *in vitro*

Transferrin is a blood plasma glycoprotein that binds iron and transports it to different parts of the body via the bloodstream (Gkouvatsos *et al*., 2012). This iron binding capacity of transferrin plays an important role in body's defence mechanism against invading microorganisms. Transferrin restricts the supply of free iron to different invading pathogens and discourages microbial infections (Cassat and Skaar, 2013), hence making it a good candidate to be screened *in vitro* for its direct effect on the growth of *mycobacteria*.

Three individual experiments were performed using different concentrations (10µg, 25µg, 50µg and 100µg/ml) of transferrin and a solvent control for transferrin (100µl water/ml bacterial suspension) according to the protocol mentioned in section 2.2. All the three experiment of transferrin with *M.smegmatis* showed the same trend (Figure 3.2b) and it was observed that transferrin protein in concentrations of up to 100µg/ml showed no inhibitory effect on the growth of *M.smegmatis* as indicated by the similar number of colony forming units (CFUs) for transferrin treated and solvent control *M.smegmatis* (Figure 3.2a, 3.2b and 3.2c). Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (p=0.77).

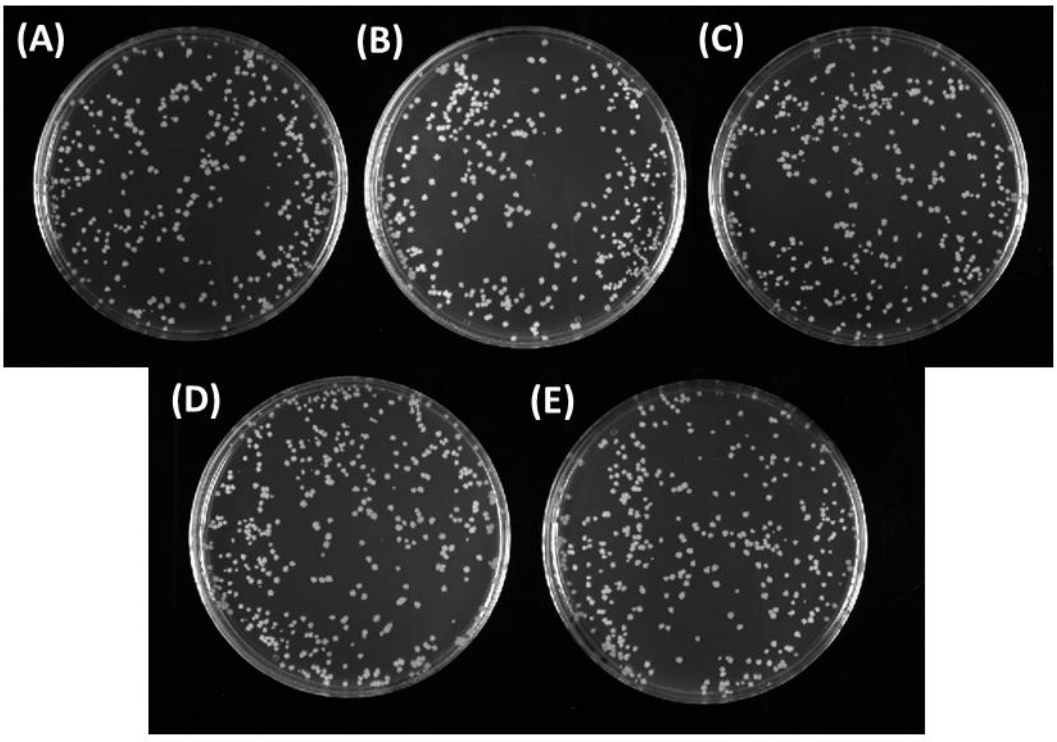


Figure 3.2a: *M.smegmatis* CFUs on LB-Agar plates after treatment with different concentrations of transferrin. Plate (A) is the solvent control for transferrin (100µl water/ml

bacterial suspension). Plate (B) Plate (C), Plate (D) and Plate (E) show the number of CFUs after treatment with 10µg, 25µg, 50µg and 100µg/ml transferrin respectively. The picture shows one representative plate from the triplicate, for solvent control and different concentrations of transferrin used during one of the experiment.

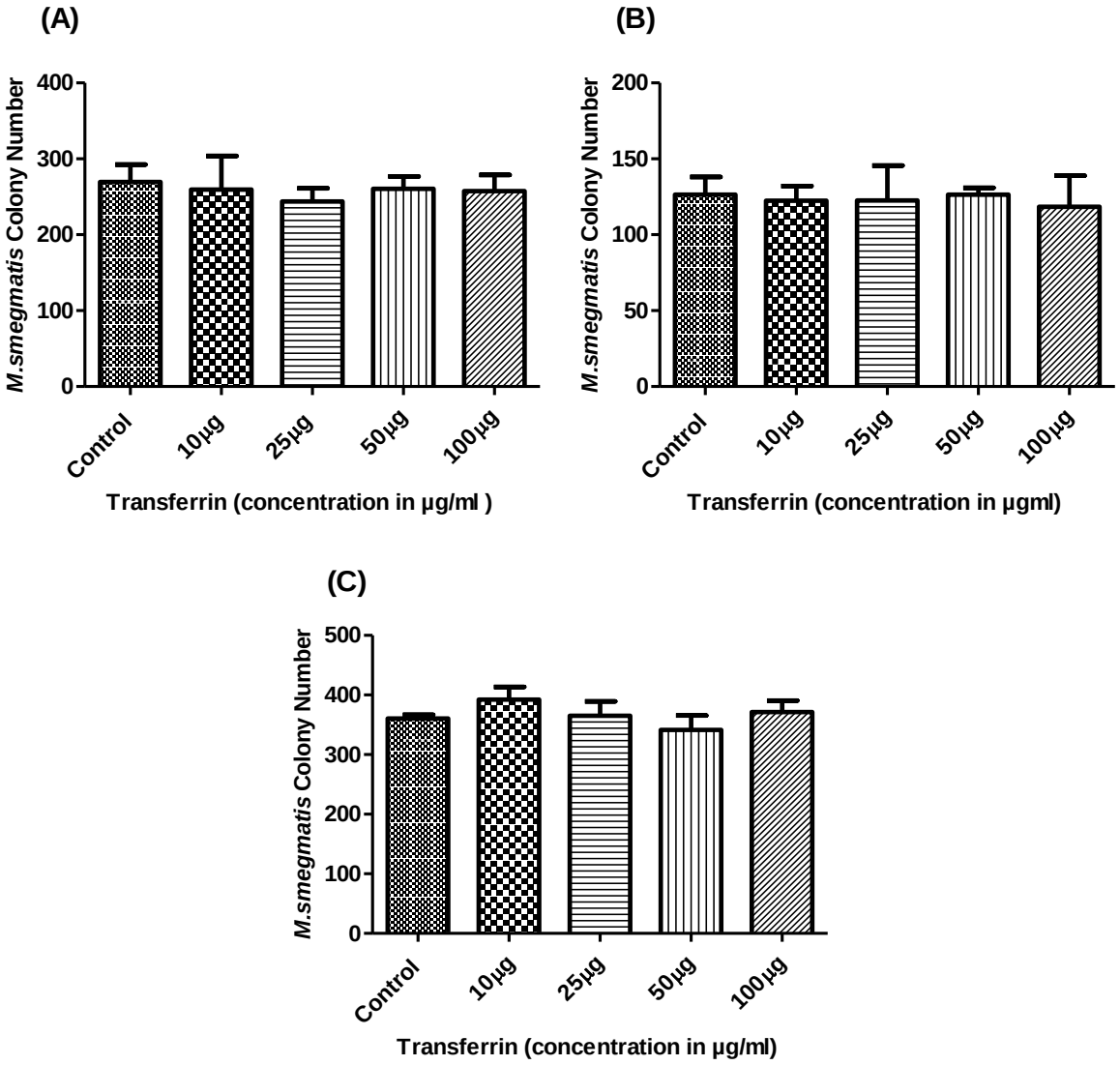


Figure 3.2b: Three individual experiments (A, B and C) for direct effect of transferrin on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

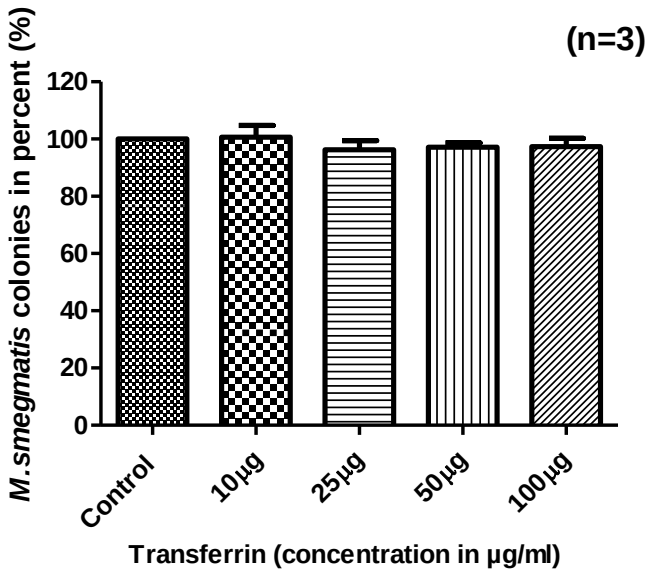


Figure 3.2c: Direct effect of transferrin protein on *M.smegmatis* growth. Each bar represents the average of three individual experiments and the error bars show standard error of means (SEM). The data is presented in percentage, where control is taken as 100% survival and different test conditions are compared to it. The graph shows that *in vitro* transferrin protein has no direct effect on the growth of *M.smegmatis.* Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (p=0.77).

3.3 Direct effect of Lactoferrin on *M. smegmatis* growth *in vitro*

Lactoferrin is an iron-binding protein belonging to the transferrin family. The increased level of lactoferrin during inflammation (Legrand, 2011), its ability to bind free iron (García-Montoya *et al*., 2012) and its growth inhibitory effect on a variety of pathogenic microorganisms including bacteria (Yamauchi *et al*., 1993; Arnold *et al*., 1977), viruses (Berlutti *et al*., 2011) and fungi (Nikawa *et al*., 1993) made it a good candidate to be screened for its direct effect on the growth of *M.smegmatis*.

Three individual experiments were carried out to find the direct effect of lactoferrin on *M.smegmatis* growth using different concentrations (10µg, 25µg, 50µg and 100µg) of lactoferrin protein and a solvent control for lactoferrin (100µl water/ml bacterial suspension) according to the protocol mentioned in section 2.2. All these experiments of lactoferrin with *M.smegmatis* showed the same trend (Figure 3.3b) and it was observed that lactoferrin protein in concentrations of up to 100µg/ml had no direct inhibitory effect on the growth of

M.smegmatis as indicated by the number of CFUs for lactoferrin treated and solvent control *M.smegmatis* (Figure 3.3a, 3.3b and 3.3c). Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (p=0.33).

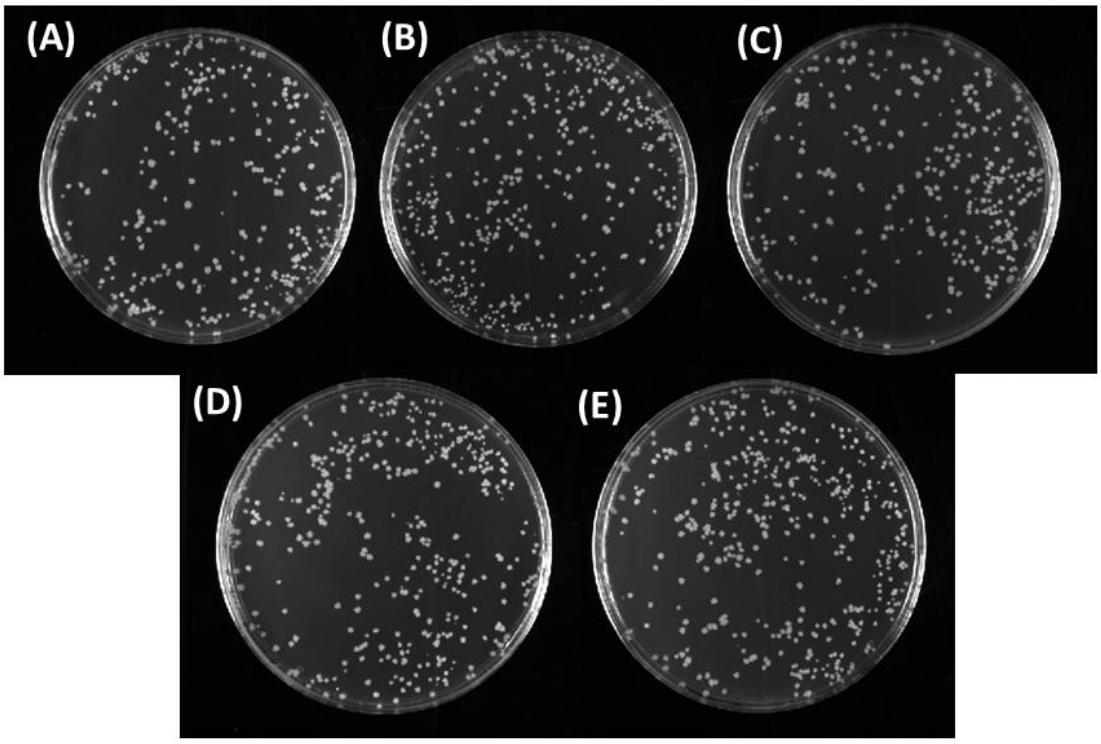


Figure 3.3a: *M.smegmatis* CFUs on LB-Agar plates after treatment with different concentrations of lactoferrin. Plate (A) is the solvent control for lactoferrin (100µl water/ml bacterial suspension), Plate (B), Plate (C), Plate (D) and Plate (E) shows the number of *M.smegmatis* CFUs after treatment with 10µg, 25µg, 50µg and 100µg/ml of lactoferrin respectively. The picture shows one representative plate from the triplicates for solvent control and different test concentrations of lactoferrin used during one of the experiment.

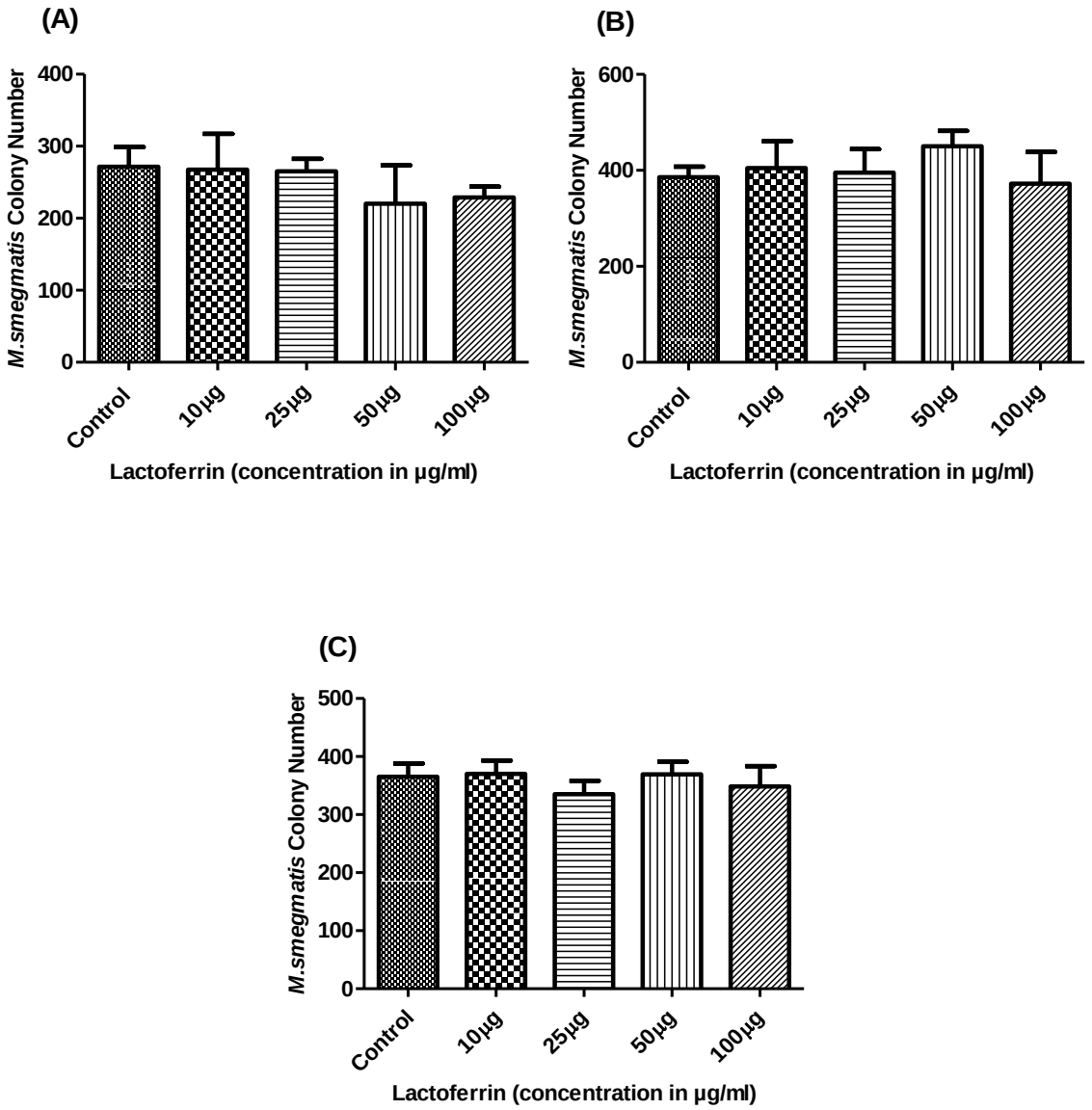


Figure 3.3b: Three individual experiments (A, B and C) for the direct effect of lactoferrin protein on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

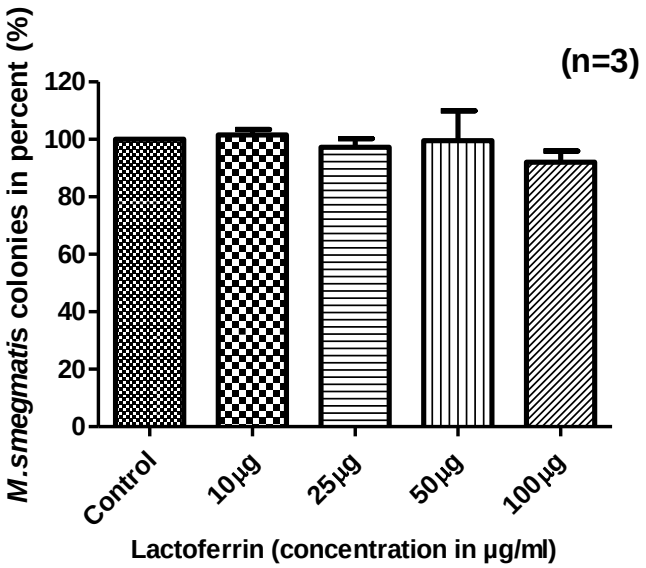


Figure 3.3c: Direct effect of lactoferrin on the growth of *M.smegmatis*. Each bar represents the average of three individual experiments and the error bars show standard error of means (SEM). The data is presented in percentage, where solvent control is taken as 100% survival and different test conditions are compared to it. The graph shows that *in vitro* lactoferrin protein has no direct effect on the growth of *M.smegmatis*. Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (p=0.33).

3.4 Direct effect of Fibrinogen on *M. smegmatis* growth *in vitro*

Fibrinogen is a blood plasma glycoprotein that plays an important role in blood clotting. Increased levels of fibrinogen can be detected in the human body during infection and inflammation (Madden *et al*., 2008; Holm and Godal, 1984). This made fibrinogen a good candidate to be screened for its direct effect on mycobacterial growth.

Three independent experiments using different concentrations (10µg, 25µg, 50µg and 100µg/ml) of fibrinogen protein and solvent control for fibrinogen (100µl water/ml bacterial suspension) were carried out according to the protocol mentioned in section 2.2. All these three experiments showed the same trend (Figure 3.4b) and it was observed that *in vitro* fibrinogen protein in concentrations of up to 100µg/ml had no direct inhibitory effect on the growth of *M.smegmatis* as determined by counting the number of CFUs for fibrinogen treated and solvent control *M.smegmatis* (Figure 3.4a, 3.4b, and 3.4c). Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (P=0.81).

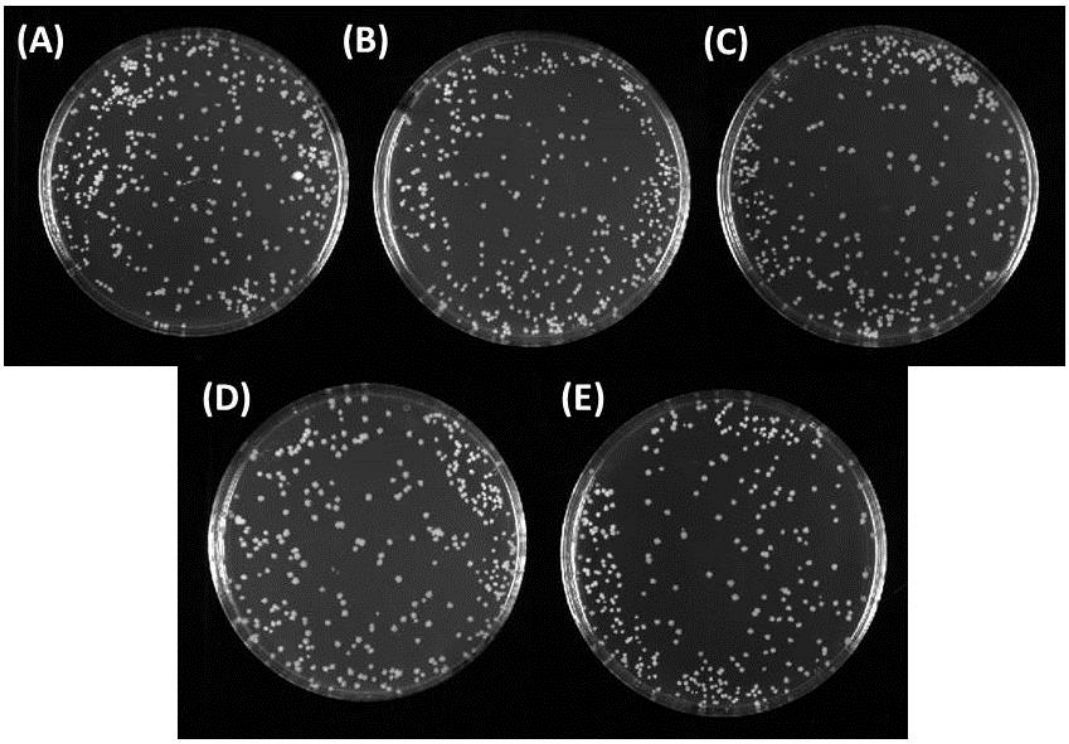


Figure 3.4a: *M.smegmatis* CFUs on LB-Agar plates after treatment with different concentrations of fibrinogen. Plate (A) is solvent control for fibrinogen protein (100µl water/ml bacterial suspension), Plate (B), Plate (C), Plate (D) and Plate (E) shows the number of *M.smegmatis* CFUs after treatment with 10µg, 25µg, 50µg and 100µg/ml of fibrinogen respectively. The picture shows one representative plate from the triplicates for solvent control and different concentrations of fibrinogen used during one of the experiment.

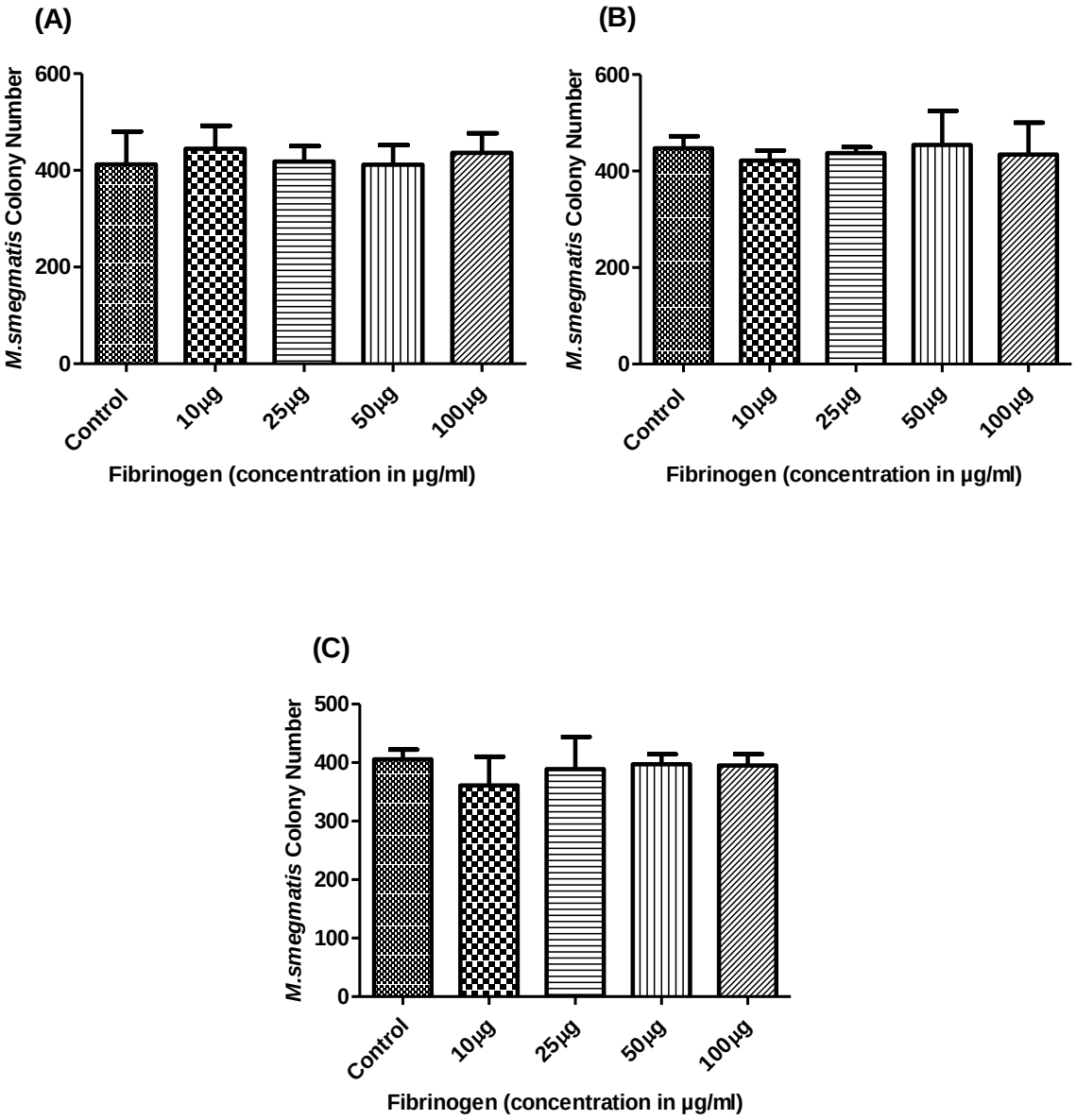


Figure 3.4b: Three individual experiments (A, B and C) for the direct effect of fibrinogen protein on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

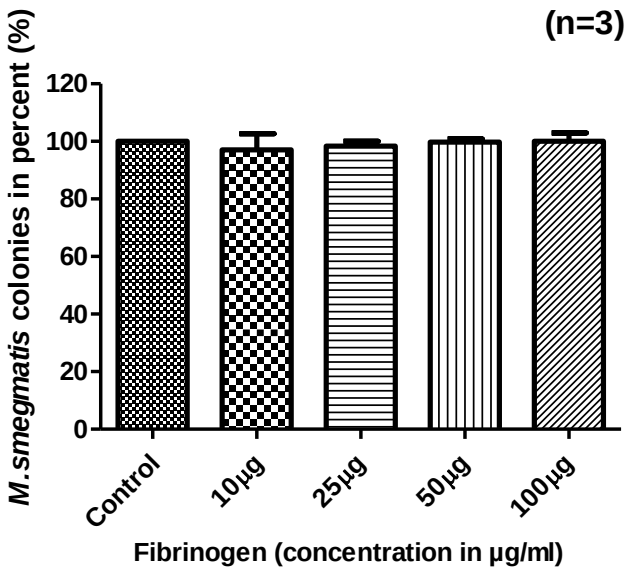


Figure 3.4c: Direct effect of fibrinogen on the growth of *M.smegmatis*. Each bar represents the average of three individual experiments and the error bars show standard error of means (SEM). The data is presented in percentage, where solvent control is taken as 100% survival and different test conditions are compared to it. The graph shows that *in vitro* fibrinogen protein has no direct growth inhibitory on *M.smegmatis.* Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (P=0.81).

3.5 Direct effect of C-reactive protein (CRP) on *M.smegmatis* growth *in vitro*

C-reactive protein (CRP) is a blood plasma protein with standard concentration ranging from 5-10mg/L of human serum, however, its concentration is increased several folds during pathogen infection, tissue injury and inflammation (Marnell *et al*., 2005). Therefore, CRP was investigated *in vitro* for its direct effect on the growth of *M.smegmatis*. All the experiments were carried out in the presence of calcium as CRP requires the presence of Ca^{+2} for its optimal functioning (Agarwal *et al*., 2002).

Three individual experiments were carried out in the presence of 1mM $CaCl_2$ to find the direct effect of CRP on *M.smegmatis* at different concentrations (10µg, 25µg and 50µg/ml) of CRP and a solvent control for CRP (10µl of 140mM NaCl+20mM Tris-HCl/ml of bacterial suspension) according to the protocol mentioned in section 2.2. All the experiments of CRP with *M.smegmatis* showed the same trend (Figure 3.5b) and it was observed that CRP in concentrations of up to 50µg/ml had no inhibitory effect on the growth of *M.smegmatis* as

indicated by the number of CFUs for CRP treated and solvent control *M.smegmatis* as shown in figures 3.5a, 3.5b and 3.5c. Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (p=0.78)

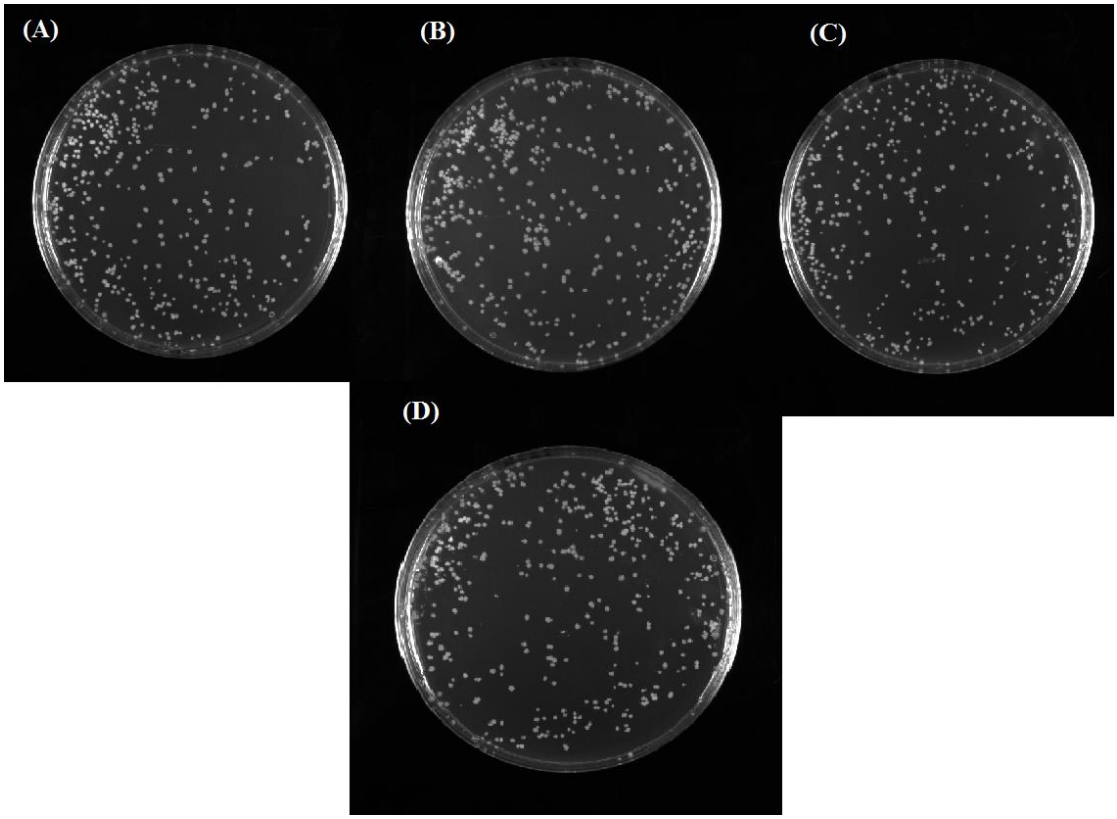


Figure 3.5a: *M.smegmatis* CFUs on LB-Agar plates after treatment with different concentrations of CRP. Plate (A) is solvent control for CRP (10µl of 140mM NaCl+20mM Tris-HCl/ml of bacterial suspension), Plate (B), Plate (C), and Plate (D) shows the number of *M.smegmatis* CFUs after treatment with 10µg, 25µg, and 50µg/ml of CRP respectively in the presence of 1mM $CaCl_2$. The picture shows one representative plate from the triplicates for solvent control and different test concentrations of CRP used during one of the experiment.

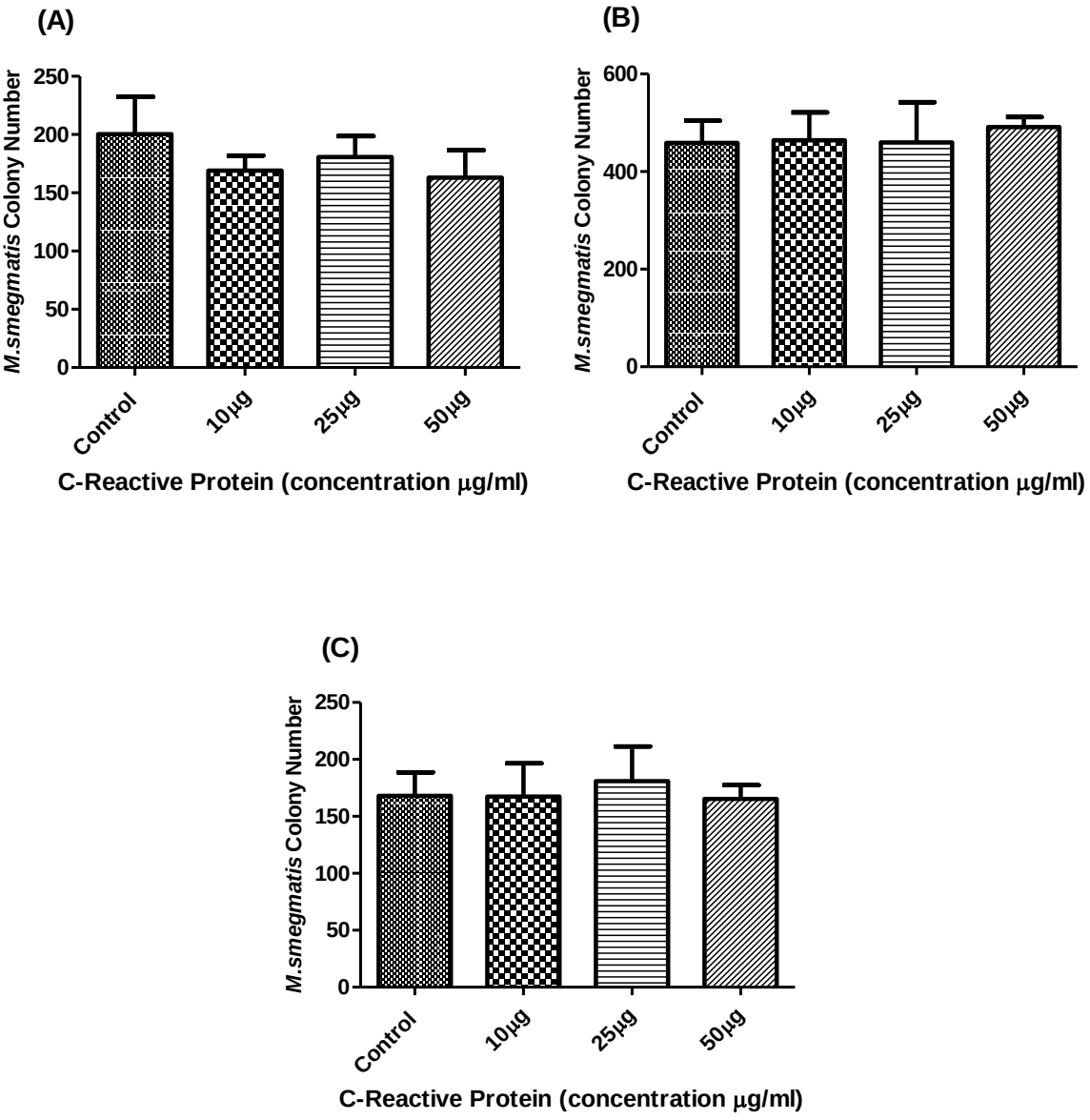


Figure 3.5b: Three individual experiments (A, B and C) for the direct effect of CRP on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

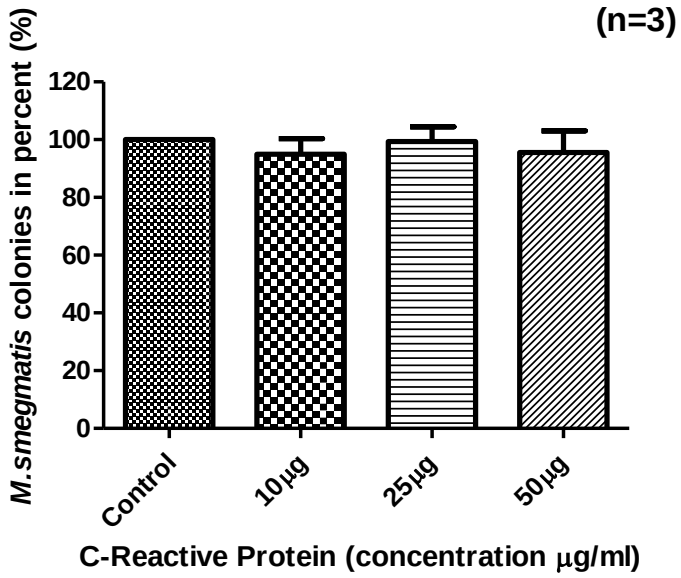


Figure 3.5c: Direct effect of CRP on *M.smegmatis* growth. Each bar represents the average of three individual experiments and the error bars show standard error of means (SEM). The data is presented in percentage, where solvent control is taken as 100% survival and different test conditions are compared to it. The graph shows that *in vitro* CRP has no direct effect on inhibiting the growth of *M.smegmatis*. Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (p=0.78)

3.6 Direct effect of Alpha-2-macroglobulin (α2M) on *M. smegmatis* growth *in vitro*

Alpha-2-macroglobulin (α2M) is a blood plasma protein that is mainly synthesized in the liver (Feldman *et al*., 1985) however, other cells including macrophages (White *et al*., 1980), fibroblasts (Mosher and Wing, 1976) can also synthesize α2M. This plasma protein can capture and bind a variety of self and foreign peptides and particles using its high-affinity sites and play an important role in the host defence (Borth, 1992). α2M was, therefore, used during the *in vitro* screening to check if this plasma protein can directly inhibit the growth of *mycobacteria*.

Three individual experiments were carried out to find using different concentrations (10µg, 25µg, 50µg and 100µg/ml) of α2M and a solvent control for α2M (100µl water/ml bacterial suspension) according to the protocol mentioned in section 2.2. All the experiments of α2M with *M.smegmatis* showed the same trend (Figure 3.6b). From these experiments, it was observed that *in vitro* α2M in concentrations of up to 100µg/ml had no direct effect on the

growth of *M.smegmatis* as indicated by the similarity in the number of CFUs for α2M treated and solvent control *M.smegmatis* shown in figures 3.6a, 3.6b and 3.6c. Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (p=0.47).

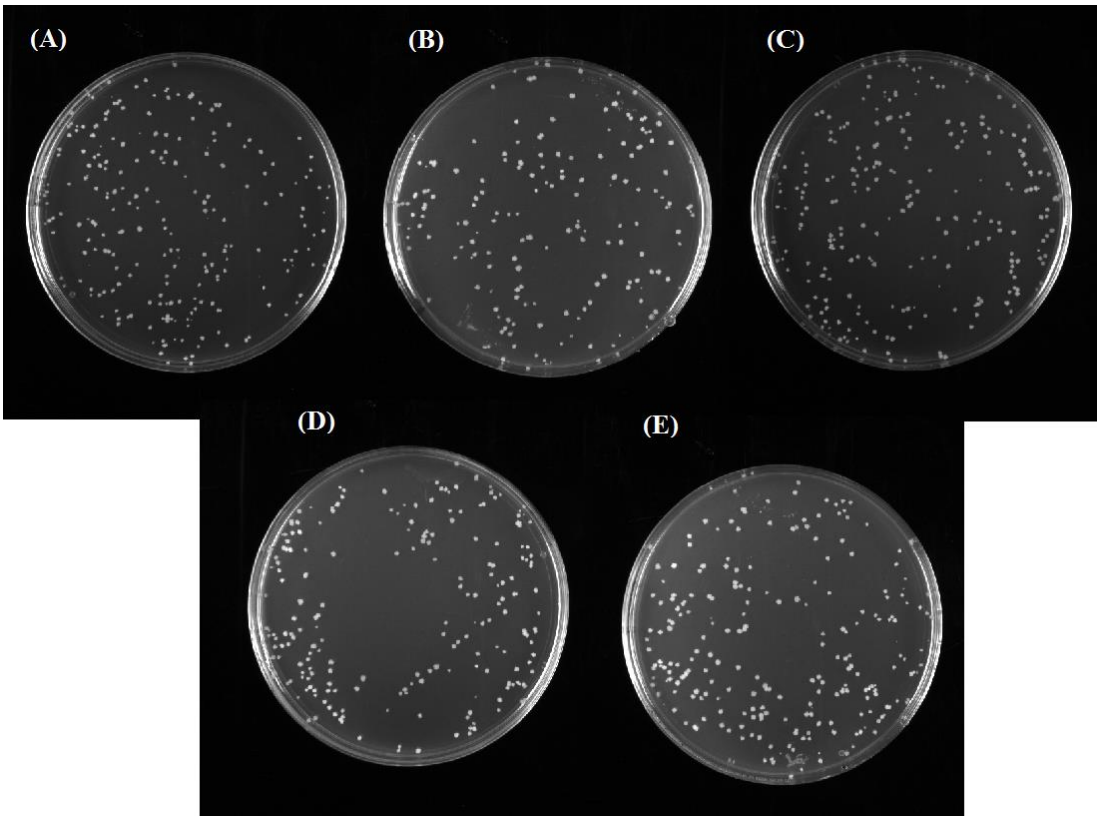


Figure 3.6a: *M.smegmatis* CFUs on LB-Agar plates after treatment with different concentrations of α2M. Plate (A) is solvent control for α2M (100µl water/ml bacterial suspension), Plate (B), Plate (C), Plate (D) and Plate (E) shows the number of *M.smegmatis* CFUs after treatment with 10µg, 25µg, 50µg and 100µg/ml of α2M respectively. The picture shows one representative plate from the triplicates for solvent control and different test concentrations of α2M used during one of the experiment.

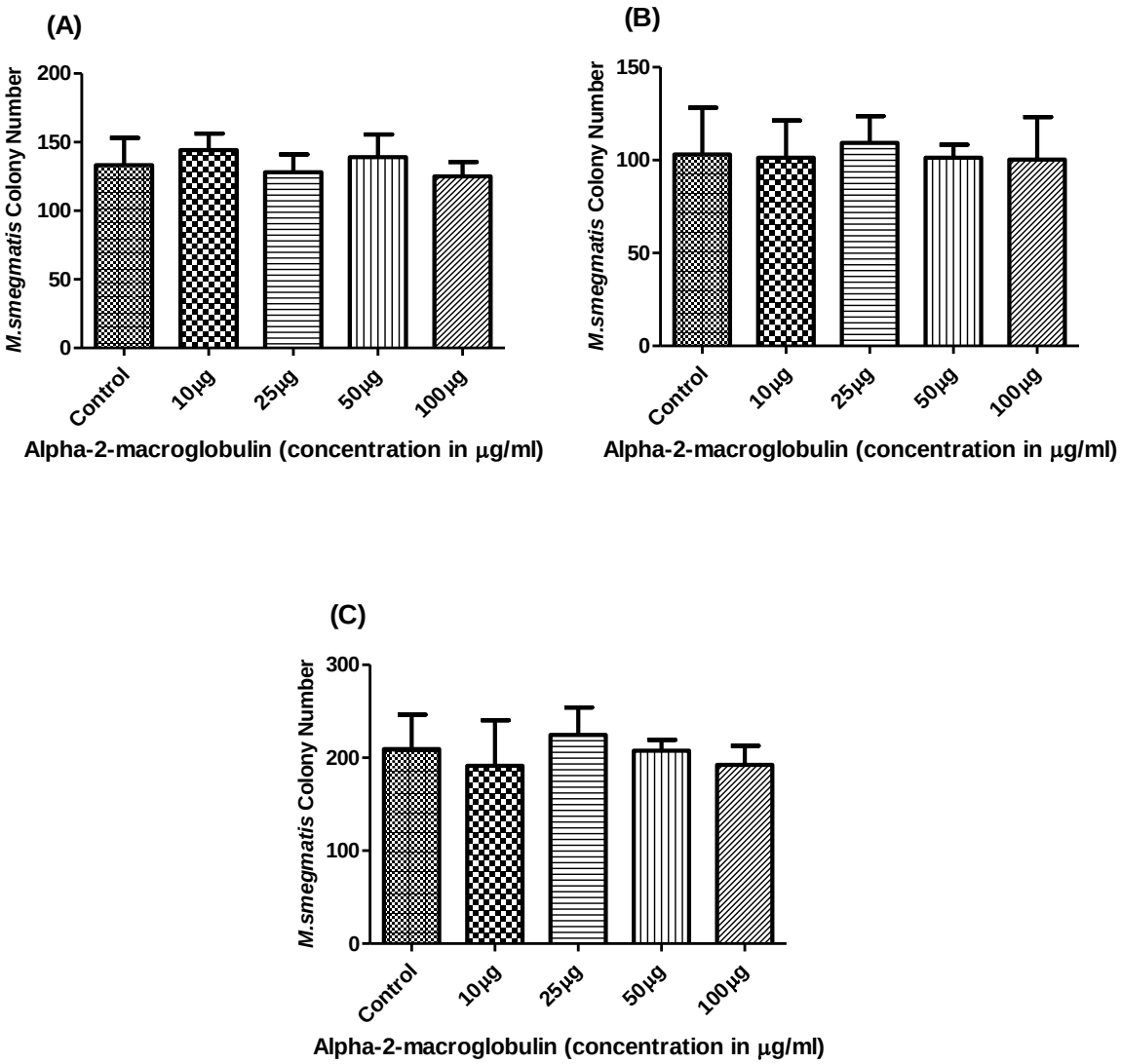


Figure 3.6b: Three individual experiments (A, B and C) for the direct effect of α2M on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

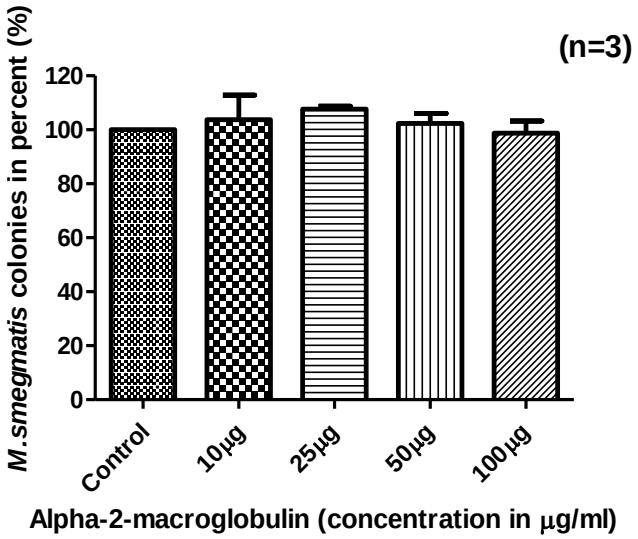


Figure 3.6c: Direct effect of α-2-Macroglobulin on the growth of *M.smegmatis*. Each bar represents the average of three individual experiments and the error bars show standard error of means (SEM). The data is presented in percentage, where solvent control is taken as 100% survival and different test conditions are compared to it. The graph shows that α2M protein *in vitro* has no direct inhibiting effect on *M.smegmatis*. Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (p=0.47).

3.7 Direct effect of Vitronectin on *M. smegmatis* growth *in vitro*

Vitronectin is a 75kDa glycoprotein present at a concentration of 200-400µg/ml in human serum and plays important role in cell adhesion and regulatory processes like fibrinolysis (Preissner, 1991). Vitronectin has been shown to prevent the deposition of membrane attack complex (MAC) on the surface of gram-negative bacteria helping them in survival, while in case of gram-positive bacteria vitronectin facilitated their attachment to the host immune cells and their subsequent internalization by the host immune cells (Singh *et al*., 2010). Therefore, this plasma protein was investigated for its direct effect on the growth of *mycobacteria*.

Three individual experiments were performed using different concentrations (10µg, 25µg, 50µg and 100µg/ml) of vitronectin protein and solvent control for vitronectin (100µl water/ml bacterial suspension) in order to investigate the *in vitro* effect of vitronectin on the growth of *M.smegmatis*. All the three experiments showed the same trend (Figure 3.7b) and it was observed that vitronectin in concentrations of up to 100µg/ml showed no direct inhibitory effect on the growth of *M.smegmatis* as indicated the number of CFUs for vitronectin treated and

solvent control *M.smegmatis* (Figure 3.7a, 3.7b and 3.7c). Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (p=0.25).

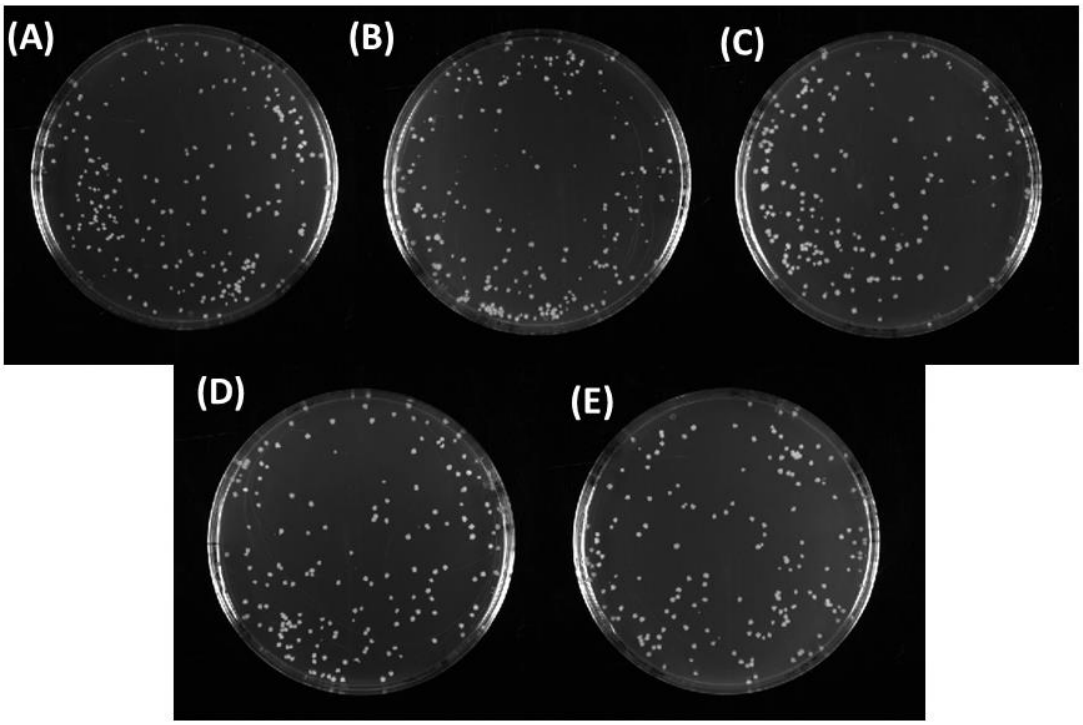


Figure 3.7a: *M.smegmatis* CFUs on LB-Agar plates after treatment with different concentrations of vitronectin. Plate (A) is solvent control for vitronectin (100µl water/ml bacterial suspension), Plate (B), Plate (C), Plate (D) and Plate (E) shows the number of *M.smegmatis* CFUs after treatment with 10µg, 25µg, 50µg and 100µg/ml of vitronectin respectively. The picture shows one representative plate from the triplicates for solvent control and different test concentrations of vitronectin used during one of the experiment.

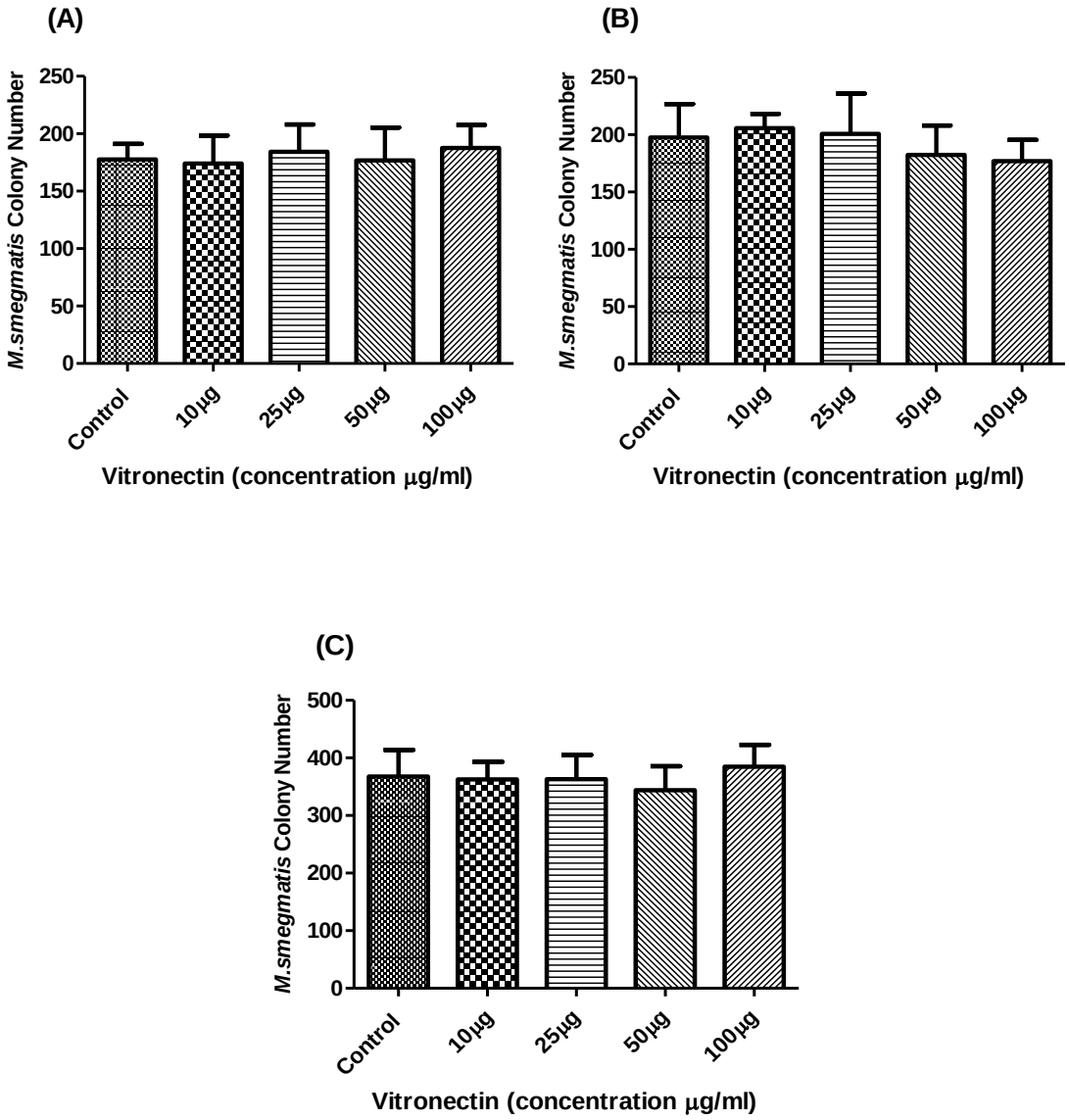


Figure 3.7b: Three individual experiments (A, B and C) for the direct effect of vitronectin on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

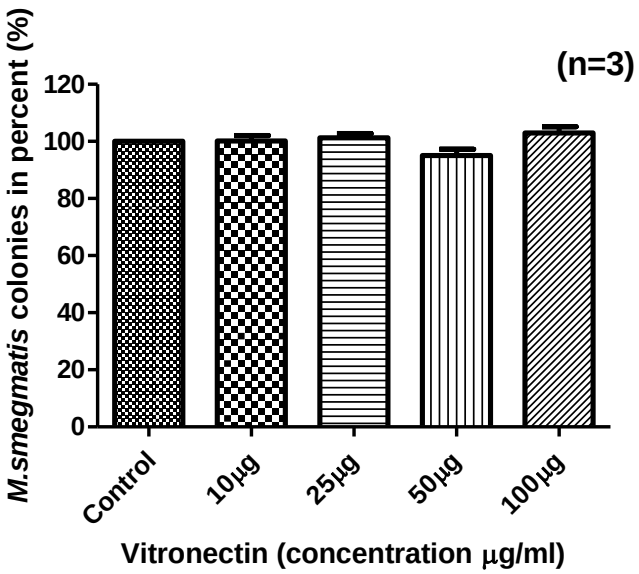


Figure 3.7c: Direct effect of Vitronectin on the growth of *M.smegmatis*. Each bar represents the average of three individual experiments and the error bars show standard error of means (SEM). The data is presented in percentage, where solvent control is taken as 100% survival and different test conditions are compared to it. The graph shows that *in vitro* vitronectin protein has no direct growth inhibiting effect on *M.smegmatis*. Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (p=0.25).

3.8 Direct effect of Plasminogen on *M. smegmatis* growth *in vitro*

Plasminogen is a precursor blood plasma protein, synthesized in the liver. It is converted by the enzymes urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) into an active form, plasmin which helps in the dissolution of fibrin blood clots (Cesarman-Maus and Hajjar, 2005) and has also been shown play important roles in other physiological processes including inflammation (Syrovets *et al*., 2012). Therefore, plasminogen was tested *in vitro* for its ability to inhibit or enhance the growth of *mycobacteria*.

Three individual experiments were carried out to find the direct effect of plasminogen on the growth of *M.smegmatis* using different concentrations (10µg, 25µg, 50µg and 100µg/ml) of plasminogen protein and solvent control for plasminogen (10µl of 50mM Tris-HCl+100mM NaCl/ml of bacterial suspension) according to the protocol mentioned in section 2.2. All the three experiments of plasminogen with *M.smegmatis* showed the same trend (Figure 3.8b) and

it was observed that plasminogen protein in concentrations of up to 100µg/ml had no direct inhibitory effect on the growth of *M.smegmatis* as indicated by the number of CFUs for plasminogen treated and solvent control *M.smegmatis* (Figures 3.8a, 3.8b and 3.8c). Non-parametric Kruskal-Wallis test was used to determine the *p*-value, which was found to be non-significant (P=0.41).

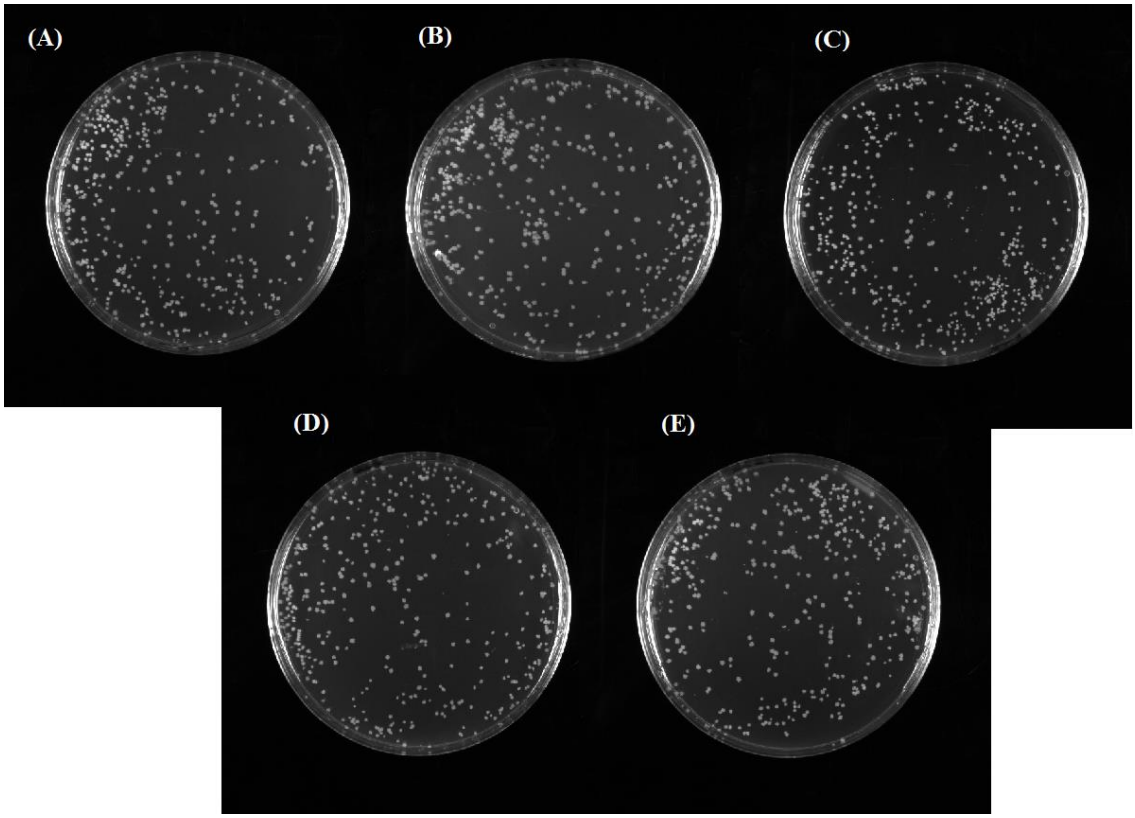


Figure 3.8a: *M.smegmatis* CFUs on LB-Agar plates after treatment with different concentrations of plasminogen. Plate (A) is the solvent control for plasminogen (10µl of 50mM Tris-HCl+100mM NaCl/ ml of bacterial suspension), Plate (B), Plate (C), Plate (D) and Plate (E) shows the number of *M.smegmatis* CFUs after treatment with 10µg, 25µg, 50µg and 100µg/ml of plasminogen respectively. The picture shows one representative plate from the triplicates for solvent control and different test concentrations of plasminogen used during one of the experiment.

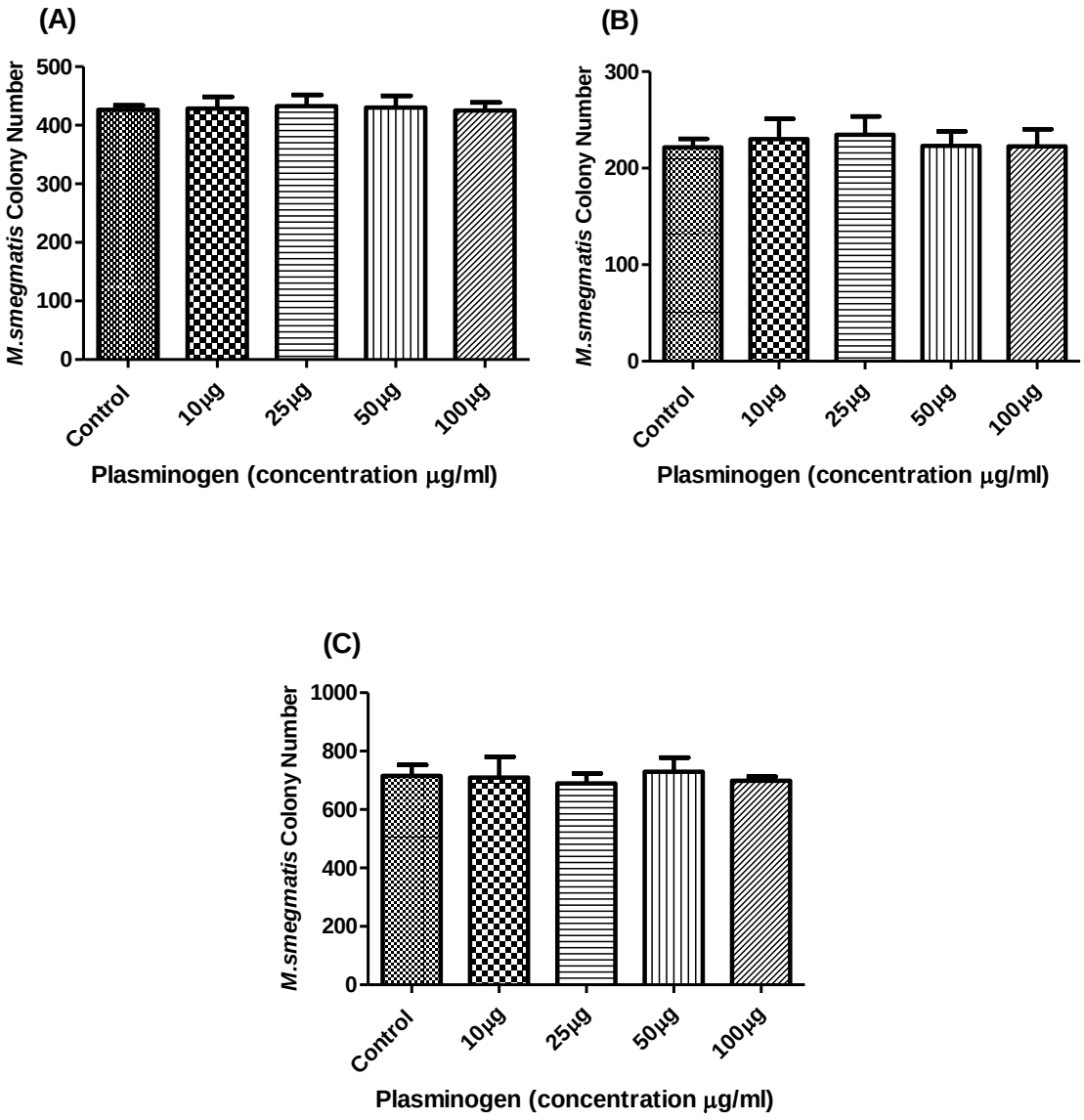


Figure 3.8b: Three individual experiments (A, B and C) for the direct effect of plasminogen protein on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

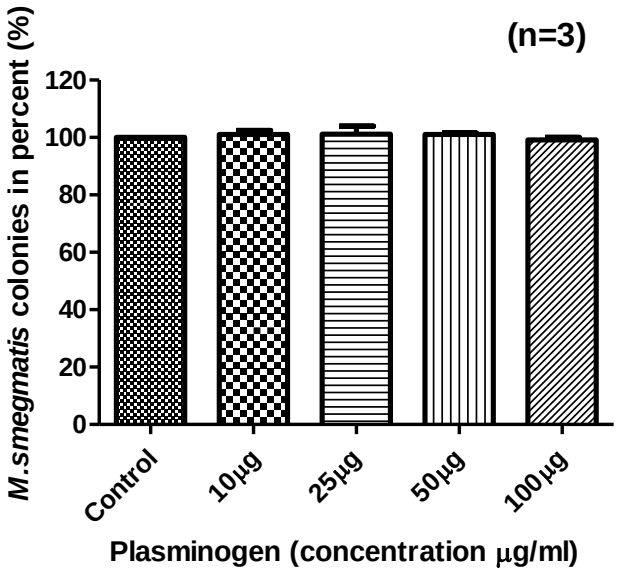


Figure 3.8c: Direct effect of plasminogen on the growth of *M.smegmatis*. Each bar represents the average of three individual experiments and the error bars show standard error of means (SEM). The data is presented in percentage, where solvent control is taken as 100% survival and different test conditions are compared to it. The graph shows that *in vitro* plasminogen has no direct growth inhibiting effect on *M.smegmatis*. Non-parametric Kruskal-Wallis test was used to determine the *p*-value, which was found to be non-significant (P=0.41).

3.9 Direct effect of Low-Density Lipoproteins (LDL) on *M. smegmatis* growth *in vitro*

Low-density lipoproteins (LDL) are composed of multiple proteins along with cholesterol, phospholipids and triglycerides. The major protein in the LDL is apolipoprotein B-100 (apo B-100), which is a 514 kDa protein (Hevonoja *et al*., 2000). Peptides derived from apo B-10 of LDL were shown to possess antimicrobial properties (Kelly *et al*., 2010). LDL was investigated in different concentration for its direct inhibitory effect on the growth of *mycobacteria*.

Three individual experiments were carried out to find the direct effect of LDL on *M.smegmatis* growth using different concentrations (1µg, 2.5µg, 5µg, 10µg and 25µg/ml) of LDL and a solvent control for LDL (25µl of 150mM NaCl+0.5M EDTA/ml bacterial suspension) according to the protocol mentioned in section 2.2. From these experiments, it was observed that *in vitro* LDL in concentrations of up to 25µg/ml showed no direct inhibitory effect on the growth of *M.smegmatis* as indicated by the similarity in the number of CFUs for LDL treated and solvent *M.smegmatis* shown in figures 3.9a, 3.9b and 3.9c. Non-parametric Kruskal-Wallis

test was applied on ranks to determine the *p*-value, which was found to be non-significant (*p*=0.14)

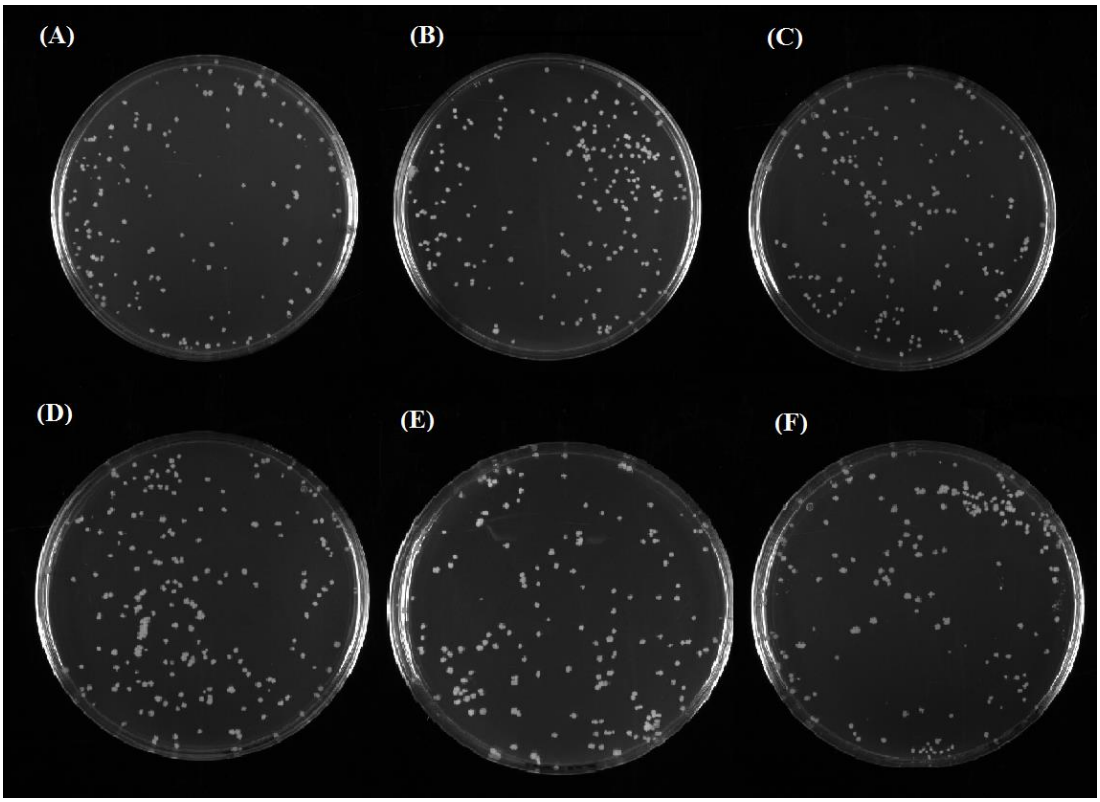


Figure 3.9a: *M.smegmatis* CFUs on LB-Agar plates after treatment with different concentrations of LDL. Plate (A) is the solvent control for LDL (25µl of 150mM NaCl+0.5M EDTA/ml bacterial suspension), Plate (B), Plate (C), Plate (D), Plate (E) and Plate (F) show the number of *M.smegmatis* CFUs after treatment with 1µg, 2.5µg, 5µg, 10µg and 25 µg/ml of LDL respectively. The picture shows one representative plate from the triplicates for solvent control and different test concentrations of LDL used during one of the experiment.

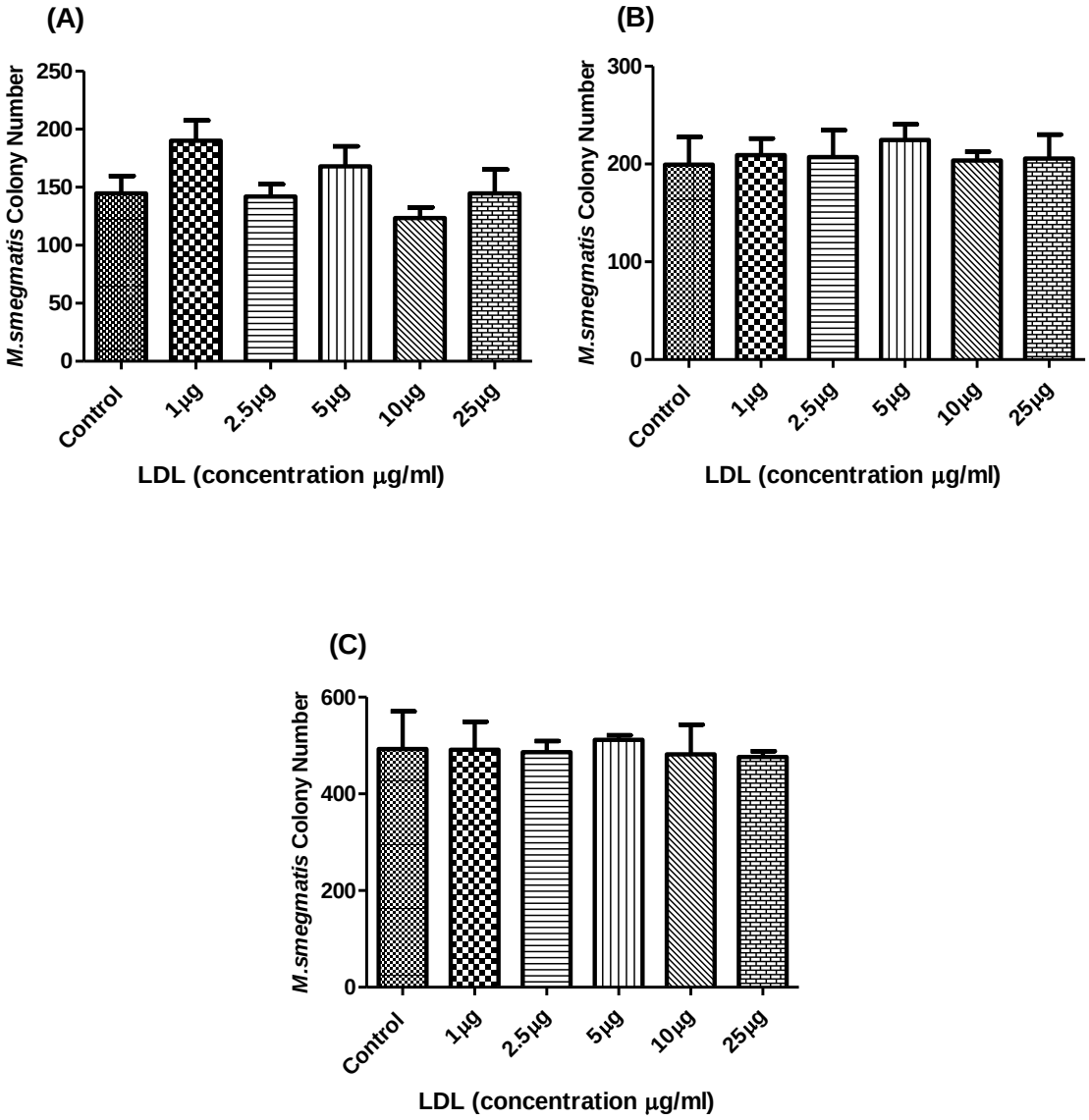


Figure 3.9b: Three individual experiments (A, B and C) for the direct effect of LDL on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

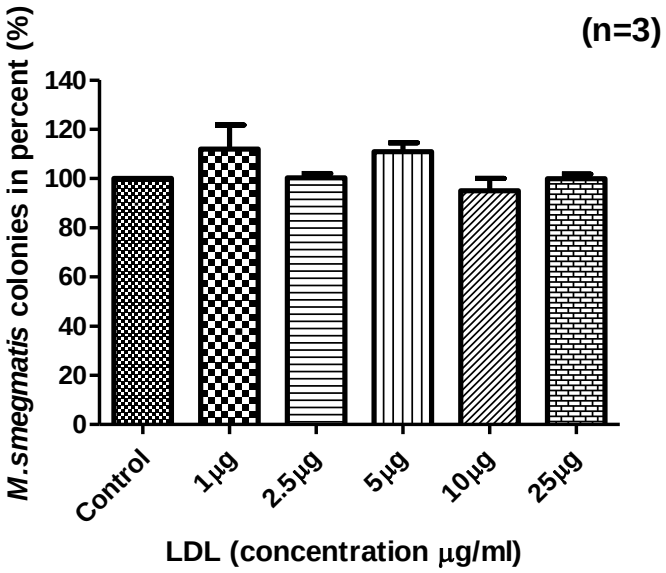


Figure 3.9c: Direct effect of LDL on the growth of *M.smegmatis*. Each bar represents the average of three individual experiments and the error bars show standard error of means (SEM). The data is presented in percentage, where solvent control is taken as 100% survival and different test conditions are compared to it. The graph shows that *in vitro* LDL has no direct growth inhibitory effect on *M.smegmatis* growth. Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (p=0.14)

3.10 Direct effect of High-Density Lipoproteins (HDL) on *M. smegmatis* growth *in vitro*

High-Density Lipoproteins (HDL) are small lipoprotein particles that are rich in proteins. The most abundant protein present in the HDL is apolipoprotein A-1 (apoA-I), a 234 amino acid protein synthesized in the liver (Phillips, 2013). High-density lipoproteins from the blood plasma have been shown to suppress the growth of gram-positive bacteria *in vitro* and the growth inhibitory property was suggested to be linked with the apoA-I part of the HDL (Tada *et al*., 1993). Therefore, HDL was investigated in different concentration for its direct inhibitory effect on the growth of mycobacteria.

Three individual experiments were carried out to find the direct effect of HDL on *M.smegmatis* growth using different concentrations (1µg, 2.5µg, 5µg, 10µg and 25µg/ml) of HDL and solvent control for HDL (25µl of 150mM NaCl+0.5M EDTA/ml bacterial suspension) according to the protocol mentioned in section 2.2. From these experiments, it was observed that *in vitro* HDL in concentrations of up to 25µg/ml showed no direct inhibitory effect on the

growth of *M.smegmatis* as indicated by the similarity in the number of CFUs for HDL treated and untreated *M.smegmatis* (Figures 3.10a, 3.10b and 3.10c). Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (p=0.87).

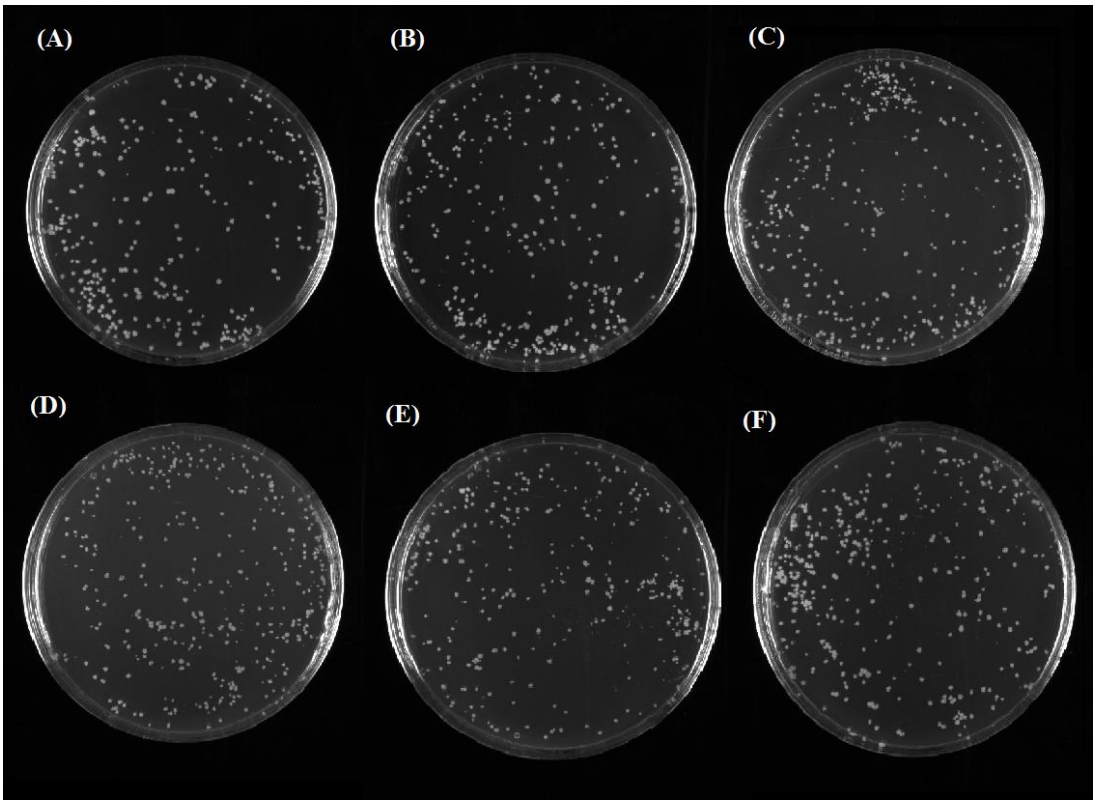


Figure 3.10a: *M.smegmatis* CFUs on LB-Agar plates after treatment with different concentrations of HDL. Plate (A) is solvent control for HDL (25µl of 150mM NaCl+0.5M EDTA/ml bacterial suspension), Plate (B), Plate (C), Plate (D), Plate (E) and Plate (F) show the number of CFUs after treatment with 1µg, 2.5µg, 5µg, 10µg and 25 µg/ml of HDL respectively. The picture shows one representative plate from the triplicates for solvent control and different test concentrations of HDL used during one of the experiment.

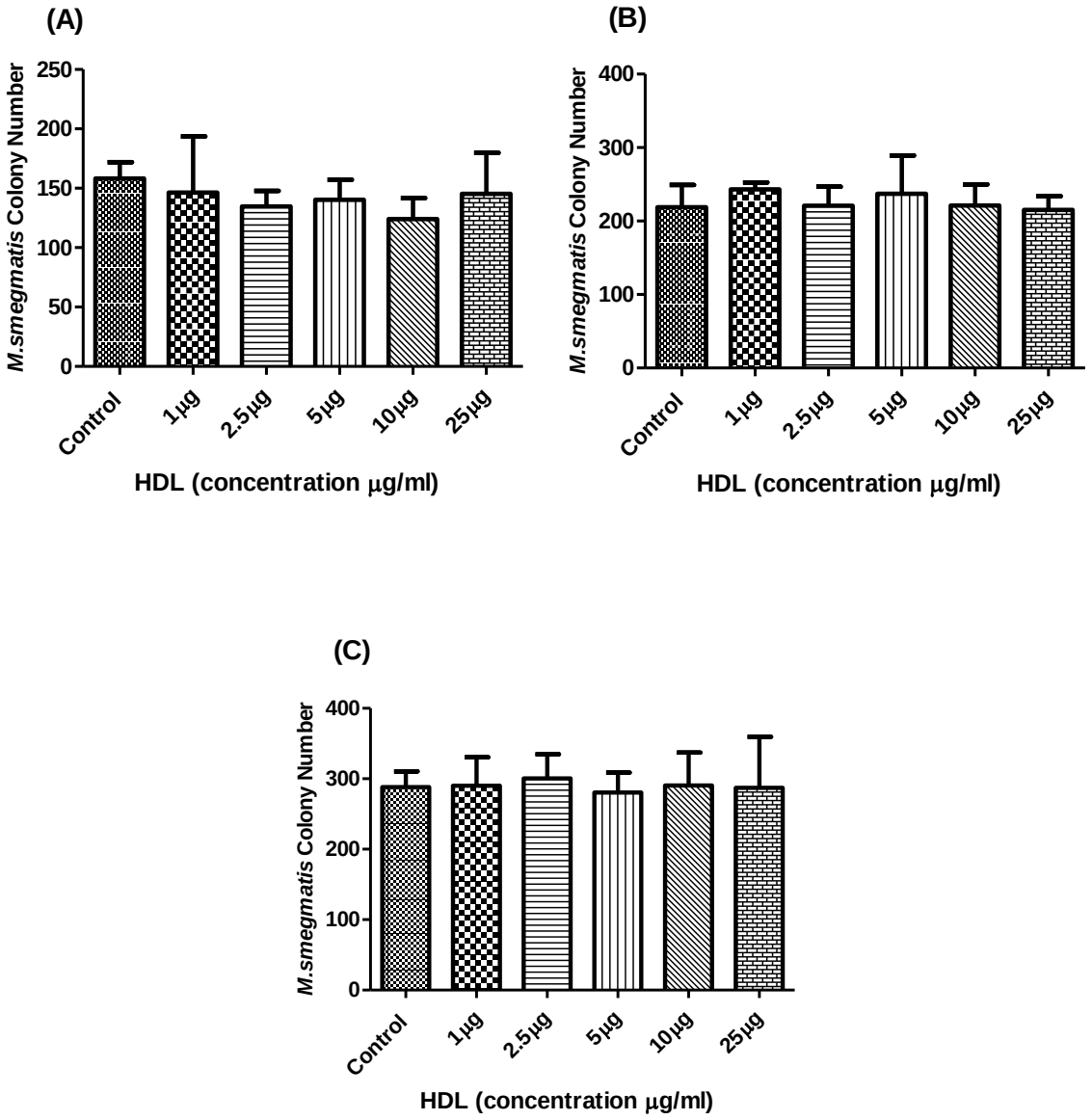


Figure 3.10b: Three individual experiments (A, B and C) for the direct effect of HDL on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

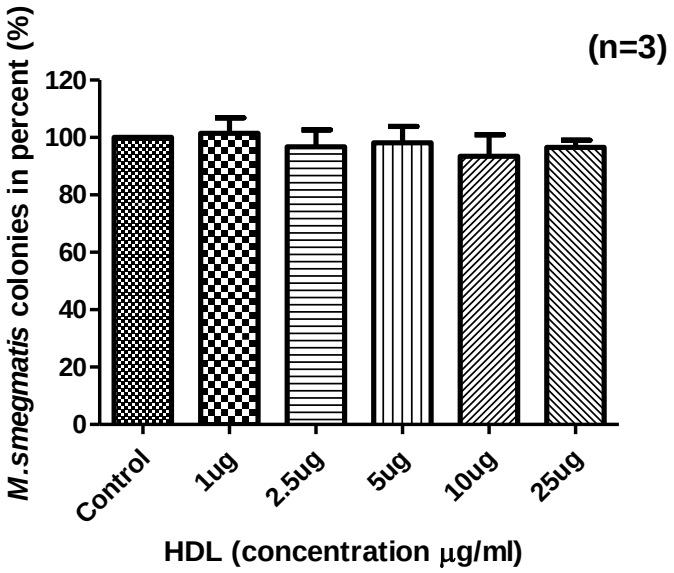


Figure 3.10c: Direct effect of HDL on the growth of *M.smegmatis*. Each bar represents the average of three individual experiments and the error bars show standard error of means (SEM). The data is presented in percentage, where solvent control is taken as 100% survival and different test conditions are compared to it. The graph shows that *in vitro* HDL has no direct growth inhibitory effect on *M.smegmatis* growth. Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (p=0.87).

3.11 Direct effect of Serotonin on *M.smegmatis* growth *in vitro*

Serotonin is chemically known as 5-Hydroxytryptamine (5-HT) or 3-(β-Aminoethyl)-5-hydroxyindole. During the process of inflammation, serotonin is released in greater quantities due to the activation of different cells such as platelets and mast cells by certain pro-inflammatory compounds such as PAF (Rubio *et al*., 2007) and is involved in leukocytes recruitment and cell adhesion to the blood vessels (Duerschmied *et al*., 2013).

Three individual experiments were carried out to find the direct effect of serotonin on *M.smegmatis* growth using different concentrations (10µg, 25µg, 50µg and 100µg/ml) of serotonin and a solvent control for serotonin (10µl DMSO/ml bacterial suspension) according to the protocol mentioned in section 2.2. All the experiments show the same trend (Figure 3.11b) and it was observed that *in vitro* serotonin in concentrations of up to 100µg/ml showed no direct inhibitory effect on the growth of *M.smegmatis* as indicated by the similarity in the number of CFUs for serotonin treated and untreated *M.smegmatis* (Figure 3.11a, 3.11b and

3.11c). Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (p=0.72).

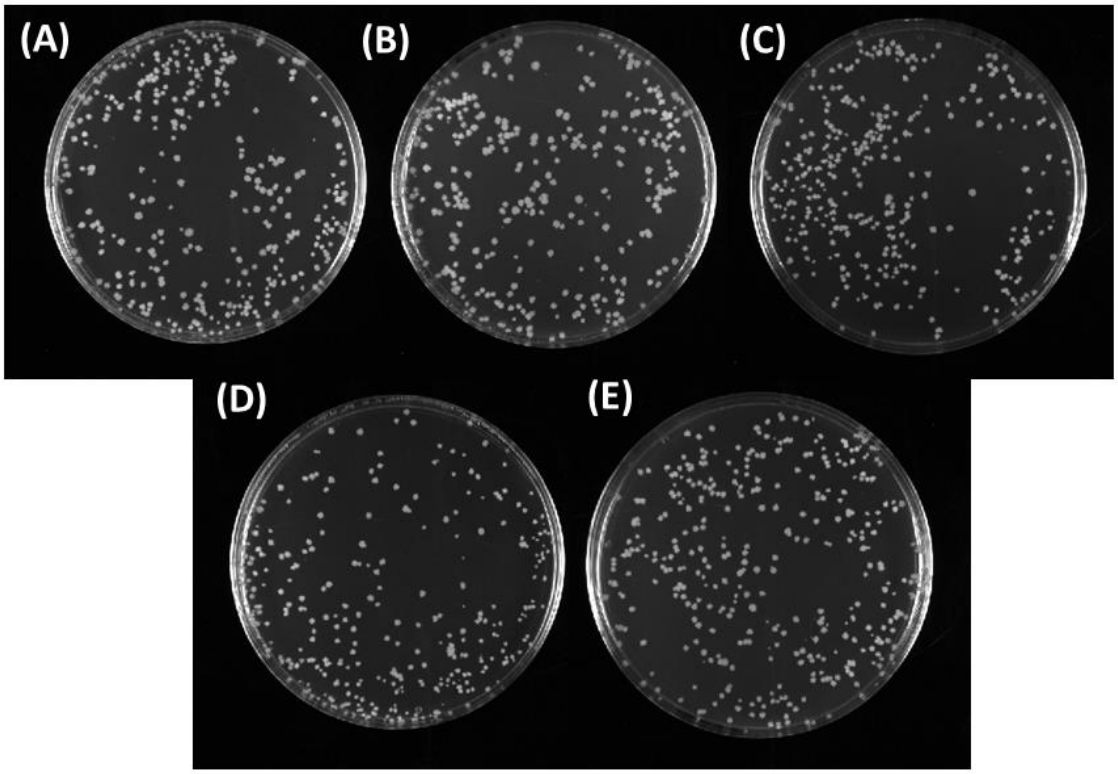


Figure 3.11a: *M.smegmatis* CFUs on LB-Agar plates after treatment with different concentrations of serotonin. Plate (A) is solvent control for serotonin (10µl DMSO/ml bacterial suspension), Plate (B), Plate (C), Plate (D) and Plate (E) shows the number of CFUs after treatment with 10µg, 25µg, 50µg and 100µg/ml of serotonin respectively. The picture shows one representative plate from the triplicates for solvent control and different test concentrations of serotonin used during one of the experiment.

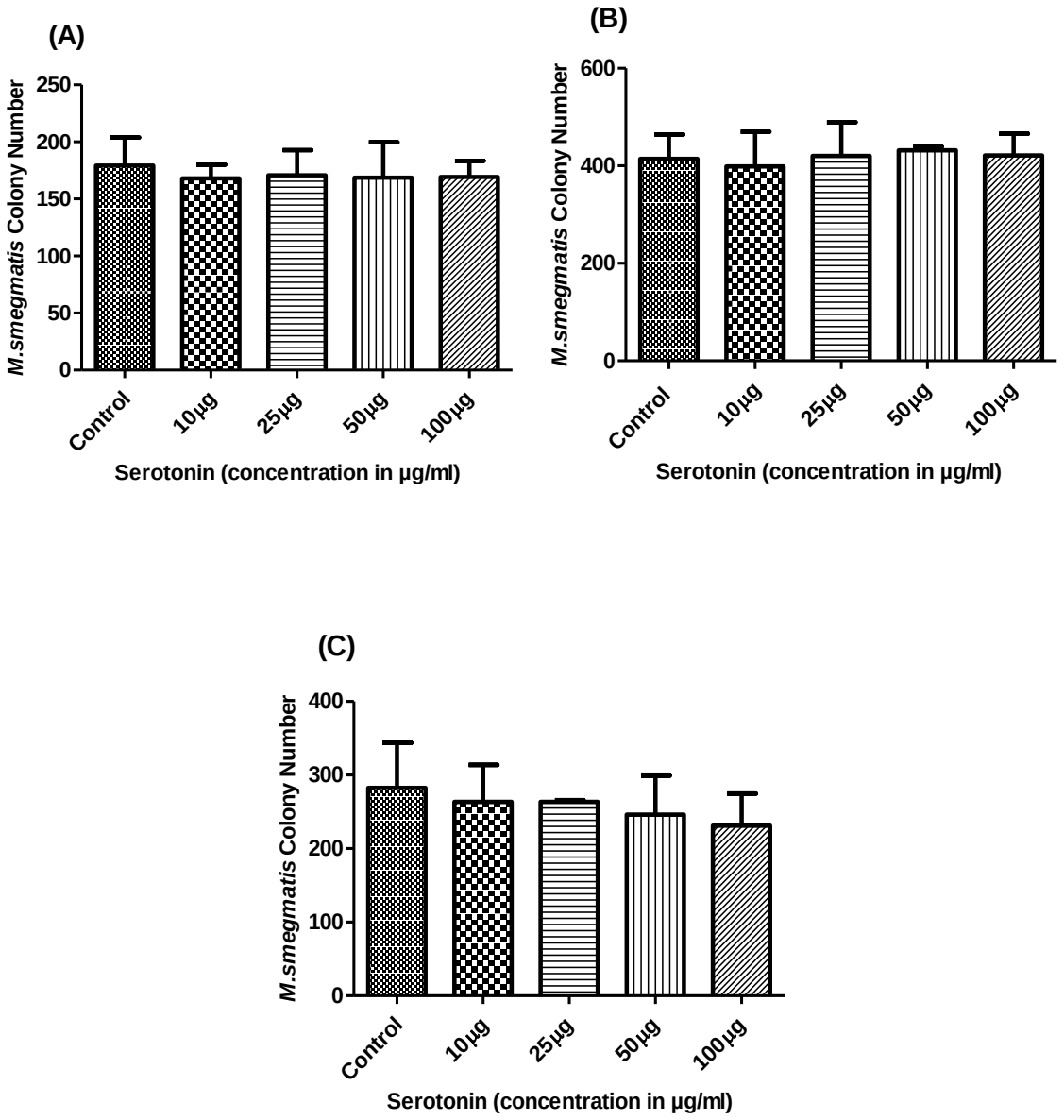


Figure 3.11b: Three individual experiments (A, B and C) for the direct effect of serotonin on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

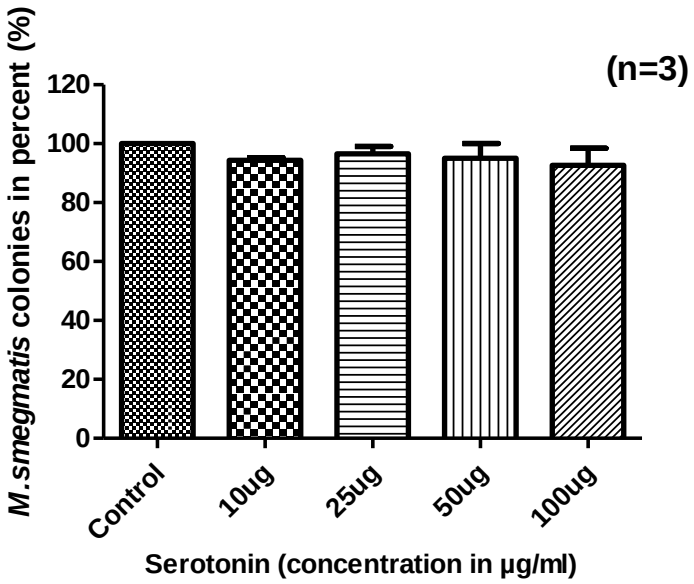


Figure 3.11c: Direct effect of serotonin on the growth of *M.smegmatis*. Each bar represents the average of three individual experiments and the error bars show standard error of means (SEM). The data is presented in percentage, where solvent control is taken as 100% survival and different test conditions are compared to it. The graph shows that *in vitro* serotonin has no direct inhibitory effect on the growth *M.smegmatis.* Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (p=0.72).

3.12 Direct effect of L-alpha dipalmitoylphosphatidylcholine (DPPC) on *M.smegmatis* growth *in vitro*

L-alpha dipalmitoylphosphatidylcholine (DPPC) is the most abundant lung surfactant phospholipid (Noutsios & Floros, 2013). DPPC was selected during the *in vitro* screening to investigate if this major alveolar phospholipid has any direct inhibitory effect on mycobacterial growth.

Three independent experiments were carried out to find the direct effect of DPPC on *M.smegmatis* growth using different concentrations (10µg, 25µg, 50µg and 100µg/ml) of DPPC and a solvent control for DPPC (10µl ethanol/ml bacterial suspension) according to the protocol mentioned in section 2.2. All the experiment showed the same trend (Figure 3.12b) and it was observed that *in vitro* DPPC in concentrations of up to 100µg/ml had no direct inhibitory effect on the growth of *M.smegmatis* (Figures 3.12a, 3.12b and 3.12c). Non-

parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be non-significant (p=0.35)

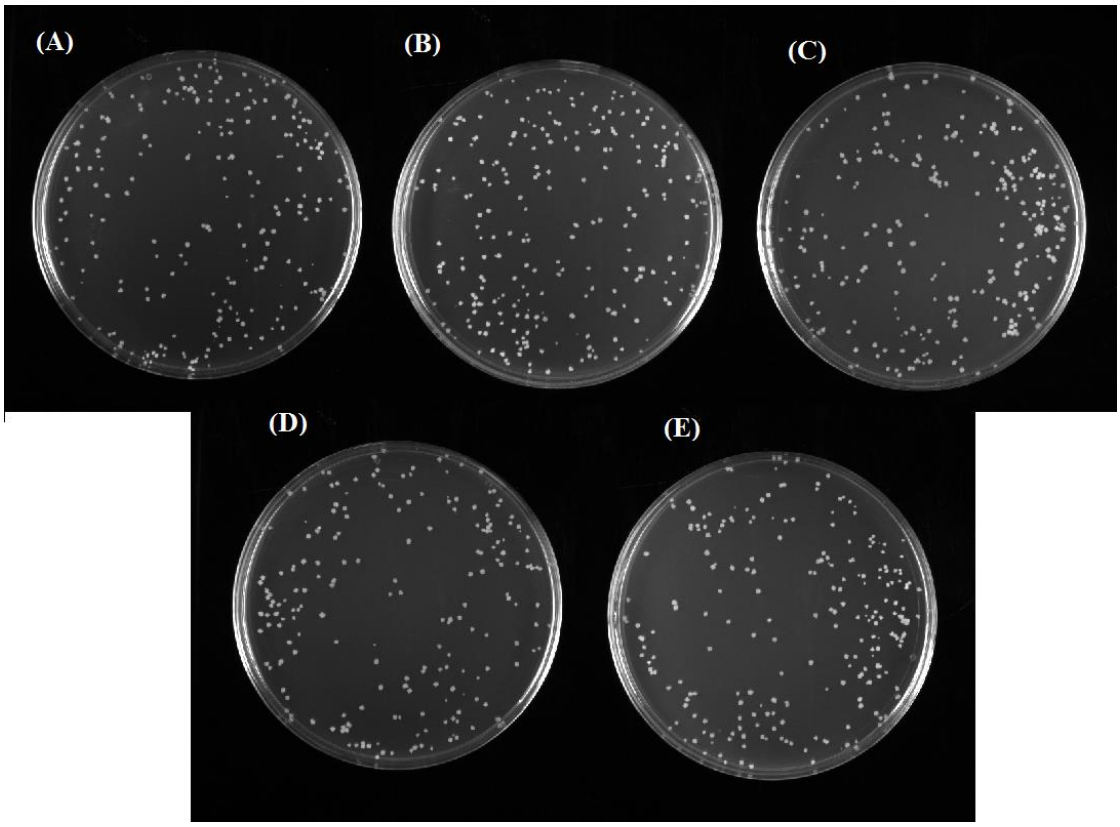


Figure 3.12a: *M.smegmatis* CFUs on LB-Agar plates after treatment with different concentrations of DPPC. Plate (A) is the solvent control for DPPC (10µl ethanol/ml bacterial suspension), Plate (B), Plate (C), Plate (D) and Plate (E) shows the number of *M.smegmatis* CFUs after treatment with 10µg, 25µg, 50µg and 100µg/ml of DPPC respectively. The picture shows one representative plate from the triplicates for solvent control and different test concentrations of DPPC used during one of the experiment.

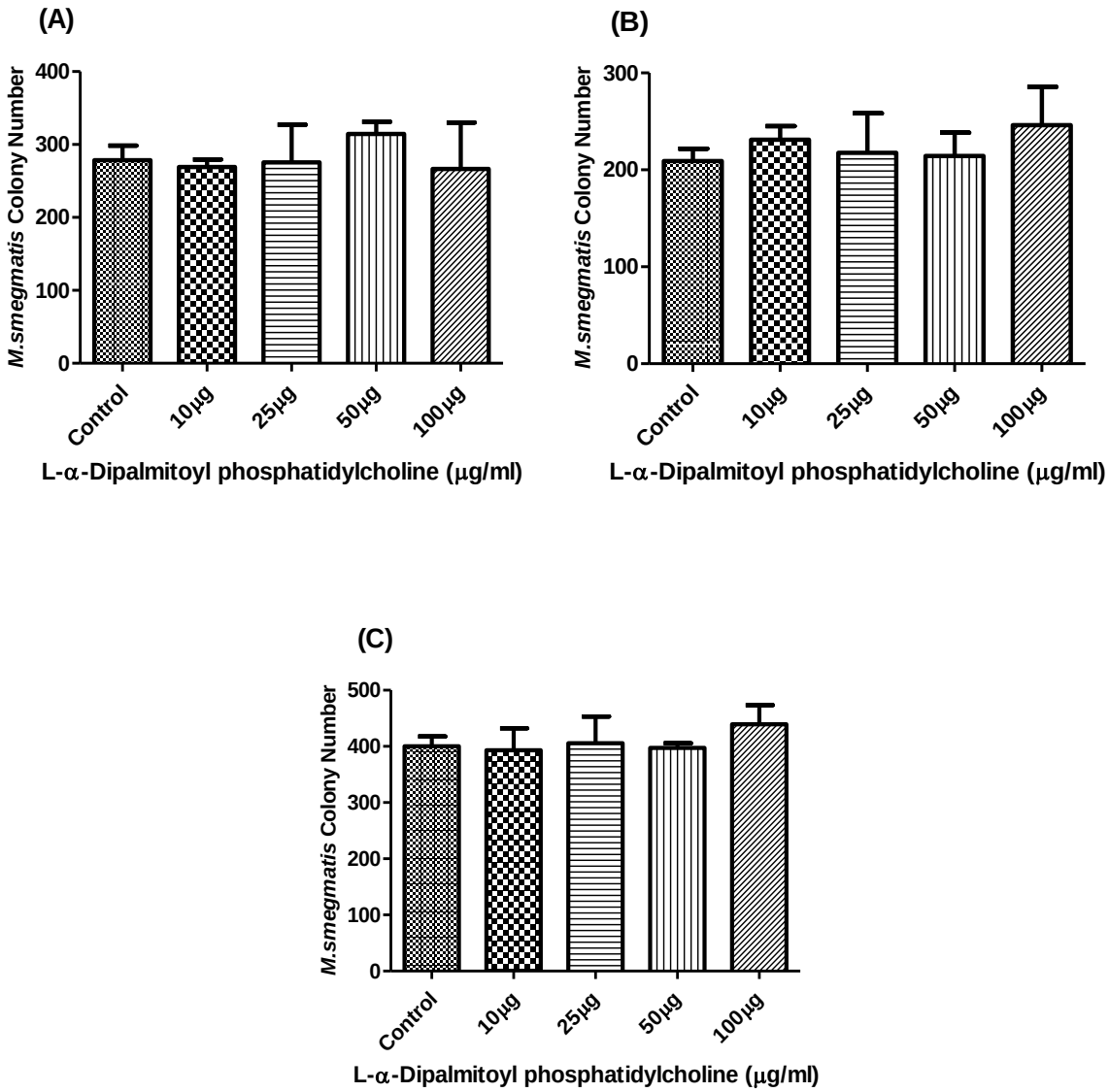


Figure 3.12b: Three individual experiments (A, B and C) for the direct effect of DPPC on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

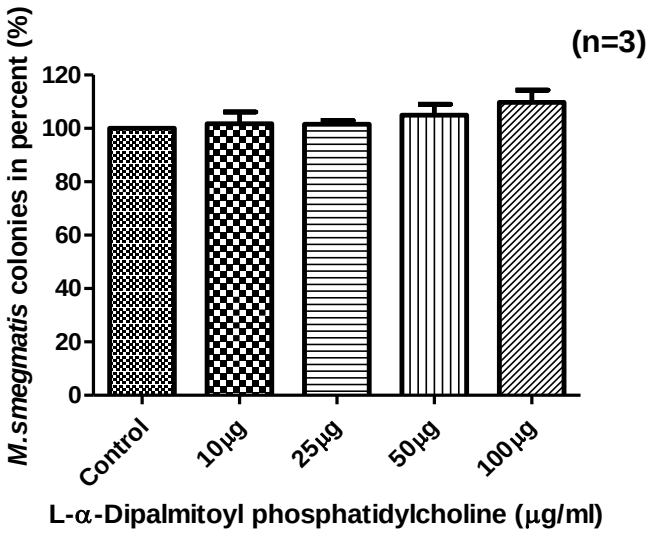


Figure 3.12c: Direct effect of DPPC on the growth of *M.smegmatis*. Each bar represents the average of three individual experiments and the error bars show standard error of means (SEM). The data is presented in percentage, where control is taken as 100% survival and different test conditions are compared to it. The graph shows that DPPC has no direct growth inhibiting effect on *M.smegmatis* growth. Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p* value, which was found to be non-significant (p=0.35).

3.13 Direct effect of Platelet Activating Factor C-16 on *M. smegmatis* growth *in vitro*

Platelet activating factor C-16 (PAF C-16) is a membrane derived alkyl-ether phospholipid chemically known as 1-O-hexadecyl-2-O-acetyl-*sn*-glyceryl-3-phosphocholine (Figure 3.13a). During inflammation, PAF C-16 is produced by different and is involved in important inflammatory processes such as changes in vascular permeability (Evans *et al*., 1987). Therefore, PAF C-16 was investigated for its direct effect on the growth of *mycobacteria*.

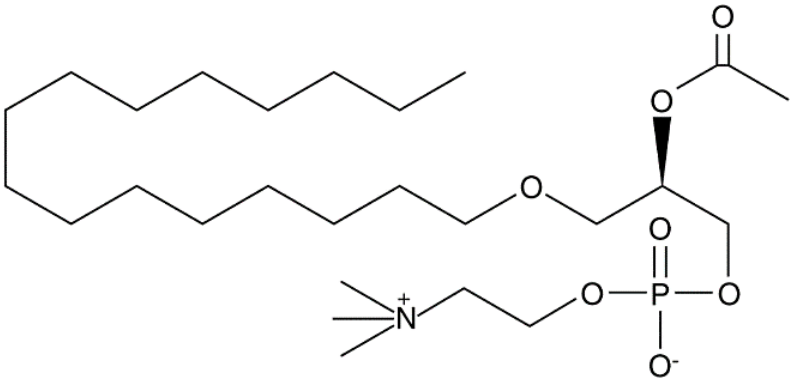


Figure 3.13a: Chemical structure of Platelet activating factor C-16 (PAF C-16).

Six individual experiments were carried out *in vitro* to find the direct effect of PAF C-16 on *M.smegmatis* growth using different concentrations (10µg, 25µg, 50µg and 100µg/ml) of PAF C-16 and a solvent control for PAF C-16 (10µl ethanol/ml bacterial suspension) according to the protocol mentioned in section 2.2. It was observed that PAF C-16 was able to inhibit the growth of *M.smegmatis* in a dose-dependent manner after treatment for 2 hours (Figure 3.13b). All the experiments of PAF C-16 with *M.smegmatis* showed the same trend of growth inhibition (Figure 3.13c). The growth inhibitory effect of PAF C-16 against *M.smegmatis* was more prominent at higher concentrations of 50µg/ml and 100µg/ml, which on average caused ~70% and ~97% reduction in the number of *M.smegmatis* CFUs respectively when compared to solvent control (Figure 3.13d). Level of significance was determined by applying non-parametric multiple comparison Kruskal Wallis test on ranks and the *p*-value was found to be significant, $p \leq 0.0001$. Individual data sets were compared by applying post hoc Dunn's multiple comparison test and the results were found to be significant only for 50µg/ml PAF C-16 vs Solvent control ($p \leq 0.01$) and 100µg/ml PAF C-16 vs Solvent control ($p \leq 0.001$).

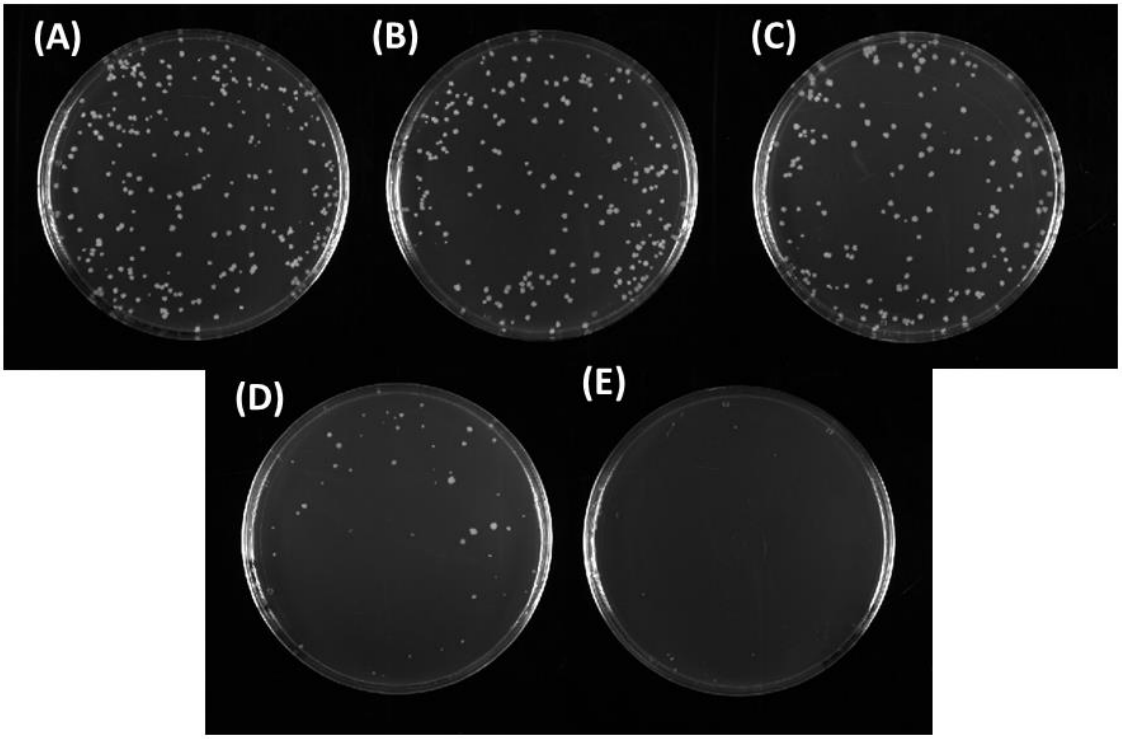


Figure 3.13b: *M.smegmatis* CFUs on LB-Agar plates after treatment with different concentrations of PAF C-16. Plate (A) is a solvent control for PAF C-16(10µl ethanol/ml bacterial suspension), Plate (B), Plate (C), Plate (D) and Plate (E) shows the number of bacterial CFUs after treatment with 10µg, 25µg, 50µg and 100µg/ml of PAF C-16 respectively. The picture shows one representative plate from the triplicates for solvent control and different test concentrations of PAF C-16 used during one of the experiment. It can be observed that PAF C-16 treatment resulted in a decrease in the number of *M.smegmatis* CFUs.

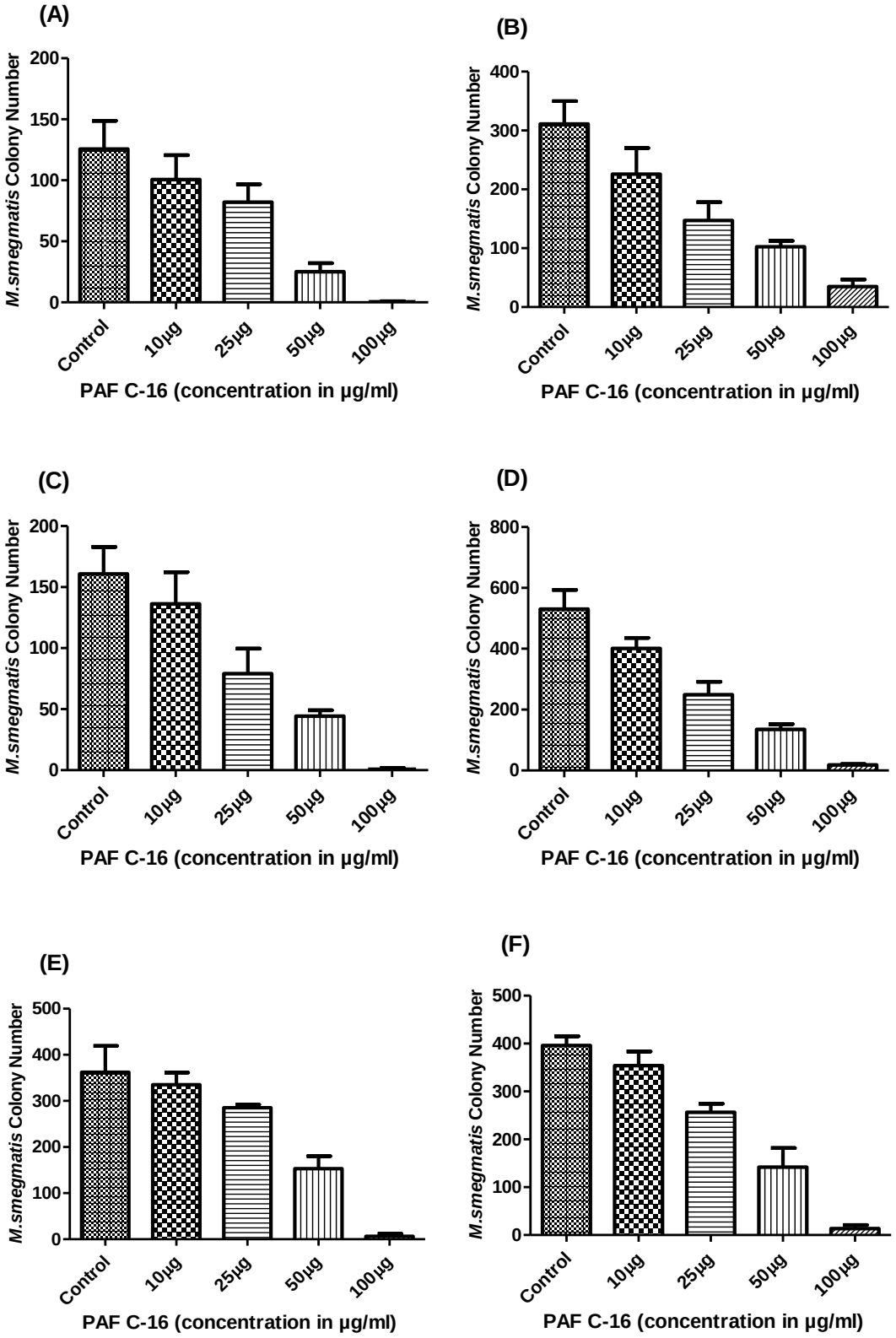


Figure 3.13c: Six individual experiments (A, B, C, D, E and F) for the direct effect of PAF C-16 on *M.smegmatis* growth. Each bar represents the average *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicates. All the graphs show the same

trend, where an increase in the concentration of PAF C-16 caused decrease in the number of bacterial colonies.

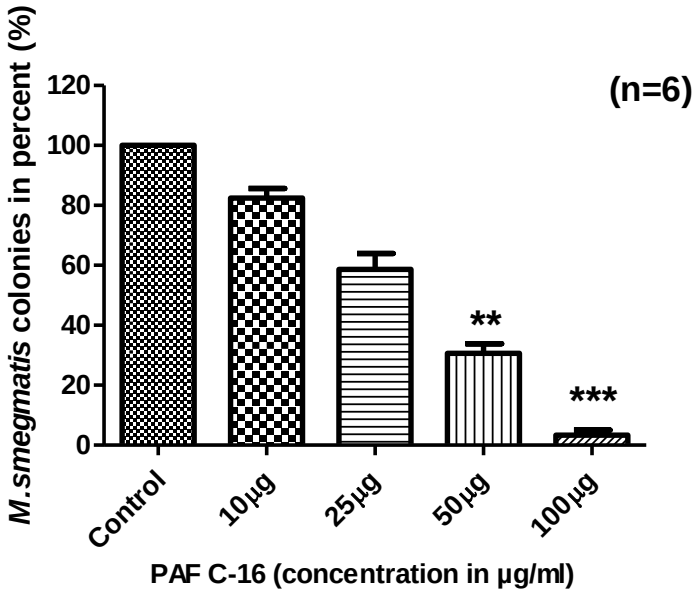


Figure 3.13d: Direct effect of PAF C-16 on *M.smegmatis* growth. Each bar represents the average of six individual experiments and the error bars show the standard error of means (SEM). Data is expressed in terms of percentage survival where solvent control is considered as 100% survival and different test conditions are compared to it. Multiple-comparison of the data sets was performed using non-parametric Kruskal-Wallis test on ranks and the *p* value was found to be significant ($p \leq 0.0001$). Individual data sets were compared using Dunn's multiple comparison test and the results were significant for 50µg/ml PAF C-16 vs Solvent control **($p \leq 0.01$) and 100µg/ml PAF C-16 vs Solvent control ***($p \leq 0.001$).

3.14 Direct effect of Platelet Activating Factor PAF C-16 on growth of *M.bovis BCG in vitro*

The direct effect of PAF C-16 on the *M.bovis BCG* growth *in vitro* was investigated by treating *M.bovis BCG* with different concentrations of PAF C-16 (10µg, 25µg, 50µg and 100µg/ml) and a solvent control for PAF C-16 (10µl ethanol/ml bacterial suspension) according to the protocol mentioned in section 2.2. Six independent experiments of PAF C-16 with *M.bovis BCG* were carried out in triplicates. It was observed that PAF C-16 was able to inhibit the

growth of *M.bovis BCG* in a dose-dependent manner after treatment for 2 hours (Figure 3.14a). All the experiments of PAF C-16 with *M.bovis BCG* showed the same trend of growth inhibition (Figure 3.14b). The growth inhibitory effect of PAF C-16 against *M.bovis BCG* was more prominent at higher concentrations of 50µg/ml and 100µg/ml, which on average caused ~65% and ~87% reduction in the number of *M.bovis BCG* CFUs respectively when compared to solvent control (Figure 3.14c). Level of significance was determined by applying non-parametric multiple comparison Kruskal Wallis test on ranks and the *p*-value was found to be significant, $p \leq 0.0001$. Individual data sets were compared by applying post hoc Dunn's multiple comparison test and the results were found to be significant only for 50µg/ml PAF C-16 vs Solvent control ($p \leq 0.01$) and 100µg/ml PAF C-16 vs Solvent control ($p \leq 0.001$).

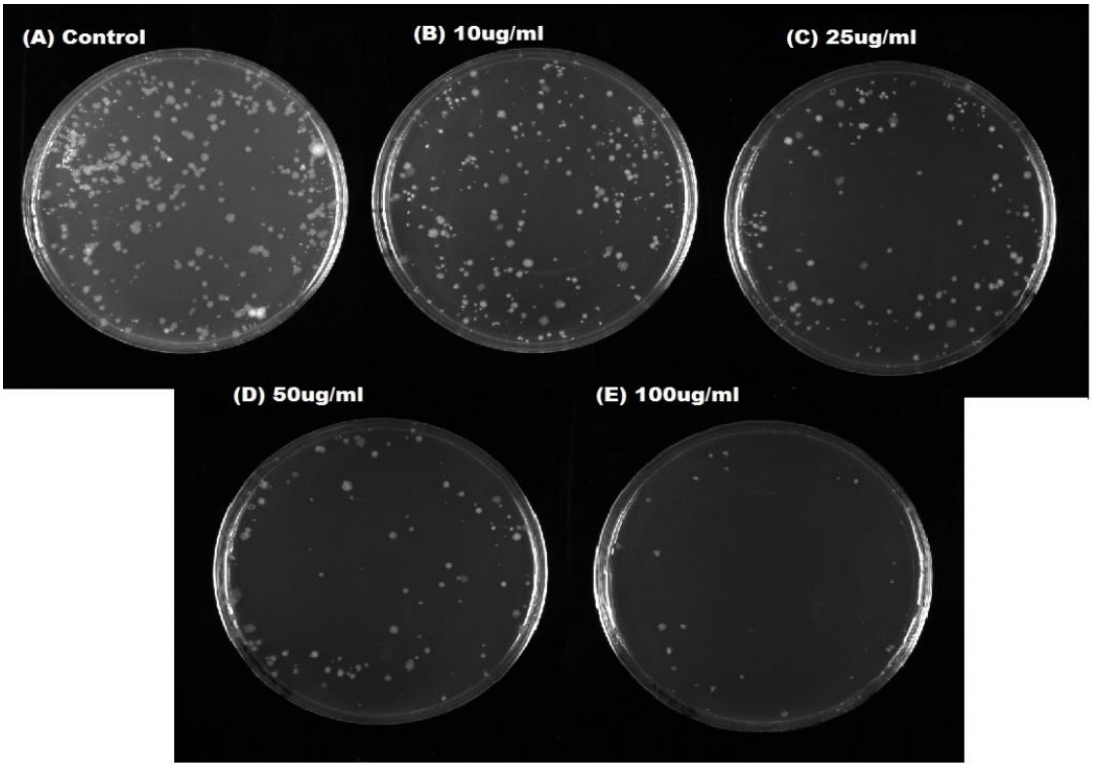


Figure 3.14a: *M.bovis BCG* CFUs on 7H10 plates after treatment with different concentrations of PAF C-16. Plate (A) is a solvent control for PAF C-16(10µl ethanol/ml bacterial suspension), Plate (B), Plate (C), Plate (D) and Plate (E) show the number of *M.bovis BCG* CFUs after treatment with 10µg, 25µg, 50µg and 100µg/ml of PAF C-16 respectively. The picture shows one representative plate from the triplicates for solvent control and different test concentrations of PAF C-16 used during one of the experiment. It can be observe that PAF C-

16 treatment inhibited the growth of *M.bovis BCG* as indicated by the decrease in the number of bacterial CFUs on the 7H10 plates.

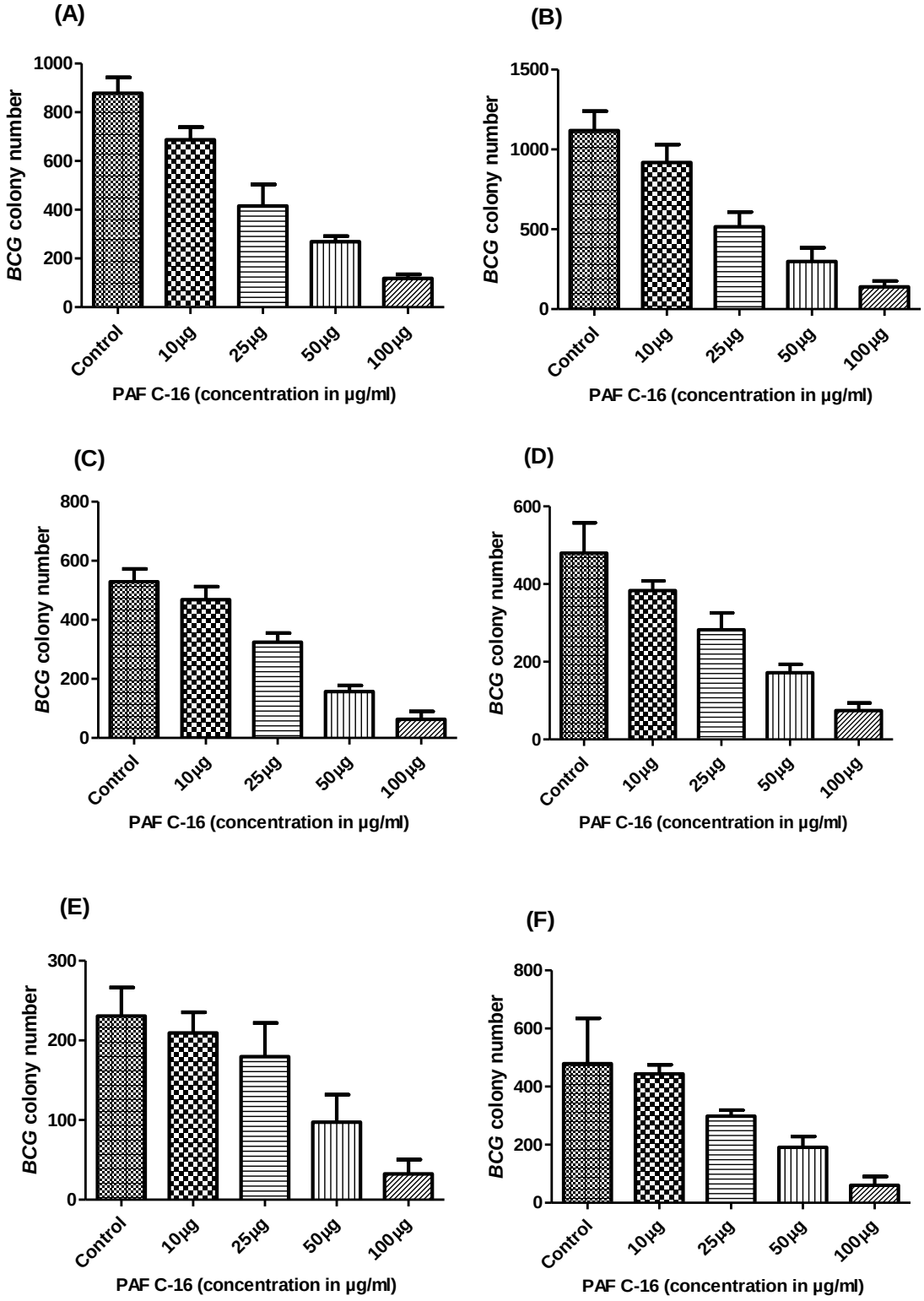


Figure 3.14b: Six individual experiments (A, B, C, D, E and F) for the direct effect of PAF C-16 on *M.bovis BCG* growth. Each bar represents the average number of *M.bovis BCG* CFUs

from triplicate plates and error bar shows standard deviation (SD) of triplicates. All the graphs show the same trend, where an increase in the concentration of PAF C-16 causes a decrease in the number of *M.bovis BCG* CFUs.

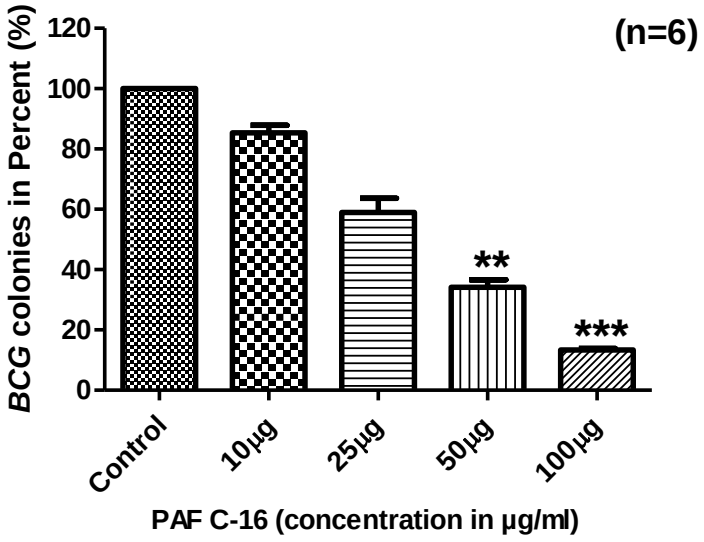


Figure 3.14c: Direct effect of PAF C-16 on *M.bovis BCG* growth. Each bar represents the average of six individual experiments and the error bars show the standard error of means (SEM). Data is expressed in terms of percentage survival where control is taken as 100% survival and different test conditions are compared to it. Multiple-comparison of the data sets was performed by applying non-parametric Kruskal-Wallis test on ranks and the *p*-value was found to be significant ($p \leq 0.0001$). Individual data sets were compared using Dunn's multiple comparison test and the results were significant for 50µg/ml PAF C-16 vs Solvent control **($p \leq 0.01$) and 100µg/ml PAF C-16 vs Solvent control ***($p \leq 0.001$).

3.15 Effect of increase in PAF C-16 incubation time on *M.smegmatis* growth

In order to investigate the effect of increase in PAF C-16 treatment time on the growth inhibition, *M.smegmatis* was treated with lower concentrations of PAF C-16 ranging from 1µg/ml-25µg/ml for extended durations of 6, 12 and 24 hours. Four independent experiments were performed *in vitro* using PAF C-16 in concentrations of 1µg, 2.5µg, 5µg, 10µg and 25µg/ml and a solvent control for PAF C-16 (2.5µl ethanol/ml bacterial suspension) according to the protocol mentioned in section 2.2.

PAF C-16 was able to inhibit the growth of *M.smegmatis* both in a time and dose-dependent manner (Figures 3.15a, 3.15b and 3.15c). It was observed that PAF C-16 at a concentration of 5µg/ml showed a reduction of 55% in the number of surviving *M.smegmatis* CFUs after 6 hours treatment whereas, the same concentration of 5µg/ml resulted in more than 95% reduction in the number of CFUs after increased treatment durations of 12 and 24 hours as compared to solvent control (Figure 3.15d).

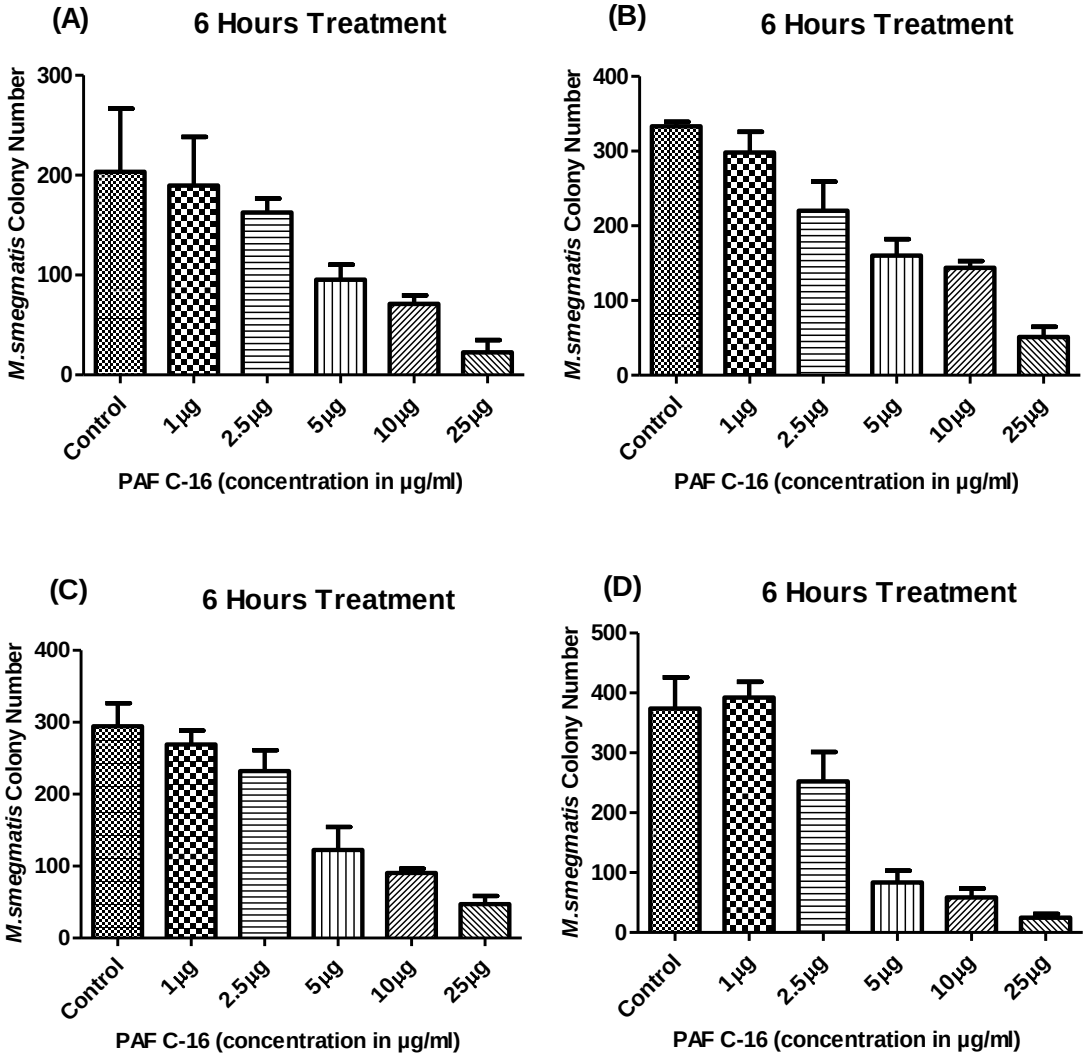


Figure 3.15a: Four individual experiments (A, B, C and D) for the effect of PAF C-16 on the growth of *M.smegmatis* after 6 hours of treatment. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicates.

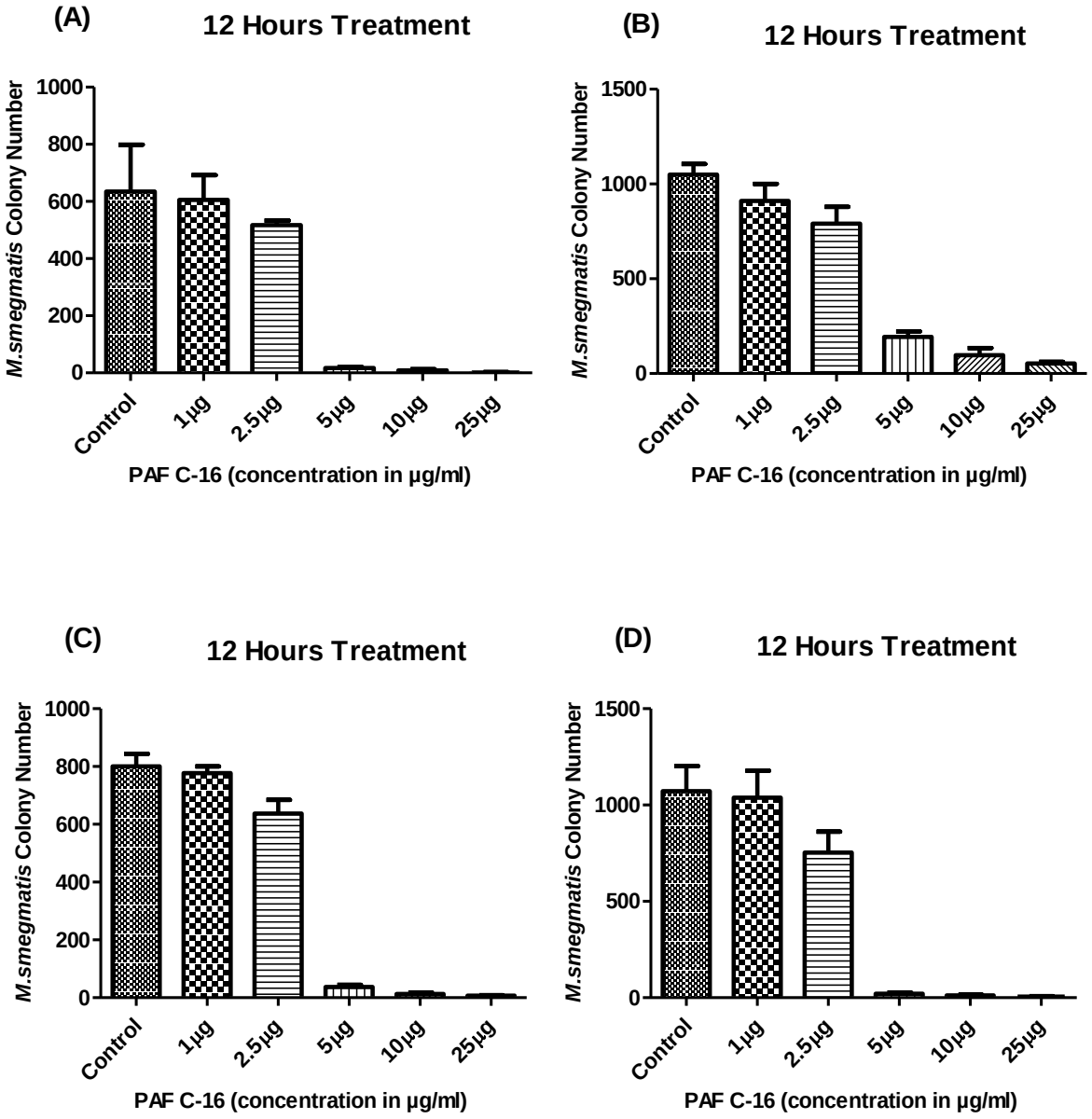


Figure 3.15b: Four individual experiments (A, B, C and D) for the effect of PAF C-16 on the growth of *M.smegmatis* after 12 hours of treatment. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicates.

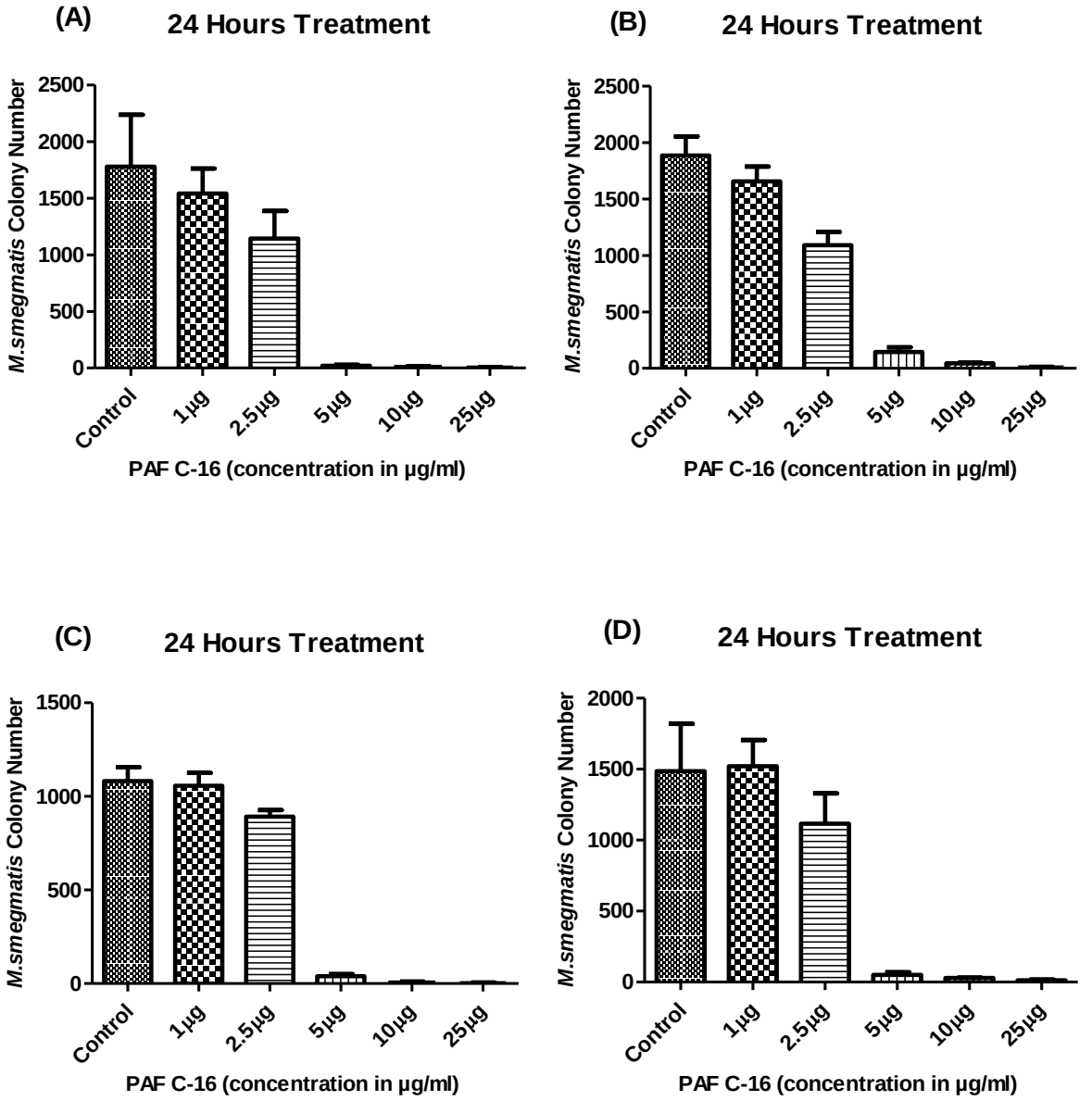


Figure 3.15c: Four individual experiments (A, B, C and D) for the effect of PAF C-16 on the growth of *M.smegmatis* after 24 hours of treatment. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicates.

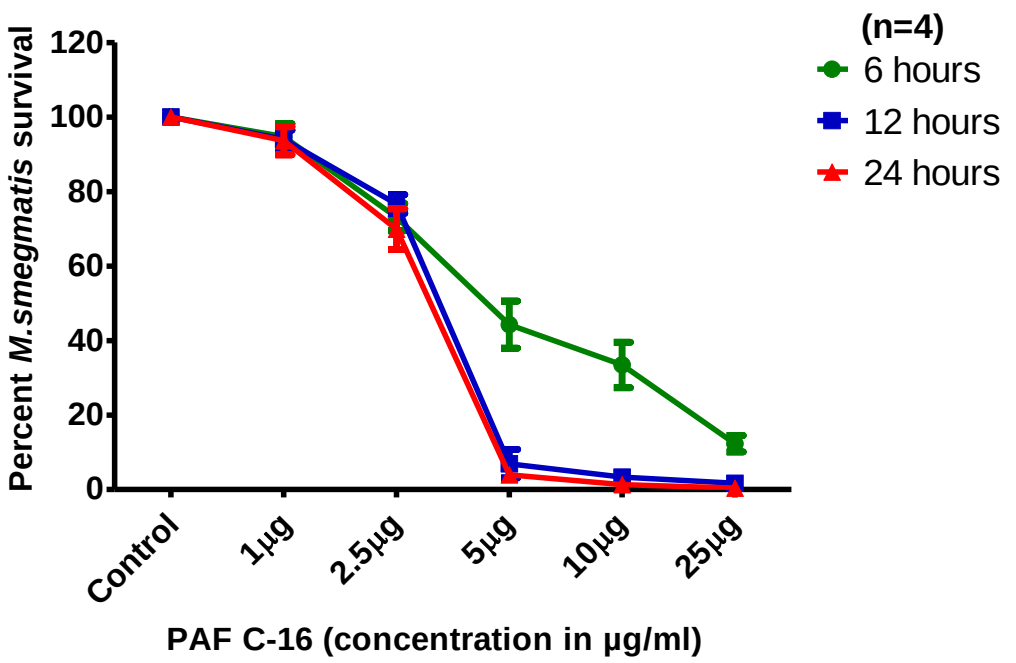


Figure 3.15d: Comparison of *M.smegmatis* survival after treatment with PAF C-16 for 6, 12 and 24 hours. The data is expressed in terms of percentage where solvent control is taken as 100% survival. Each data point on the graph represent the average CFUs form four independent experiments at a particular concentration and the error bars show the standard error of means (SEM).

3.16 Effect of increase in PAF C-16 incubation time *M.bovis BCG* growth

The effect of increase in PAF C-16 treatment time was also investigated on the growth inhibition of *M.bovis BCG*. Lower concentrations of PAF C-16 ranging from 1µg/ml-25µg/ml were used for extended durations of 6, 12 and 24 hours. Four independent experiments were performed *in vitro* using PAF C-16 in concentrations of 1µg, 2.5µg, 5µg, 10 µg and 25µg/ml and a solvent control for PAF C-16 (2.5µl ethanol/ml bacterial suspension) according to the protocol mentioned in section 2.2.

PAF C-16 was also able to inhibit the growth of *M.bovis BCG* in both time and dose-dependent manner (Figures 3.16a, 3.16b and 3.16c). It was observed that PAF C-16 at a concentration of 5µg/ml showed a reduction of 51% in the number of surviving *M.bovis BCG* CFUs after 6 hours treatment, which increased to 83% and 95% reduction treatment durations of 12 and 24 hours respectively as compared to solvent control (Figure 3.16d).

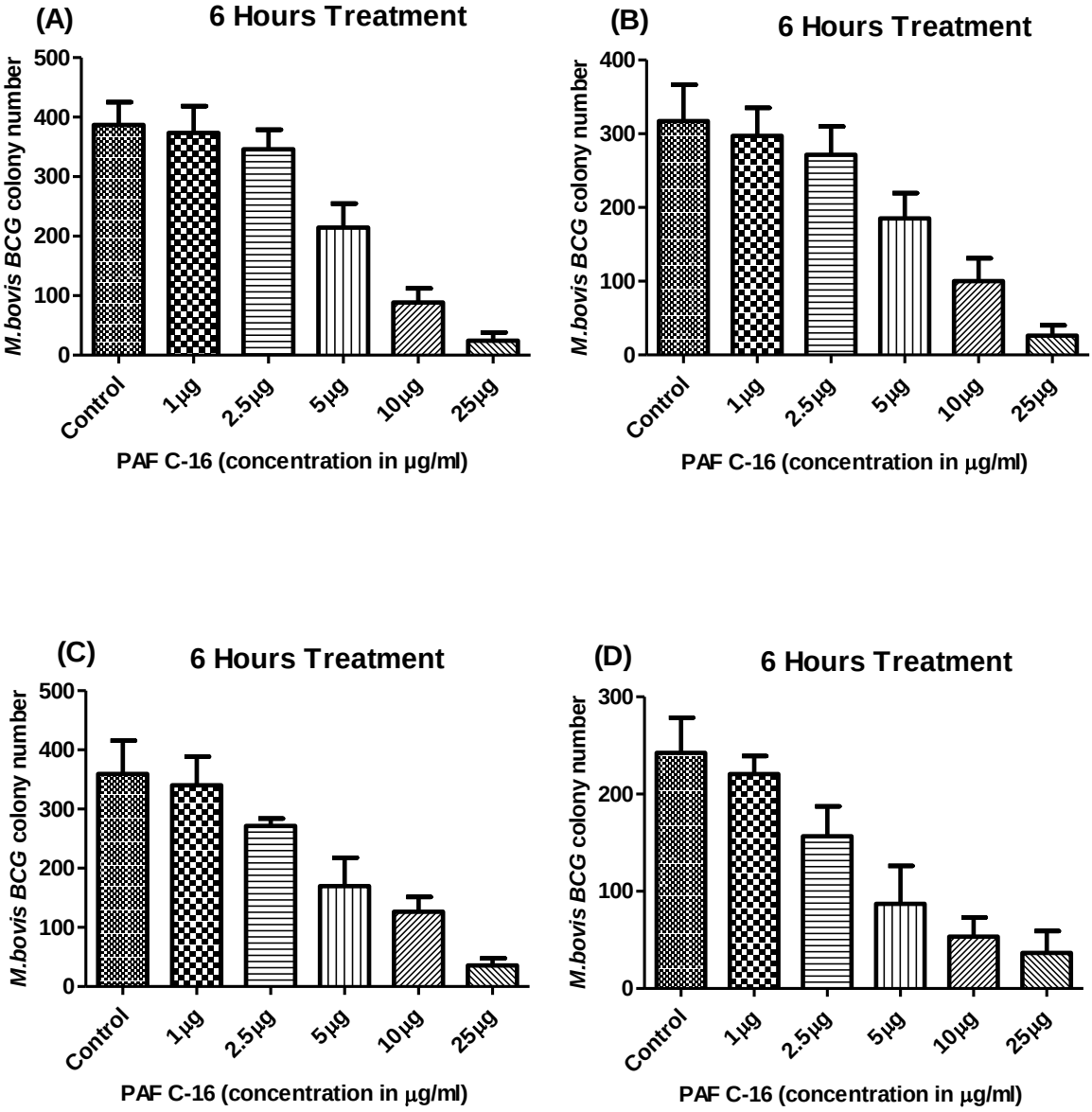


Figure 3.16a: Four individual experiments (A, B, C and D) for the direct effect of PAF C-16 on *M.bovis BCG* growth after 6 hours treatment. Each bar represents the average number of *M.bovis BCG* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicates.

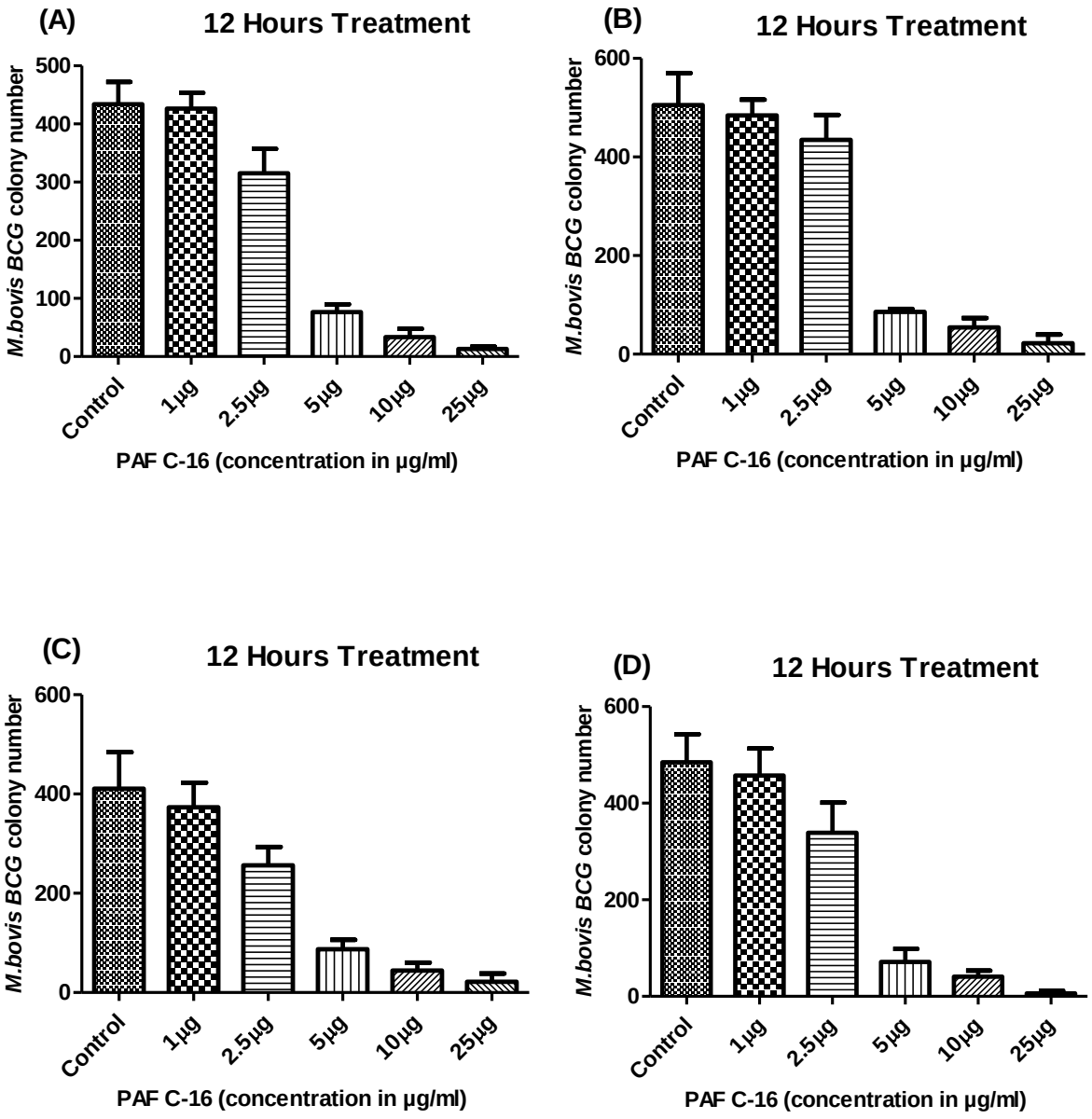


Figure 3.16b: Four individual experiments (A, B, C and D) for the direct effect of PAF C-16 on *M.bovis BCG* growth after 12 hours treatment. Each bar represents the average number of *M.bovis BCG* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicates.

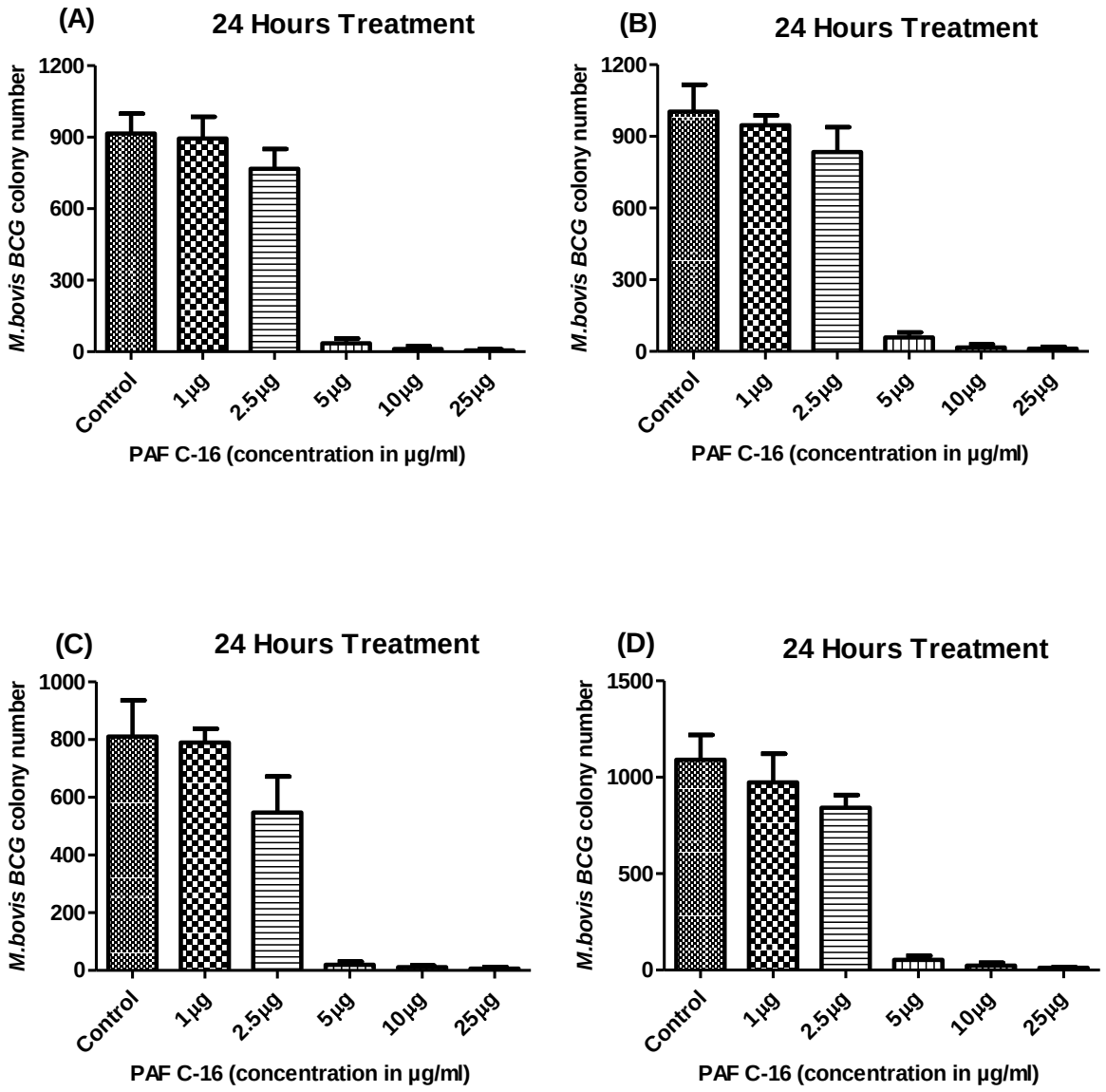


Figure 3.16c: Four individual experiments (A, B, C and D) for the direct effect of PAF C-16 on *M.bovis BCG* growth after 24 hours treatment. Each bar represents the average number of *M.bovis BCG* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicates.

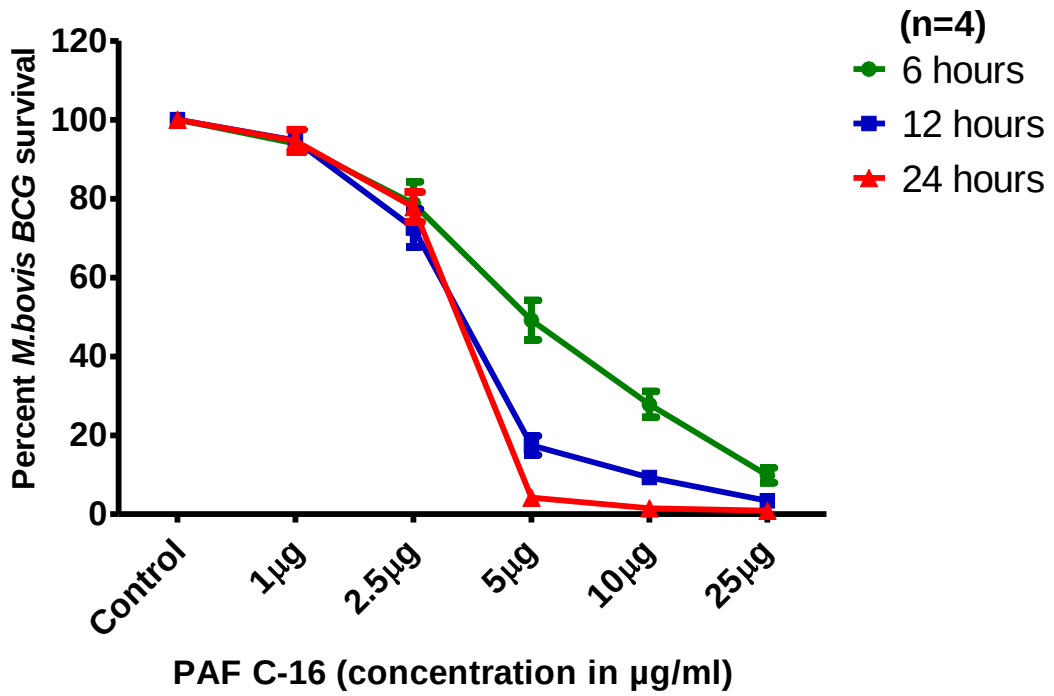


Figure 3.16d: Comparison of *M.bovis BCG* survival after treatment with PAF C-16 for 6, 12 and 24 hours. The data is expressed in terms of percentage where solvent control is taken as 100% survival. Each data point on the graph represent the average CFUs form four independent experiments at a particular concentration and the error bars show the standard error of means (SEM)

3.17 Determining the Minimum Inhibitory Concentration (MIC) for PAF C-16 against *M.smegmatis*

To determine the lowest concentration of PAF C-16 that can inhibit the growth of *M.smegmatis*, minimum inhibitory concentration (MIC) was determined for PAF C-16. Approximately $1x10^6$ *M.smegmatis* was resuspended in 2ml culture media were incubated at 37°C for 48 hours with PAF C-16 in 2-fold dilutions, ranging from concentrations100µg/ml-0.78µg/ml and a solvent control for PAF C-16 (10µl ethanol/ml bacterial suspension) was also included in the experiment. The MIC of PAF C-16 against *M.smegmatis* was found to lie between concentrations 3.12µg/ml and 1.56µg/ml of PAF C-16 (Figure 3.17). Optical density ($O.D_{600nm}$) was measured after 48 hours for the control and PAF C-16 treated *M.smegmatis* cultures. The experiment was repeated three times and the optical density ($O.D_{600nm}$) values for control and different test conditions as shown in table 3.1.

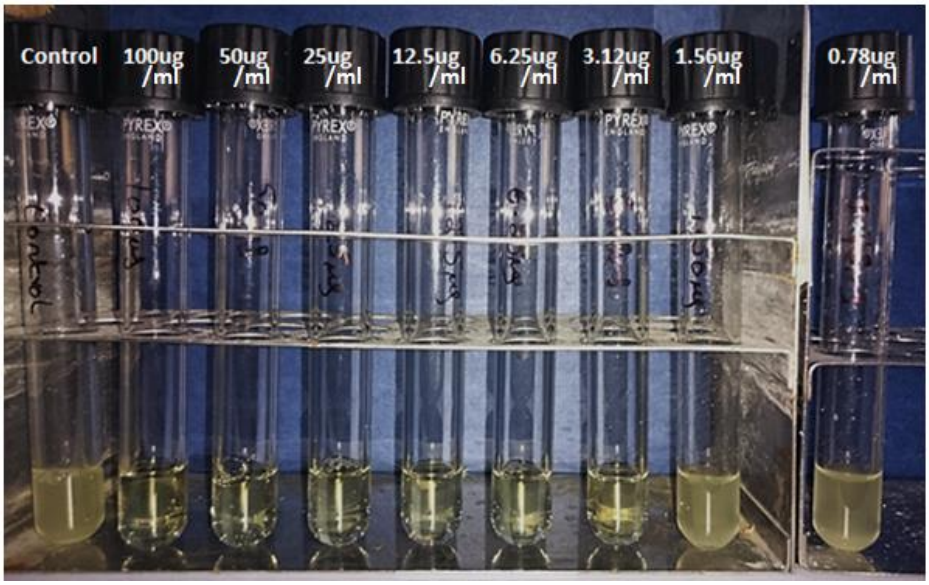


Figure 3.17: Minimum inhibitory concentration (MIC) of PAF C-16 against *M.smegmatis*. The *M.smegmatis* cultures tubes containing 100µg, 50µg, 25µg, 12.5µg, 6.25µg and 3.12µg/ml PAF C-16 are clear indicating no visible bacterial growth. The last two tubes with 1.56µg/ml and 0.78µg/ml PAF C-16 are turbid indicating *M.smegmatis* growth at those concentrations.

Table 3.1: Optical density ($O.D_{600nm}$) values for *M.smegmatis* treated with different concentration of PAF C-16 after 48 hours.

PAF C-16 concentration	**Optical density of *M.smegmatis* ($O.D_{600nm}$)**		
	Experiment 1	**Experiment 2**	**Experiment 3**
Control (10µl/ml Ethanol)	**0.82**	**1.04**	**0.89**
0.78µg/ml	**0.82**	**0.98**	**0.85**
1.56µg/ml	**0.70**	**0.96**	**0.74**
3.12µg/ml	**0.05**	**0.16**	**0.11**
6.25µg/ml	**0.03**	**0.01**	**0.06**
12.50µg/ml	**0.01**	**0.02**	**0.01**
25.00µg/ml	**0.02**	**0.01**	**0.02**
50.00µg/ml	**0.01**	**0.02**	**0.01**
100.00µg/ml	**0.01**	**0.01**	**0.01**

3.18 Discussion

Mycobacterium tuberculosis (*M.tb*) infections are the leading cause of human mortality among all bacterial pathogens and result in more than 1 million human deaths every year. According to WHO, approximately 10 million new cases of *M.tb* infections were reported worldwide, during 2015 (WHO, Global TB report 2016). New challenges such as HIV-TB coinfections (Daley *et al*., 1992) and the emergence of multi-drug resistant stains (Espinal *et al*., 2003) and extensively-drug resistant strains (Tang *et al*., 2011; Jain and Mondal, 2008) of *M.tb* have amplified the severity of the problem. Furthermore, there are currently a limited number of drugs available for treating tuberculosis and the treatment requires a minimum of 6 months duration. On the prophylactic side, the current vaccine against TB, Bacillus Calmette Gu´erin (BCG) has showed variable efficacy in controlling tuberculosis, especially in the adult population (Aronson *et al*., 2004; Colditz *et al*., 1994; Palmer and Long, 1966). Therefore, novel interventions are required both on the therapeutic and preventive fronts to control the global menace of *M.tb*.

Infection with pathogen causes a disturbance in the host homeostasis and the host responds by producing a non-specific, acute phase inflammatory response to the invading pathogen (Cooper and Flynn, 1995). As a result, there is a local reaction at the site of infection characterized by rapid blood flow, blood vessels dilation and increase in vascular permeability causing the leakage of blood plasma fluid. This exudate is rich in both protein and non-protein factors, which are most likely to come in direct contact with the pathogen and immune cells at the site of infection. Different immune cells such as polymorphonuclear cells (PMCs) and monocytes/macrophages also migrate to the site of infection to eliminate the invading pathogen (Shi and Pamer, 2011; Toossi, 2001). These immune cells can also synthesize proteins such as complement protein C1q (Kaul and Loos 2001; Loos *et al*., 1989) phospholipids such as PAF C-16 (Camussi *et al*., 1987) and release acute phase cytokines such as TNF-α, IL-6 and IL-1 (Gruys *et al*., 2005) at the site of infection. The acute phase cytokines can later act on different target cells and thus lead to a systemic reaction. These cytokines also control the concentration of different plasma proteins known as the acute phase proteins (APP) by regulating their synthesis rate in the liver (van Miert, 1995).

In this study, a number of host factors including both proteins and non-proteins present in the blood plasma and/or locally at the site of infection were used. We hypothesized that nature has given multiple functions to these natural factors apart from their known functions and therefore, we explored the unknown effect of these host factors on the growth of *mycobacteria* by using

M.smegmatis and *M.bovis BCG* as model organisms for *M.tb*. This information will help in understanding the role of these factors in bacterial pathogenesis and their therapeutic utility against *M.tb*. The advantage of screening these factors is that they are produced within the body and therefore, they will not excite immunological reactions causing minimum or no side effects. The factors screened in the current study for their direct inhibitory effect on the growth of *mycobacteria* included Transferrin, Lactoferrin, Fibrinogen, C-reactive protein (CRP), Vitronectin, Plasminogen, Alpha-2-Macroglobulin (α2M), High Density Lipoproteins (HDL), Low Density Lipoproteins (LDL), L-alpha dipalmitoyl phosphocholine (DPPC), Serotonin, and Platelet Activating Factor (PAF C-16).

Some of the host factors included in this study such as transferrin, lactoferrin and platelet activating factor have previously been shown to possess direct growth inhibitory effect against a number of gram positive and/or gram negative bacteria. Lin *et al* used recombinant human transferrin protein at concentrations ranging from 0.6µg-60µg/ml and showed 6µg/ml transferrin as the minimum inhibitory concentration for gram positive bacteria *S.aureus* as well as gram negative bacteria *A.baumannii*, which is substantially lower than the physiological concentration of transferrin protein (Lin *et al*., 2010). Singh *et al* used lactoferrin protein at a concentration range of 10-100µg/ml and showed that lactoferrin protein was able to inhibit the growth of gram negative *P.aeruginosa* significantly at a concentration of 100µg/ml (Singh *et al*., 2002). Similar results were obtained by Bortner *et al*, as they showed that lactoferrin protein at a concentration of 0.09mg/ml (90µg/ml) was able to reduce the viability of a pathogenic gram negative bacteria *L.pneumophila* by approximately 99.99% (Bortner *et al*., 1989). Steel *et al* screened PAF C-16 and Lyso PAF for their direct growth inhibitory effect against both gram positive and gram negative bacteria at a concentration range of 0.25-20µg/ml and showed both these phospholipid compounds were able to significantly inhibit the growth of gram positive bacteria at concentration as low as 2.5µg/ml, however no significant inhibitory effect was observed on gram negative bacteria at any of the concentrations used (Steel *et al*., 2002). Therefore, we followed a similar range of concentrations (1-100µg/ml) during the screening experiments for all the test compounds keeping in view the range of concentrations from the previously published literature as mentioned above.

In humans, transferrins are an important class of proteins that consist of iron binding proteins (Lambert *et al*., 2005). Two important members of this group include Transferrin protein and Lactoferrin/Lactotransferrin protein. Transferrin protein is abundant in human serum (Cazzola *et al*., 1985), whereas lactoferrin is abundant in milk, mucosal fluids and inside

polymorphonuclear leukocytes (Conesa *et al*., 2008; Levay, and Viljoen, 1995; Broxmeyer *et al*., 1978). In humans, these proteins play a strategic role by maintaining homeostasis as they hamper the microbial growth by creating an environment of low iron (Rogan *et al*., 2004; von Bonsdorff *et al*., 2003). In the current study, the direct effect of both iron binding transferrin protein and lactoferrin protein was investigated on the growth of *mycobacteria* using *M.smegmatis* as a model. However, both these proteins in concentrations of up to 100µg/ml failed to show any direct inhibitory effect on the growth of *M.smegmatis*. Human transferrin has previously been shown to possess antimicrobial properties both *in vitro* and *in vivo*. Lin *et al* showed that transferrin protein can inhibit the growth of both gram-negative (*Acinetobacter baumannii*) and gram-positive (*Staphylococcus aureus*) bacteria as well as fungi (*Candida albicans*) *in vitro*. This transferrin induced growth inhibition was shown to be as a result of damage to the cell membrane potential and ATP generation (Lin *et al*., 2014). *In vivo*, administration of human transferrin in infected mice improved survivability by controlling the growth of the above mentioned pathogens (Lin *et al*., 2014). Similarly, a number of previous studies have shown that *in vitro* lactoferrin also possess direct antibacterial properties bacteria such as *Escherichia coli*, *Salmonella typhimurium*, *Vibrio cholerae*, and *Streptococcus pneumoniae*. Lactoferrin inhibits the growth of these bacteria by mechanisms including inhibition of the bacterial H^+-ATPase complex and damage to the outer membrane in gram-negative bacteria due to LPS removal (Nibbering *et al*., 2001; Naidu *et al*., 1993; Arnold *et al*., 1981; Arnold *et al*., 1977).

A panel of three positive acute phase proteins comprising of Fibrinogen, C-reactive protein (CRP) and Alpha-2-macroglobulin (α2M) was also investigated *in vitro* for their direct growth inhibitory on *M.smegmatis*.

Fibrinogen is a precursor plasma protein, which upon activation with thrombin is converted to fibrin. The concentration of fibrinogen increases in blood during inflammation and infections (Toss *et al*., 1998). Therefore, we investigated the direct effect of this acute phase protein on the growth of *mycobacteria*. Our experiments with fibrinogen and *M.smegmatis* showed that *in vitro* fibrinogen in concentrations of up to 100µg/ml was unable to inhibit the growth of the *M.smegmatis*. Påhlman *et al* showed that treatment of Group A Streptococci such as *Streptococcus pyogenes* with exogenous fibrinogen in combination with thrombin resulted in the growth inhibition of these bacteria. Furthermore, this direct growth inhibitory effect was shown to be associated with a peptide fragment "GHR28" released from the β chain of fibrinogen (Påhlman *et al*., 2013).

CRP is an acute phase plasma protein whose concentration increases by several folds in response to stimuli such as infection or tissue injury (Povoa *et al*., 2005). Increase in concentration of CRP in blood during infection and inflammation made CRP an interesting candidate to be investigated for its direct effect on the growth of the *mycobacteria* during the screening. However, the results showed that *in vitro* CRP in concentrations of up to 50µg/ml had no direct inhibitory effect on *M.smegmatis* growth. Previously it has been shown that recombinant horseshoe crab C-reactive protein (rCRP2) possess direct bactericidal activity against gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* but was unable affect the growth of gram-positive bacteria such as *Staphylococcus aureus* (Tan *et al*., 2005).

The plasma α2M protein acts a non-specific proteinase inhibitor and is produced in increased quantities during infection and inflammation (Rehman *et al*., 2013). Our experiments with α2M and *M.smegmatis* showed that *in vitro* α2M in concentrations of up to 100µg/ml had no direct inhibitory effect on *M.smegmatis* growth. Khan *et al* suggested a protective role for α2M during pathogen infection and showed that administration of exogenous α2M resulted in improved survivability in *Pseudomonas aeruginosa* infected guinea pigs as compared to untreated control. The protective effect of α2M was due to its neutralizing effect on the bacterial elastase enzyme which are important for bacterial virulence (Khan *et al*., 1994). However, other reports suggested a protective role for α2M on bacterial growth. α2M was shown to bind to different pathogenic bacteria such as Streptococci via a bacterial surface protein known as GRAB. This binding of α2M to bacterial surfaces is thought to be helpful for bacteria as α2M can provide protection to the bacteria from different antibacterial proteinases inside the body (Rasmussen *et al*., 1999).

Plasma proteins vitronectin and plasminogen were also included in the screening and investigated for their direct effect on the growth of *M.smegmatis*. There is limited information about the direct effect of these plasma proteins on the growth of bacteria. Our results showed that *in vitro* both vitronectin and plasminogen proteins had no direct inhibitory effect on the growth of *M.smegmatis* at different concentrations ranging from 10µg-100µg/ml. Vitronectin has previously been shown to bind to different bacteria such as *Haemophilus influenzae, Streptococcus pneumoniae* and *Moraxella catarrhalis* and prevent them from the lytic effects of host complement membrane attack complex (MAC) by preventing it deposition on the bacterial surface (Hallström *et al*., 2006; Chhatwal *et al*., 1987). Plasminogen is an important precursor plasma protein that is converted into active form plasmin, which plays important role

in fibrinolysis (Aĭsina and Mukhametova, 2014). To the best of our knowledge there is currently no information about the direct effect of plasminogen on the growth of *mycobacteria*. Previously it has been shown that plasminogen can interact with bacterial proteases such as Streptokinase (SK) and Staphylokinase (SAK) enzymes from *Streptococci* and *Staphylococci* and may augment the proteolytic invasion of the host by these bacteria (Peetermans *et al*., 2016).

Bacterial infection accompanied by inflammation results in the production of different cytokines and some of these cytokines such as IL-6, TNF-α, and IL-1 cause profound changes in the concentration, composition and function of the plasma lipid and lipoproteins (Nassaji and Ghorbani, 2012; Khovidhunkit *et al*., 2004). We investigated two blood plasma lipoproteins, High-density lipoprotein (HDL) and Low-density Lipoprotein (LDL) for their direct inhibitory effect on *M.smegmatis* growth. However, our results showed that both HDL and LDL had no direct inhibitory effect in concentrations up to 25µg/ml on *M.smegmatis* growth. Previously, Apolipoprotein A-1 (Apo A-1), a major protein of HDL was shown to possess bactericidal activity against gram-negative bacteria *Yersinia enterocolitica* (Biedzka-Sarek *et al*., 2011). Similarly, transgenic mice lacking plasma LDL Apolipoprotein B-100 (Apo B-100) were shown to be more susceptible to *Staphylococcus aureus* infection (Peterson *et al*., 2008) indicating a protective role this LDL protein component against bacterial infection of the host.

Non-protein factors including the major lung surfactant L-alpha dipalmitoyl phosphocholine (DPPC) and two pro-inflammatory mediators, serotonin and platelet activating factor (PAF C-16) were also screened for their direct inhibitory effect on mycobacterial growth.

L-alpha dipalmitoyl phosphocholine (DPPC) is the most abundant lung surfactant phospholipid (Noutsios and Floros, 2013). This lung surfactant was investigated for its direct growth inhibitory effect on *M.smegmatis*. However, our results showed that *in vitro* DPPC in concentrations of up to 100µg/ml was unable to inhibit the growth of *M.smegmatis*. There are previous studies demonstrating the bactericidal activity of leukocyte free bronchoalveolar lavage and purified lavage surfactants from rats against *Staphylococcus pneumoniae* suggesting that lung surfactants are involved in protection against pathogens (Coonrod *et al*., 1984; O'Neill *et al*., 1984).

Serotonin (5-hydroxytryptamine) is released in increased amounts during inflammation from different cells such as mast cells (Kushnir-Sukhov *et al*., 2007) and platelets (Ge *et al*., 2009)

and causes changes in vascular permeability (Cloutier *et al*., 2012). Our experiments with serotonin and *M.smegmatis* showed that *in vitro* this compound was unable to inhibit the growth of *M.smegmatis* in concentrations of up to 100µg/ml. A previous study with serotonin showed that serotonin possesses direct *in vitro* antifungal activity and was able to inhibit the growth of clinically isolated species of *Candida* (Lass-Flörl *et al*., 2002).

During the screening, PAF C-16, a pro-inflammatory phospholipid was the only compound that inhibited the growth of *mycobacteria*. Treatment of both *M.smegmatis* and *M.bovis BCG* with exogenous PAF C-16 for 2 hours resulted in the growth inhibition of these bacteria in a dose-dependent manner. The growth inhibitory effect of PAF C-16 was more noticeable at higher concentrations of 50µg/ml and 100µg/ml and caused significant levels ($p \leq 0.01$-≤ 0.001) of reduction in the number of *M.smegmatis* and *M.bovis BCG* CFUs when compared with the solvent control. PAF C-16 treatment also affected the colony morphology of the surviving bacteria on agar plates as the colonies appeared thinner when compared to the control indicating that the surviving bacteria were under stress. To the best of our knowledge, there is no previous study that shows the direct inhibitory effect of PAF C-16 on the growth of *mycobacteria*. A single previous study by Steel *et al* showed that exogenous PAF C-16 was able to directly inhibit the growth of gram-positive bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus* and *Streptococcus mutans* in cultures and had no inhibitory effect on the growth of gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Klebsiella pneumoniae*. The mechanism for PAF C-16 induced bacterial growth inhibition was suggested to be through disruption of microbial K^+ transport (Steel *et al*., 2002). However, the exact mechanism of PAF C-16 induced growth inhibition in gram-positive bacteria and its ineffectiveness against gram-negative requires further investigation. Furthermore, PAF C-16 was also shown to inhibit the growth of lower eukaryotes such as yeast in a dose-dependent manner when added to the growth medium and this growth inhibitory effect of PAF C-16 was partially mitigated by pre-treatment with PAF receptor antagonists suggesting that the growth inhibitory activity of PAF C-16 was mediated through its receptor (Nigam *et al*., 2013).

Although PAF C-16 was able to inhibit the growth of *mycobacteria* during the initial screening, the concentrations of PAF C-16 that caused significant growth inhibition of *M.smegmatis* and *M.bovis BCG* were very high. There are a number of adverse side effects associated with higher concentrations of PAF C-16 such as increased vascular permeability, hypotension, decreased cardiac output, asthma and gastrointestinal and inflammatory disorders. (Stafforini *et al*.,

2003). Therefore, we investigated the growth inhibitory effect of PAF C-16 at lower concentrations and increased incubation duration on *mycobacteria*. It was observed that by increasing the treatment duration to 6 hours, PAF C-16 at a concentration of 5µg/ml caused ~50% reduction in *M.smegmatis* and *M.bovis BCG* CFUs ($p \leq 0.01$) as compared to the solvent control. The growth inhibition at 5µg/ml PAF C-16 increased to ≥80% ($p \leq 0.001$) when the treatment times were extended to 12 and 24 hours. These results indicated that the growth inhibitory effect of PAF C-16 was also dependent on treatment time. Minimum inhibitory concentration (MIC) for PAF C-16 against *M.smegmatis* was also determined and was found to lie between concentrations 3.12µg/ml and 1.56µg/ml of PAF C-16. For the sake of convenience, all further experiments with PAF C-16 or its structural analogues were performed at concentrations 10µg, 25µg, 50µg and 100µg/ml with 2 hours treatment time as in principle we have established that PAF C-16 can inhibit the growth of both *M.smegmatis* and *M.bovis BCG* at the lower concentration as well.

In conclusion, as a result of our screening, novel direct mycobacterial growth inhibition activity was detected for PAF C-16. The inhibitory effect of PAF C-16 on the growth of *M.smegmatis* and *M.bovis BCG* was shown to be both dose-dependent and time-dependent.

Chapter 4: Investigating the effect of changes in structure of PAF C-16 on the growth of *mycobacteria* and the mechanism of PAF C-16 induced mycobacterial growth inhibition

4.1 Introduction

After showing *in vitro* the direct inhibitory effect of PAF C-16 on the growth of *M.smegmatis* and *M.bovis BCG* in a dose and time-dependent manner, we focused on the structure-activity relationship of PAF C-16. For this purpose, different PAF C-16 structural analogues were used and their effect on mycobacterial growth was investigated *in vitro*. Previous research has shown that small changes in the structure of PAF C-16 can alter its biological activity in mammals, with effects ranging from complete inactivation to decreased potency during different physiological processes (Stewart and Grigoriadis, 1991).

Structurally PAF C-16 consists of a glycerol backbone with a phosphocholine head at position *sn*-3, an acetyl group at position *sn*-2 and a 16-carbon atoms aliphatic tail attached via ether bond at position *sn*-1 (Hanahan *et al*., 1980) (Figure 3.13a). In order to localize the active portion of PAF C-16 that is responsible for its growth inhibitory effect against *mycobacteria*, compounds with structures similar to different portions of PAF C-16 were used.

PAF C-16 performs its diverse biological activities in eukaryotic organisms by binding to G-protein coupled transmembrane receptors known as PAF receptors (PAFR) on the cell membrane (Ishii *et al*., 2002). However, there is currently no information about the interaction of PAF C-16 with *mycobacteria* or the presence of PAFR on *mycobacteria* or any other bacteria. Also, limited research has been done to establish the mechanism through which PAF C-16 may inhibit the growth of prokaryotic organisms (Steel *et al*., 2002). Therefore, in this chapter we further investigated the effects of small modifications in PAF C-16 structure on its growth inhibition activity, structurally active portion of PAF C-16 and the mechanism for PAF C-16 induced mycobacterial growth inhibition.

Aims:

1) Investigating *in vitro* the direct effect of PAF C-16 structure analogues on the growth of *mycobacteria*.
2) Localization of active portion of PAF C-16 involved in mycobacterial growth inhibition.
3) Understanding the mechanism of PAF C-16 induced direct growth inhibition of *mycobacteria*.

4.2 PAF C-16 analogues and their growth inhibition potency against *mycobacteria*

A number of PAF C-16 structure analogues were investigated for their direct inhibitory effect on the growth of *mycobacteria*. These PAF C-16 structure analogues were selected in a way that each analogue represented a change of different functional group. Various PAF C-16 analogues used in this study included:

a) Lyso-PAF
b) PAF C-18
c) Hexanolamino PAF
d) 2-O-Methyl PAF
e) Pyrrolidino PAF

4.2.1 Direct effect of Lyso-Platelet Activating Factor (Lyso-PAF) on *M.smegmatis* growth *in vitro*

Lyso-Platelet Activating Factor (Lyso-PAF) is the precursor form of naturally occurring PAF C-16, chemically known as 1-O-hexadecyl-*sn*-glyceryl-3-phosphorylcholine (Figure 4.2.1a). This precursor form is considered to be biologically inactive and the direct effect of this de-acylated PAF on the growth of *mycobacteria* has not been investigated.

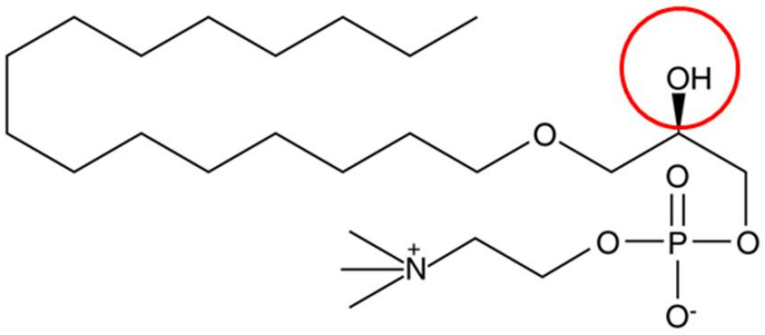


Figure 4.2.1a: Chemical structure of Lyso-PAF. Lyso-PAF contains a hydroxyl group at position *sn*-2 instead of an acetyl group, which is shown in the red circle.

The direct effect of Lyso-PAF on the growth of *M.smegmatis* was investigated according to the protocol in chapter 2.2. Six individual experiments (Exp1, 2, 3, 4, 5 and 6) were performed by treating *M.smegmatis* with different concentrations of Lyso-PAF (10µg, 25µg, 50µg and

100µg/ml of bacterial suspension) for 2 hours. A solvent control for Lyso-PAF (10µl ethanol/ml bacterial suspension) was also included in the experiment.

It was observed that Lyso-PAF was able to inhibit the growth of *M.smegmatis* in a dose-dependent manner as indicated by the reduction in the number of colony forming units (CFUs) when compared with the solvent control (Figure 4.2.1b). All the experiments of Lyso-PAF with *M.smegmatis* showed the same trend of growth inhibition (Figure 4.2.1c). The growth inhibitory effect of Lyso-PAF against *M.smegmatis* was more prominent at higher concentrations of 50µg/ml and 100µg/ml, which on average caused ~68% and ~95% reduction in the number of *M.smegmatis* CFUs respectively (Figure 4.2.1d). Non-parametric multiple comparison Kruskal Wallis test was applied on ranks and the *p*-value was found to be significant, $p \leq 0.0001$. Individual data sets were compared by applying post hoc Dunn's multiple comparison test and the results were found to be significant only for 50µg/ml Lyso-PAF vs Solvent control ($p \leq 0.01$) and 100µg/ml Lyso-PAF vs Solvent control ($p \leq 0.001$).

These results suggest that substituting the acetyl group (CH_3O) of PAF C-16 at position *sn*-2 with a hydroxyl group (OH) did not affect the growth inhibitory activity against *M.smegmatis*.

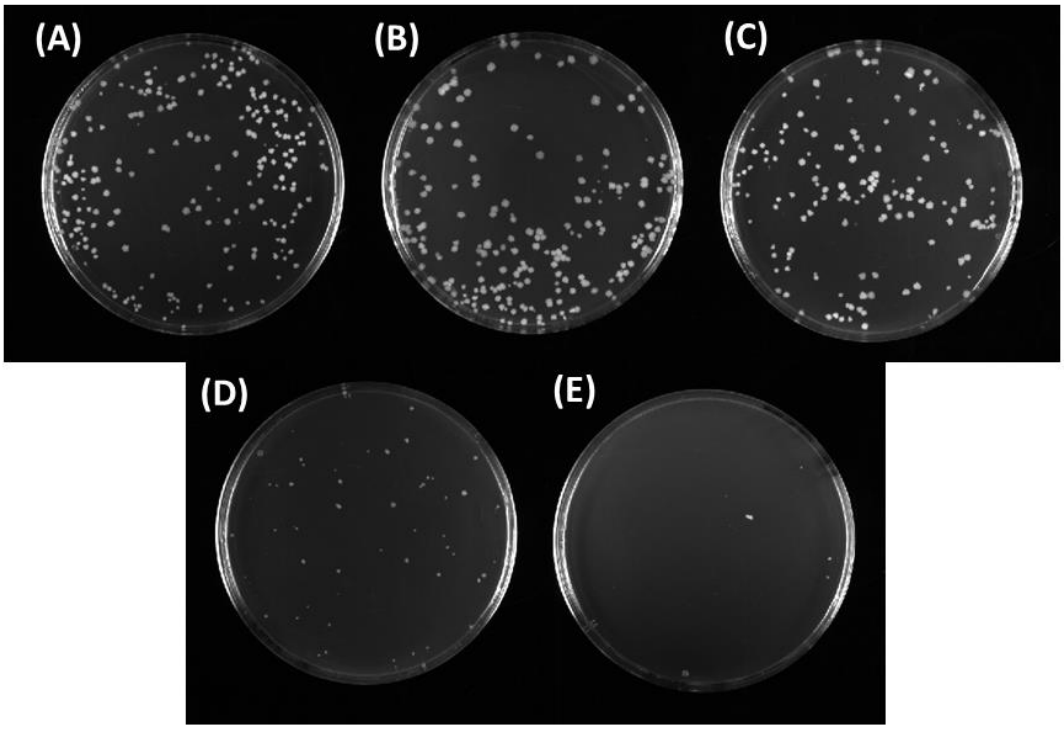
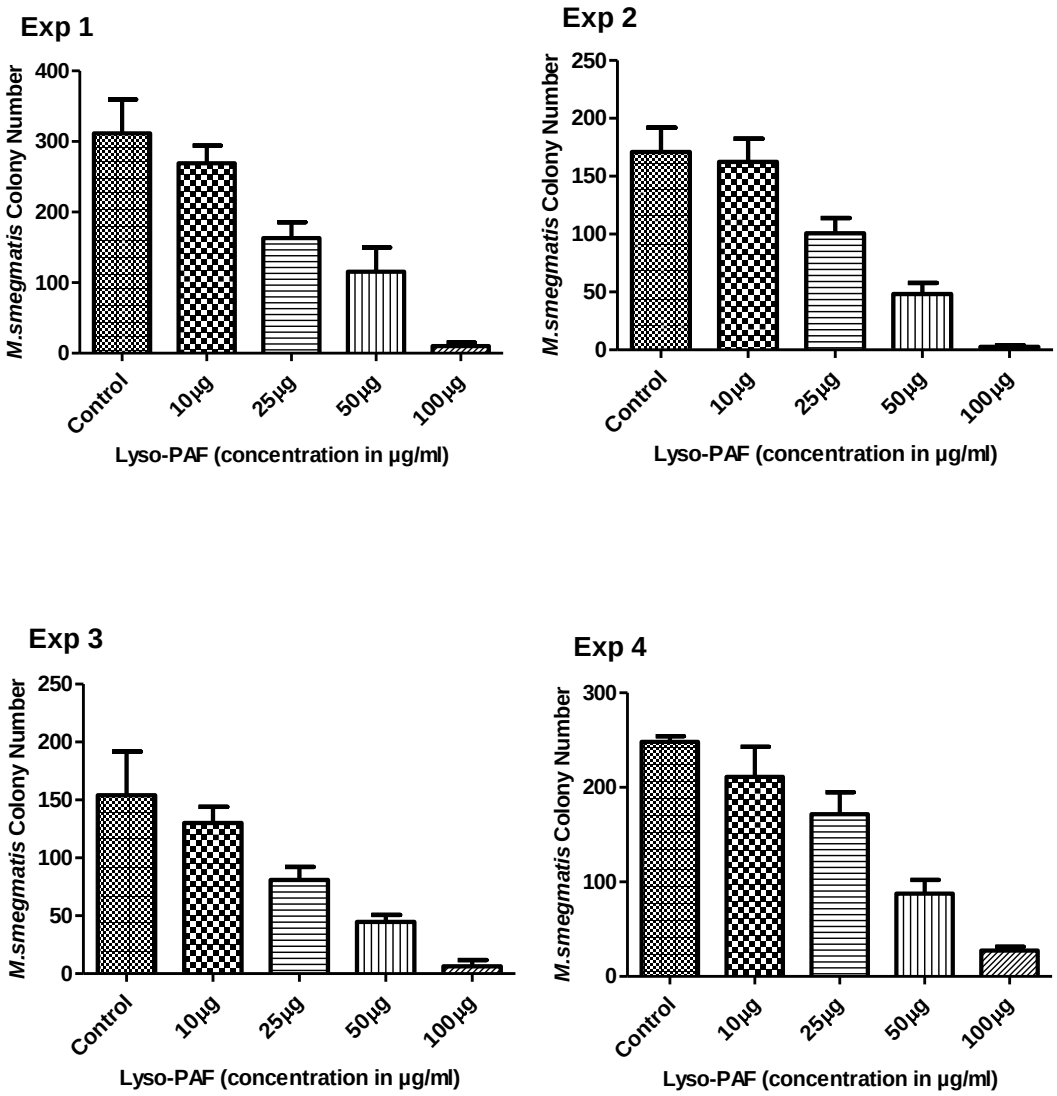


Figure 4.2.1b: *M.smegmatis* CFUs on LB-Agar plates after treatment with different concentrations of Lyso-PAF. Plate (A) is a solvent control for Lyso-PAF (10µl ethanol/ml

bacterial suspension), Plate (B), Plate (C), Plate (D) and Plate (E) shows the number of CFUs after treatment with 10µg, 25µg, 50µg and 100µg/ml of Lyso-PAF respectively. The picture shows one representative plate from the triplicate for solvent control and different concentrations of Lyso-PAF used during one of the experiment.



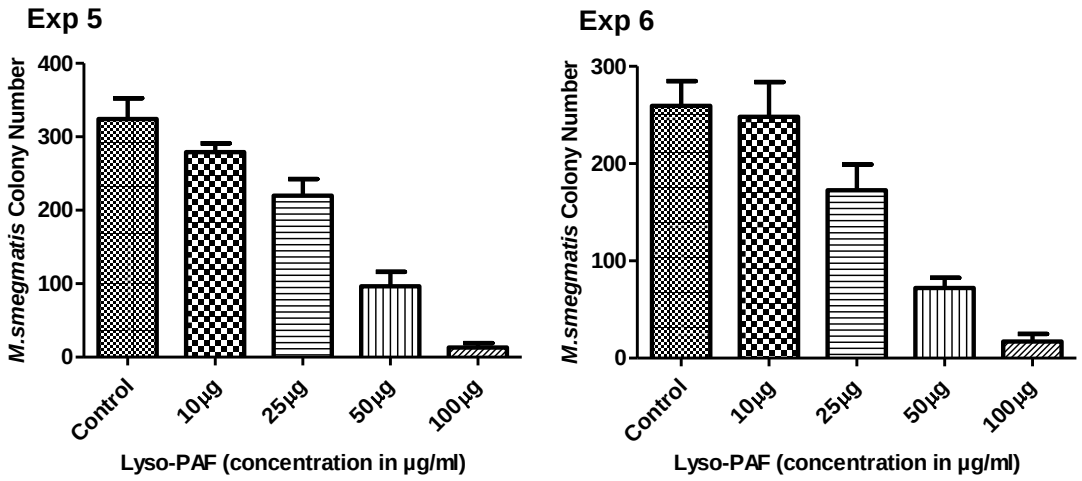


Figure 4.2.1c: Six independent experiments (Exp1, 2, 3, 4, 5 and 6) showing the direct effect of Lyso-PAF on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

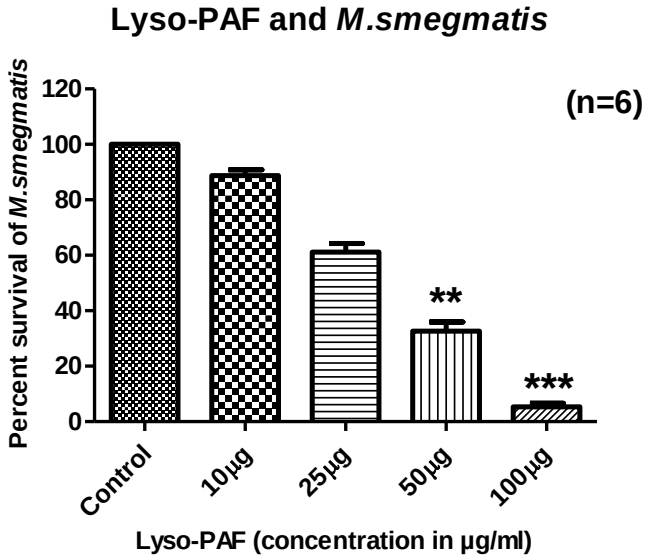


Figure 4.2.1d: Direct effect of Lyso-PAF on *M.smegmatis* growth. Data is expressed in terms of percentage, where solvent control is considered as 100% survival and different Lyso-PAF treated test conditions are compared to it. Each bar represents the average of six independent experiments and the error bars show the standard error of means (SEM). The graph represents the numbers of *M.smegmatis* CFUs after treatment with different concentrations of Lyso-PAF for 2 hours. Non-parametric multiple-comparison Kruskal-Wallis test was applied on ranks to

calculate the *p*-value which was found to be significant ($p \leq 0.0001$). Individual data sets were compared using Dunn's multiple comparison test, where 50µg/ml Lyso-PAF vs Solvent control **($p \leq 0.01$) and 100µg/ml Lyso-PAF vs Solvent control ***($p \leq 0.001$).

4.2.2 Direct effect of Lyso-Platelet Activating Factor (Lyso-PAF) on *M.bovis BCG* growth *in vitro*

Lyso-PAF was also investigated for its direct inhibitory effect on slow growing *M.bovis BCG*.

Six individual experiments (Exp1, 2, 3, 4, 5 and 6) were performed using different concentrations of Lyso-PAF (10µg, 25µg, 50µg and 100µg/ml bacterial suspension) and a solvent control for Lyso-PAF (10µl ethanol/ml bacterial suspension) according to the protocol mentioned in section 2.2. These experiments showed that Lyso-PAF was able to inhibit the growth of *M.bovis BCG* in a dose-dependent manner as indicated by a decrease in the number of *M.bovis BCG* CFUs when compared with the solvent control (Figure 4.2.2a). All the experiments of Lyso-PAF with *M.bovis BCG* showed the same trend of growth inhibition (Figure 4.2.2b). Lyso-PAF treatment of *M.bovis BCG* at concentrations of 50µg/ml and 100µg/ml on average caused ~64% and ~88% reduction in the number of CFUs respectively when compared with the solvent control (Figure 4.2.2c). The level of significance was determined by applying non-parametric multiple comparison Kruskal Wallis test on ranks and the *p*-value was found to be significant, $p \leq 0.0001$. Individual data sets were compared through post hoc Dunn's multiple comparison test and the results were found to be significant only for 50µg/ml Lyso-PAF vs Solvent control ($p \leq 0.01$) and 100µg/ml Lyso-PAF vs Solvent control ($p \leq 0.001$).

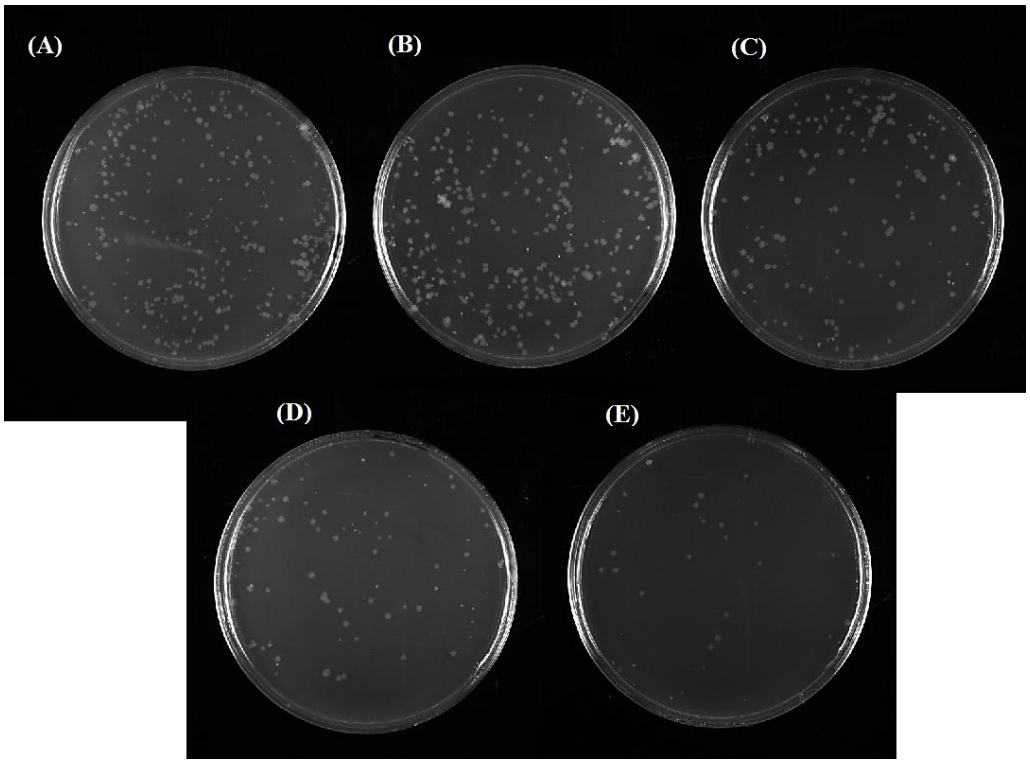


Figure 4.2.2a *M.bovis BCG* CFUs on 7H10 plates after treatment with different concentrations of Lyso-PAF. Plate (A) is a solvent control for Lyso-PAF (10µl ethanol/ml bacterial suspension), Plate (B), Plate (C), Plate (D) and Plate (E) shows the number of *M.bovis BCG* CFUs after treatment with 10µg, 25µg, 50µg and 100µg/ml of Lyso-PAF respectively. The picture shows one representative plate from the triplicates, for solvent control and different concentrations of Lyso-PAF used during one of the experiment.

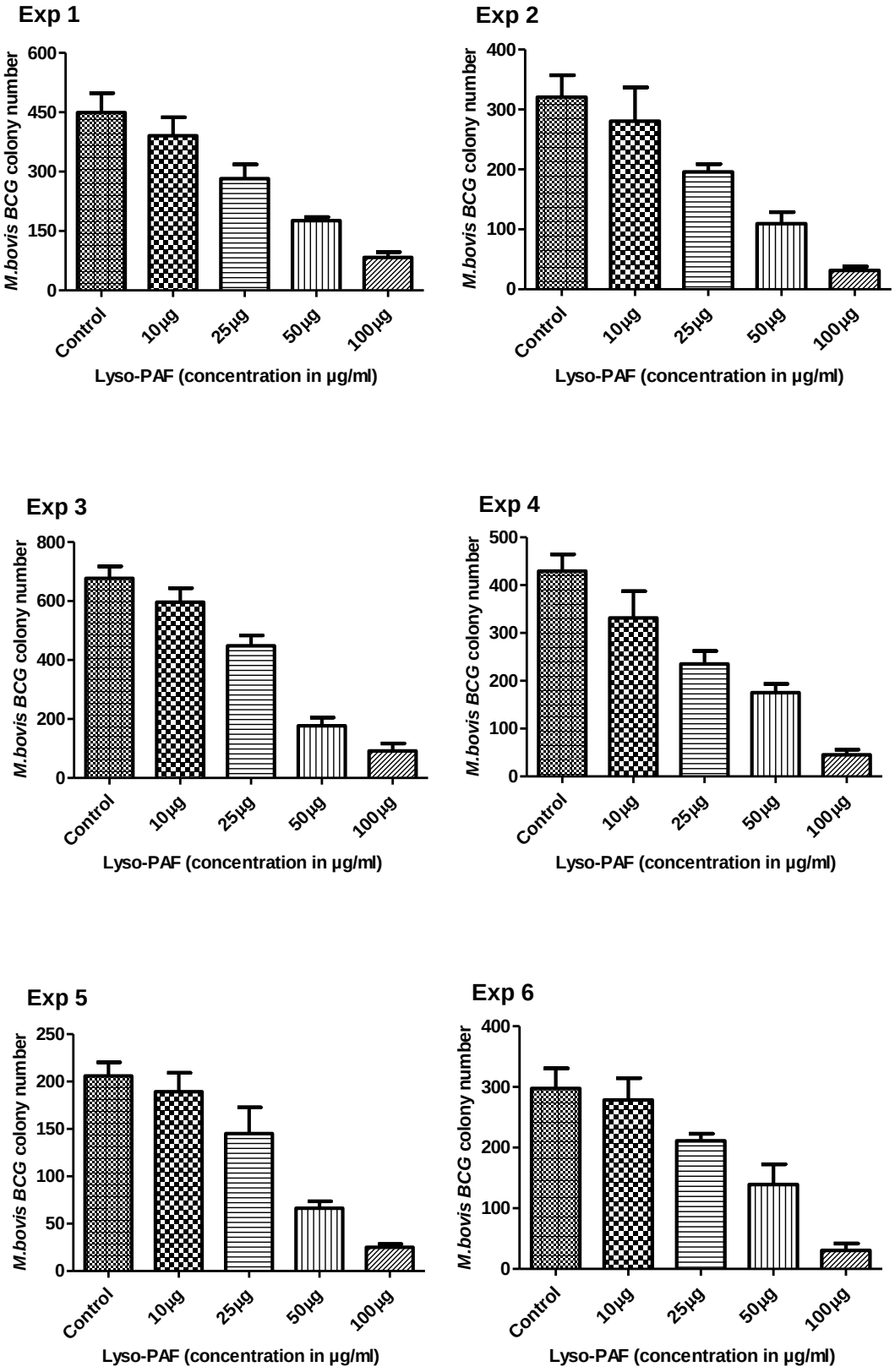


Figure 4.2.2b: Six independent experiments (Exp1, 2, 3, 4, 5 and 6) showing the direct effect of Lyso-PAF on *M.bovis BCG* growth. Each bar represents the average number of *M.smegmatis*

CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

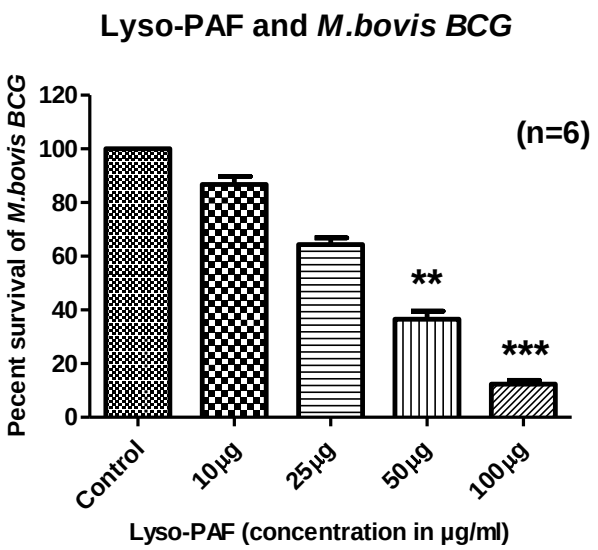


Figure 4.2.2c: Direct effect of Lyso-PAF on *M.bovis BCG* growth. Each bar represents the average of six individual experiments and the error bars show the standard error of means (SEM). Data is expressed in terms of percentage survival where solvent control is considered as 100% survival and different test conditions are compared to it. Non-parametric multiple-comparison Kruskal-Wallis test was applied on ranks to calculate the *p*-value which was found to be significant ($p \leq 0.0001$). Individual data sets were compared using Dunn's multiple comparison test, where 50µg/ml Lyso-PAF vs Solvent control **($p \leq 0.01$) and 100µg/ml Lyso-PAF vs Solvent control ***($p \leq 0.001$).

4.2.3 Direct effect of Platelet Activating Factor C-18 (PAF C-18) on *M.smegmatis* and *M.bovis BCG* growth *in vitro*

PAF C-18 is a naturally produced PAFC-16 analogue, chemically known as, 1-O-octadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine. The difference between PAF C-16 and PAF C-18 is the longer carbon tail with two additional carbon atoms in PAF C-18 (Figure 4.2.3a).

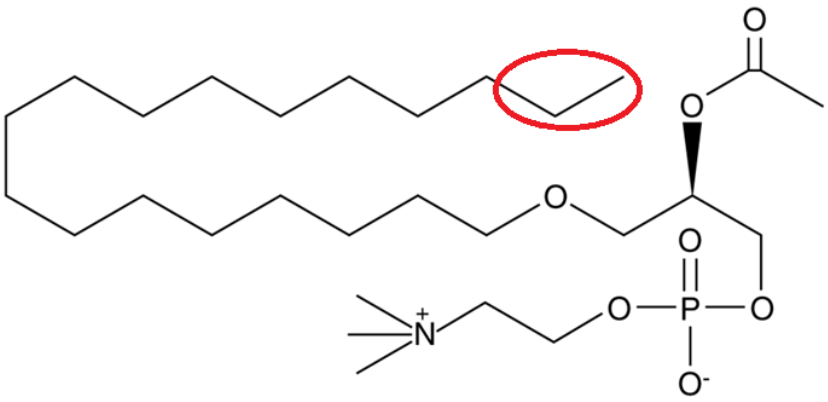


Figure 4.2.3a: Chemical structure of PAF C-18. The carbon tail in PAF C-18 is octadecyl, having two additional carbons shown in the red circle as compared to PAF C-16.

The direct effect of PAF C-18 on the growth of *M.smegmatis* and *M.bovis BCG* was investigated according to the protocol in chapter 2.2. Three independent experiments (Exp1, 2 and 3) were performed for each *M.smegmatis* and *M.bovis BCG* by treating them with different concentrations of PAF C-18 (10µg, 25µg, 50µg and 100µg/ml of bacterial suspension) for 2 hours. A solvent control for PAF C-18 (10µl ethanol/ml bacterial suspension) was included in each experiment.

It was observed that PAF C-18 was able to inhibit the growth of both *M.smegmatis* and *M.bovis BCG* in a dose-dependent manner after treatment for 2 hours as indicated by a reduction in the number of CFUs when compared with the solvent control. The experiments of PAF C-18 with *M.smegmatis* and PAF C-18 with *M.bovis BCG* showed the same trend of growth inhibition (Figures 4.2.3b and 4.2.3c). The growth inhibitory effect of PAF C-18 was more prominent at higher concentrations of 50µg/ml and 100µg/ml. On average, PAF C-18 treatment of *M.smegmatis* at concentrations of 50µg/ml and 100µg/ml resulted in ~69% and ~98% reduction in the number of CFUs respectively when compared with the solvent control (Figure 4.2.3d graph (i)). In case of *M.bovis BCG*, PAF C-18 treatment at concentrations 50µg/ml and 100µg/ml on average caused ~57% and ~93% growth inhibition respectively (Figure 4.2.3d graph (ii)). Non-parametric multiple comparison Kruskal Wallis test was applied on ranks and the *p*-value was found to be significant in case of both *M.smegmatis* (p=0.008) and *M.bovis BCG* (p=0.008). Individual data sets were compared by post hoc Dunn's multiple comparison test and the results were found to be significant only for 100µg/ml PAF C-18 vs Solvent control ($p \leq 0.01$).

These results showed that increase in the length of the aliphatic carbon tail in PAFC-16 has no effect on its growth inhibitory properties against *M.smegmatis* and *M.bovis BCG*.

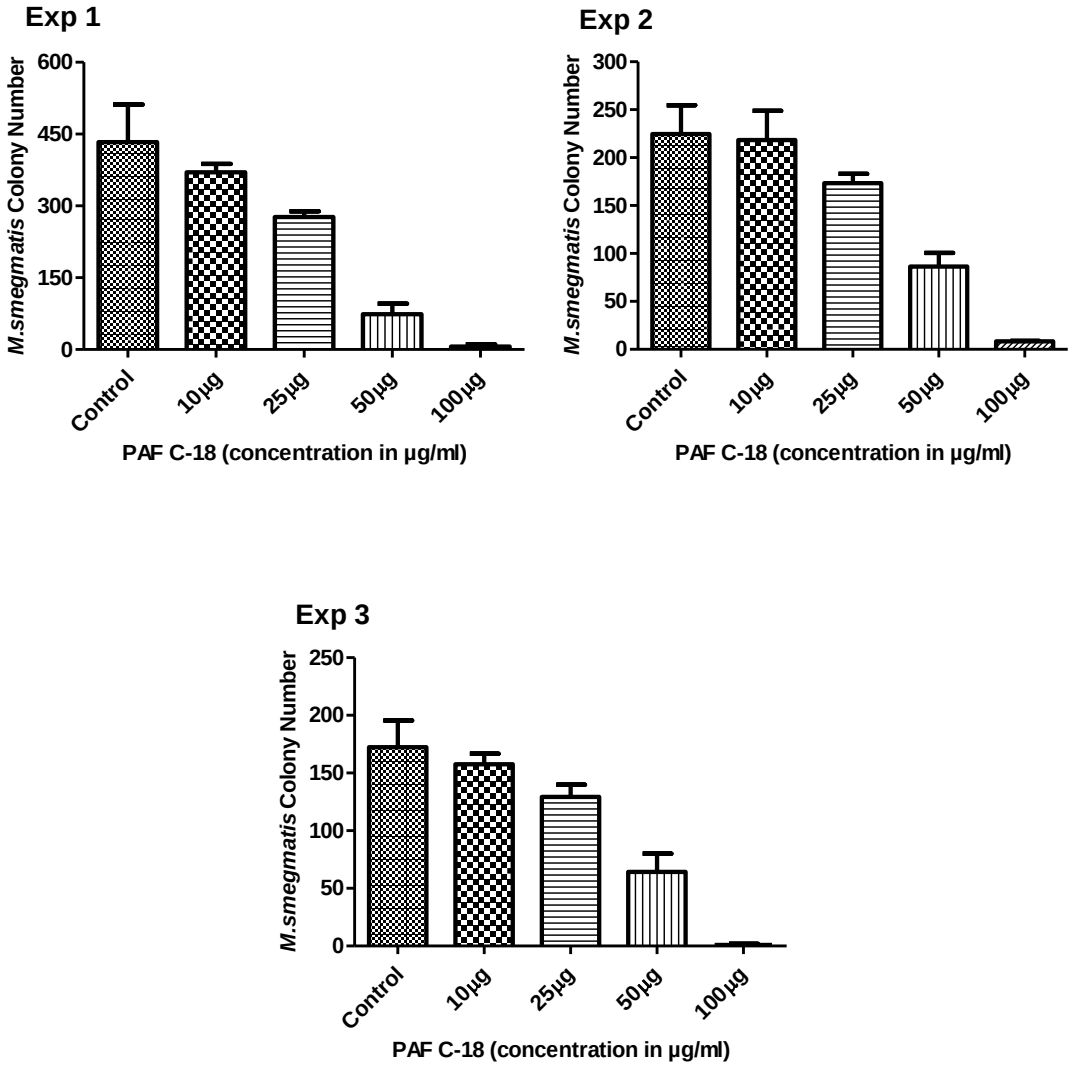


Figure 4.2.3b: Three independent experiments (Exp1, 2 and 3) for the direct effect of PAF C-18 on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

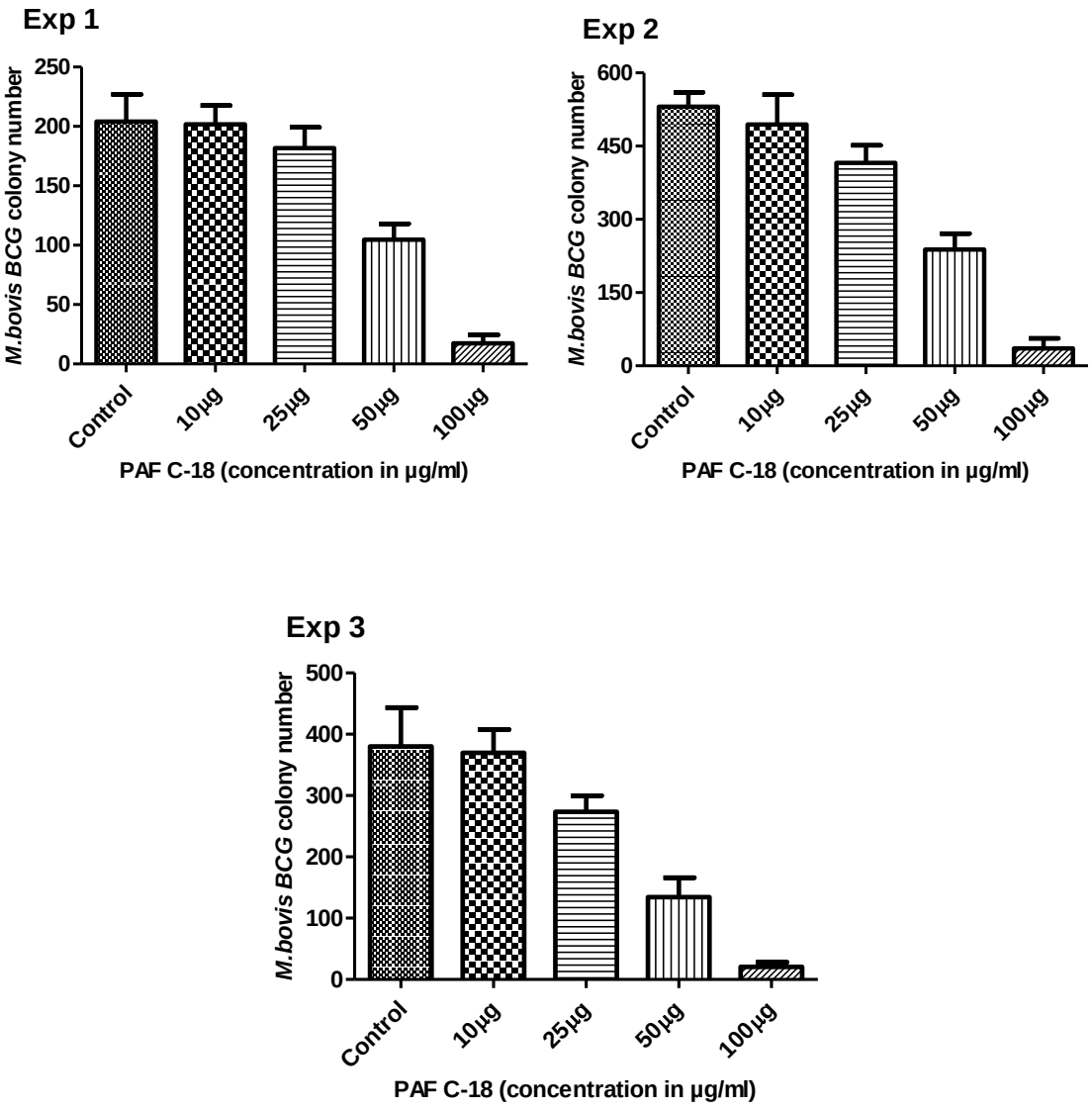


Figure 4.2.3c: Three independent experiments (Exp1, 2 and 3) for the direct effect of PAF C-18 on *M.bovis BCG* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

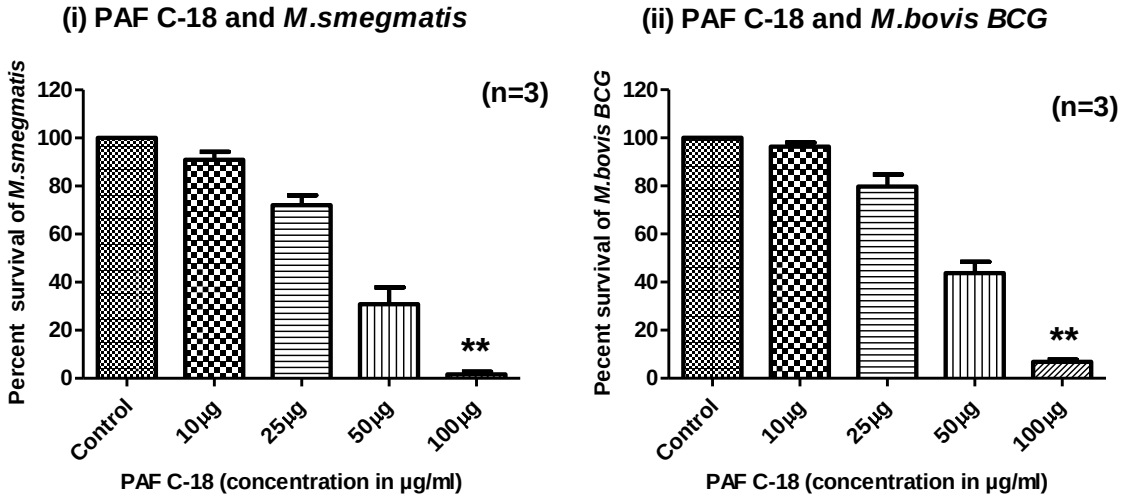


Figure 4.2.3d: Effect of PAF C-18 on *M.smegmatis* and *M.bovis BCG* growth. Each bar represents the average of three independent experiments and the error bars show the standard error of means (SEM). Data is expressed in terms of percentage survival where solvent control is taken as 100% survival and different PAF C-18 treated test conditions are compared to it. The *p*-value was found to be significant in case of both *M.smegmatis* (p=0.008) and *M.bovis BCG* (p=0.008) and was determined by applying non-parametric Kruskal-Wallis test on ranks. Individual data sets were compared using Dunn's multiple comparison test and 100µg/ml PAF C-18 vs Solvent control was found significant for both *M.smegmatis* and *M.bovis BCG* **($p \leq 0.01$).

4.2.4 Direct effect of Hexanolamino PAF on *M.smegmatis* and *M.bovis BCG* growth *in vitro*

Hexanolamino PAF is chemically known as 1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phospho (N,N,N-trimethyl). This analogue differs from naturally occurring PAF C-16 in the position of the terminal amino group, which is attached by 4 additional carbon atoms to the phosphate group of the phosphocholine head portion (Figure 4.2.4a)

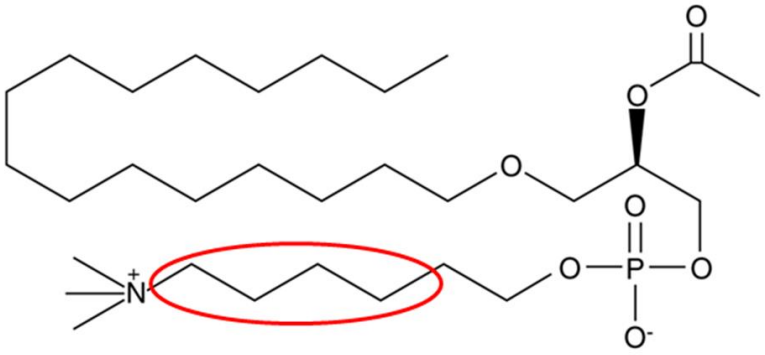


Figure 4.2.4a: Structure of Hexanolamino PAF. Red circle shows the 4 additional carbon atoms that attaches the amino group to the phosphate group in the phosphocholine head portion of the compound.

The direct effect of Hexanolamino PAF on the growth of *M.smegmatis* and *M.bovis BCG* was investigated according to the protocol in chapter 2.2. Three independent experiments (Exp1, 2 and 3) were performed for each *M.smegmatis* and *M.bovis BCG* by treating them with different concentrations of Hexanolamino PAF (10µg, 25µg, 50µg and 100µg/ml of bacterial suspension) for 2 hours. A solvent control for Hexanolamino PAF (10µl ethanol/ml bacterial suspension) was also included in each experiment.

The results showed that Hexanolamino PAF was able to inhibit the growth of both *M.smegmatis* and *M.bovis BCG* in a dose-dependent manner after treatment for 2 hours as indicated by a reduction in the number of CFUs when compared with the solvent control. The experiments of Hexanolamino PAF with *M.smegmatis* and Hexanolamino PAF with *M.bovis BCG* showed the same trend of growth inhibition (Figures 4.2.4b, 4.2.4c). The growth inhibitory effect of Hexanolamino PAF was more prominent at higher concentrations of 50µg/ml and 100µg/ml. On average, Hexanolamino PAF treatment of *M.smegmatis* at concentrations of 50µg/ml and 100µg/ml resulted in ~62% and ~95% reduction in the number of CFUs respectively when compared to the solvent control (Figure 4.2.4d, graph (i)). In case of *M.bovis BCG*, Hexanolamino PAF treatment at concentrations 50µg/ml and 100µg/ml on average caused ~65% and ~94% reduction in the number of CFUs respectively when compared to the solvent control (Figure 4.2.4d, graph (ii)). Non-parametric multiple comparison Kruskal Wallis test was applied on ranks and the *p*-value was found to be significant in case of both *M.smegmatis* (p=0.009) and *M.bovis BCG* (p=0.008). Individual data sets were compared by

applying post hoc Dunn's multiple comparison test and the results were found to be significant only for 100µg/ml Hexanolamino PAF vs Solvent control ($p \leq 0.01$).

These results showed that changing the position terminal amino group in the phosphocholine head region of PAF C-16 did not affect the growth inhibitory properties against *M.smegmatis* and *M.bovis BCG.*

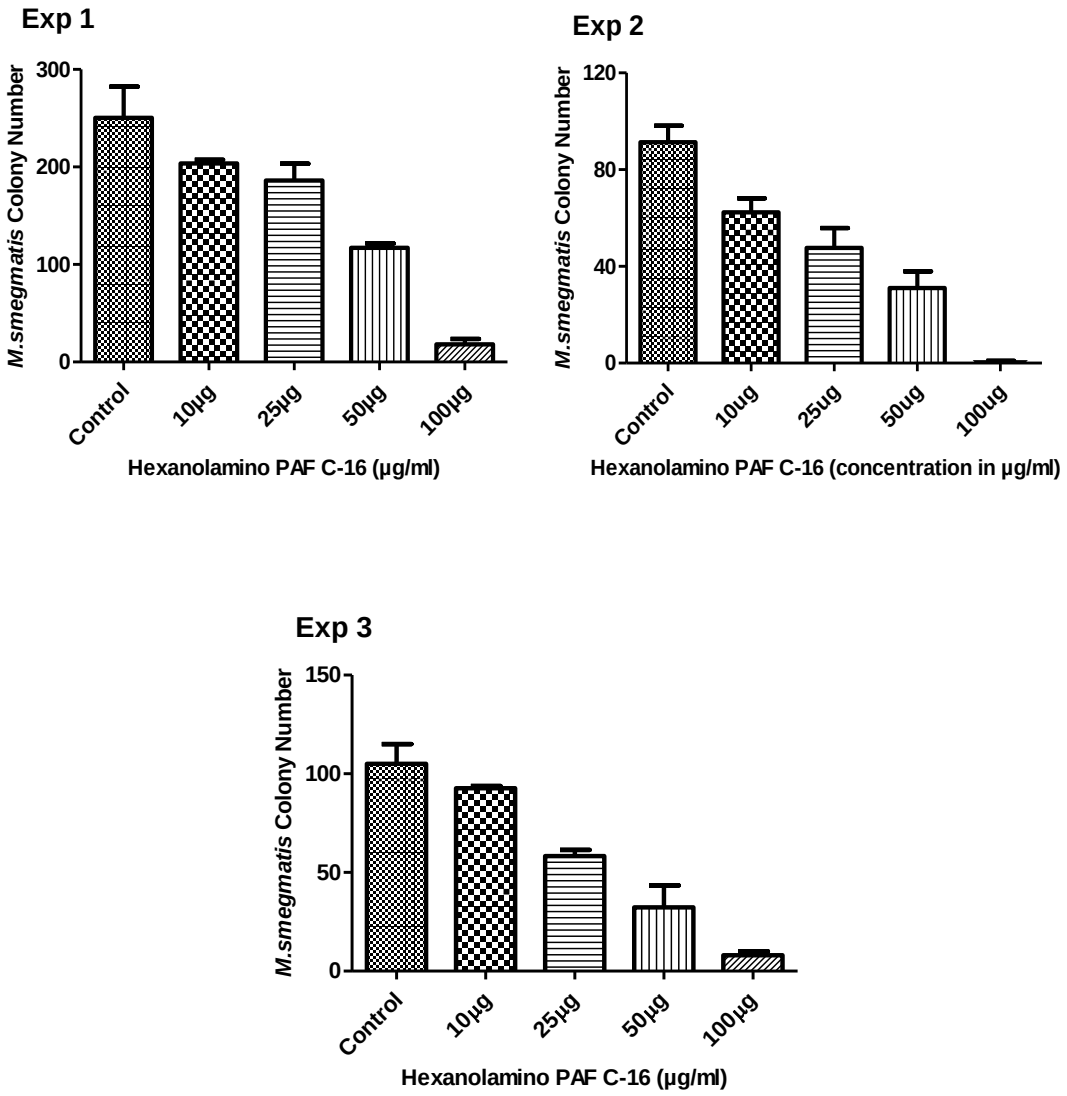


Figure 4.2.4b: Three independent experiments (Exp1, 2 and 3) for the direct effect of Hexanolamino PAF on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicates.

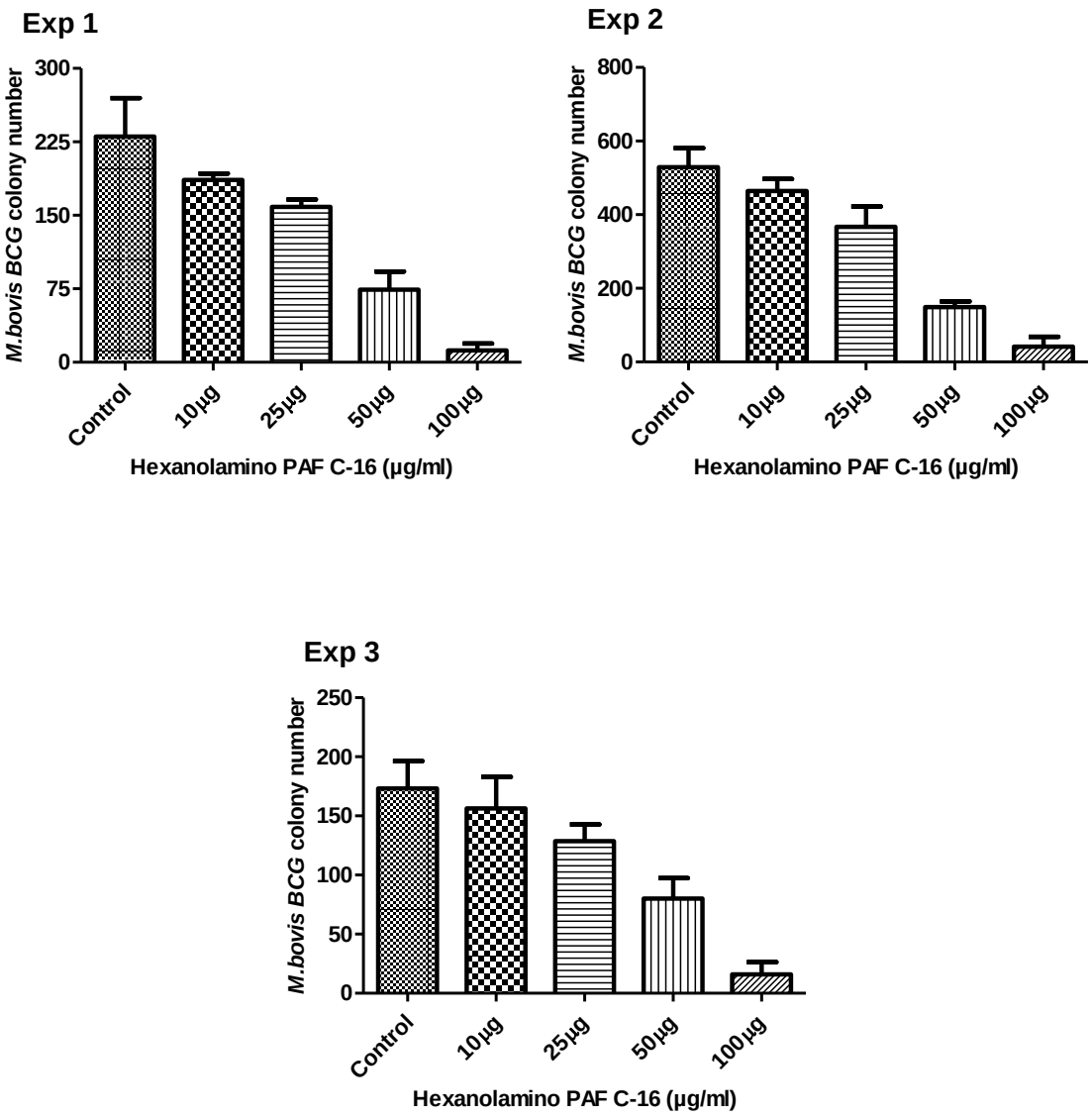


Figure 4.2.4c: Three independent experiments (Exp1, 2 and 3) for the direct effect of Hexanolamino PAF on *M.bovis BCG* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

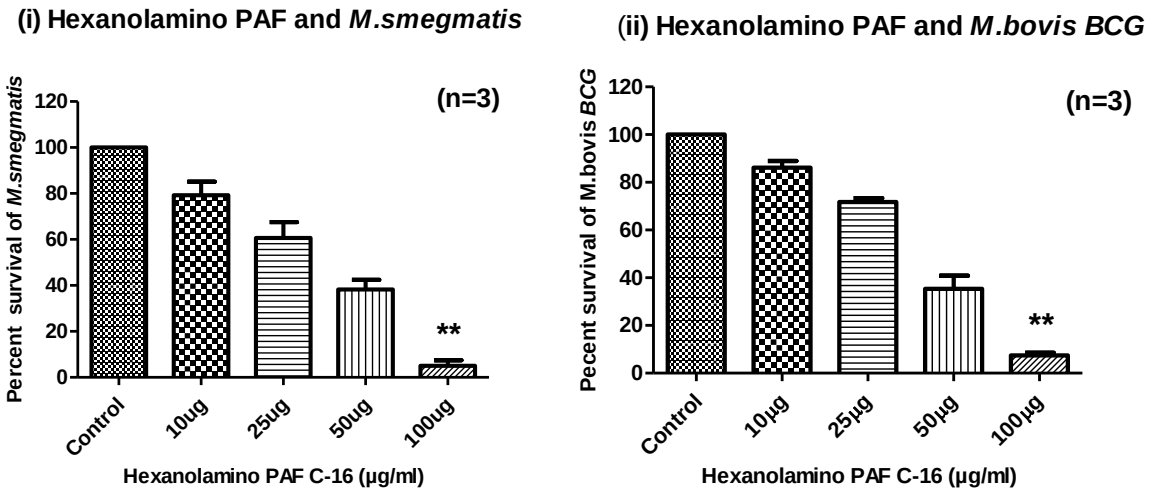


Figure 4.2.4c: Direct effect of Hexanolamino PAF on *M.smegmatis* and *M.bovis BCG* growth. Each bar represents the average of three independent experiments and the error bars show the standard error of means (SEM). Data is expressed in terms of percentage survival where solvent control is taken as 100% survival and different test conditions are compared to it. The *p*-value was found to be significant in case of both *M.smegmatis* (p=0.009) and *M.bovis BCG* (p=0.008) and was determined by applying non-parametric Kruskal-Wallis test on ranks. Individual data sets were compared using Dunn's multiple comparison test and 100µg/ml Hexanolamino PAF vs Solvent control was significant for both *M.smegmatis* and *M.bovis BCG* **($p \leq 0.01$).

4.2.5 Direct effect of 2-O-methyl PAF C-16 on *M.smegmatis* and *M.bovis BCG* growth *in vitro*

2-O-methyl PAF C-16 is a synthetic PAF C-16 analogue. This structure analogue has a methyl group at the *sn*-2 position instead of acetyl group. Chemically it is known as 1-O-hexadecyl-2-O-methyl-*sn*-glyceryl-3-phosphocholine (Figure 4.2.5a)

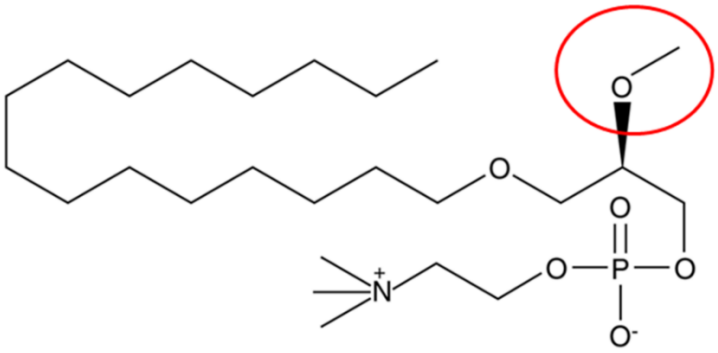


Figure 4.2.5a: Chemical structure of 2-O-methyl PAF. The red circle shows a methyl group attached at position *sn*-2 instead of an acetyl group.

The direct effect of 2-O-methyl PAF on the growth of *M.smegmatis* and *M.bovis BCG* was investigated according to the protocol in chapter 2.2. Three independent experiments (Exp1, 2 and 3) were performed with each *M.smegmatis* and *M.bovis BCG,* by treating them with different concentrations of 2-O-methyl PAF (10µg, 25µg, 50µg and 100µg/ml of bacterial suspension) for 2 hours. A solvent control for 2-O-methyl PAF (10µl ethanol/ml bacterial suspension) was included in each experiment.

It was observed that 2-O-methyl PAF was able to inhibit the growth of both *M.smegmatis* and *M.bovis BCG* in a dose-dependent manner after treatment for 2 hours as indicated by a reduction in the number of CFUs when compared to the solvent control. All the experiments of 2-O-methyl PAF with *M.smegmatis* and 2-O-methyl PAF with *M.bovis BCG* showed the same trend of growth inhibition (Figures 4.2.5b, 4.2.5c). The growth inhibitory effect of 2-O-methyl PAF was more prominent at higher concentrations of 50µg/ml and 100µg/ml. On average, 2-O-methyl PAF treatment of *M.smegmatis* at concentrations of 50µg/ml and 100µg/ml resulted in ~61% and ~92% reduction in the number of CFUs respectively as compared to the solvent control (Figure 4.2.5d, graph (i)). In case of *M.bovis BCG,* 2-O-methyl PAF treatment at concentrations 50µg/ml and 100µg/ml on average caused ~70% and ~97% growth inhibition respectively (Figure 4.2.5d, graph (ii)). The level of significance was determined by applying non-parametric multiple comparison Kruskal Wallis test on ranks and the *p*-value was found to be significant in case of both *M.smegmatis* (p=0.01) and *M.bovis BCG* (p=0.008). Individual data sets were compared by applying post hoc Dunn's multiple

comparison test and the results were found to be significant only for 100µg/ml 2-O-methyl PAF vs Solvent control ($p \leq 0.01$).

These results suggested that substituting the acetyl group (CH_3O) of PAF C-16 with methyl group (CH_3) did not affect its growth inhibitory properties against both *M.smegmatis* and *M.bovis BCG.*

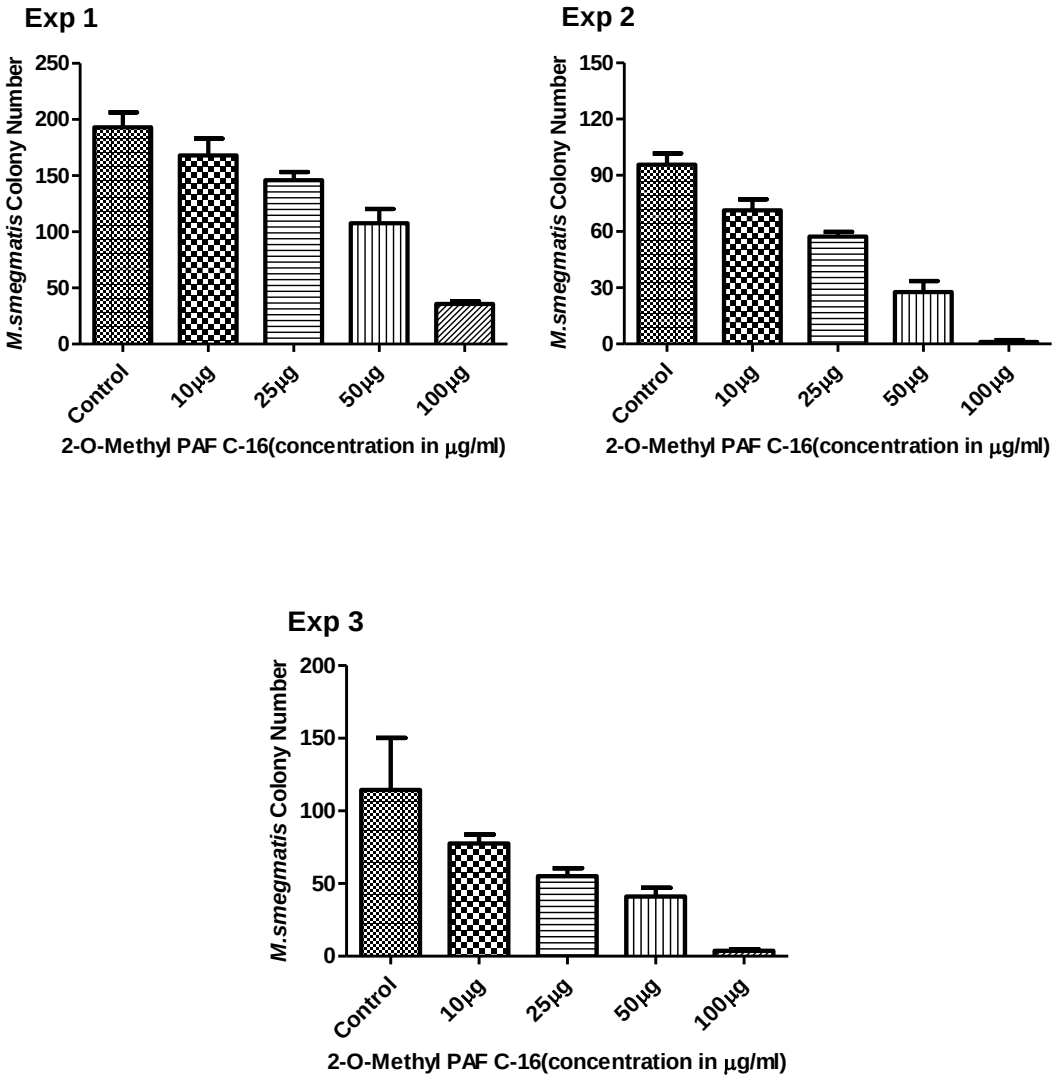


Figure 4.2.5b: Three independent experiments (Exp1, 2 and 3) for the direct effect of 2-O-methyl PAF on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

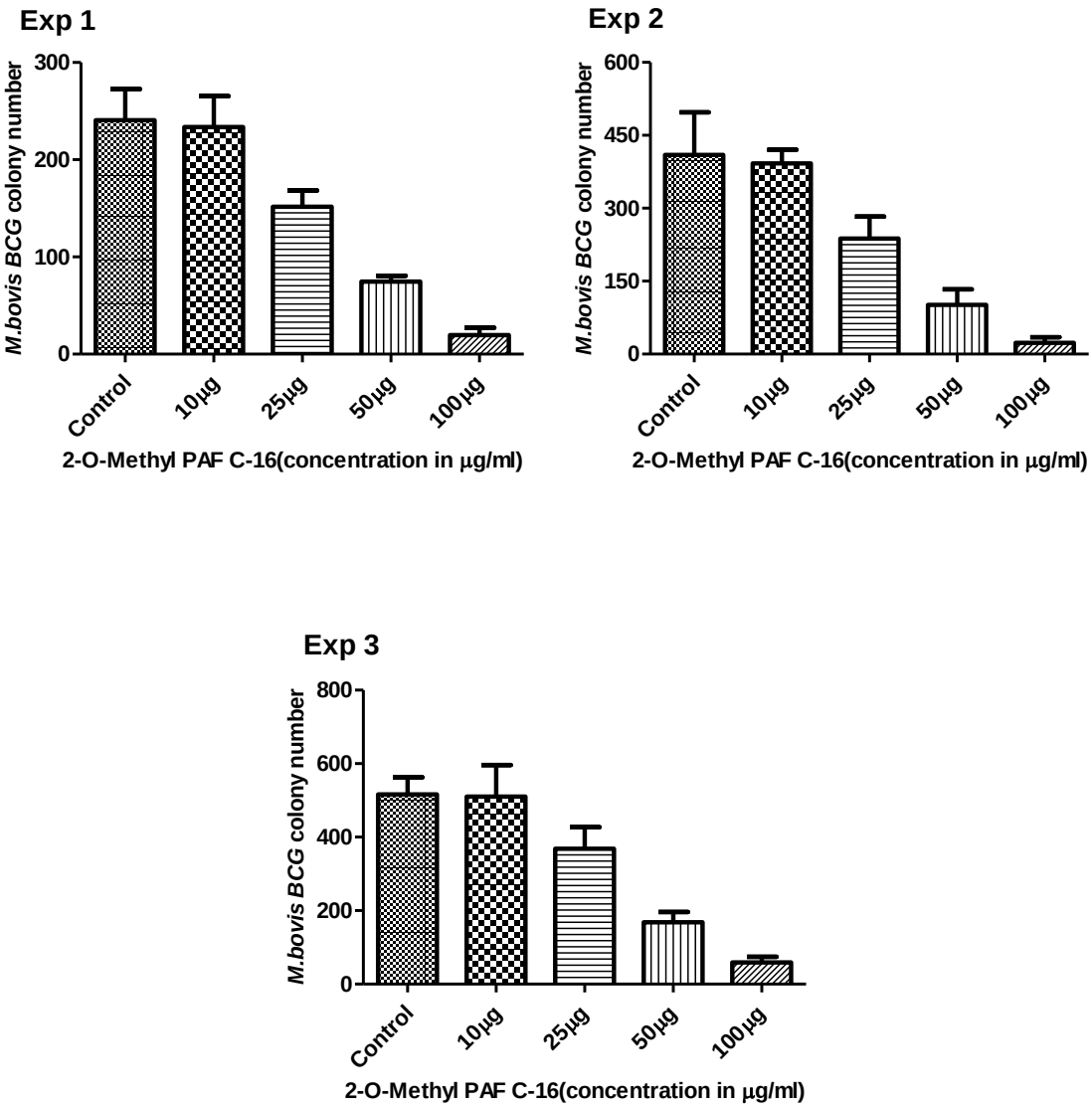


Figure 4.2.5c: Three independent experiments (Exp1, 2 and 3) for the direct effect of 2-O-methyl PAF on *M.bovis BCG* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

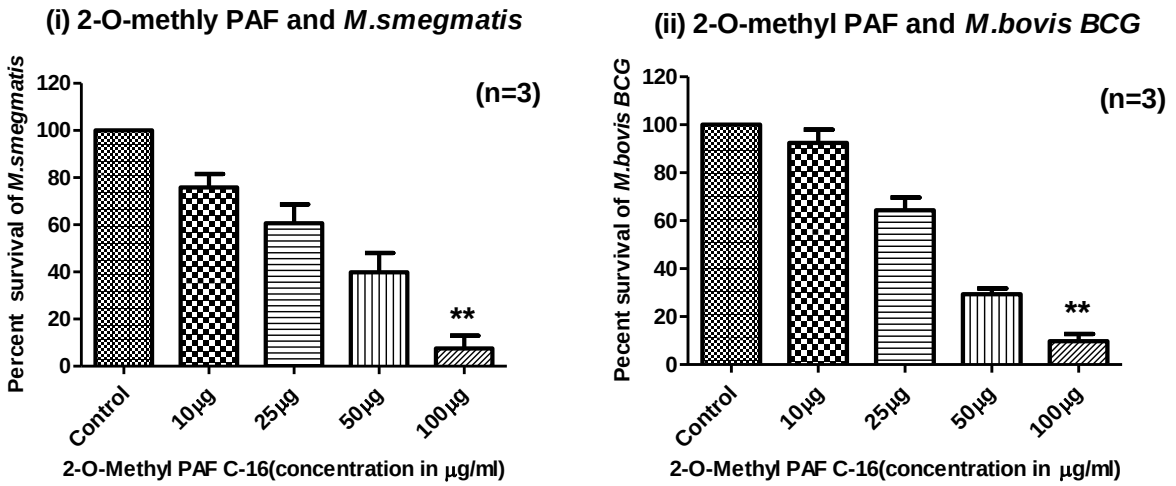


Figure 4.2.5d: Direct effect of 2-O-methyl PAF C-16 on *M.smegmatis* and *M.bovis BCG* growth. Each bar represents the average of three independent experiments and the error bars show the standard error of means (SEM). Data is expressed in terms of percentage survival where solvent control is considered as 100% survival and different test conditions are compared to it. *p*-value was determined by applying non-parametric Kruskal-Wallis test on ranks and was found to be significant in case of both *M.smegmatis* (p=0.01) and *M.bovis BCG* (p=0.008). Individual data sets were compared using Dunn's multiple comparison test and 100µg/ml 2-O-methlyl PAF vs Solvent control was significant for both *M.smegmatis* and *M.bovis BCG* **($p \leq 0.01$).

4.2.6 Direct effect of Pyrrolidino PAF on *M.smegmatis* growth *in vitro*

Pyrrolidino PAF C-16 is a synthetic analogue of PAF C-16. Chemically it is known as 1-O-hexadecyl-2-O-acetyl-*sn*-glyceryl-3-phosphoryl-N-methyl-pyrrolodinium ethanol and has 5-member lactam ring attached to the phosphate group (Figure 4.2.6)

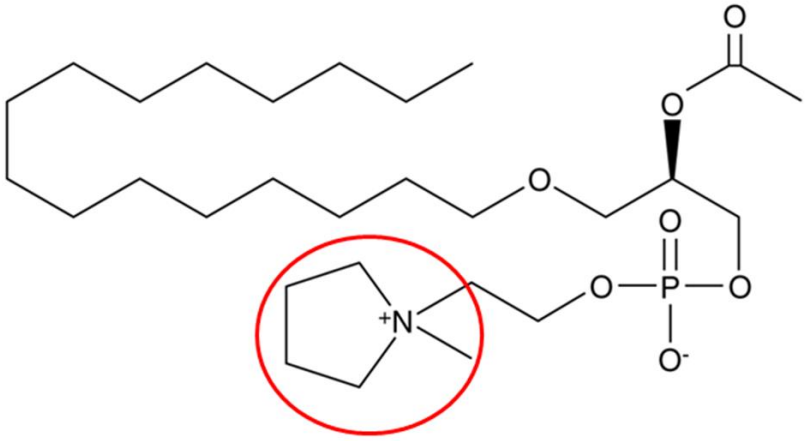


Figure 4.2.6a: Chemical structure of Pyrrolidino PAF. The red circle shows the 5-membered lactam ring attached to the phosphate group instead of an amino group in the phosphocholine head portion of the compound.

The direct effect of Pyrrolidino PAF on the growth of *M.smegmatis* was investigated according to the protocol in chapter 2.2. Three independent experiments (Exp1, 2 and 3) were performed by treating *M.smegmatis* with different concentrations of Pyrrolidino PAF (10µg, 25µg, 50µg and 100µg/ml of bacterial suspension) for 2 hours. A solvent control for Pyrrolidino PAF (10µl ethanol/ml bacterial suspension) was also included in the experiment.

It was observed that Pyrrolidino PAF was able to inhibit the growth of *M.smegmatis* in a dose-dependent manner after treatment for 2 hours as indicated by the reduction in the number of CFUs when compared with the solvent control. All the experiments of Pyrrolidino PAF with *M.smegmatis* showed the same trend of growth inhibition (Figure 4.2.6b). The growth inhibitory effect of Pyrrolidino PAF against *M.smegmatis* was more prominent at higher concentrations of 50µg/ml and 100µg/ml and on average caused ~58% and ~98% growth inhibition of *M.smegmatis* respectively (Figure 4.2.6c). The level of significance was determined by applying non-parametric multiple comparison Kruskal Wallis test on ranks and the *p*-value was found to be significant, $p=0.008$. Individual data sets were compared by applying post hoc Dunn's multiple comparison test and the results were found to be significant only for 100µg/ml Pyrrolidino PAF vs Solvent control ($p \leq 0.01$).

These experiments suggested that replacing the terminal group of PAF C-16 with a 5 member lactam ring did not affect the growth inhibitory properties of PAF C-16.

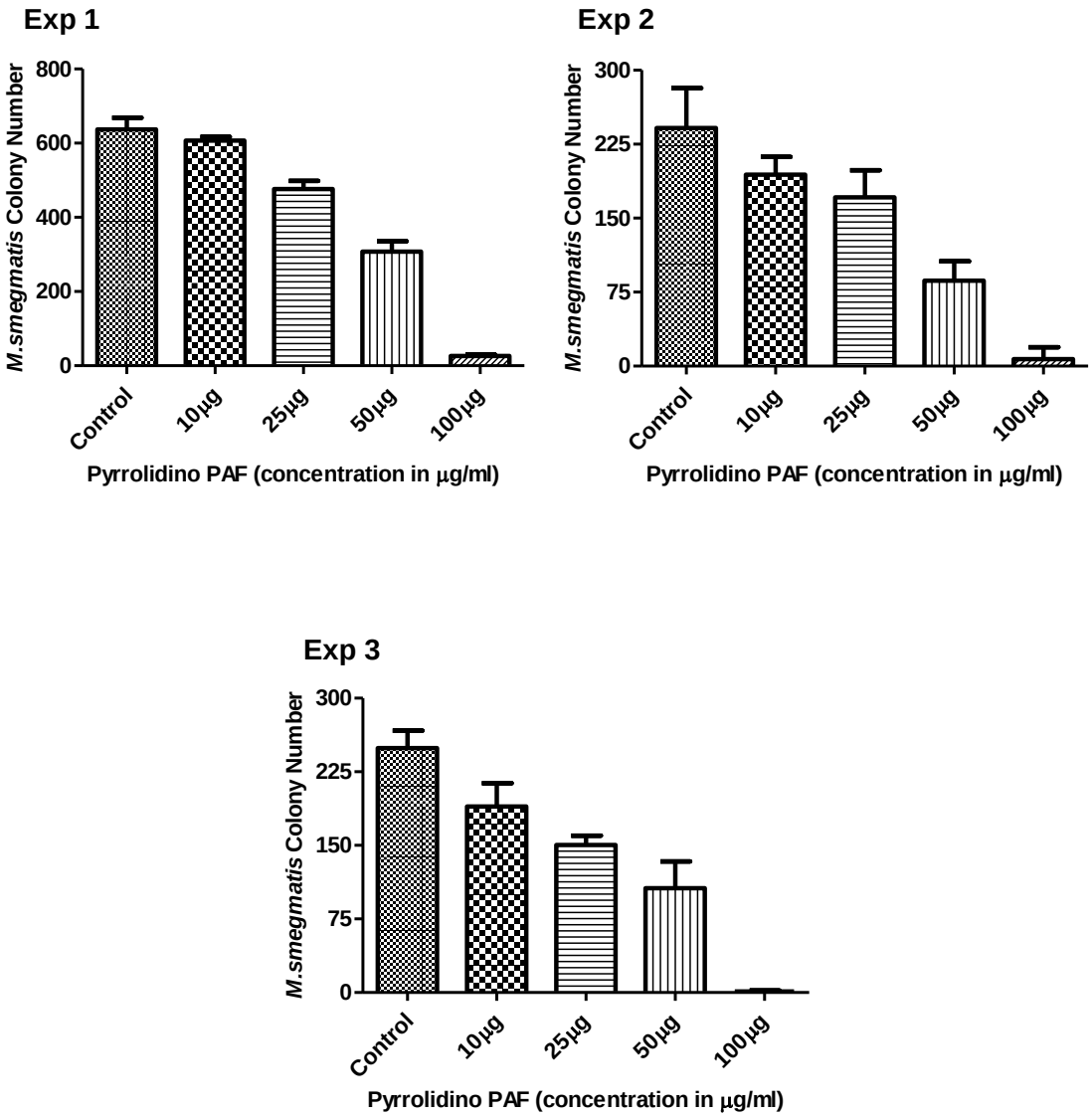


Figure 4.2.6b: Three independent experiments (Exp1, 2 and 3) for the direct effect of Pyrrolidino PAF on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

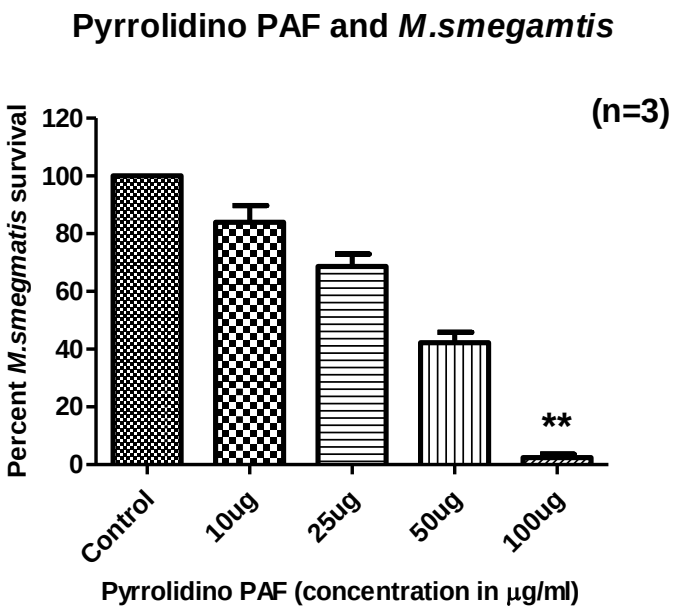


Figure 4.2.6c: Direct effect of Pyrrolidino PAF on *M.smegmatis* growth. Each bar represents the average of three independent experiments and the error bars show the standard error of means (SEM). Data is expressed in terms of percentage survival where solvent control is taken as 100% survival and different test conditions are compared to it. *p*-value was determined by applying non-parametric Kruskal-Wallis test on ranks and was found to be significant (p=0.008). Individual data sets were compared using Dunn's multiple comparison test and 100μg/ml Pyrrolidino PAF vs Solvent control was found to be significant **($p \leq 0.01$).

4.3 Comparison of growth inhibitory potential of PAF C-16 and various PAF C-16 structural analogues against *M.smegmatis* and *M.bovis BCG*

PAF C-16 and different PAF C-16 structure analogues were compared to check the effect of small modifications in structure of PAF C-16 on the growth inhibitory potency against both *M.smegmatis* and *M.bovis BCG*. However, similar levels of *M.smegmatis* and *M.bovis BCG* growth inhibition were observed for PAF C-16 and all the analogues tested, suggesting that these small modification in structure of PAF C-16 do not decrease its mycobacterial growth inhibition potency (Figure 4.3a and 4.3b).

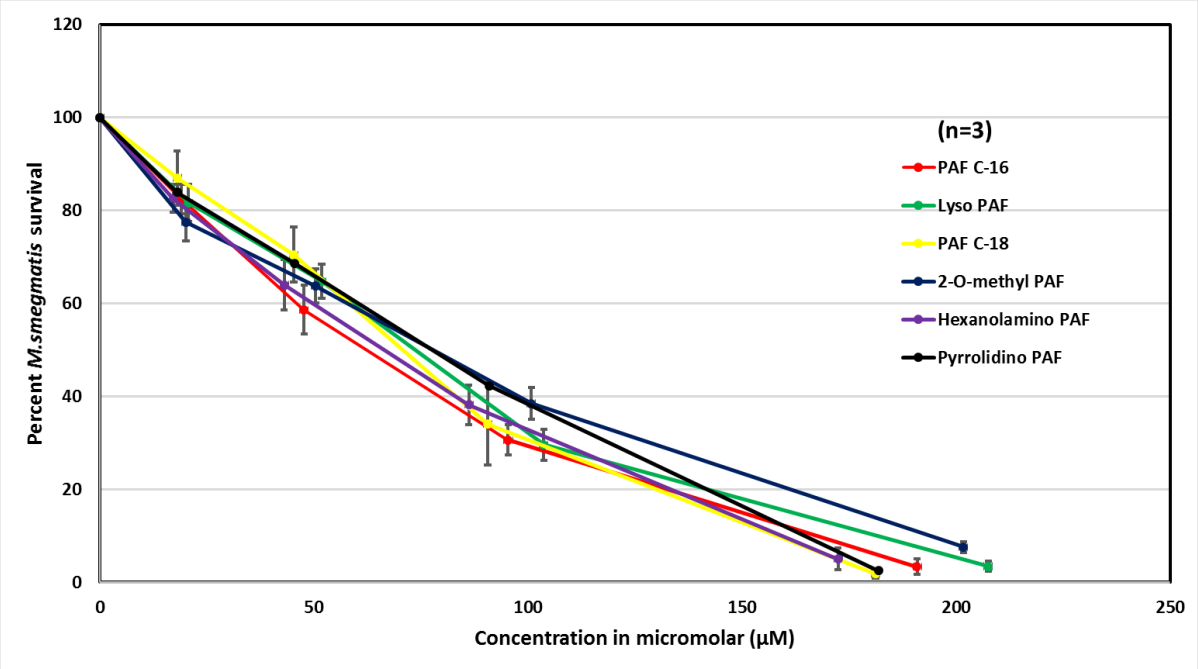


Figure 4.3a: Comparison of *M.smegmatis* growth inhibition by PAF C-16 and different PAF analogues *in vitro*. The data is expressed as percentage where solvent control is considered as 100% survival. Each data point represents the average number of *M.smegmatis* CFUs from three individual experiments for test compounds (in µM concentrations) and solvent control and the error bars show the standard errors of mean (SEM).

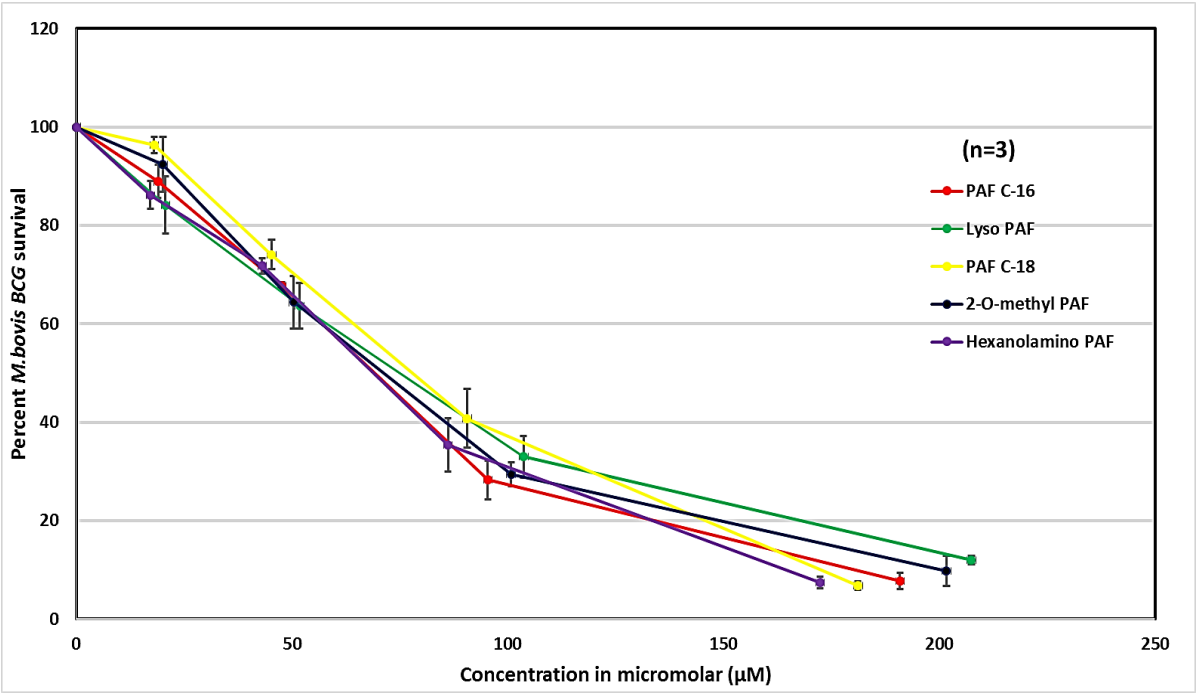


Figure 4.3b: Comparison of *M.bovis BCG* growth inhibition by PAF C-16 and different PAF structural analogues *in vitro*. The data is expressed as percentage where solvent control is

considered as 100% survival. Each data point represents the average number of *M.bovis BCG* CFUs from three individual experiments at different concentrations of test compounds (in µM concentrations) and a solvent control and the error bars show the standard errors of mean (SEM).

4.4 Structural dissection of PAF C-16 to localize the active portion involved in mycobacterial growth inhibition

In order to localize the active portion of PAF C-16 that contributes to its growth inhibitory properties against *mycobacteria*, compounds with chemical structures similar to different portions of PAF C-16 were investigated for their direct growth inhibitory activity using *M.smegmatis* as model organism. Different compounds tested in this study for their direct growth inhibitory activity included:

a) Palmitic acid
b) Phosphocholine chloride calcium tetrahydrate salt
c) 1-O-Hexadecyl-*sn*-glycerol
d) Miltefosine
e) Hexadecyl lactate
f) Palmitoleic Acid and Oleic Acid

4.4.1 Direct effect of Palmitic acid on *M.smegmatis* growth *in vitro*

Palmitic acid is also known as hexadecanoic acid and comprises of a linear 16carbon atoms chain attached to a carboxyl group (Figure 4.4.1a). The carbon chain in palmitic acid does not contain any double bonds chain and is similar to the carbon tail of PAF C-16 in the number of carbon atoms. Therefore, palmitic acid was tested for its growth inhibition activity against *M.smegmatis* to investigate the role of PAF C-16 carbon tail in its growth inhibition activity.

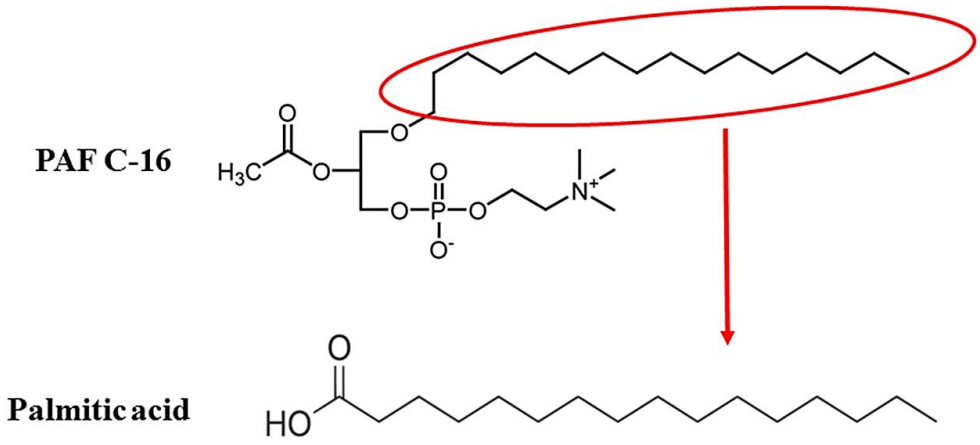


Figure 4.4.1a: Chemical structure of palmitic acid. The carbon chain contains 16 carbon atoms with no double bonds and is similar to that of PAF C-16.

Three independent experiments (Exp1, 2 and 3) were performed *in vitro* to investigate the direct inhibitory effect of palmitic acid on *M.smegmatis* growth using different concentrations of palmitic acid (10µg, 25µg, 50µg and 100µg/ml of bacterial suspension) and a solvent control for palmitic acid (10µl ethanol/ml of bacterial suspension) according to the protocol mentioned in section 2.2.

All the three experiments showed the same trend and it was observed that palmitic acid in concentrations of up to 100µg/ml was unable to directly inhibit the growth of *M.smegmatis* as indicated by the number of CFUs which was similar to the solvent control. Treatment of palmitic acid with *M.smegmatis* in fact caused in a slight increase in the number of *M.smegmatis* CFUs as compared to the solvent control (Figures 4.4.1b and 4.4.1c). These results suggested that the carbon tail of PAF C-16 on its own does not possess bacterial growth inhibition potential.

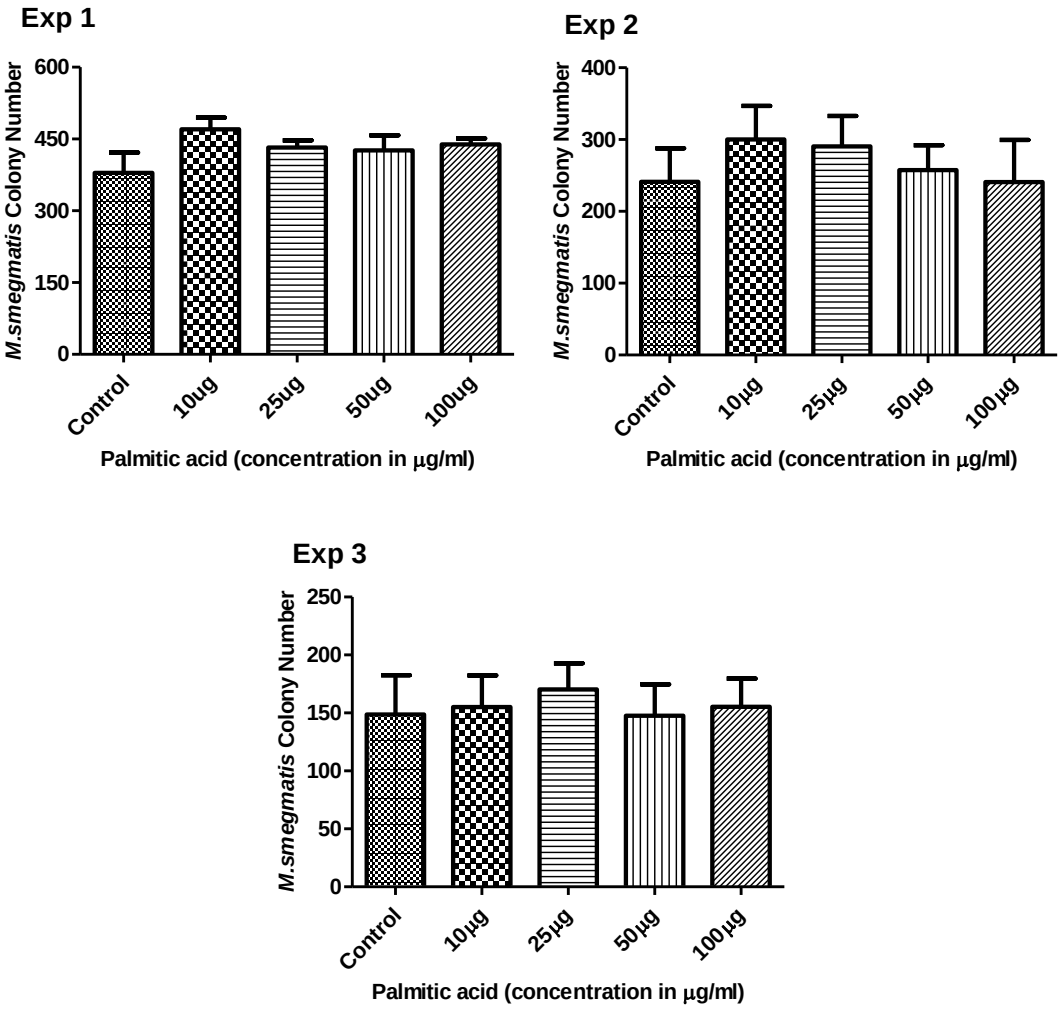


Figure 4.4.1b: Three individual experiments (Exp1, 2 and 3) for the direct effect of palmitic acid on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

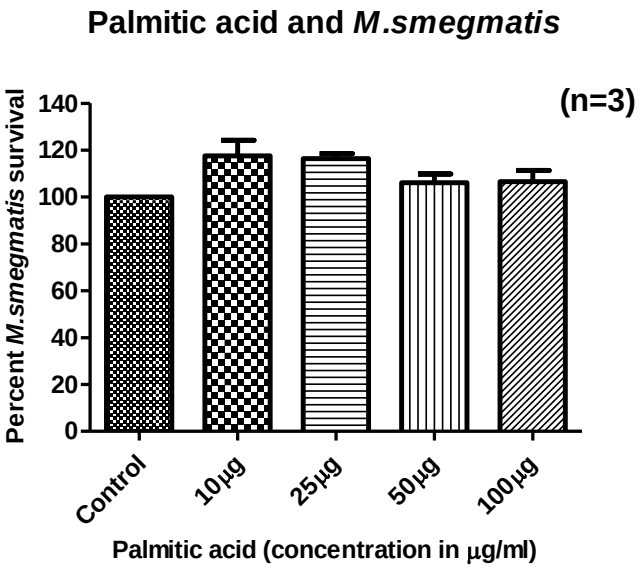


Figure 4.4.1c: Direct effect of palmitic acid on *M.smegmatis* growth. Each bar represents the average of three independent experiments and the error bars show the standard error of means (SEM). Data is expressed in terms of percentage where solvent control is considered as 100% survival and different test conditions are compared to it. *p*-value was calculated by applying non-parametric Kruskal-Wallis test on ranks and was found to be non-significant (p=0.14).

4.4.2 Direct effect of Phosphocholine chloride calcium tetrahydrate on *M.smegmatis* growth *in vitro*

Phosphocholine chloride calcium tetrahydrate is similar in structure to the phosphocholine head portion of PAF C-16 that is attached at position *sn*-3 of the glycerol backbone (Figure 4.4.2a). Phosphocholine chloride calcium tetrahydrate was, therefore, tested for its direct inhibitory effect on *M.smegmatis* to investigate the role of phosphocholine head portion of PAF C-16 in its growth inhibition activity against *mycobacteria*.

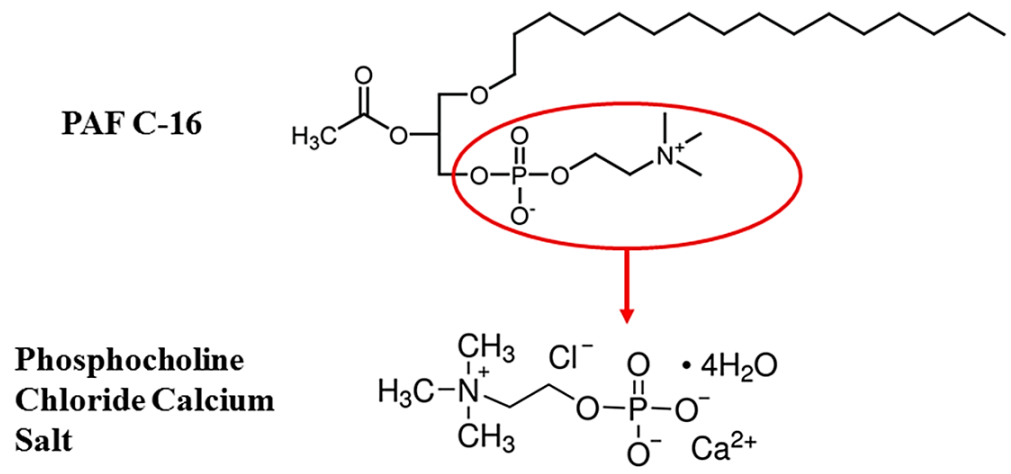


Figure 4.4.2a: Chemical structure of Phosphocholine chloride calcium tetrahydrate. This compound has a similar structure to the phosphocholine head portion of PAF C-16.

Three independent experiments (Exp1, 2 and 3) were performed according to the protocol in section 2.2 to investigate the direct inhibitory effect of phosphocholine chloride calcium tetrahydrate on *M.smegmatis* growth in different concentrations (10µg, 25µg, 50µg and 100µg/ml of bacterial suspension). A solvent control for phosphocholine chloride calcium tetrahydrate (20µl water/ml of bacterial suspension) was also included in each experiment.

All the three experiments showed that phosphocholine chloride calcium tetrahydrate had no direct inhibitory effect on *M.smegmatis* growth in concentrations of up to 100µg/ml as indicated by the similar number of CFUs when compared with the solvent control. These results suggested that the phosphocholine head portion of PAF C-16 on its own does not possess the bacterial growth inhibition activity of PAF C-16 (Figure 4.4.2b and 4.4.2c).

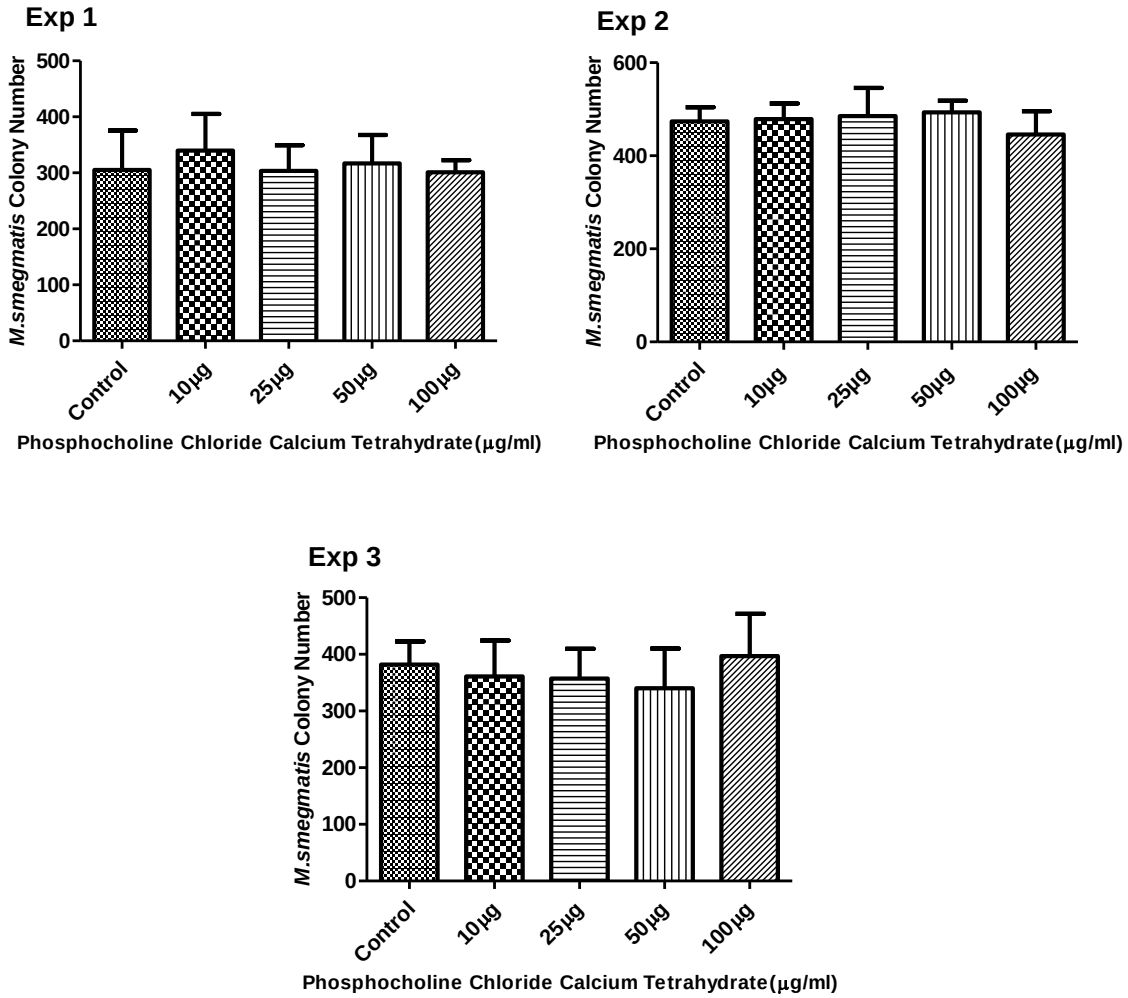


Figure 4.4.2b: Three individual experiments (Exp1, 2 and 3) for the direct effect of phosphocholine chloride calcium tetrahydrate on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

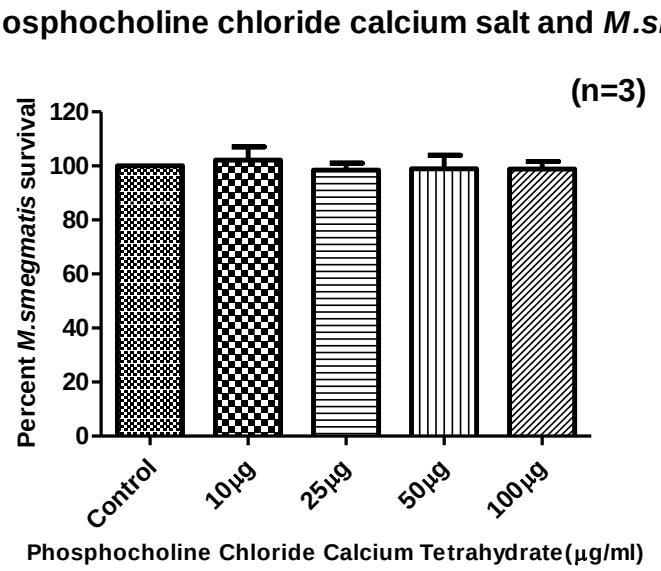


Figure 4.4.2c: Direct effect of Phosphocholine chloride calcium salt on *M.smegmatis* growth. Each bar represents the average of three independent experiments performed and the error bars show the standard error of means (SEM). Data is expressed in terms of percentage where solvent control is considered as 100% survival and different test conditions are compared to it. Multiple-comparison non-parametric Kruskal-Wallis test was applied on ranks and the *p*-value was non-significant (p=0.88).

4.4.3 Direct effect of 1-O-hexadecyl-*sn*-glycerol on *M.smegmatis* growth *in vitro*

1-O-hexadecyl-*sn*-glycerol is an ether lipid with a 16 carbon atoms chain. This compounds is similar to PAF C-16 as it contains a glycerol backbone with a 16 carbon atoms chain attached to it via an ether bond (Figure 4.4.3a).

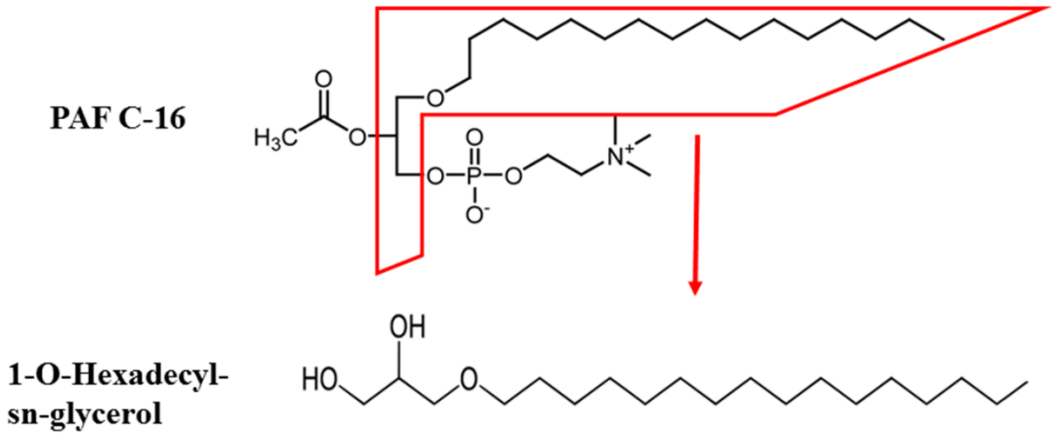


Figure 4.4.3a: Chemical structure of 1-O-hexadecyl-*sn*-glycerol. The compound contains a 16 carbon atoms chain attached to the glycerol backbone via an ether bond.

The direct effect of 1-O-hexadecyl-*sn*-glycerol on the growth of *M.smegmatis* was investigated according to the protocol in chapter 2.2. Three independent experiments (Exp1, 2 and 3) were performed by treating different concentrations of 1-O-hexadecyl-*sn*-glycerol (10µg, 25µg, 50µg and 100µg/ml of bacterial suspension) with *M.smegmatis* for 2 hours. A solvent control for 1-O-hexadecyl-*sn*-glycerol (10µl ethanol/ml bacterial suspension) was also included in each experiment.

It was observed that 1-O-hexadecyl-*sn*-glycerol was able to inhibit the growth of *M.smegmatis* in a dose-dependent manner as indicated by a reduction in the number of CFUs when compared to the solvent control. All the experiments of 1-O-hexadecyl-sn-glycerol with *M.smegmatis* showed the same trend of growth inhibition (Figure 4.4.3b). On average, 1-O-hexadecyl-*sn*-glycerol at concentrations of 25µg/ml, 50µg/ml and 100µg/ml caused a reduction of ~42% ~63% and ~83% in the number of *M.smegmatis* CFUs respectively (Figure 4.4.3c). The level of significance was determined by applying non-parametric multiple comparison Kruskal Wallis test on ranks and the *p*-value was found to be significant, $p=0.008$. Individual data sets were compared by applying post hoc Dunn's multiple comparison test and the results were found to be significant only for 100µg/ml 1-O-hexadecyl-*sn*-glycerol vs Solvent control ($p \leq 0.01$).

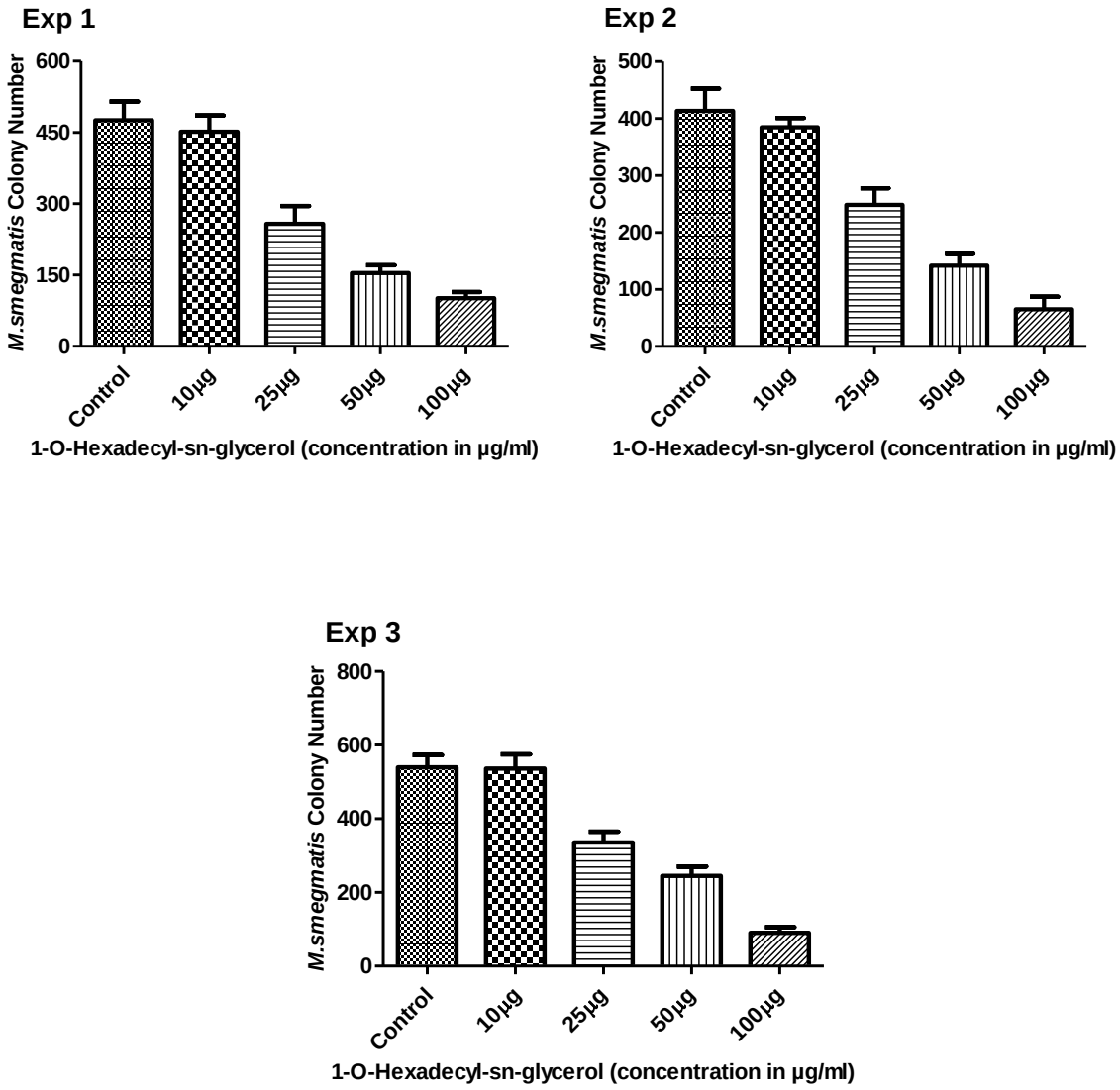


Figure 4.4.3b: Three individual experiments (Exp1, 2 and 3) for the direct effect of 1-O-hexadecyl-*sn*-glycerol on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

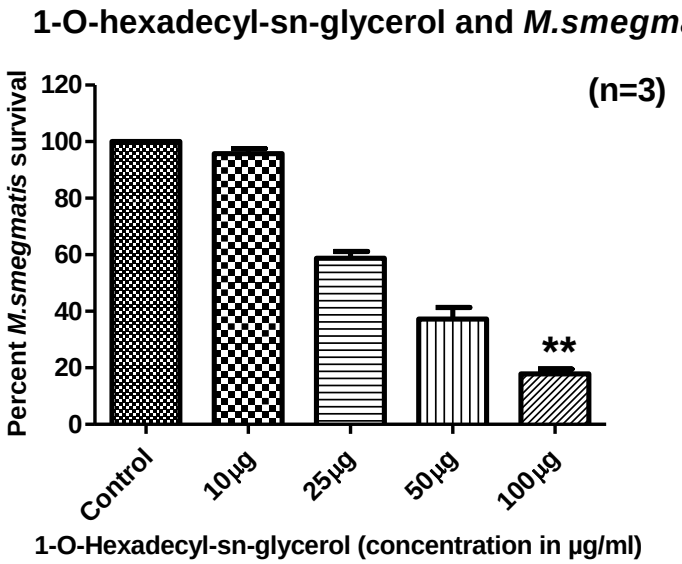


Figure 4.3.3c: Direct effect of 1-O-hexadecyl-*sn*-glycerol on *M.smegmatis* growth. Each bar represents the average of three independent experiments and the error bars show the standard error of means (SEM). Data is expressed in terms of percentage survival where control is taken as 100% survival and different test conditions are compared to it. Multiple-comparison of the data sets was performed using non-parametric Kruskal-Wallis test (p=0.008). Individual data sets were compared using Dunn's multiple comparison test and only for 100µg/ml 1-O-hexadecyl-*sn*-glycerol vs Solvent control was found to be significant **($p \leq 0.01$).

4.4.4 Direct effect of Miltefosine on *M.smegmatis* growth *in vitro*

Miltefosine is an alkyl-phosphocholine, chemically known as hexadecyl phosphocholine or Hexadecyl 2-(trimethylammonio)ethyl phosphate. Miltefosine consists of a 16 carbon atoms chain with no double bonds attached to the phosphate group of a phosphocholine head by ester bond (Figure 4.4.4a). Structurally miltefosine is similar to PAF C-16 as it contains a 16 carbon atoms chain and a phosphocholine portion, however, it lacks the glycerol backbone and the acetyl group of PAF C-16.

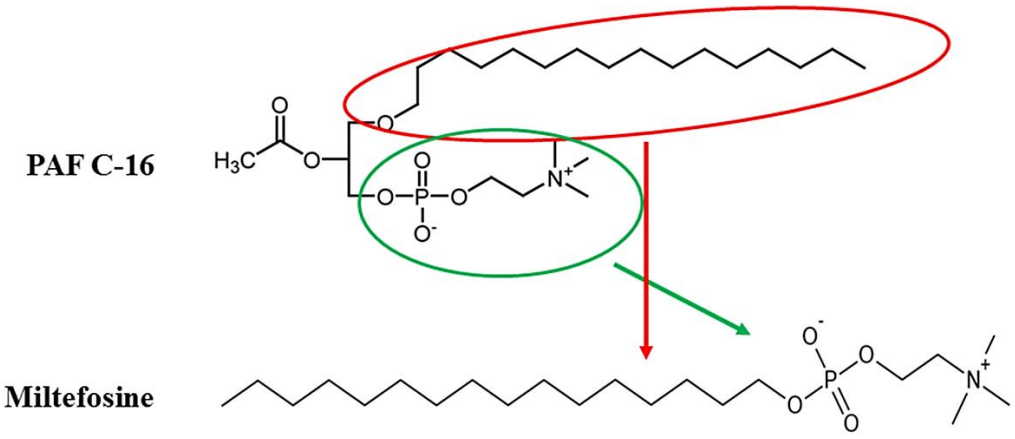


Figure 4.4.4a: Chemical structure of miltefosine. Miltefosine has a 16 carbon atoms chain attached via ester bond to the phosphate group of phosphocholine portion.

The direct effect of miltefosine on the growth of *M.smegmatis* was investigated according to the protocol in chapter 2.2. Three independent experiments (Exp1, 2 and 3) were performed by treating different concentrations of miltefosine (10µg, 25µg, 50µg and 100µg/ml of bacterial suspension) with *M.smegmatis* for 2 hours. A solvent control for miltefosine (20µl water/ml bacterial suspension) was also included in each experiment.

It was observed that miltefosine was able to inhibit the growth of *M.smegmatis* in a dose-dependent manner as indicated by a reduction in the number of CFUs when compared with the solvent control. All the experiments of miltefosine with *M.smegmatis* showed the same trend of growth inhibition (Figure 4.4.4b). On average, miltefosine at concentrations of 25µg/ml, 50µg/ml and 100µg/ml caused ~70% ~89% and ~99% reduction in the number of *M.smegmatis* CFUs respectively when compared with the number of CFUs for solvent control (Figure 4.4.4c). The level of significance was determined by applying non-parametric multiple comparison Kruskal Wallis test on ranks and the *p*-value was found to be significant, $p=0.008$. Individual data sets were compared by applying post hoc Dunn's multiple comparison test and the results were found to be significant only for 50µg/ml miltefosine vs Solvent control ($p \leq 0.05$) and 100µg/ml miltefosine vs Solvent control ($p \leq 0.01$).

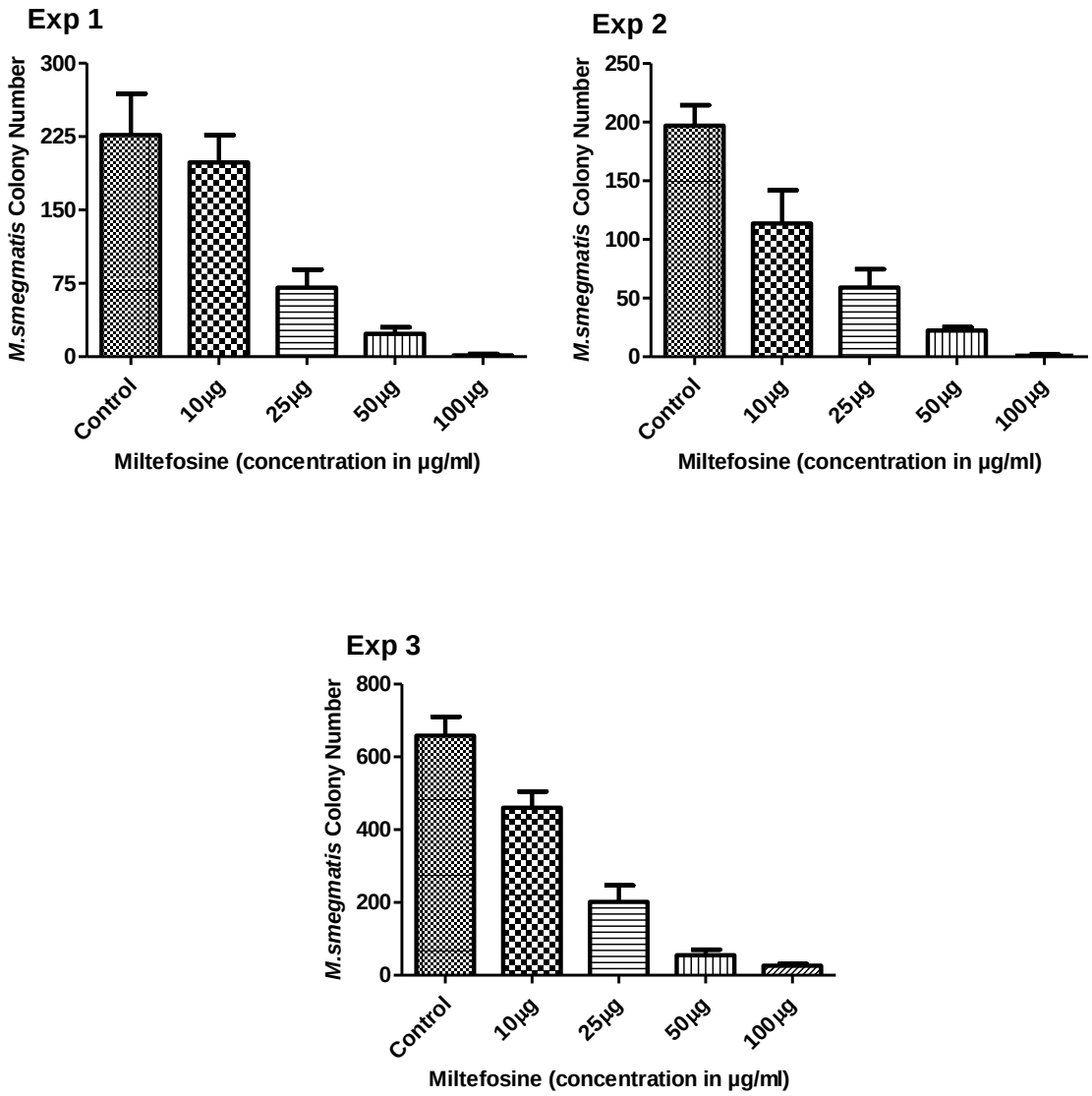


Figure 4.4.4b: Three independent experiments (Exp1, 2 and 3) for the direct effect of miltefosine on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

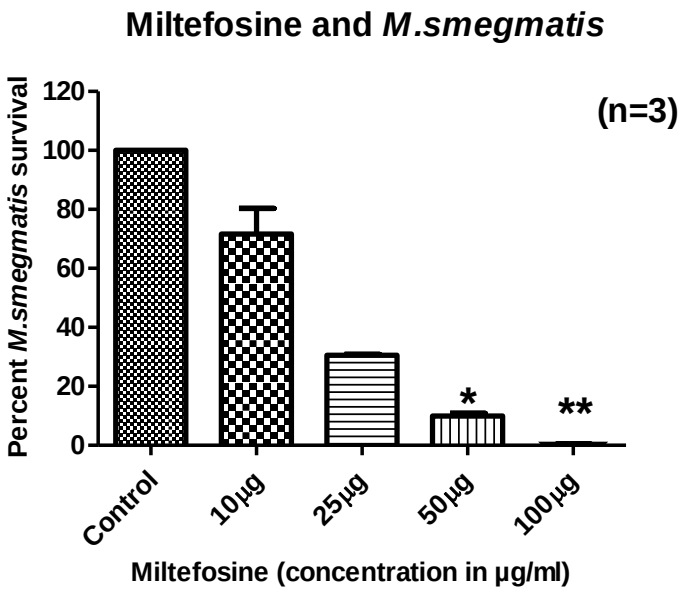


Figure 4.4.4c: Direct effect of miltefosine on the growth of *M.smegmatis*. Each bar represents the average of three independent experiments and the error bars show the standard error of mean (SEM). Data is expressed in terms of percentage survival where control is taken as 100% and different test conditions are compared to it. Non-parametric multiple comparison Kruskal-Wallis test was applied on ranks and was *p*-value was found to be significant, p=0.008. Individual data sets were compared using Dunn's multiple comparison test, where 50µg/ml miltefosine vs Solvent control *($p \leq 0.05$) and 100µg/ml miltefosine vs Solvent control **($p \leq 0.01$) were found to be significant.

4.4.5 Direct effect of Hexadecyl lactate on *M.smegmatis* growth *in vitro*

Hexadecyl lactate consists of a 16 carbon atoms chain with no double bonds attached to a lactyl group via ester linkage (Figure 4.4.5a). This compounds contains a similar length carbon chain to PAF C-16, however, the carbon chain differs from PAF C-16 in its attachment as it is attached via ester linkage.

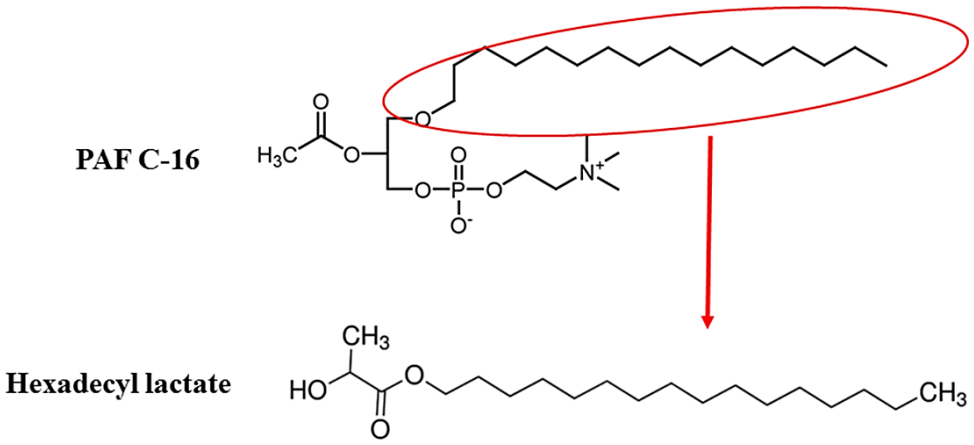


Figure 4.4.5a: Chemical structure of hexadecyl lactate. Hexadecyl lactate has a 16 carbon atoms chain attached by ester bond to a lactyl group.

Three independent experiments (Exp1, 2 and 3) were performed to investigate the effect of hexadecyl lactate on the growth of *M.smegmatis* according to the protocol in chapter 2.2. Hexadecyl lactate in different concentrations (10µg, 25µg, 50µg and 100µg/ml of bacterial suspension) was treated with *M.smegmatis* for 2 hours. A solvent control for hexadecyl lactate (10µl DMSO/ml bacterial suspension) was also included in all the experiments.

It was observed that hexadecyl lactate was able to inhibit the growth of *M.smegmatis* in a dose-dependent manner as indicated by a reduction in the number of CFUs when compared with the solvent control. All the experiments of hexadecyl lactate with *M.smegmatis* showed the same trend of growth inhibition (Figure 4.4.5b). On average, hexadecyl lactate at concentrations of 50µg/ml and 100µg/ml caused a reduction of ~50% and ~83% in the number of *M.smegmatis* CFUs respectively (Figure 4.4.5c). The level of significance was determined by applying non-parametric multiple comparison Kruskal Wallis test on ranks and the *p*-value was found to be significant, $p=0.008$. Individual data sets were compared by applying post hoc Dunn's multiple comparison test and the results were found to be significant only for 100µg/ml hexadecyl lactate vs Solvent control ($p \leq 0.01$).

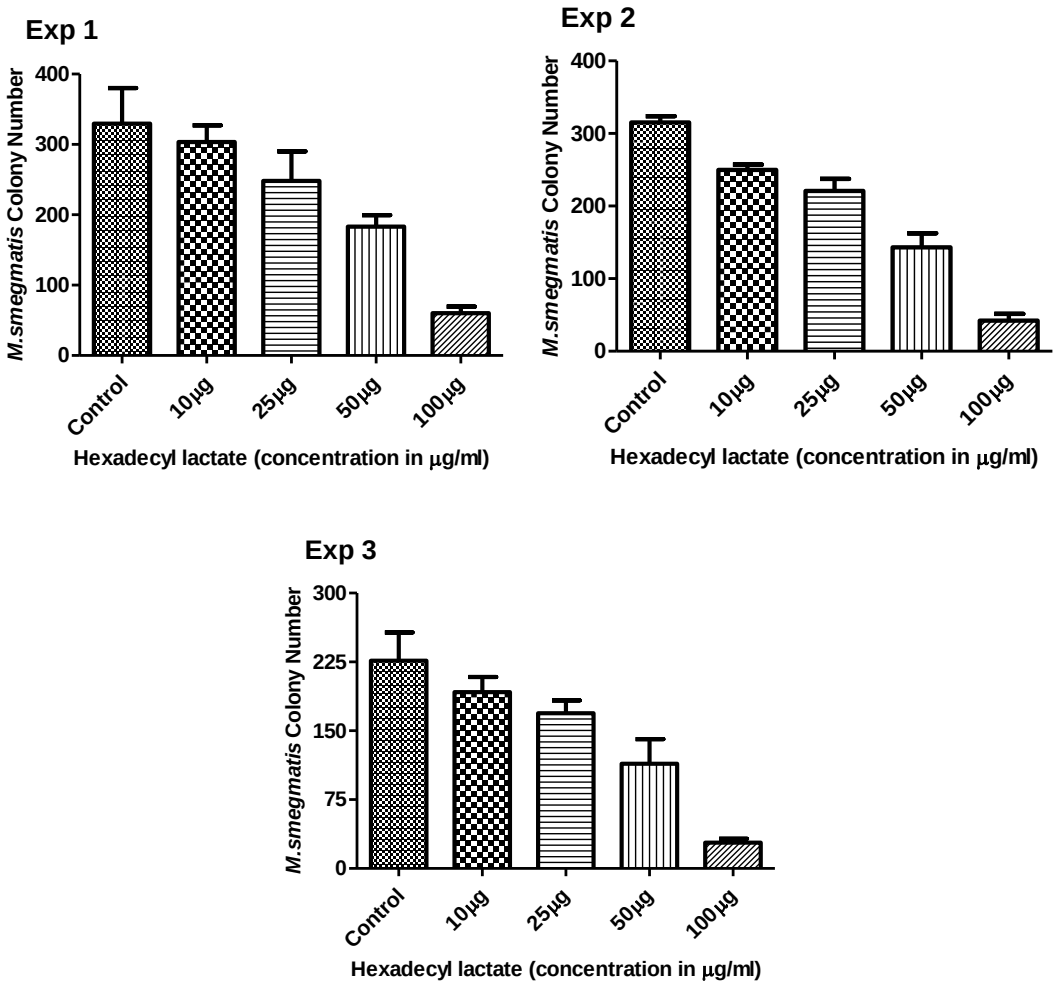


Figure 4.4.5b: Three independent experiments (Exp1, 2 and 3) for the direct effect of hexadecyl lactate on the growth of *M.smegmatis*. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates for solvent control and test conditions and error bar shows standard deviation (SD) of the triplicates.

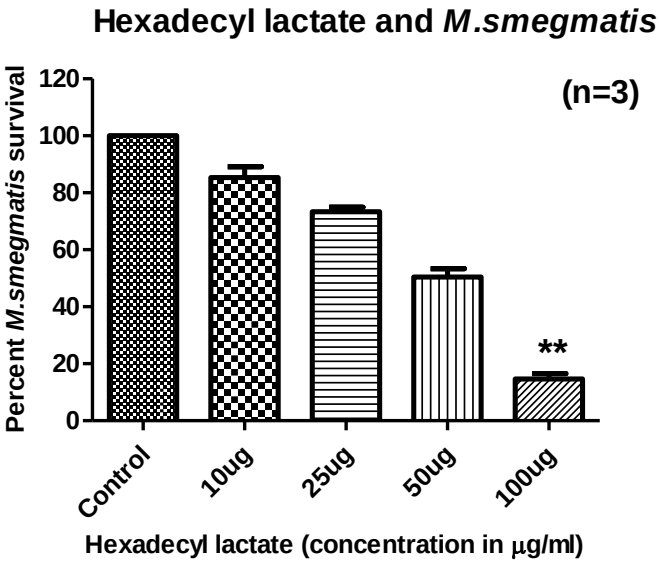


Figure 4.3.5c: Direct effect of Hexadecyl lactate on the growth of *M.smegmatis*. Each bar represents the average of three individual experiments and the error bars show the standard error of means (SEM). Data is expressed in terms of percentage survival where control is taken as 100% and different test conditions are compared to it. Non-parametric multiple comparison Kruskal-Wallis test was applied on ranks and was *p*-value was found to be significant, p=0.008. Individual data sets were compared using Dunn's multiple comparison test, where 100μg/ml hexadecyl lactate vs Solvent control **($p \leq 0.01$) was found to be significant.

4.4.6 Direct effect of Palmitoleic acid and Oleic acid on *M.smegmatis* growth *in-vitro*

To investigate effect of conformational changes in the aliphatic carbon chain on the bacterial growth inhibition activity, two unsaturated fatty acids, palmitoleic acid and oleic acid were used. These compounds comprise of 16 carbon atoms and 18 carbon atoms chains respectively which are attached to a carboxyl group. Both these compounds contain a single double bond which changes the linear structure of the carbon chain to bend and induces a kink in these compounds (Figure 4.4.6a).

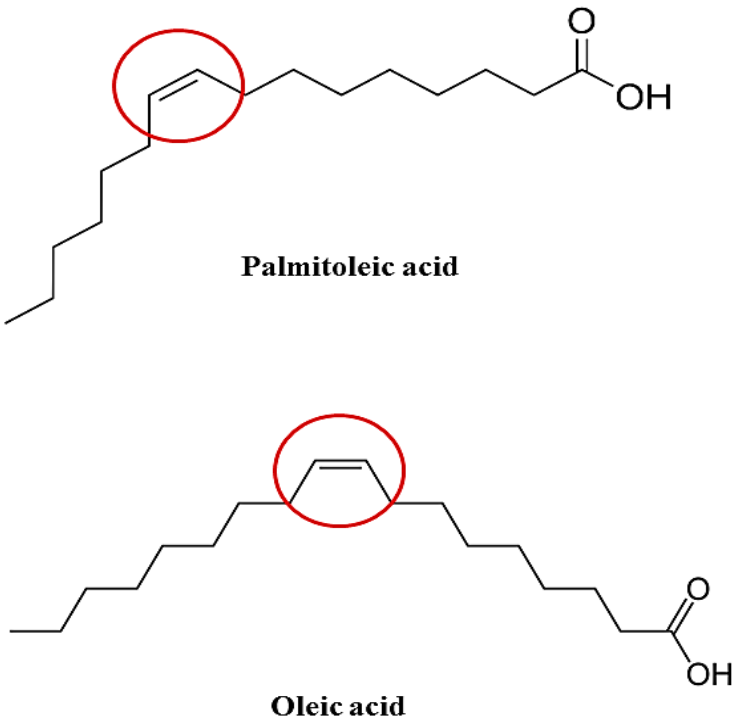


Figure 4.4.6a: Chemical structure of Palmitoleic acid and Oleic acid. The portion in red circle shows the double bond between two carbon atoms of the aliphatic tail which causes the carbon chain to bend.

To check the effect of presence of double bond in the carbon tail on growth of *M.smegmatis*, palmitoleic acid and oleic acid in concentrations of 10µg, 25µg, 50µg and 100µg/ml and a solvent control (10µl ethanol/ml of bacterial suspension) were directly treated with *M.smegmatis* according to the protocol in section 2.2. It was observed that both these compounds were able to inhibit *M.smegmatis* growth even at the lowest concentration of 10µg/ml (Figure 4.4.5b). These results suggested that changes in the confirmation of the carbon chain can contribute to it bacterial growth inhibition potential of compounds.

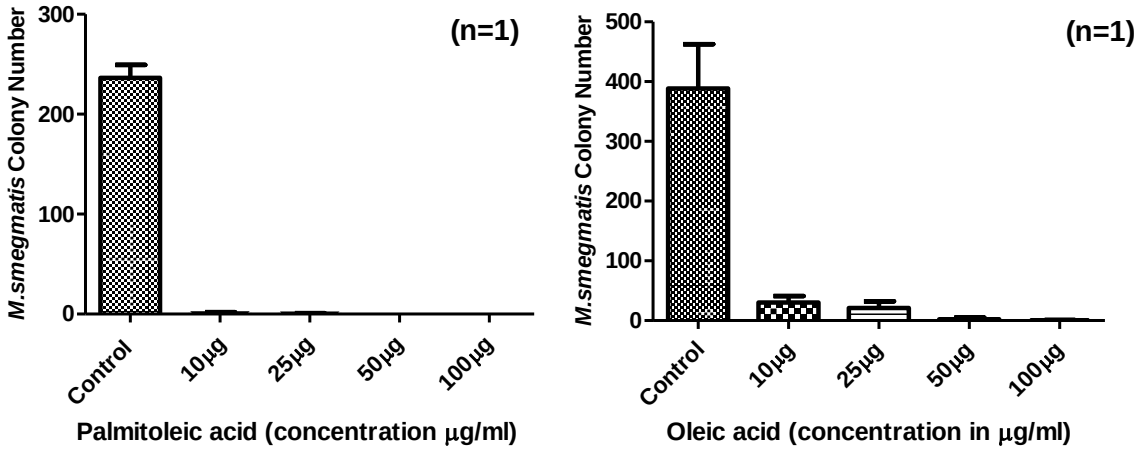


Figure 4.4.6b: Direct effect of palmitoleic acid and oleic acid on *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicates. The graphs show that both palmitoleic acid and oleic acid are effective inhibitors of *M.smegmatis* growth.

4.5 Effect of PAF C-16 treatment on mycobacterial cell membrane

Microscopy and flow cytometry using cell viability dyes such as Trypan blue and Propidium iodide (PI) were performed to investigate the effect of PAF C-16 treatment on mycobacterial cell membrane.

4.5.1 Light Microscopy with PAF C-16 treated *M.smegmatis* and *M.bovis BCG*

Trypan blue is a cell viability dye that stains only the bacteria with damaged cell membranes. *M.smegmatis* and *M.bovis BCG* were treated with PAF C-16 (100µg/ml) and solvent control for PAF C-16 (10µl ethanol/ml) for 4 hours were stained with trypan blue according the protocol mentioned in chapter 2.3.1.

The effect of PAF C-16 treatment on the mycobacterial cell membrane was detected by visualizing both PAF C-16 treated and solvent control *M.smegmatis* and *M.bovis BCG* at a 400X magnification using a light microscope. PAF C-16 treated *M.smegmatis* and *M.bovis BCG* showed positive staining with trypan blue whereas the bacteria in the solvent control remained unstained (Figure 4.5.1a and 4.5.1b). These results suggested that PAF C-16 treatment has a damaging effect on the mycobacterial cell membrane that makes the bacteria permeable to the otherwise impermeable trypan blue dye. Moreover, the PAF C-16 treated *mycobacteria* appeared fragmented suggesting cell death.

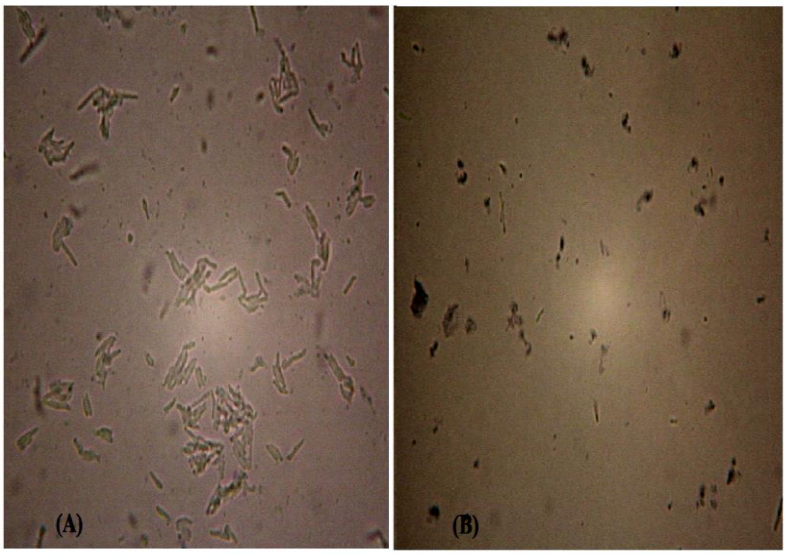


Figure 4.5.1a: Trypan blue staining of *M.smegmatis* to detect cell membrane damage. Microscopic images of *M.smegmatis* at 400X magnification. Slide (A) is the solvent control: 10µl ethanol/ml of bacterial suspension. The rod shaped *M.smegmatis* have not taken any dye, suggesting that the cell membrane is intact. Slide (B) is PAF C-16 treated test: 100µg PAF C-16/ml of bacterial suspension. PAF C-16 treated *M.smegmatis* appeared blue indicating entry of trypan blue in to the cell suggesting damage to the cell membrane of *M.smegmatis.* Moreover, the bacteria appeared fragmented indicating cell death.

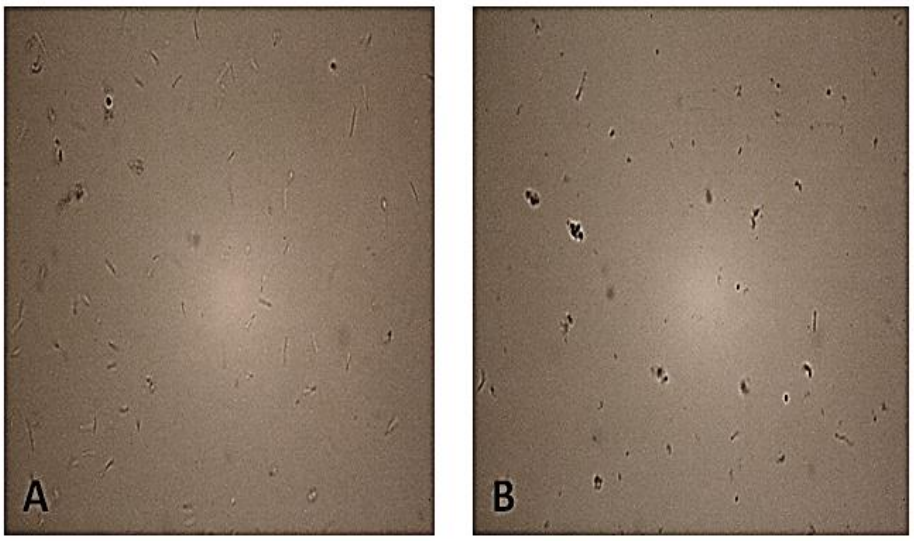


Figure 4.5.1b: Trypan blue staining of *M.bovis BCG* to detect cell membrane damage. Microscopic images of *M.bovis BCG* at 400X magnification. Slide (A) is the solvent control:

10µl ethanol/ml of bacterial suspension. There is no positive staining of the bacteria suggesting the cell membrane is intact. Slide (B) is PAF C-16 treated test: 100µg PAF C-16/ml of bacterial suspension. PAF C-16 treated *M.bovis BCG* appeared blue due entry of trypan blue suggesting damage to the cell membrane and also appeared to be fragmented.

4.5.2 Fluorescence microscopy with PAF C-16 treated *M.smegmatis* and *M.bovis BCG*

The results from light microscopy indicating that PAF C-16 treatment has a damaging effect on *M.smegmatis* and *M.bovis BCG* cell membrane were re-confirmed by performing fluorescence microscopy. PAF C-16 treated (100µg/ml) and control *M.smegmatis* and *M.bovis BCG* were stained with a nucleic acid binding dye, Propidium Iodide (PI) according to the protocol in chapter 2.3.2 and the bacteria were visualised by using the bright field and the CY3 channels of a Leica DM4000® microscope.

It was observed that most of the *M.smegmatis* and *M.bovis BCG* treated with PAF C-16 (100µg/ml) for 2 hours were positively stained with PI whereas, no PI staining was detected in the solvent control *M.smegmatis* and *M.bovis BCG* (Figure 4.5.2a and 4.5.2b). Heat treated (100°C for 10min) *M.smegmatis* and *M.bovis BCG* were included as positive control for PI staining in each experiment. These fluorescent microscopy results showing the entry of PI in PAF C-16 treated *mycobacteria* suggested that PAF C-16 had a damaging effect on the mycobacterial cell membrane.

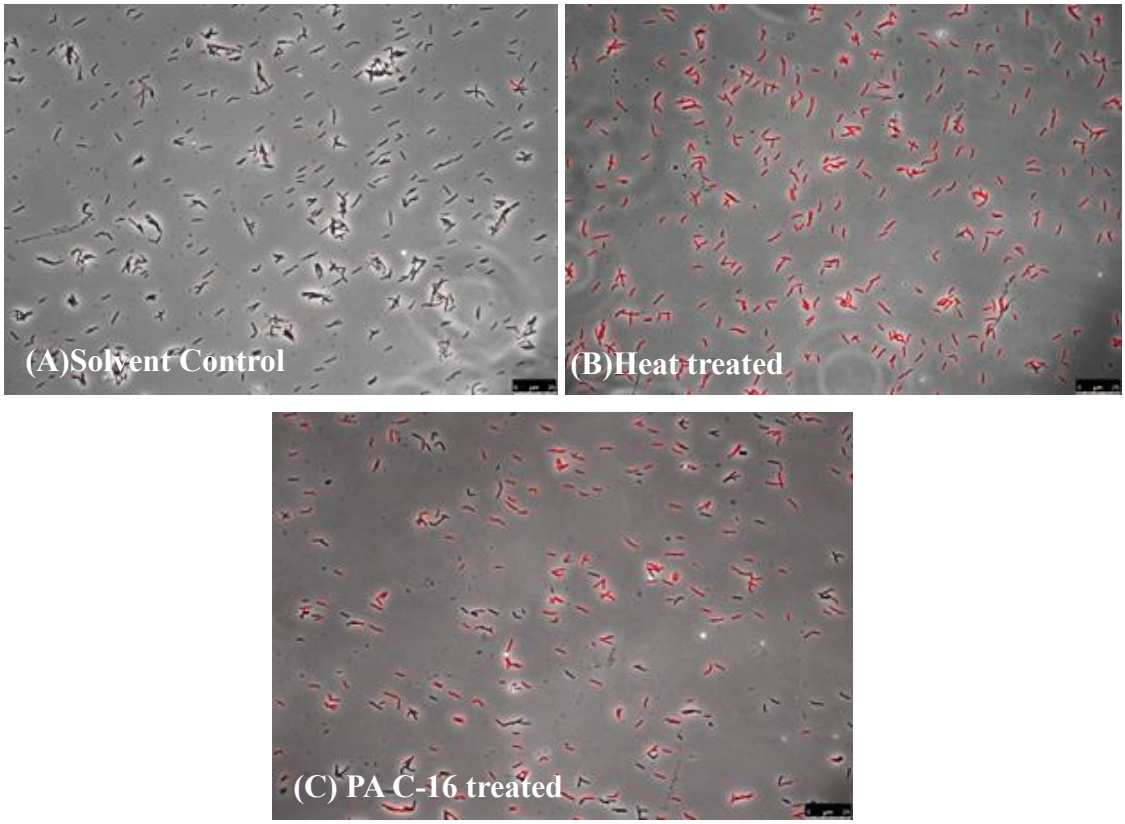


Figure 4.5.2a: PI staining of *M.smegmatis* to detect cell membrane damage. Each slide shows a merger for *M.smegmatis* in Bright field channel and CY3 channel (PI) at 400X magnification. Side A is the solvent control (10µl ethanol/ml of bacterial suspension) where majority of the bacteria remained unstained. Slide B shows heat treated (100^{o}C for 10 minutes) *M.smegmatis*, used as a positive control and almost all *M.smegmatis* showed positive staining for PI. Slide C shows *M.smegmatis* after treatment with 100µg/ml PAF C-16 for 2 hours and most of *M.smegmatis* showed positive staining for PI suggesting PAF C-16 has damaging effect on *M.smegmatis* cell membrane.

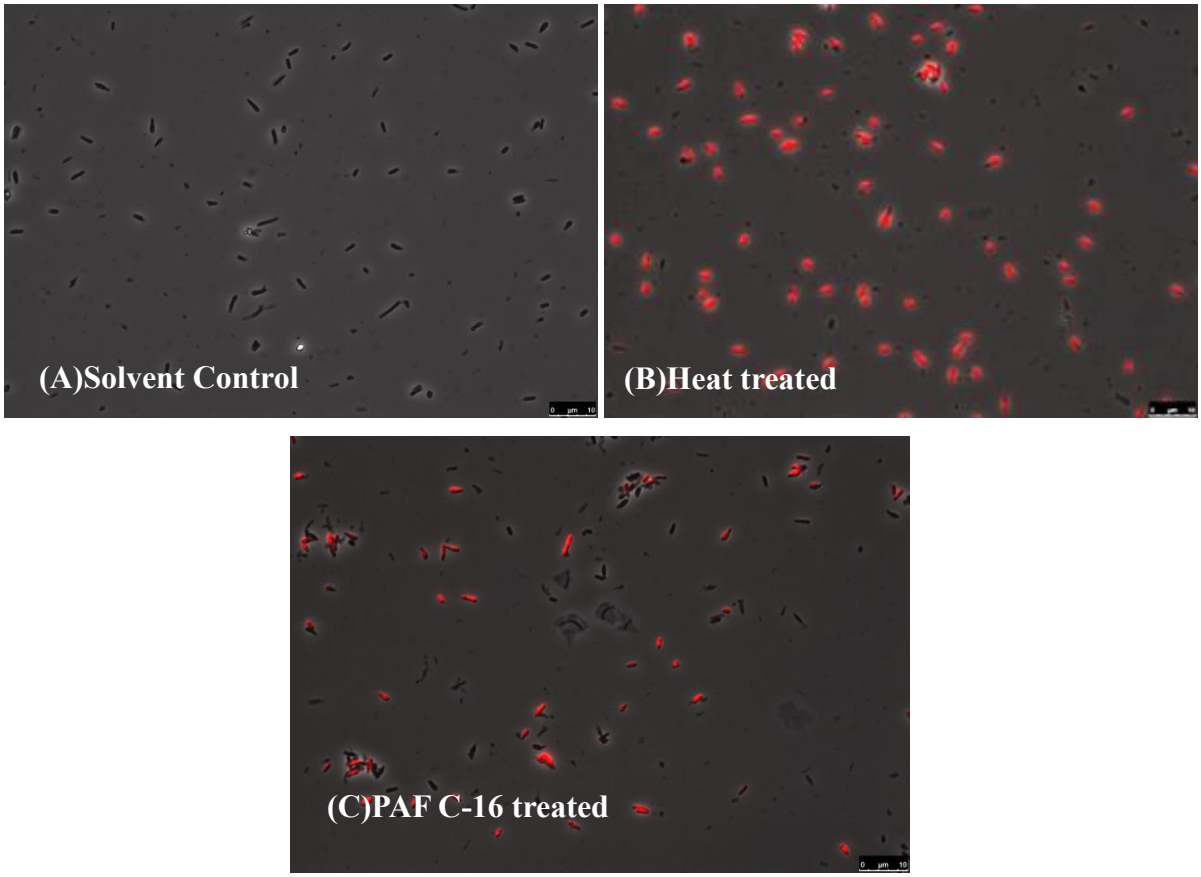


Figure 4.5.2b: PI staining of *M.bovis BCG* to detect cell membrane damage. Each slide shows a merger of Bright field channel and CY3 channel (PI) for *M.bovis BCG* at 400X magnification. Slide A is the solvent control (10µl ethanol/ml of bacterial suspension). The bacterial cell membrane is intact and no PI staining was observed. Slide B shows heated treated (100^{o}C for 10 minutes) *M.bovis BCG* used as a positive control where majority of bacteria appeared red indicating the entry of PI into the cell. Slide C shows PAF C-16 (100µg/ml) treated *M.bovis BCG*. PAF C-16 treatment caused an increase in number of *M.bovis BCG* to be stained positively with PI as compared to solvent control suggesting that PAF-C16 causes membrane damage in *M.bovis BCG*.

4.5.3 Flow cytometry analysis of PAF-C16 treated *M.smegmatis* and *M.bovis BCG*

To quantitate the number of *M.smegmatis* and *M.bovis BCG* with damaged cell membrane due PAF C-16 treatment, flow cytometry was performed. Nucleic acid binding fluorescent dye, PI was used to detect the cell membrane integrity of *M.smegmatis* and *M.bovis BCG*. Bacteria that showed positive staining for PI were considered dead or injured due to the damage to their cell membrane and all the unstained cells with intact cell membrane were considered healthy.

Three independent flow cytometry experiments were performed with both *M.smegmatis* and *M.bovis BCG* according to the protocol in chapter 2.4. For each PAF C-16 (100µg/ml) treated, solvent control (10µl ethanol/ml) and heat (100^{o}C for 10 minutes) treated *M.smegmatis* or *M.bovis BCG*, 20,000- 30,000 events were collected and the data was analysed using NovoExpress® software.

Density plots showing the percentage of PI stained and unstained bacteria were generated for PAF C-16 treated and control conditions. It was observed that PAF C-16 treatment of *M.smegmatis* at a concentration of 100µg/ml for 2 hours resulted in 49.8% *M.smegmatis* to be positively stained with PI as compared to the solvent control where only 2.0% of *M.smegmatis* were PI positive (Figure 4.5.3a). Similarly, 41.0% *M.bovis BCG* were stained positively with PI after treatment with PAF C-16 (100µg/ml) for 2 hours as compared to the solvent control where only 2.0% of *M.bovis BCG* showed positive staining for PI (Figure 4.5.3b).

Furthermore, the histogram overlay showed peak shift in case of both PAF C-16 treated *M.smegmatis* and *M.bovis BCG* when compared to solvent control (Figure 4.5.3c). The values of mean channel fluorescence for PI (MeanX) were higher for PAF C-16 treated bacteria as compared to the solvent control. For *M.smegmatis*, the MeanX for solvent control was 74 which increased to 318 after treatment with 100µg/ml PAF C-16. Similarly for *M.bovis BCG*, the MeanX for solvent control was 7 which increased to 135 when the bacteria were treated with 100µg/ml PAF C-16.

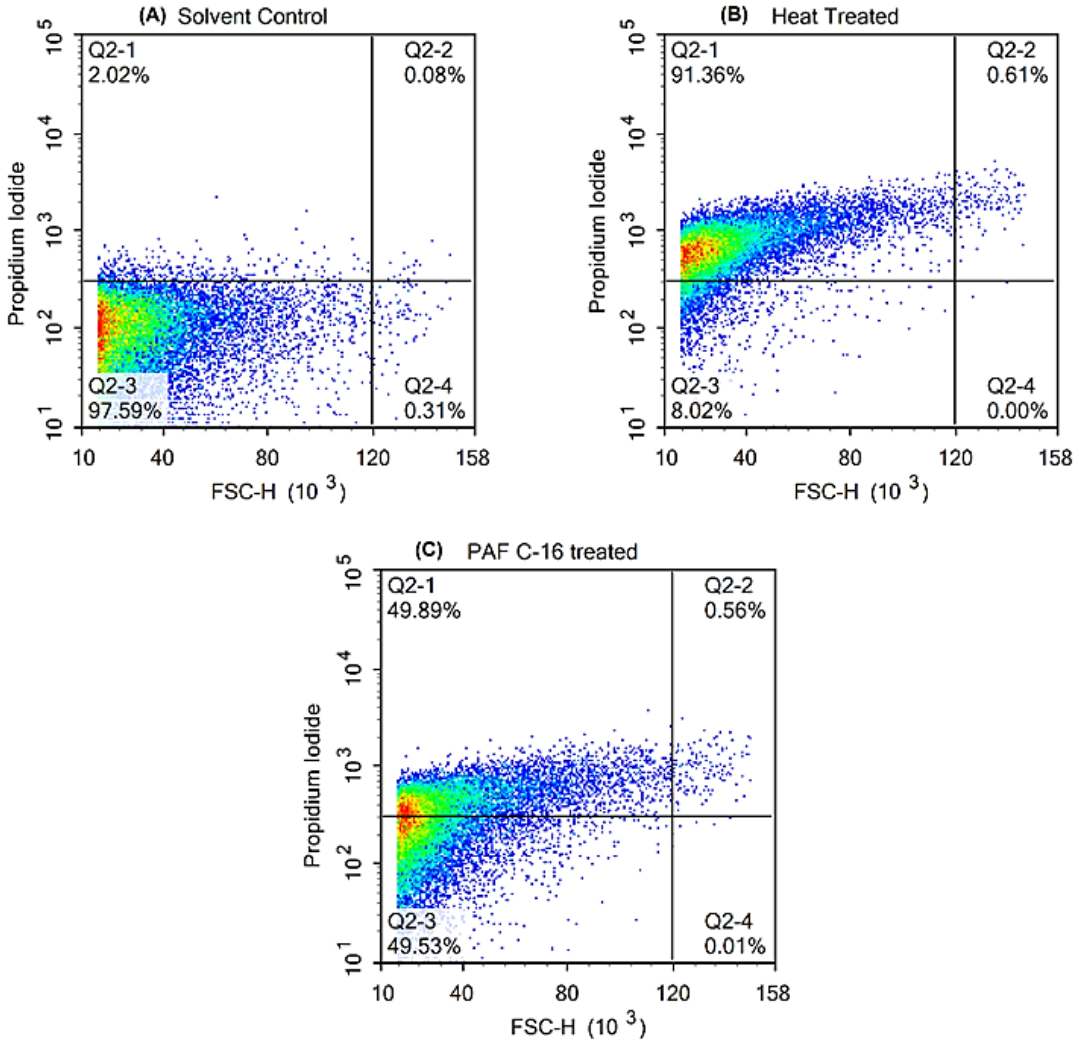


Figure 4.5.3a: Flow cytometry density plots for PI stained *M.smegmatis*. Slide A is the density plot for solvent control *M.smegmatis* (10µl ethanol/ml). Slide B is the density plot for heat treated (100^{o}C for 10 minutes) positive control *M.smegmatis*. Slide C is the density plot for PAF C-16 treated *M.smegmatis* (100µg/ml). Each plot shows the percentage of PI stained (Q2-1) and unstained (Q2-3) *M.smegmatis* in quadrants. X-axis shows the forward scatter indicating cell size and Y-axis shows the fluorescence intensity for PI.

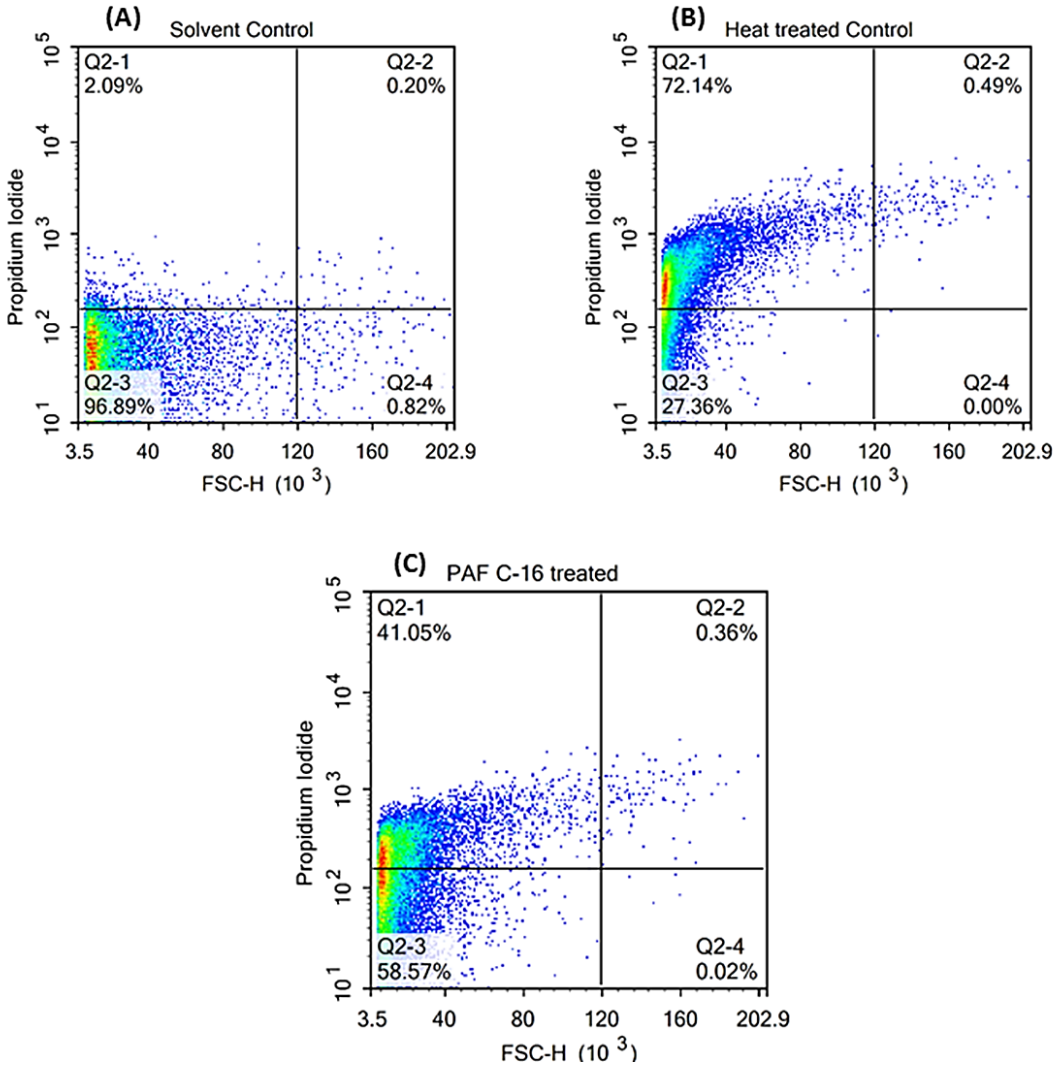


Figure 4.5.3b: Flow cytometry density plots for PI stained *M.bovis BCG*. Slide A is the density plot for solvent control *M.bovis BCG* (10µl ethanol/ml). Slide B is the density plot for heat treated (100ºC for 10 minutes) positive control *M.bovis BCG*. Slide C is the density plot for PAF C-16 treated *M.bovis BCG* (100µg/ml). Each plot shows the percentage of PI stained (Q2-1) and unstained (Q2-3) *M.smegmatis* in quadrants. X-axis shows the forward scatter indicating cell size and Y-axis shows the fluorescence intensity for PI.

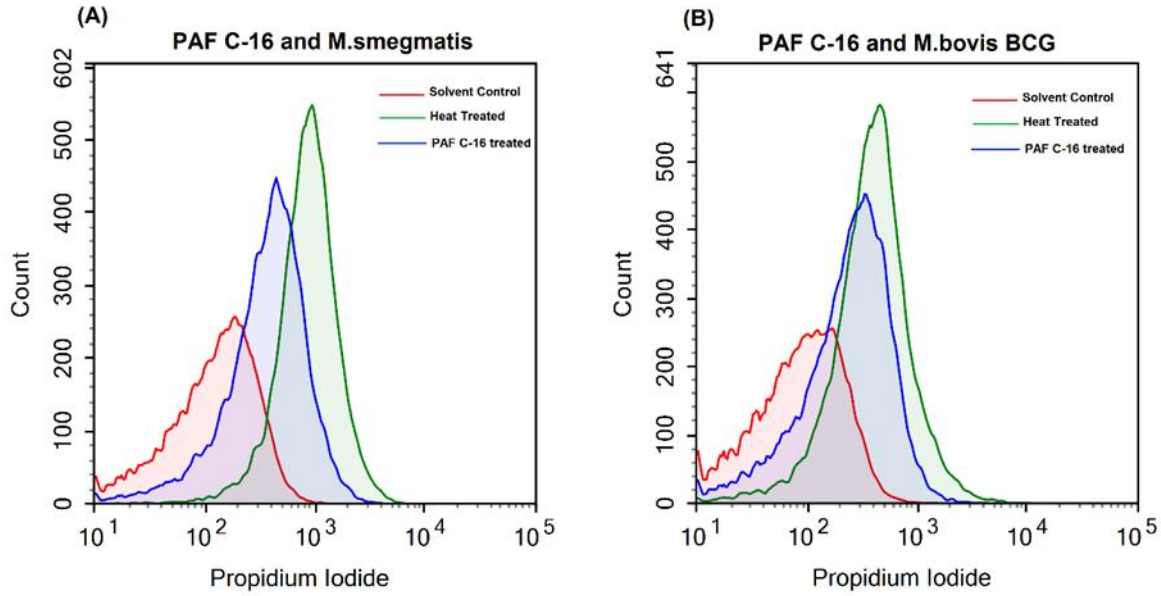


Figure 4.5.3c: Histogram overlay for PAF C-16 treated and control *M.smegmatis* and *M.bovis BCG*. The slides A and B shows histogram overlay for controls and PAF C-16 (100µg/ml) treated *M.smegmatis* and *M.bovis BCG* respectively. X-axis represents the fluorescence intensity of PI and Y-axis is the number of events at each intensity value. The red colour graph represent solvent control, the green colour graph is for positive control comprising of heat treated and the blue colour graph for PAF C-16 treated *M.smegmatis* or *M.bovis BCG*.

4.6 Effect of PAF receptor antagonists on PAF-C16 induced growth inhibition

There are a number of naturally occurring and synthetic compounds that can bind *in vivo* and *in vitro* to PAF receptor (PAFR) on mammalian cells with high affinity. These compounds are known as PAFR antagonists and can potentially abolish the actions of PAF C-16 by blocking the PAFR (Singh *et al*., 2013).

Two different PAFR antagonists, ABT-491 and WEB-2086 were used *in vitro* to check if they can prevent PAF C-16 induced growth inhibition of *M.smegmatis*. Three independent experiments (Exp1, 2 and 3) were performed with each ABT-491 and WEB-2086 according to the protocol in chapter 2.5. *M.smegmatis* was treated with 100µg/ml of ABT-491 or WEB-2086 for 1 hour prior to treatment with 50µg/ml and 100µg/ml PAF C-16 for 2 more hours.

It was observed that both PAFR antagonists, ABT-491 and WEB-2086 were unable to prevent PAF C-16 induced growth inhibition of *M.smegmatis*. The number of CFUs for *M.smegmatis* treated with PAFR antagonist (ABT-491 or WEB-2086) as well as PAF C-16 was similar to

the number of CFUs for PAF C-16 only treated bacteria (Figure 4.6a and 4.6b). Furthermore, both PAFR antagonists, ABT-491 and WEB-2086 showed no effect on the growth of *M.smegmatis* on their own.

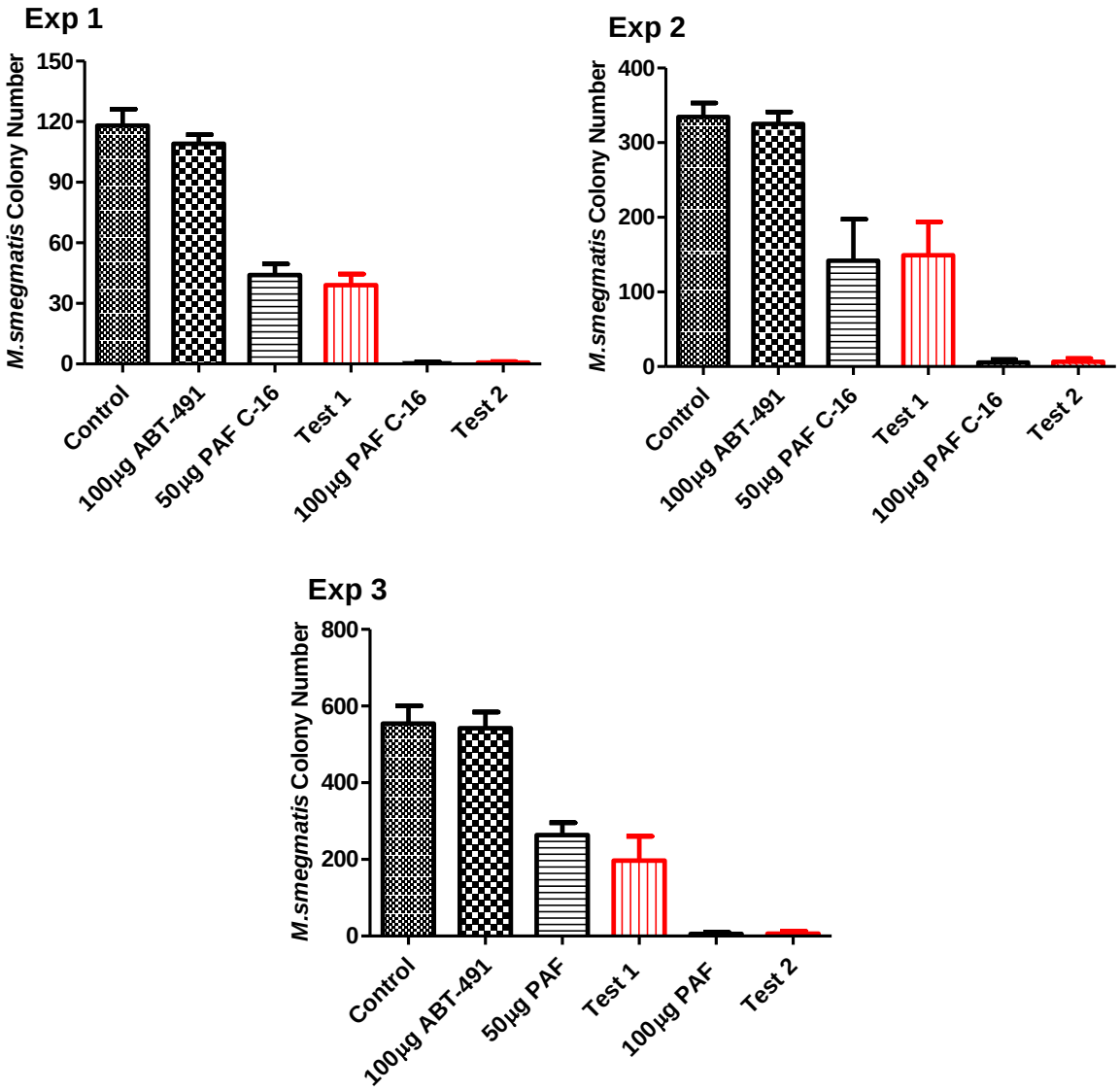


Figure 4.6a: Three individual experiments (Exp1, 2 and 3) showing the effect of ABT-491 on PAF C-16 induced *M.smegmatis* growth inhibition. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicate. **Test 1** consists of 100µg/ml ABT-491 and 50µg/ml PAF C-16. **Test 2** consists of 100µg/ml ABT-491 and 100µg/ml PAF C-16.

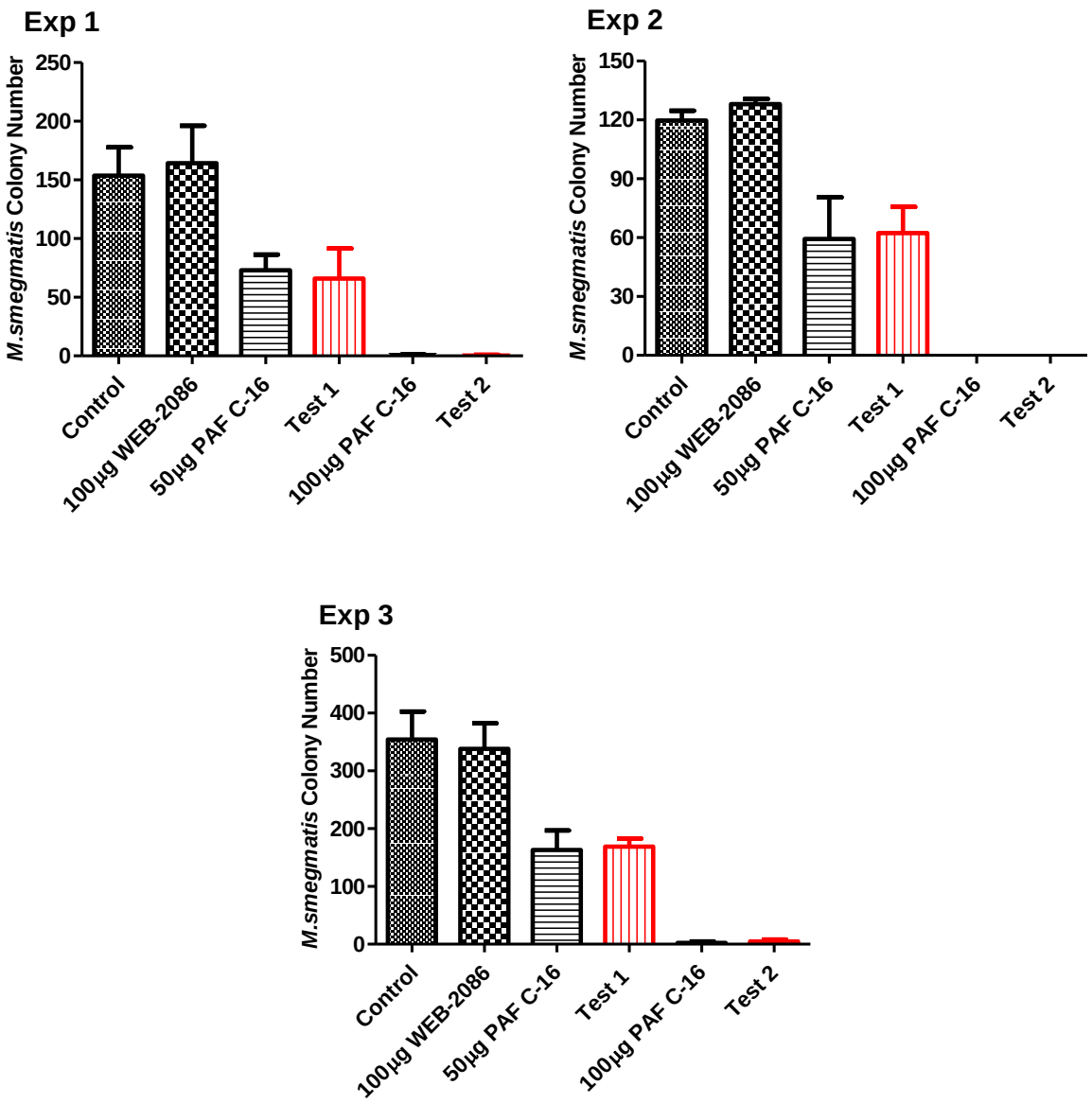


Figure 4.6b: Three individual experiments (Exp1, 2 and 3) showing the effect of WEB-2086 on PAF C-16 induced *M.smegmatis* growth inhibition. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicate. **Test 1** consists of 100µg/ml WEB-2086 and 50µg/ml PAF C-16. **Test 2** consists of 100µg/ml WEB-2086 and 100µg/ml PAF C-16.

These results suggested that PAF C-16 might be acting via a different receptor on bacteria as compared to mammalian cells or the growth inhibition of bacteria can be through a receptor independent mechanism. BLAST search was performed for human PAFR complete coding sequence (CDS) against mycobacteria using BLSAT utility tool at NCBI to find a similar

nucleotide sequences. However, the results showed no significant nucleotide similarity between human PAFR CDS and mycobacteria genome (Figure 4.6c).

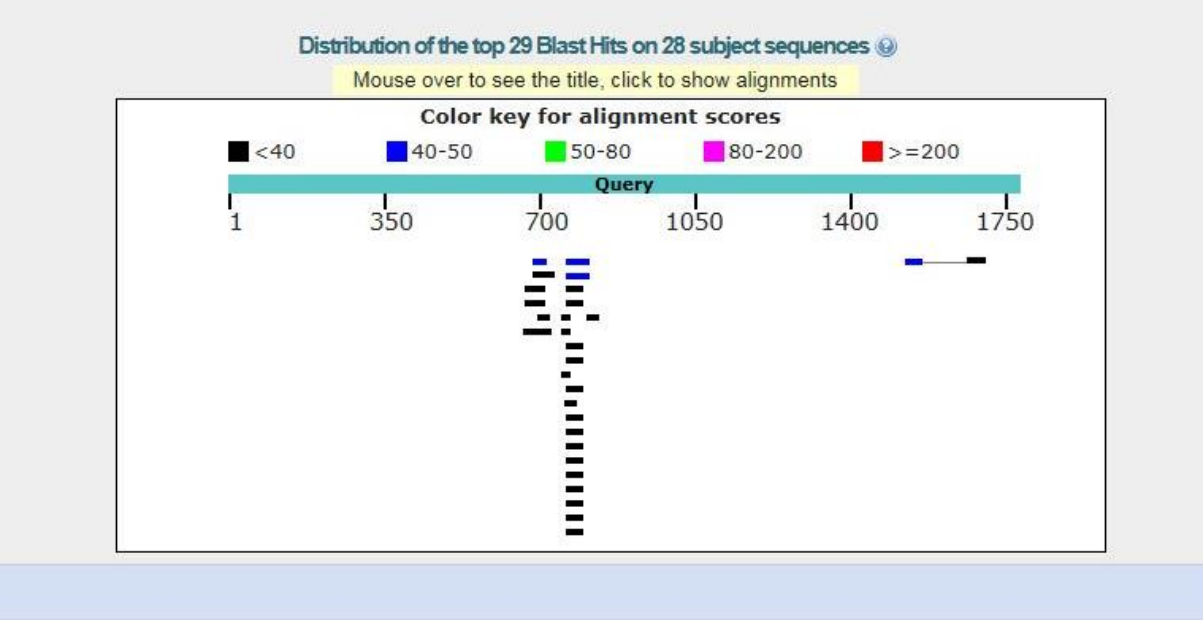


Figure 4.6c: Nucleotide BLAST for Human Platelet Activing Factor Receptor (PAFR) against mycobacteria genome. The figure shows the level of similarity between human PAFR and nucleotide sequences similar in *mycobacteria*. The number of identical nucleotide sequences between the genomes is indicated by different colours.

4.7 Effect of phospholipase inhibitors on PAF C-16 induced growth inhibition

Phospholipase A_2 enzyme hydrolyses phospholipids in to short chain fatty acids. These short chain fatty acids have a damaging effect on the cell membrane (Balsinde *et al*., 2002). In order to investigate the role of phospholipases in PAF C-16 induced growth inhibition, two Phospholipase A_2 inhibitors, dexamethasone and benzenesulfonamide were used.

Three individual experiments (Exp1, 2 and 3) were performed with each dexamethasone and benzenesulfonamide respectively according to the protocol in chapter 2.5 to check whether these phospholipase inhibitors can prevent PAF C-16 induced growth inhibition in *M.smegmatis*. *M.smegmatis* was treated with 100µg/ml of dexamethasone or benzenesulfonamide for 1 hour prior to treatment with 50µg/ml and 100µg/ml PAF C-16 for 2 more hours.

It was observed that both dexamethasone and benzenesulfonamide were unable to prevent PAF C-16 induced growth inhibition of *M.smegmatis* as indicated by the similarity in number of *M.smegmatis* CFUs for Phospholipase A_2 inhibitor (dexamethasone or benzenesulfonamide) as well as PAF C-16 treated to the number of CFUs for PAF C-16 only treated condition (Figure 4.7a and 4.7b). Furthermore, dexamethasone and benzenesulfonamide showed no effect on the growth of *M.smegmatis* on their own.

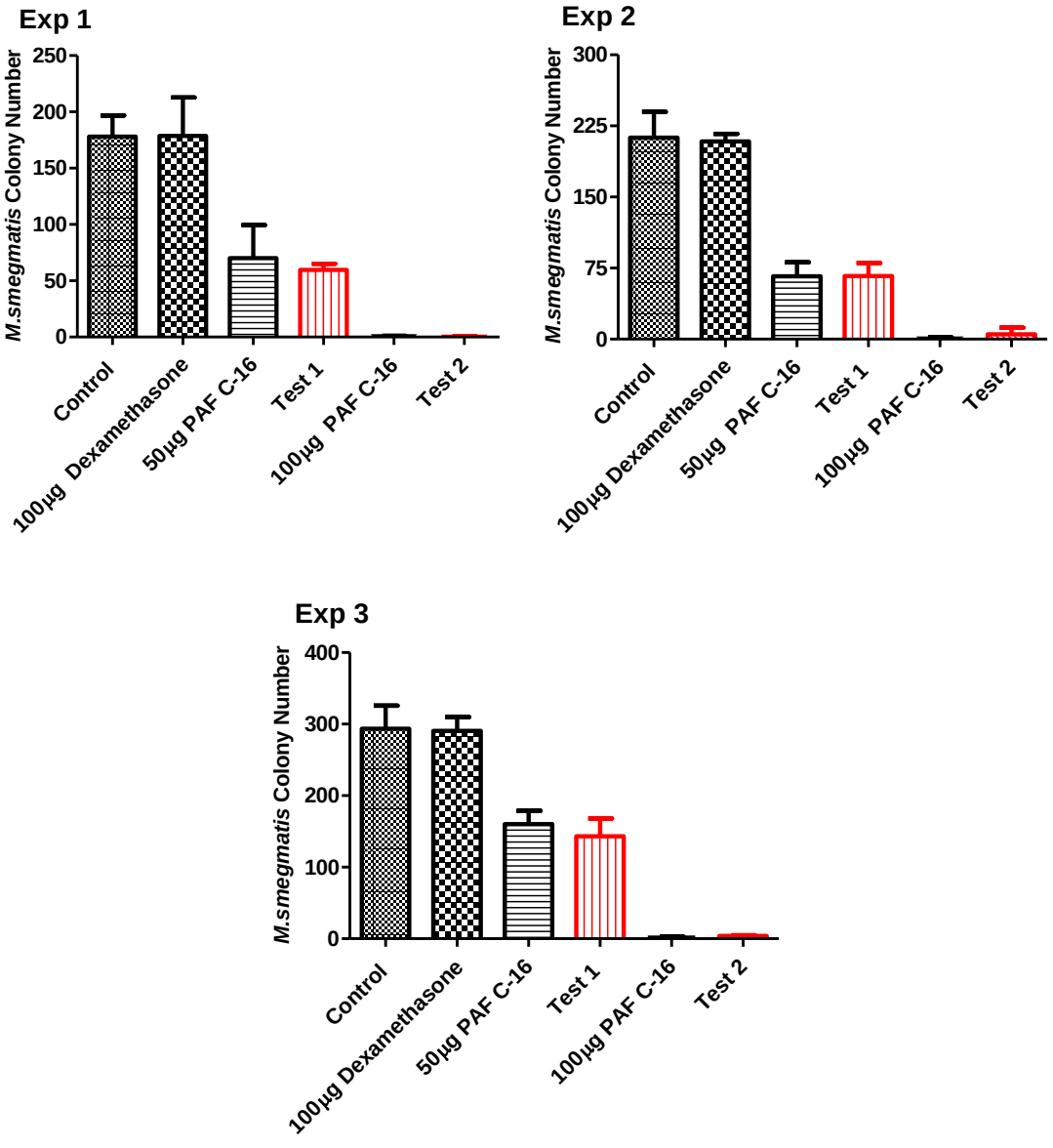


Figure 4.7a: Three individual experiments (Exp1, 2 and 3) showing the effect of dexamethasone on PAF C-16 induced growth inhibition of *M. smegmatis*. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicate. **Test 1** consists of 100µg/ml dexamethasone and 50µg/ml PAF C-16. **Test 2** consists of 100µg/ml dexamethasone and 100µg/ml PAF C-16.

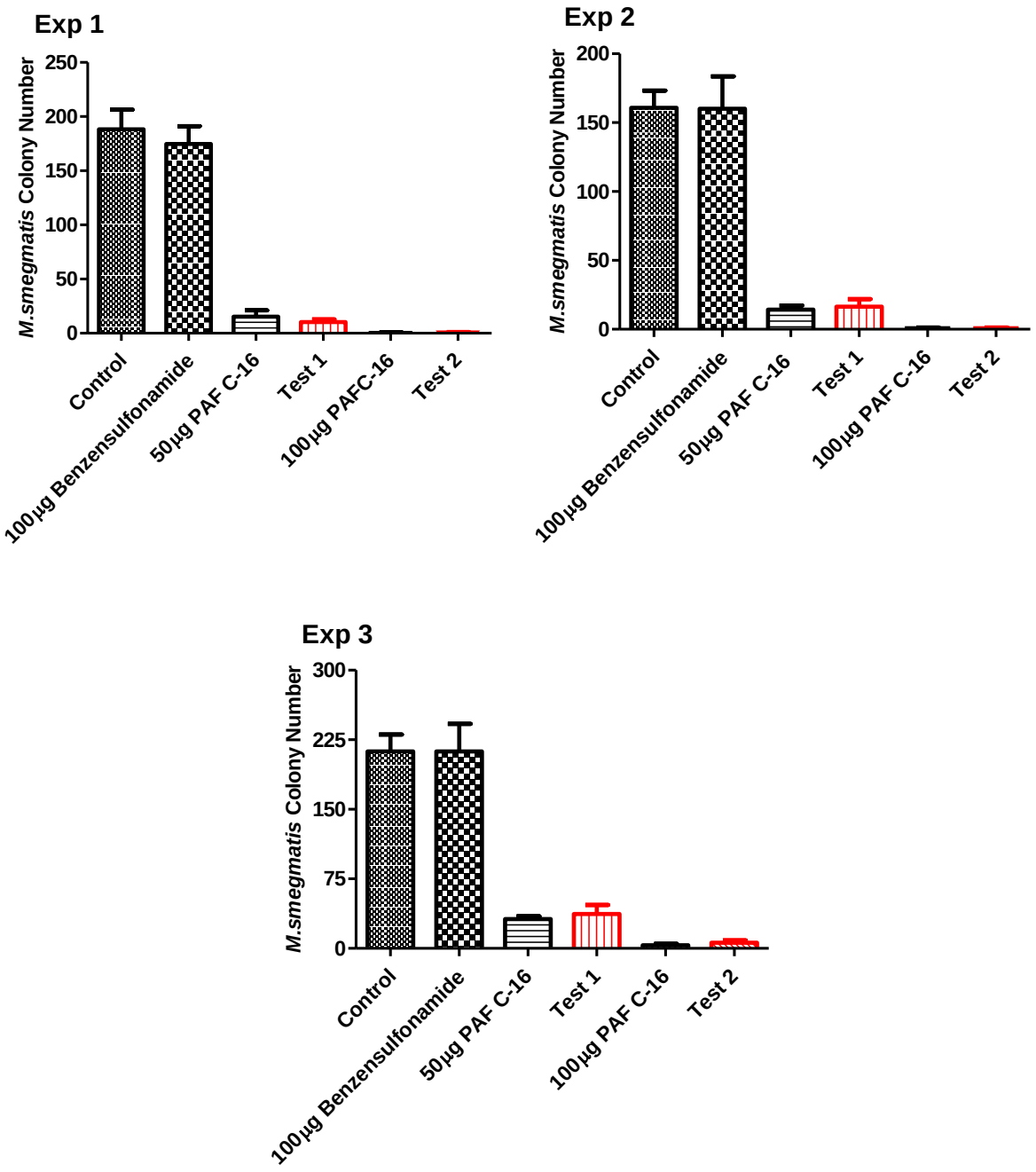


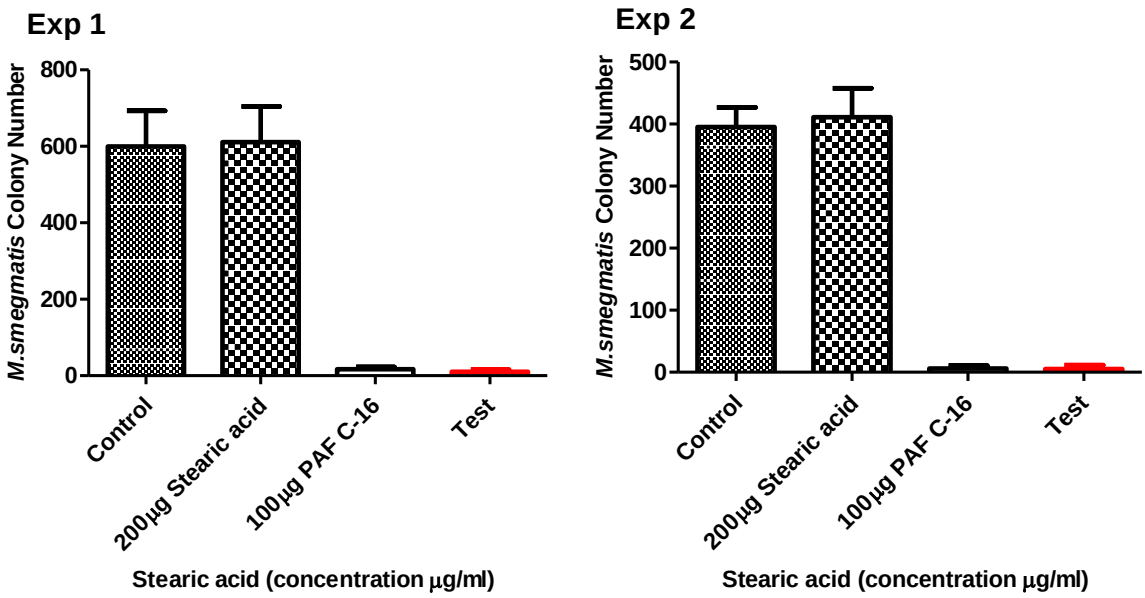
Figure 4.7b: Three individual experiments (Exp1, 2 and 3) showing the effect of benzenesulfonamide on PAF C-16 induced *M. smegmatis* growth inhibition. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicate. **Test 1** consists of 100µg/ml benzenesulfonamide and 50µg/ml PAF C-16. **Test 2** consists of 100µg/ml benzenesulfonamide and 100µg/ml PAF C-16.

4.8 Effect of exogenous fatty acids on PAF C-16 induced *M.smegmatis* growth inhibition

Fatty acid are essential for bacterial survival and are used during the synthesis of bacterial membranes (Zheng *et al*., 2005). Two saturated fatty acids, stearic acid and palmitic acid were used to check their effect on the PAF C-16 induced growth inhibition of *M.smegmatis*.

Three individual experiments (Exp1, 2 and 3) were performed with each stearic acid and palmitic acid respectively according to the protocol in chapter 2.5. *M.smegmatis* was treated with 200µg/ml of stearic acid or palmitic acid for 1 hour prior to treatment with 100µg/ml PAF C-16 for 2 more hours.

It was observed that prior addition of stearic acid or palmitic acid had no effect on the PAF C-16 induced growth inhibition of *M.smegmatis.* The results showed that the number of CFUs for *M.smegmatis* treated with both fatty acid (stearic acid or palmitic acid) and PAF C-16 was similar to the number of CFUs for PAF C-16 only treated *M.smegmatis*, suggesting that exogenous fatty acids were unable to prevent PAF C-16 induced *M.smegmatis* growth inhibition (Figure 4.8a and 4.8b).



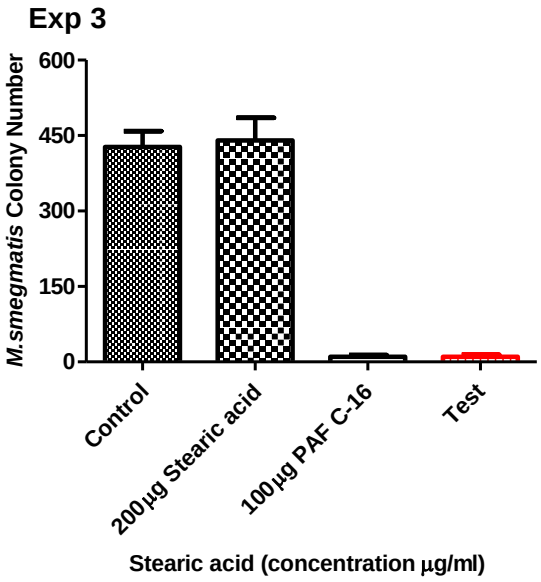
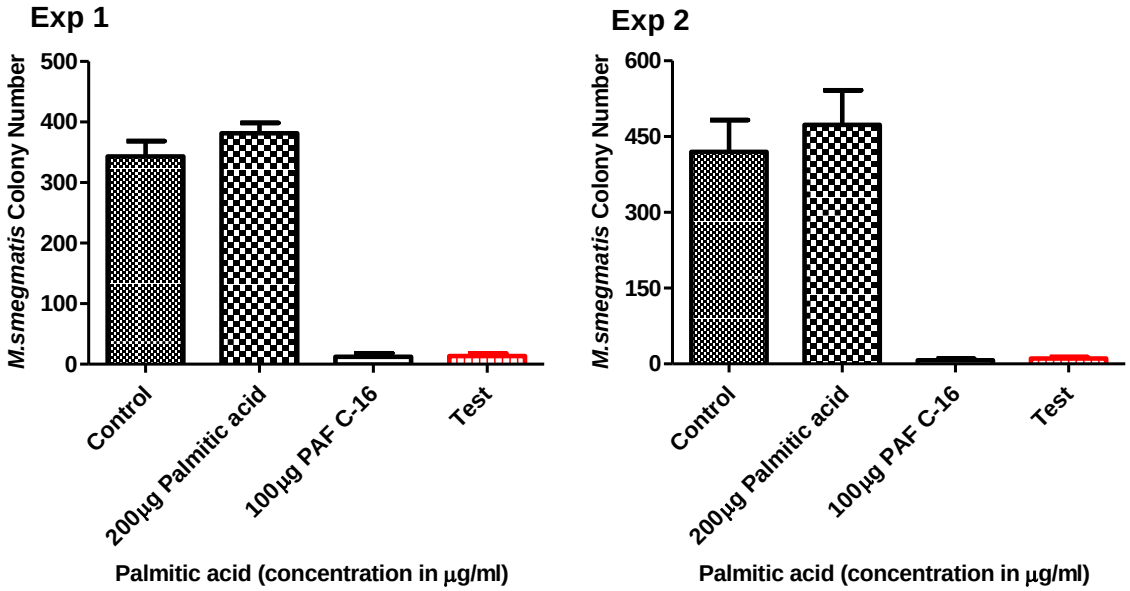


Figure 4.8a: Three individual experiments (Exp1, 2 and3) showing the direct effect of stearic acid on PAF C-16 induced *M.smegmatis* growth inhibition. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicate. **Test** consists of 200µg/ml stearic acid and 100µg/ml PAF C-16.



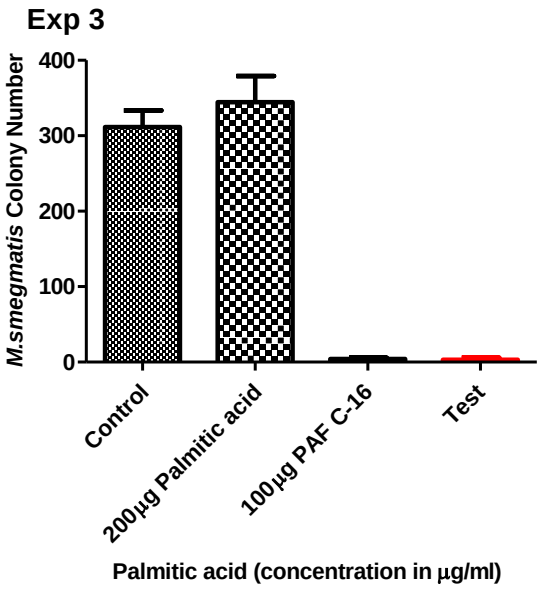


Figure 4.8b: Three individual experiments (Exp1, 2 and 3) showing the effect of palmitic acid PAF C-16 induced *M.smegmatis* growth inhibition. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicate. **Test** consists of 200µg/ml palmitic acid and 100µg/ml PAF C-16.

4.9 Effect of α-Tocopherol (Vitamin E) on PAF C-16 induced *M.smegmatis* growth inhibition

α-tocopherol serves as an antioxidant and can protect cell membrane from free radicals. This compound also acts as a membrane stabilizer by forming physiochemical association with the fatty acids in the cell membrane giving stability to the membrane and preventing damage of the membrane permeability barrier (Topinka *et al*., 1989; Kagan, 1989).

The effect of α-tocopherol on PAF C-16 induced *M.smegmatis* growth inhibition was investigated according to the protocol in chapter 2.5. Three independent experiments (Exp1, 2 and 3) were performed by treating *M.smegmatis* with 100µg/ml α-tocopherol for 1 hour prior to treatment with 50µg/ml and 100µg/ml PAF C-16 for 2 more hours.

It was observed that prior treatment of *M.smegmatis* with α-tocopherol was able to partially mitigate the inhibitory effect of PAF C-16 on the growth of *M.smegmatis*. The same trend was observed during all three experiments where treatment of *M.smegmatis* with α-tocopherol prior to PAF C-16 increased the number of *M.smegmatis* CFUs when compared to the number CFUs from PAF C-16 only treated *M.smegmatis* (Figure 4.9a). On average, α-tocopherol treatment

at concentrations of 100µg/ml increased the number of *M.smegmatis* CFUs by 58-60% when compared to the number of CFUs from PAF C-16 (100µg/ml) only treated *M.smegmatis* (Figure 4.9b). The level of significance was determined by applying non-parametric Mann Whitney test on individual data sets and was found significant for "Test 1" vs 50µg/ml PAF C-16 only (*p=0.02)* and "Test 2" vs 100µg/ml PAF C-16 only (*p=0.02)*.

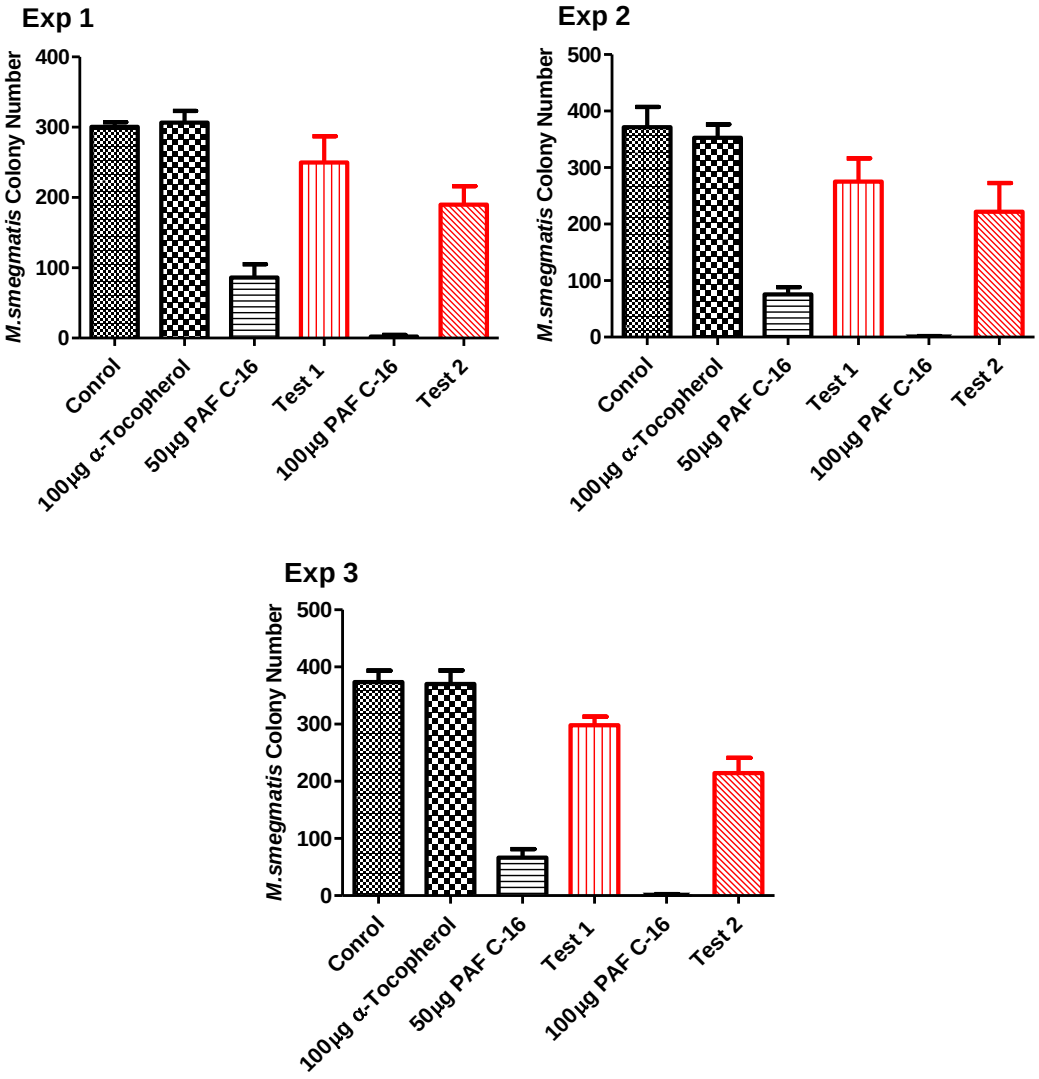


Figure 4.9a: Three individual experiments (Exp1, 2 and 3) showing the effect of α-tocopherol on PAF C-16 induced *M.smegmatis* growth inhibition. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicate. **Test 1** consists of 100µg/ml α-tocopherol and 50µg/ml PAF C-16. **Test 2** consists of 100µg/ml α-tocopherol and 100µg/ml PAF C-16.

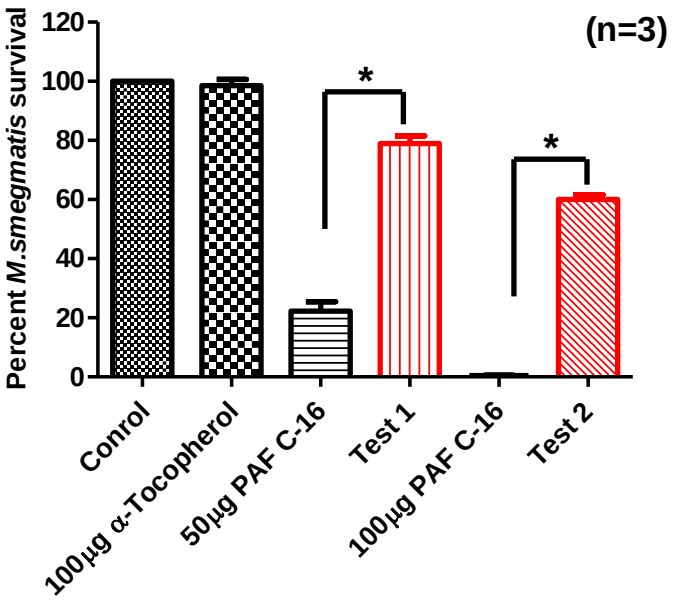


Figure 4.9b: Effect of α-tocopherol on PAF C-16 induced growth inhibition of *M.smegmatis*. Each bar represents the average of three independent experiments and the error bars show the standard error of mean (SEM). Data is expressed in terms of percentage survival where control is taken as 100% and different test conditions are compared to it. **Test 1** consists of 100µg/ml α-tocopherol and 50µg/ml PAF C-16. **Test 2** consists of 100µg/ml α-tocopherol and 100µg/ml PAF C-16. Non-parametric Mann Whitney test was used to determine the level of significance between individual data sets and the *p*-value was significant for "Test 1" vs 50µg/ml PAF C-16 only *(*p=0.02)* and "Test 2" vs 100µg/ml PAF C-16 only *(*p=0.02)*.

4.10 Effect of Ascorbic acid (Vitamin C) on PAF C-16 induced *M.smegmatis* growth inhibition

Ascorbic acid is antioxidant in nature and can prevent the plasma membrane from intracellular free radical attacks (Bendich *et al*., 1986).

Three individual experiments (Exp1, 2 and 3) were performed with ascorbic acid according to the protocol in chapter 2.5. *M.smegmatis* was treated with 100µg/ml ascorbic acid for 1 hour prior to treatment with 50µg/ml and 100µg/ml PAF C-16 for 2 hours. It was observed during all the experiments ascorbic acid was unable to prevent PAF C-16 induced growth inhibition of *M.smegmatis* (Figure 4.10). The results showed that the number of *M.smegmatis* CFUs after treatment with both ascorbic acid and PAF C-16 was similar to the number of CFUs for PAF

C-16 only treated *M.smegmatis*, suggesting that the inhibitory action of PAF C-16 on *M.smegmatis* is not through the oxidation of membrane fatty acids.

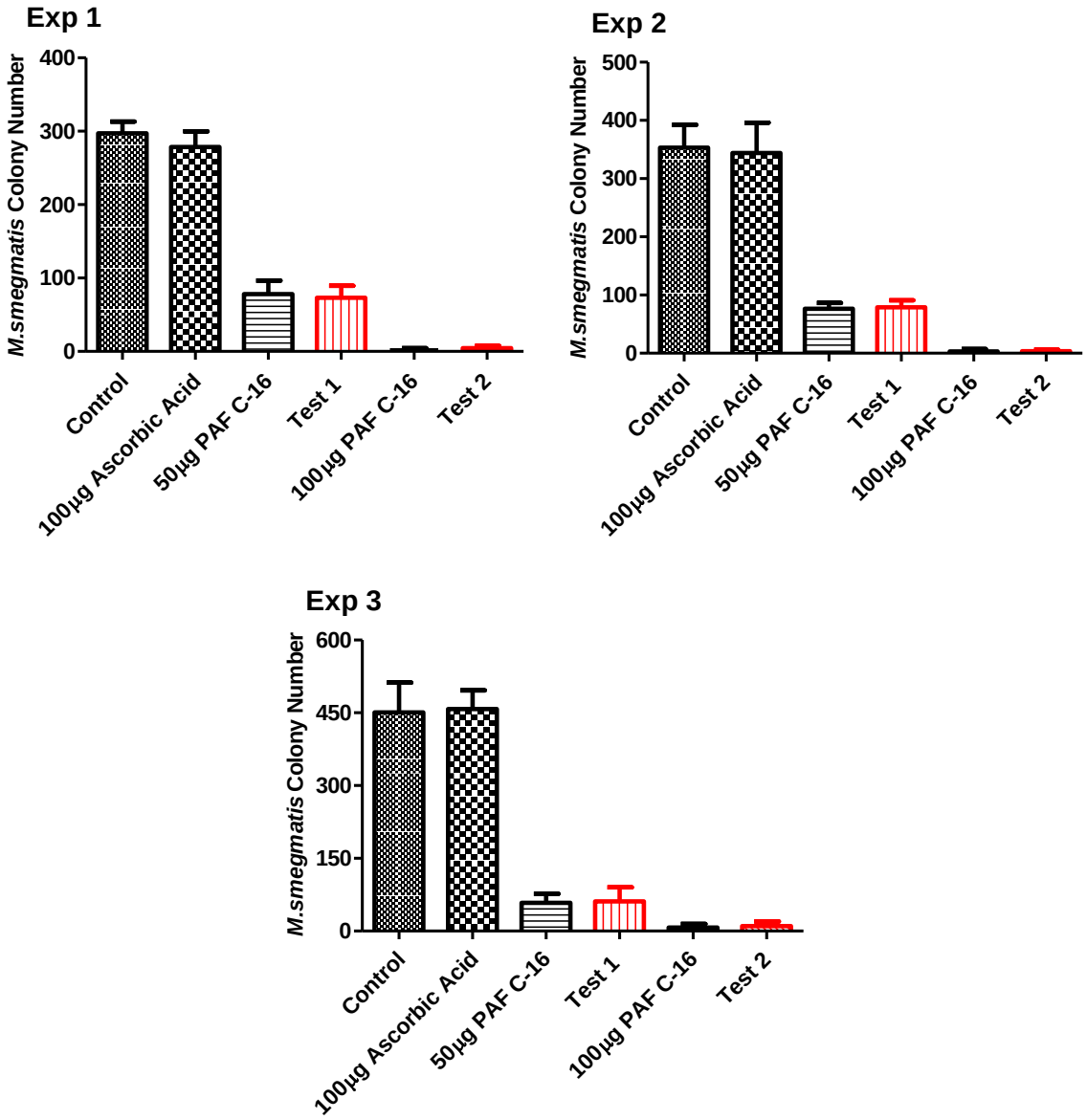


Figure 4.10: Three independent experiments (Exp1, 2 and 3) showing the effect of ascorbic acid on PAF C-16 induced *M.smegmatis* growth inhibition. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicate. **Test 1** consists of 100µg/ml ascorbic acid and 50µg/ml PAF C-16. **Test 2** consists of 100µg/ml ascorbic acid and 100µg/ml PAF C-16.

4.11 Effect of Tween-80 and Tween-20 on PAF C-16 induced *M.smegmatis* growth inhibition

Non-ionic surfactants such as Tween-80 and Tween-20 were also investigated as these compounds have been shown to reduce the antimicrobial activity of hydrophobic compounds such as essential oils and antibiotics like rifampicin (Nielsen *et al*., 2016).

The effect of Tween-80 and Tween-20 on PAF C-16 induced *M.smegmatis* growth inhibition was investigated according to the protocol in chapter 2.5. Three independent experiments (Exp1, 2 and 3) were performed by treating *M.smegmatis* with 1% v/v Tween-80 or Tween-20 for 1 hour prior to treatment with 100µg/ml PAF C-16 for 2 more hours.

It was observed that prior treatment of *M.smegmatis* with Tween-80 or Tween-20 was able to partially reverse the inhibitory effect of PAF C-16 on the growth of *M.smegmatis*. The same trend was observed during all three experiments where treatment of *M.smegmatis* with Tween-80 or Tween-20 prior to PAF C-16 increased the number of *M.smegmatis* CFUs when compared to the number CFUs from PAF C-16 only treated *M.smegmatis* (Figure 4.11a and 4.11c). On average, 1% v/v Tween-80 prior treatment increased the number of *M.smegmatis* CFUs by 87% as compared to the number CFUs from PAF C-16 only treated *M.smegmatis* (Figure 4.9b). The level of significance was determined using non-parametric Mann Whitney test on individual data sets and was found significant, $p=0.02$ for "Test" (1% v/v Tween-80 and 100µg/ml PAF C-16) vs 100µg/ml PAF C-16 only. Similarly, in case of Tween-20 prior treatment the number of *M.smegmatis* CFUs on average increased by 83% as compared to the number CFUs from PAF C-16 only treated *M.smegmatis* (Figure 4.9d). The level of significance was determined using non-parametric Mann Whitney test, and was found significant, $p=0.02$ for "Test" (1% v/v Tween-20 and 100µg/ml PAF C-16) vs 100µg/ml PAF C-16 only.

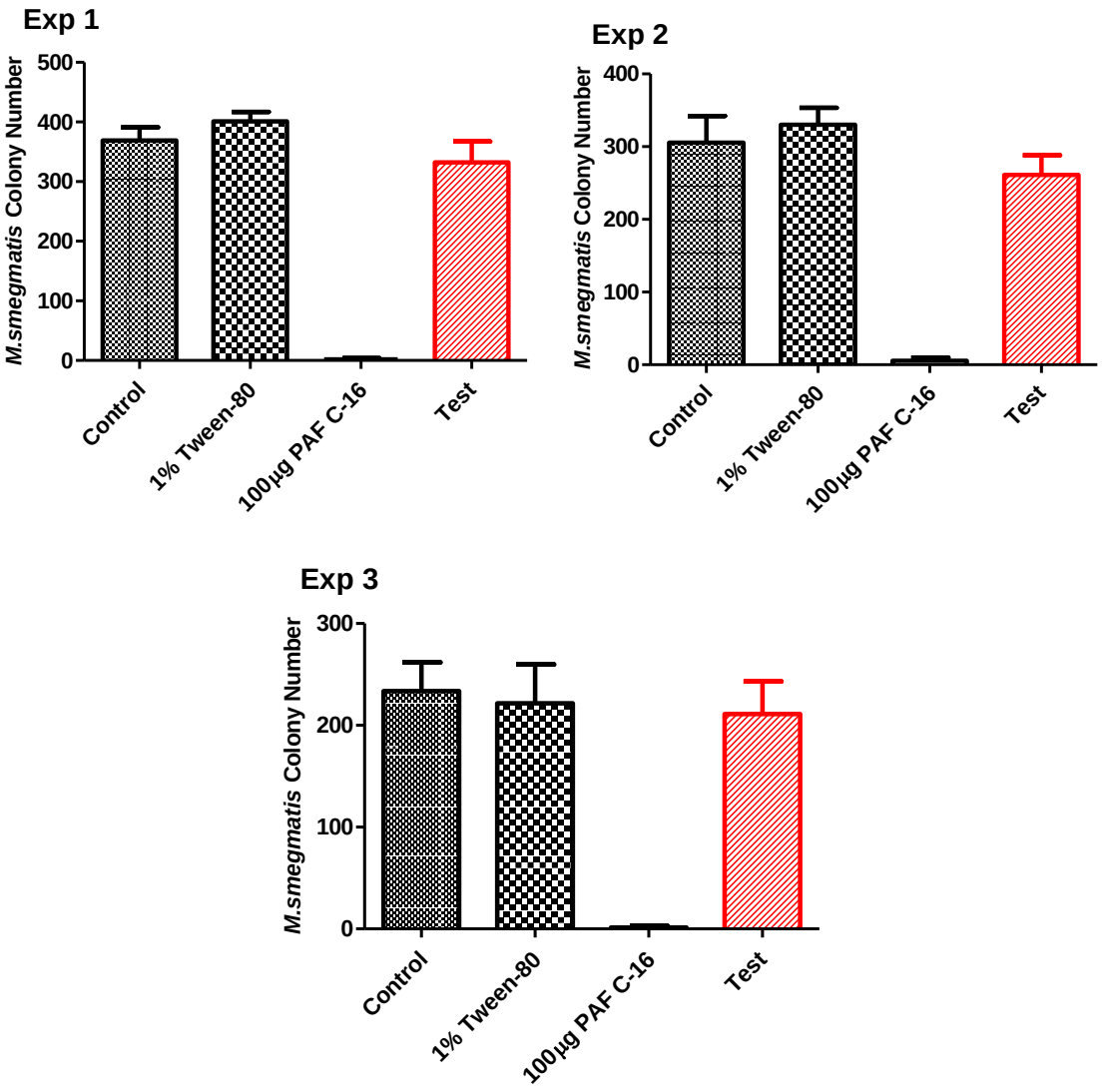


Figure 4.11a: Three independent experiments (Exp1, 2 and 3) showing the effect of Tween-80 on PAF C-16 induced *M.smegmatis* growth inhibition. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicate. **Test** consists of 1% v/v Tween-80 and 100µg/ml PAF C-16.

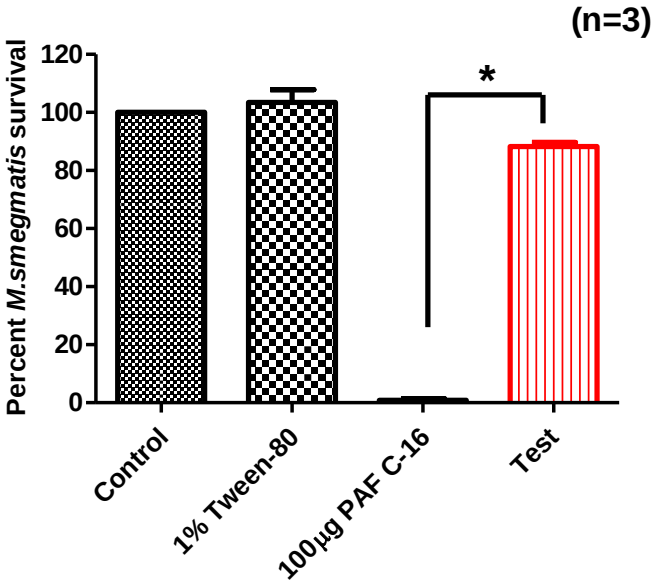


Figure 4.11b: Effect of Tween-80 on PAF C-16 induced growth inhibition of *M.smegmatis*. Data is expressed in terms of percentage survival where control is taken as 100% and different test conditions are compared to it. Each bar represents the average of three independent experiments and the error bars show the standard error of mean (SEM). **Test** consists 0f 1% v/v Tween-80 and 100µg/ml PAF C-16. Non-parametric Mann Whitney test was used to determine the level of significance between individual data sets and the *p*-value was significant for "Test" vs 100µg/ml PAF C-16 only *(*p=0.02)*

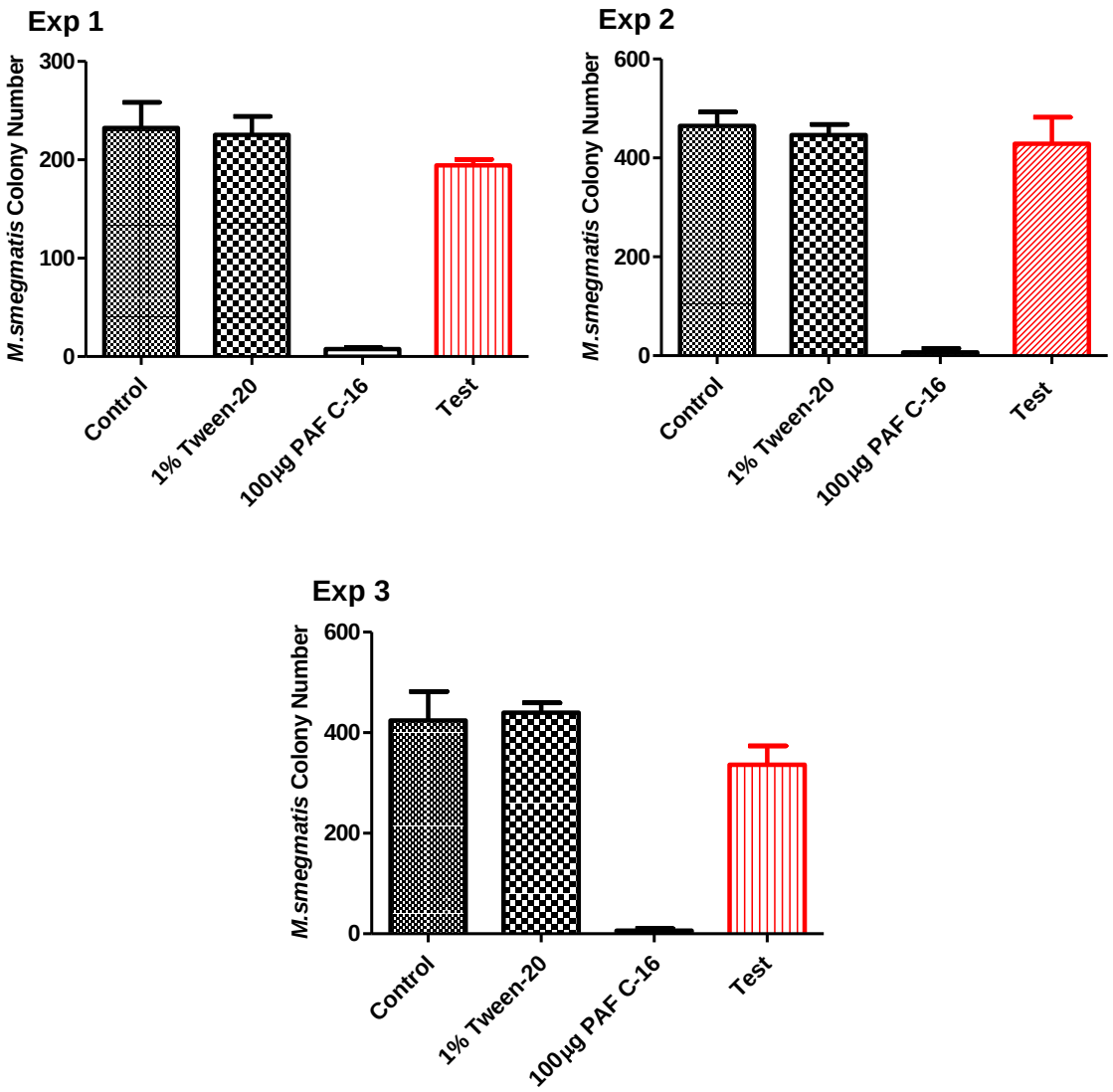


Figure 4.11c: Three independent experiments (Exp1, 2 and 3) showing the effect of Tween-20 on PAF C-16 induced growth inhibition of *M.smegmatis*. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicate. **Test** consists of 1%v/v Tween-20 and 100µg/ml PAF C-16. Non-parametric Mann Whitney test was used to determine the level of significance between individual data sets and the *p*-value was significant for "Test" vs 100µg/ml PAF C-16 only *(*p=0.02)*

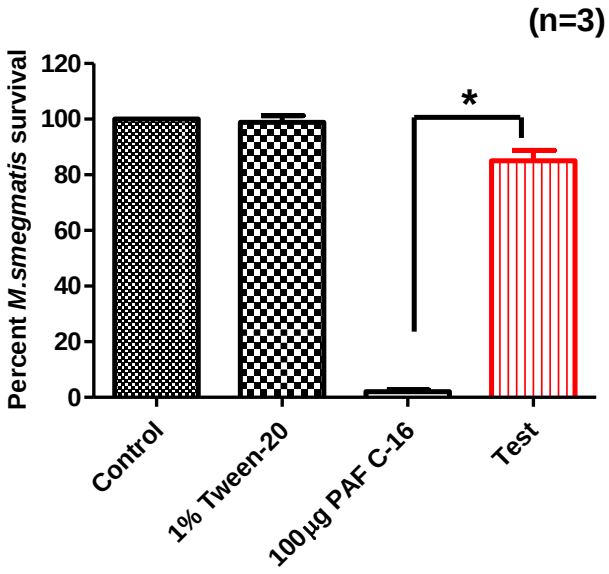


Figure 4.11d: Effect of Tween-20 on PAF C-16 induced growth inhibition of *M.smegmatis*. Data is expressed in terms of percentage survival where control is taken as 100% and different test conditions are compared to it. Each bar represents the average of three independent experiments and the error bars show the standard error of mean (SEM). Test consists 0f 1% v/v Tween-20 and 100µg/ml PAF C-16.

4.12 Discussion

After showing the direct inhibitory effect of exogenous PAF C-16 on the growth of *mycobacteria* (*M.smegmatis* and *M.bovis BCG*), we investigated the structure-activity relationship for PAF C-16 and the mechanism of PAF C-16 induced *mycobacterial* growth inhibition.

PAF C-16 structure analogues are compounds similar to PAF C-16 but with small structural modifications such as variations in functional groups or carbon tail length. Previous studies with PAF C-16 analogues in mammalian systems have shown that these small structural modifications can affect the activity of PAF C-16 during biological processes such as platelet aggregation and macrophage activation (Stewart and Grigoriadis, 1991; O'Flaherty *et al*., 1983; Ténce *et al*., 1981). To the best of our knowledge, PAF C-16 structure analogues have not been investigated for their direct effect on the growth of *mycobacteria*. Therefore, different PAF C-16 structure analogues were assessed *in vitro* for their growth inhibitory potential against *M.smegmatis* and *M.bovis BCG*. PAF C-16 structure analogues used in this study were selected in a way that each analogue represented a change of a different functional group. The results showed that all the PAF C-16 structure analogs tested were able to inhibit the growth of both *M.smegmatis* and *M.bovis* BCG and they showed comparable levels of growth inhibition to PAF C-16.

Lyso-PAF C-16 is the immediate precursor form of naturally produced PAF C-16. It has been shown that Lyso-PAF lacks most of the biological activity associated with PAFC-16 such as aggregation of platelets, release of serotonin from platelets, pulmonary functions and cardiovascular functions (McManus *et al*., 1981; Cuss *et al*., 1986). In this study, Lyso-PAF was investigated for its direct inhibitory effect on the growth of *mycobacteria*. The results showed that this precursor form was able to inhibit the growth of both *M.smegmatis* and *M.bovis BCG* in a dose-dependent manner and at levels comparable to PAF C-16. On structural level, these results suggested that replacing the acetyl functional group with a hydroxyl group at position *sn*-2 has no effect on the bacterial growth inhibition activity of PAF C-16. Since Lyso-PAF is the inactive analogue and does not possess most of the side effects associated with PAF C-16, this compound needs further investigation for its anti-M.tb properties.

Most of the naturally produced PAF occurs as hexadecyl, having a 16-carbon atoms chain attached at *sn*-1 position (Clay *et al*., 1984). However, there are naturally produced analogues, which are octadecyl with 18-carbon atoms chain (Mallet and Cunningham, 1985; Oda *et al*.,

1985). Increase in length of carbon chain at position *sn*-1 has been shown to be associated with reduced potency during different biological activities such as neutrophil chemotaxis (Carolan and Casale, 1990) and stimulation of cell proliferation in mouse embryos (Stoddart *et al*., 2001). Therefore, PAF C-18 was investigated for its direct inhibitory effect on the growth of *mycobacteria* and the results showed that *in vitro* PAF C-18 was able to inhibit the growth of both *M.smegmatis* and *M.bovis BCG* in a dose-dependent manner at comparable levels to PAF C-16. On a structural level, these results suggested that increase in carbon tail by 2 carbon atoms had a no effect on the *in vitro* bacterial growth inhibition potency.

Hexanolamino PAF is another important PAF C-16 analogue that differs from PAF C-16 in the position of the terminal amino group. In Hexanolamino PAF the terminal amino group is attached via 4 additional carbon atoms to the phosphate group. Hexanolamino PAF was investigated for its direct growth inhibitory effect against *mycobacteria*. The results showed that *in vitro* Hexanolamino PAF inhibited the growth of both *M.smegmatis* and *M.bovis BCG* in a dose-dependent manner. Hexanolamino PAF has previously been shown perform biological activities that are different from PAF C-16 and this structure analogue can act as a PAF C-16 antagonist or agonist in mammals (Merendino *et al*., 1999; Rouis *et al*., 1988). However, our result showed Hexanolamino PAF was able to inhibit the growth of both *M.smegmatis* and *M.bovis BCG* at comparable levels to PAF C-16. On structural level these results indicated that the attachment of the terminal amino group to the phosphate group via 4 additional carbon atoms did not affect the mycobacterial growth inhibition potency.

Similar results were obtained for two synthetic PAF C-16 analogues: 2-O-methyl PAF and Pyrrolidino PAF when tested for their direct growth inhibition activity against *mycobacteria*. In 2-O-methyl PAF, a methyl group substitutes for the acetyl group at position *sn*-2, whereas in Pyrrolidino PAF, the terminal amino group in the phosphocholine head is replaced by a 5-member lactam ring attached to the phosphate group. The results showed that these modifications had no effect on the bacterial growth inhibition activity and both these compounds were effective in inhibiting the growth of *mycobacteria* in a dose-dependent manner and at levels similar to PAF C-16.

Structurally, PAF C-16 consists of a glycerol backbone having a 16 carbon atoms chain attached via ether bond at position *sn*-1, an acetyl group at position *sn*-2 and a phosphocholine group attached at *sn*-3 position (Prescott *et al*., 1990). Different compounds with structures

similar to the phosphocholine head and the aliphatic carbon tail of PAF C-16 were tested to localize the structurally active portion of PAF C-16 that might be involved in the bacterial growth inhibition using *M.smegmatis* as a model. Palmitic acid is a saturated fatty acid with a 16-carbon atoms chain similar to PAF C-16 carbon tail in length and Phosphocholine chloride calcium tetra-hydrate salt is similar in structure to the phosphocholine head portion of PAF C-16. Both these compounds were tested *in vitro* for their direct growth inhibitory effect against *M.smegmatis*. However, the results showed that both these compounds in concentrations of up to 100µg/ml were unable to directly inhibit the growth of *M.smegmatis*. In fact, treatment of *M.smegmatis* with palmitic acid resulted in a slight growth-enhancing effect, which could be due to the utilization of this exogenous fatty acid by the bacteria for the synthesis of its phospholipids (Parsons *et al*., 2011). Another compounds, 1-O-hexadecyl-*sn*-glycerol was tested to investigate the role of 1-O-alkyl chain and ether bond at *sn*-1 position. 1-O-hexadecyl-*sn*-glycerol is similar to PAF C-16 as it consists of a 16 carbon atoms chain attached via ether bond to the *sn*-1 position of a glycerol backbone. The results showed that 1-O-hexadecyl-*sn*-glycerol was able to inhibit the growth of *M.smegmatis* in a dose-dependent manner after 2 hours of treatment. On structural level these results suggested that the attachment of carbon tail to glycerol backbone via ether bond (1-O-alkyl chain) in PAF C-16 might be responsible for its bacterial growth inhibition activity. Two additional compounds, miltefosine and hexadecyl lactate, each having a 16 carbon atoms chain attached by ester bond were also included in the study and investigated *in vitro* for their direct growth inhibitory effect against *M.smegmatis*. Both miltefosine and hexadecyl lactate also inhibited the growth of *M.smegmatis* in a dose-dependent manner, suggesting that the growth inhibition of *M.smegmatis* is not limited to the attachment of carbon chain via ether bond and compounds with a carbon chain attached via ester bond also possess mycobacterial growth inhibition potential. To the best of our knowledge, there is currently no information about the inhibitory effect of 1-O-hexadecyl-*sn*-glycerol, hexadecyl lactate and miltefosine on mycobacterial growth and our results show a novel activity for these compounds. Therefore, these compounds need further investigation for their direct inhibitory effect on the growth of *M.tb* and if found effective they have the potential to be used as anti-TB drugs. Miltefosine is currently used for the treatment of Leishmaniasis (Machado *et al*., 2010) and has also been shown to inhibit the growth pathogenic bacteria such as *Streptococcus pneumoniae* (Llull *et al*., 2007) and fungi such as *Aspergillus fumigatus* and *Candida* species (Biswas *et al*., 2013; Widmer *et al*., 2006).

PAF C-16 performs most of the biological functions in mammals by its binding to specific G-protein coupled receptors known as PAF receptor on target cells (Honda *et al*., 2002). PAF receptor antagonists are compounds that bind to PAF receptor on eukaryotic cells with high affinity and are effective inhibitors of PAF C-16 induced changes (Singh *et al*., 2013). Currently, there is no information about the presence of any PAF receptor on bacteria. We hypothesized that *mycobacteria* may have a similar PAF receptor to eukaryotic cells and used two high-affinity PAF receptor antagonists, ABT-491 and WEB-2086 to check whether they can mitigate PAF C-16 induced growth inhibition of *M.smegmatis*. The results showed that prior treatment of PAF receptor antagonists, ABT-491 or WEB-2086 had no effect on the PAF C-16 induced growth inhibition of *M.smegmatis*. The complete sequence of genes coding PAF receptor is known in humans (Nakamura *et al*., 1991). BLAST search for the coding sequence of human PAF receptor against *mycobacteria* genome was performed using the BLAST search utility available at NCBI to find similar nucleotide sequences. However, the results did not show any significant sequence similarity, suggesting that *mycobacteria* may possess different PAF receptor or the growth inhibition may be through a receptor-independent mechanism.

Next, the effect of PAF C-16 treatment on mycobacterial morphology was investigated by performing microscopy and flow cytometry using cell viability dyes, Trypan Blue and Propidium Iodide (PI). The results showed that treatment of *M.smegmatis* and *M.bovis BCG* with PAF C-16 at a concentration 100µg/ml caused a large number of bacteria to be stained with Trypan Blue or PI as compared to the bacteria in solvent control, where little or no staining was observed. These results indicated that PAF C-16 had a damaging effect on the cell membrane of both *M.smegmatis* and *M.bovis BCG* which could be responsible for the growth inhibition of these bacteria.

There are a number of mechanisms through which different compounds can damage bacterial cell membrane and lead to their growth inhibition. We first investigated the involvement of bacterial Phospholipase A_2 (PLA_2) enzyme in PAF C-16 induced mycobacterial growth inhibition by using PLA_2 inhibitors, dexamethasone and benzenesulfonamide. However, the results showed that treating *M.smegmatis* with PLA_2 inhibitors, dexamethasone or benzenesulfonamide prior to PAF C-16 treatment had no effect on the PAF C-16 induced growth inhibitory suggesting that the growth inhibitory effect of PAF C-16 is not through activation of bacterial PLA_2. In eukaryotes, PLA_2 enzyme is involved in lipid digestion, microbial degradation, cell signalling, membrane remodelling and inflammation (Murakami and Kudo, 2001). PAF C-16 signalling through its receptor can result in the activation of PLA_2

enzyme in eukaryotic cells (Balsinde *et al*., 2002). Membrane-bound and secretory forms of PLA_2 have also been reported in different bacteria such as *Helicobacter pylori* and *Campylobacter coli* (Istivan and Coloe, 2006; Grant *et al*., 1997; Slomiany and Slomiany, 1992). It has been shown that activated PLA_2 enzyme can hydrolyse membrane phospholipids in mammalian cells resulting in the production of polyunsaturated fatty acids, mostly arachidonic acid (Axelrod *et al*., 1988). Excess hydrolysis of the cell membrane by PLA_2 can cause damage to the cell membrane. Additionally, the unsaturated fatty acids produced during the PLA_2 hydrolysis have also been shown to possess cell membrane damaging properties (Farooqui *et al*., 1997).

Another possible mechanism of damage to the bacterial cell membrane is through inhibition of bacterial fatty acid synthase system involved in the production of fatty acid, that are used in the synthesis of membrane phospholipids by providing the acyl chains (Zhang *et al*., 2006; Heath *et al*., 2001). In this study, stearic acid and palmitic acid were used as exogenous sources of fatty acids and their effect was investigated on PAF C-16 induced growth inhibition of *M.smegmatis*. However, the results showed that both these saturated fatty acids were unable to prevent the growth inhibitory effect of PAF C-16 on *M.smegmatis* suggesting that PAF C-16 induced growth inhibition is not through disruption of fatty acid synthesis. Administration of exogenous saturated fatty acids such as palmitic acid or stearic acid have been shown to revert cell membrane damage in bacteria such as *Staphylococcus aureus* (Zheng *et al*., 2005).

α-tocopherol, a well-known antioxidant (Patra *et al*., 2001) and membrane stabilizer (Kagan, 1989) was also investigated for its effect on PAF C-16 induced growth inhibition of *M.smegmatis*. The results showed that prior treatment of *M.smegmatis* with α-tocopherol partially mitigated the growth inhibitory effect of PAF C-16. Since α-tocopherol can function both as antioxidant and membrane stabilizer, ascorbic acid another potent antioxidant (Padayatty *et al*., 2003) was also investigated for its effect on PAF C-16 induced *M.smegmatis* growth inhibition. However, ascorbic acid was unable to prevent the growth inhibitory effect of PAF C-16. These results suggested that the protective role of α-tocopherol was due to its membrane stabilizing activity. It has been shown that α-tocopherol can interact with the hydrophobic core of plasma membranes due to its lipid soluble nature through its carbon chain and can lead to changes in the physical properties of the lipid membranes such as increase in fluidity (Fukuzawa *et al*., 1980). The chromanol head of α-tocopherol binds to the plasma membrane phospholipids acyl chains and limit their mobility within the membrane resulting in plasma membrane stabilization (Urano *et al*., 1988).

Two non-ionic surfactants, tween-80 and tween-20 were also investigated for their effect on PAF C-16 induced growth of *M.smegmatis*. The results showed that treatment of *M.smegmatis* with tween-80 or tween-20 prior to PAF C-16 partially abolished the PAF C-16 induced growth inhibition of *M.smegmatis*. Nielsen *et al* showed that tween-80 treatment decreased the antimicrobial activity of hydrophobic compounds including rifampicin and isoeugenol, which acts mostly by damaging the bacterial membrane and suggested a protective effect for tween-80 on bacterial cell membrane (Nielsen *et al*., 2016). Although most of the biological functions associated with PAF C-16 are mediated through PAF receptor on target cells, there are certain PAF C-16 activities that are independent of receptor binding. One such PAF C-16 activity is its detergent-like effect during which PAF C-16 gets incorporated in lipid membranes and disturb the molecular organization of these membrane (lipid-lipid interactions and lipid-protein interactions) and hence membrane functions (Sawyer and Andersen, 1989). The mycobacterial growth inhibition by PAF C-16 can, therefore, be attributed to its detergent-like effect causing damage to the bacterial cell membrane.

Unsaturated fatty acids (C16:1, C18:1, C20:1) have previously been shown to inhibit the growth of different bacteria by damaging the cell membrane (Kanetsuna, 1985; Saito *et al*., 1984). In the current study, we also confirmed that unsaturated fatty acids such as palmitoleic acid (C16:1) and oleic acid (C18:1) were able to inhibit *M.smegmatis* growth. The exact mechanism of bacterial growth inhibition by unsaturated fatty acid is complex and is still not completely understood. One of the proposed mechanisms is the detergent-like activity of unsaturated fatty acid due to their amphipathic structure (Boyaval *et al*., 1995) and the "kink effect" (Desbois and Smith, 2010). Unsaturated fatty acids have double bonds between adjacent carbon atoms, which cause a bend in the carbon chain by up to 30 degrees. When these unsaturated fatty acids interact with the plasma membrane they get inserted in membrane, pushing the adjacent phospholipid molecules wide apart due to their kinked structure. As a result pores are produced in the cell membrane that ultimately leads to cell death due to changes in the osmotic pressure of the cell, efflux of cytoplasmic contents and the influx of water (Heipeiper *et al*., 2003). We, therefore, think that PAF C-16 might be acting through a similar mechanism where the polar phosphocholine head at *sn*-3 and the ether bond at *sn*-1 produce skewness in the PAF C-16 molecule causing the carbon tail to bend. When this skewed PAF C-16 molecule interacts with the bacteria, the aliphatic carbon tail is inserted into the bacterial cell membrane and the bend in the PAF C-16 causes the lipid molecules in the cell membrane

to move apart. As a result, pores are formed in the bacterial cell membrane and the bacterial cells are no longer able to maintain their osmotic pressure and ultimately die.

In conclusion, it was shown that small modification in structure of PAF C-16 had no effect on the direct mycobacterial growth inhibition activity of PAF C-16. The attachment of aliphatic carbon tail via ether bond at position *sn*-1 of the glycerol backbone seemed to be important for the mycobacterial growth inhibition activity of PAF C-16. PAF C-16 treatment was shown to have damaging effect on the mycobacterial cell membrane. Membrane stabilizers such as α-tocopherol, tween-80 and tween-20 were able to partially mitigate the growth inhibitory effect of PAF C-16. In addition, other compounds such as miltefosine and hexadecyl lactate having 16 carbon atoms chain attached via ester bonds were also shown to be effective in inhibiting the growth of *M.smegmatis*. The data from different compounds investigated for their direct growth inhibitory effect against *M.smegmatis* and *M.bovis* BCG is summarized in table 4.1.

Table 4.1: Summary for direct growth inhibitory effect of different compounds used against *M.smegmatis and M.bovis* BCG.

Compound	Results from our study		Results from other groups
	Direct Growth Inhibition of *M.smegmatis*	Direct Growth Inhibition of *M.bovis* BCG	Direct growth inhibitory effect on other bacteria
PAF C-16	+	+	***S.aureus*** **(Steel *et al*., 2002)**
Lyso PAF	+	+	***S.aureus*** **(Steel *et al*., 2002)**
PAF C-18	+	+	**Not investigated**
2-O-methyl PAF	+	+	**Not investigated**
Hexanolamino PAF	+	+	**Not investigated**
Pyrrolidino PAF	+	**Not investigated**	**Not investigated**
Palmitic acid	−	**Not investigated**	***N.gonorrhoeae*** **(Miller *et al*., 1977)**
Phosphocholine chloride calcium tetrahydrate	−	**Not investigated**	**Not investigated**
1-O-hexadecyl-*sn*-glycerol	+	**Not investigated**	**Not investigated**
Miltefosine	+	**Not investigated**	***S.pneumoniae*** **(Llull *et al*., 2007)**
Hexadecyl lactate	+	**Not investigated**	**Not investigated**
Palmitoleic acid	+	**Not investigated**	***S.pyogenes*** **(Zheng *et al*., 2005)**
Oleic acid	+	**Not investigated**	***S.pyogenes*** **(Zheng *et al*., 2005)**

"+" indicates intracellular growth inhibition and "–" indicates lack of intracellular growth inhibition.

Chapter 5: Investigating the effect of PAF C-16 and its structural analogues on the growth of intracellular *mycobacteria* and understanding the mechanism of growth inhibition

5.1 Introduction

PAF C-16 is a phospholipid compound that is involved in a number of important physiological processes in mammals, including its well-known function of platelet aggregation (Chesney *et al*., 1982) and key roles in inflammation and allergic responses (Henderson *et al*., 2000). The biological activity of PAF C-16 results from binding to specific G-protein coupled receptors known as PAF receptor (PAFR) on target cells (Ishii *et al*., 2002). This binding leads to the activation of different downstream signalling pathways including different kinases such as protein tyrosine kinase and protein kinase C pathways, phospholipases activation as well as the production of cytokines such as TNF-α, IL-1 etc. and prostaglandins (Honda *et al*., 2002; Ishii and Shimizu, 2000).

Exogenous PAF C-16 has previously been shown to inhibit the growth of different intracellular pathogenic protozoans such as Leishmania and Trypanosoma inside human and mouse macrophages by causing the production of reactive oxygen and nitrogen species (Borges *et al*., 2017; Lonardoni *et al*., 2000; Aliberti *et al*., 1999). Similarly, administration of exogenous PAF C-16 in mice infected with lethal doses of *Candida albicans* reduced the number of the pathogens and improved the survival by causing the production of NO and TNF-α in the infected hosts (Kim *et al*., 2008; Im *et al*., 1997).

However, to the best of our knowledge, there has been no study that shows the effect of exogenous PAF C-16 on the growth on intracellular *mycobacteria*. Therefore, in this chapter, PAF C-16 and a number of PAF C-16 structure analogues were investigated for their effect on the growth of intracellular *mycobacteria* inside human monocytes derived THP-1 cells *in vitro*.

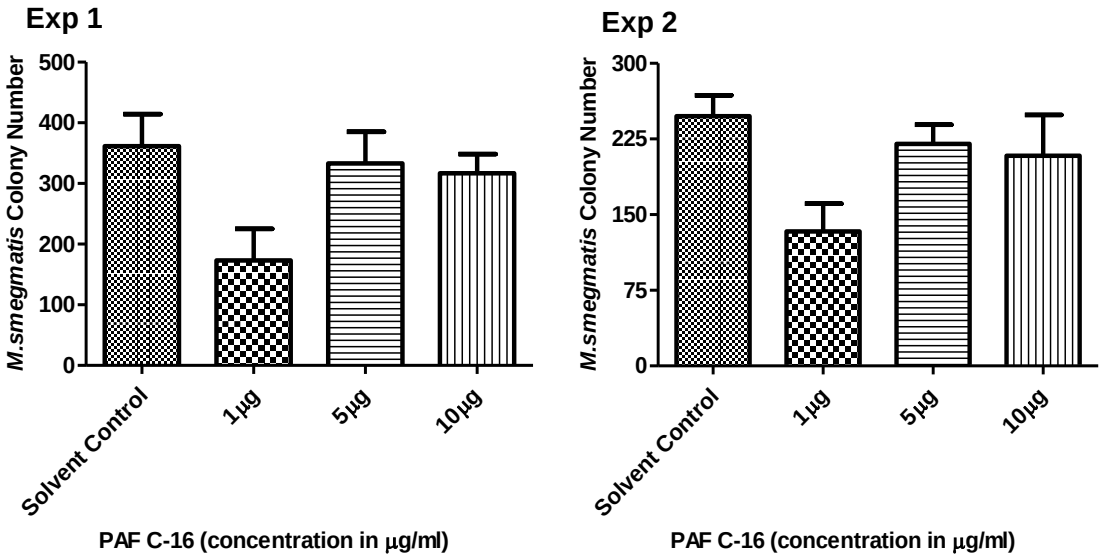
Aims:

1) Investigating *in vitro* the effect of exogenous PAF C-16 and different PAF C-16 structure analogues on the growth of intracellular *mycobacteria* inside phagocytic cells.
2) Understanding the underlying mechanism of PAF C-16 induced growth inhibition of intracellular *mycobacteria*.

5.2 Effect of PAF C-16 on *M.smegmatis* growth inside THP-1 cells

The effect of exogenous PAF C-16 on the growth of intracellular *M.smegmatis* was investigated according to the protocol in chapter 2.7. Four individual experiments (Exp1, 2, 3 and 4) were performed by treating *M.smegmatis* infected THP-1 cells with PAF C-16 in concentrations of 1µg, 5µg and 10µg/ml for 24 hours. The effect of PAF C-16 treatment on the growth of intracellular *M.smegmatis* was determined by comparing the number of CFUs from different PAF C-16 treated test conditions with the solvent control for PAF C-16 (2µl ethanol /ml cell suspension).

The results showed that PAF C-16 was able to inhibit the growth of intracellular *M.smegmatis* during all four experiments. Furthermore, PAF C-16 treatment at a concentration of 1µg/ml showed greater inhibition of intracellular *M.smegmatis* as compared to 5µg and 10µg/ml (Figure 5.2a). On average, PAF C-16 treatment of *M.smegmatis* infected THP-1 cells at concentrations 1µg, 5µg and 10µg/ml resulted in 49.1%, 7.5% and 19.7% decrease in the growth of intracellular *M.smegmatis* respectively as indicated by the number of CFUs when compared with the solvent control (Figure 5.2b). The level of significance was determined by applying non-parametric multiple comparison Kruskal Wallis test on ranks and the *p*-value was found to be significant, $p=0.002$. Individual data sets were compared by applying post hoc Dunn's multiple comparison test and 1µg/ml PAF C-16 vs Solvent Control was found to be significant, $p \leq 0.001$.



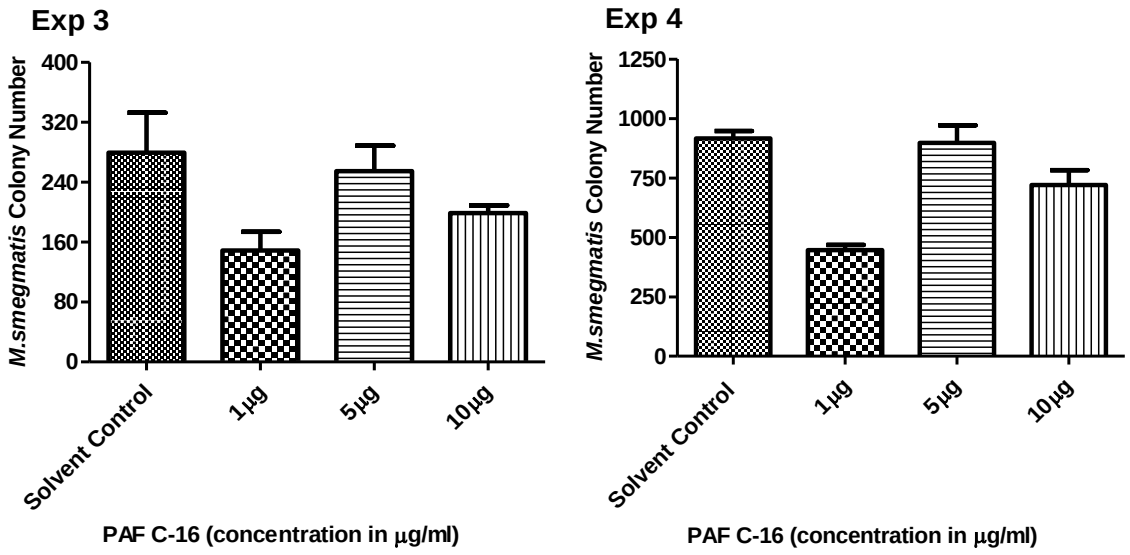


Figure 5.2a: Four individual experiments (Exp1, 2, 3, and 4) for the effect of PAF C-16 on intracellular *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bars show standard deviation (SD) of the triplicates.

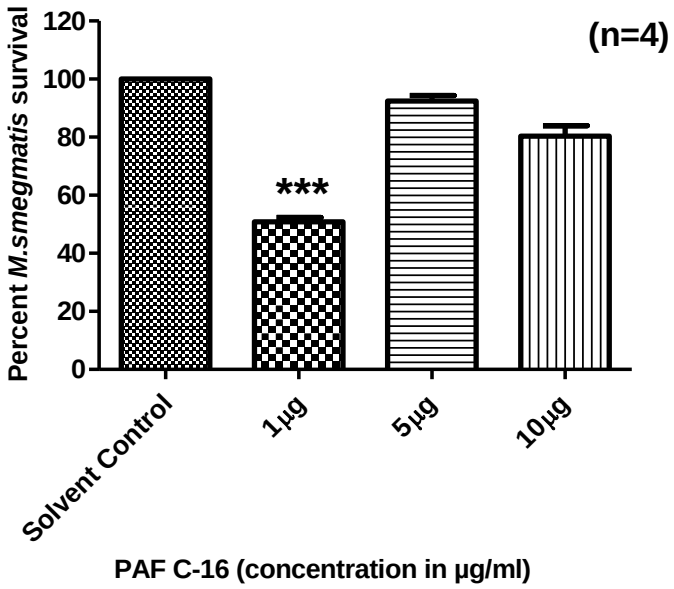
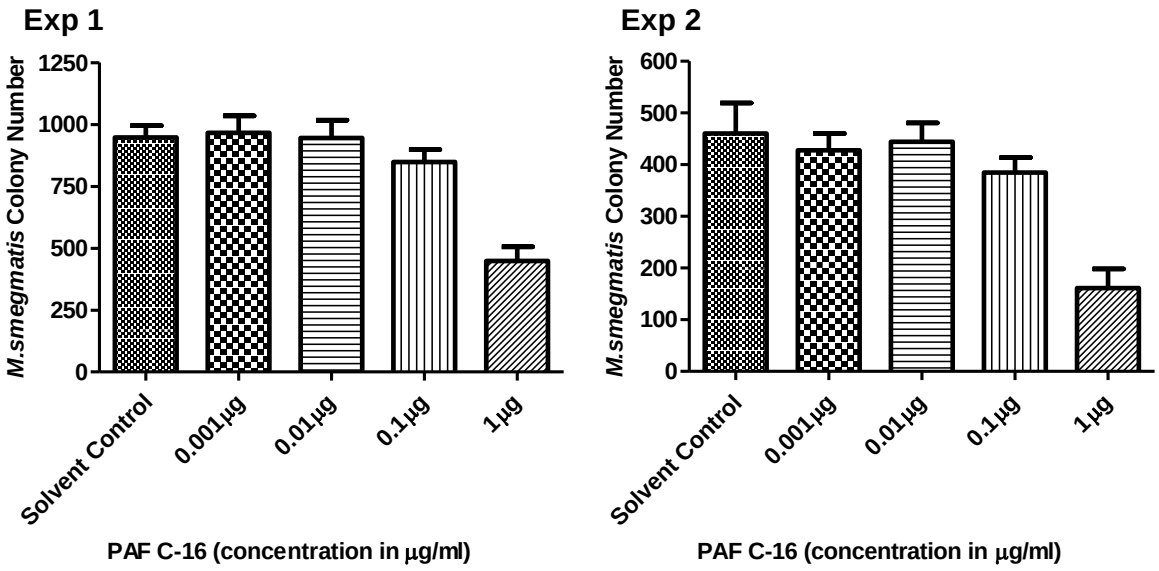


Figure 5.2b: Effect of PAF C-16 on the growth of intracellular *M.smegmatis*. Each bar represents the average of four individual experiments and the error bars show standard error of means (SEM). The data is expressed in terms of percentage, where solvent control is taken as 100% survival and different PAF C-16 treated test conditions are compared to it. Level of significance was determined by applying non-parametric multiple comparison Kruskal-Wallis

test on ranks, p=0.002. Individual data sets were compared through post hoc Dunn's multiple comparison test where PAF C-16 (1µg/ml) vs solvent control was found to be significant, ***($p \leq 0.001$).

Lower concentrations of PAF C-16 (0.001µg, 0.01µg, 0.1µg and 1µg/ml of cell suspension) were also investigated for their inhibitory effect on the growth of intracellular *M.smegmatis* inside THP-1 cells. Four individual experiments (Exp1, 2, 3 and 4) were performed according to the protocol in section 2.7. It was observed that treatment of *M.smegmatis* infected THP-1 cells with PAF C-16 at concentrations 0.001µg, 0.01µg and 0.1µg/ml for 24 hours had insignificant inhibitory effect on the growth of intracellular *M.smegmatis* as indicated by the similar number of CFUs when compared with the solvent control (Figure 5.2c). However, as seen previously, treatment of *M.smegmatis* infected THP-1 cells with PAF C-16 at 1µg/ml inhibited the growth of intracellular *M.smegmatis* during all four experiments (Figure 5.2c). On average, PAF C-16 treatment at concentrations 0.1µg/ml and 1µg/ml caused 14.3% and 58.25% reduction in the number of surviving *M.smegmatis* CFUs respectively when compared to the solvent control (Figure 5.2d). The level of significance was determined by applying non-parametric multiple comparison Kruskal Wallis test on ranks and the p-value was found to be significant, p=0.003. Individual data sets were compared by applying post hoc Dunn's multiple comparison and only 1µg/ml PAF C-16 vs Solvent Control was found to be significant, $p \leq 0.01$.



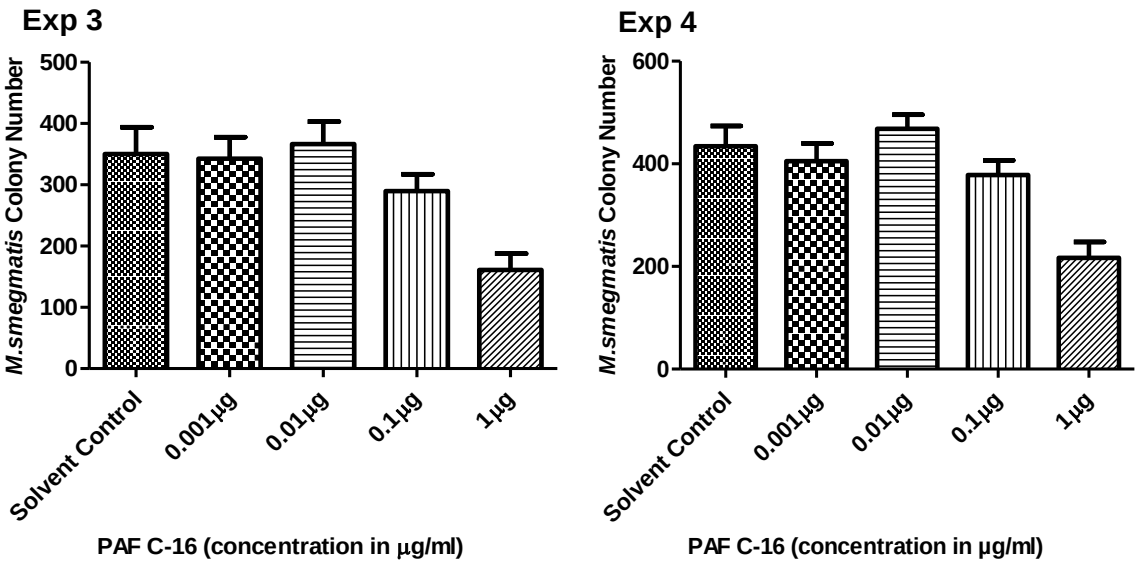


Figure 5.2c: Four individual experiments (Exp1, 2, 3 and 4) for the effect of PAF C-16 (low concentrations) on intracellular *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bars show standard deviation (SD) of the triplicates.

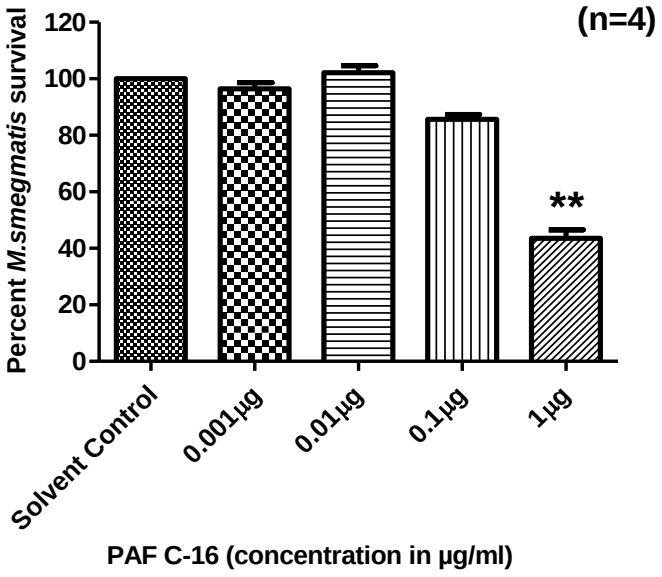


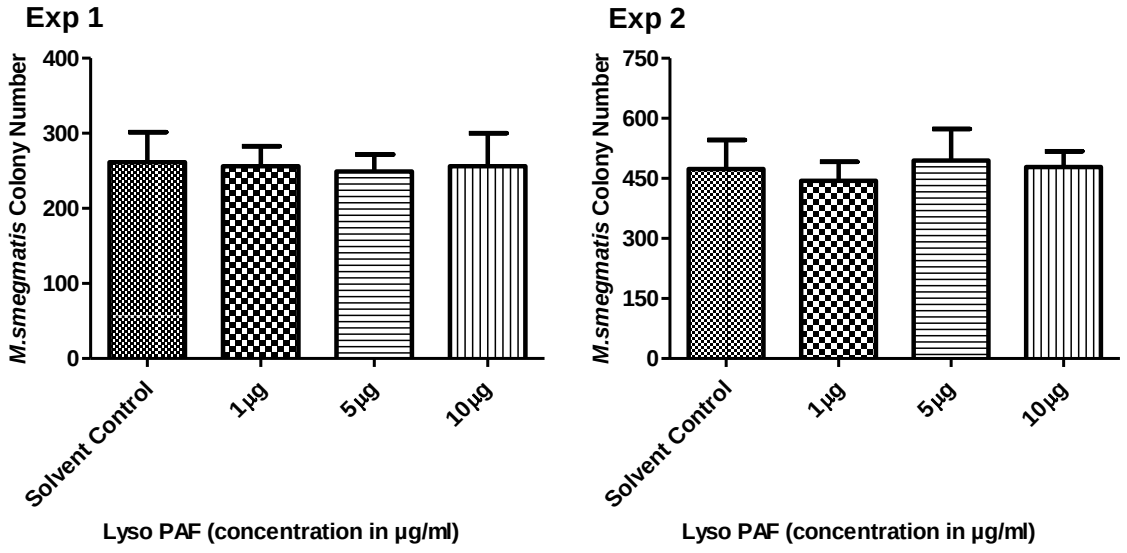
Figure 5.2d: Effect of PAF C-16 on intracellular *M.smegmatis* growth (low concentrations). Each bar represents the average of four individual experiments and the error bars show standard error of means (SEM). The data is expressed in terms of percentage, where solvent control is taken as 100% survival and different PAF C-16 treated test conditions are compared to it. Level of significance was calculated by applying multiple-comparison non-parametric Kruskal-

Wallis test on ranks, p=0.003 and individual data sets were compared through post hoc Dunn's multiple comparison test where PAF C-16 (1µg/ml) vs solvent control was found to be significant, **($p \leq 0.01$).

5.3 Effect of Lyso PAF on *M.smegmatis* growth inside THP-1 cells

Lyso PAF is the precursor form PAF C-16 and lacks most of the biological activities associated with PAF C-16 (Montrucchio *et al*., 2000; Aliberti *et al*., 1999; Pendino *et al*., 1993). This PAF C-16 structural analogue contains a hydroxyl group at position *sn*-2 instead of an acetyl group as shown in figure 4.2.1a. In results chapter 4, Lyso PAF was shown *in vitro* to inhibit the growth of both *M.smegmatis* and *M.bovis BCG* directly in a dose-dependent manner. Therefore, Lyso PAF was also investigated for its inhibitory effect on the growth of intracellular *M.smegmatis* inside THP-1 cells.

Four individual experiments (Exp1, 2, 3 and 4) were performed according to the protocol in chapter 2.7 by treating Lyso PAF in different concentrations (1µg, 5µg and 10µg/ml of cell suspension) with *M.smegmatis* infected THP-1 cells for 24 hours. During all four experiments, Lyso PAF did not show any inhibitory effect on the growth of intracellular *M.smegmatis* as indicated by the similar number of CFUs for Lyso PAF treated test conditions and solvent control (Figure 5.3a and 5.3b). Statistical analysis of the data by non-parametric multiple comparison Kruskal-Wallis test on ranks showed that the results were non-significant, p=0.18.



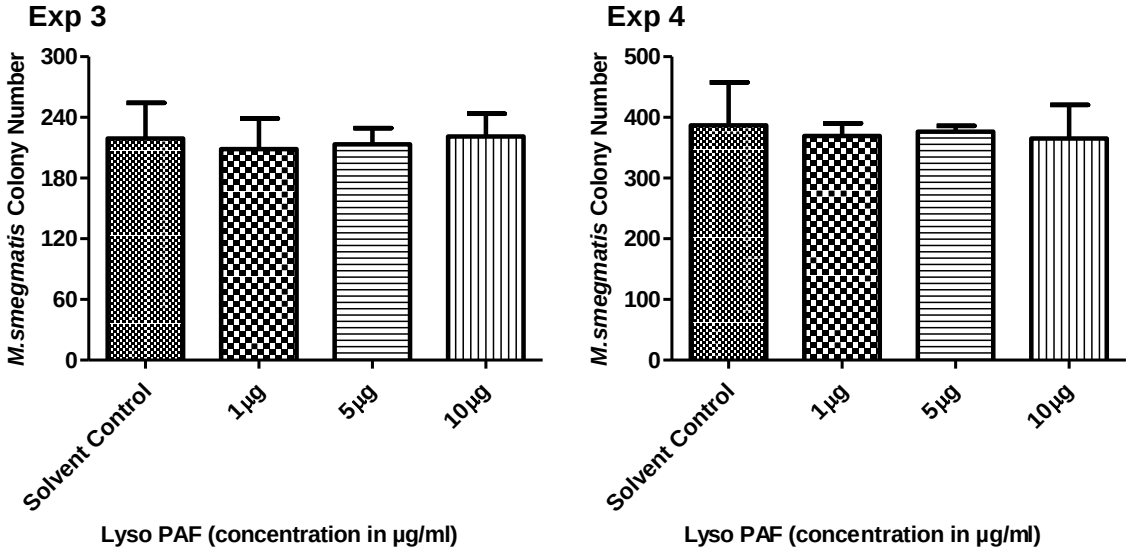


Figure 5.3a: Four individual experiments (Exp1, 2, 3 and 4) for the effect of Lyso PAF on intracellular *M.smegmatis* growth. Each bar represents the average number of CFUs from triplicate plates and error bars show standard deviation (SD) of the triplicates.

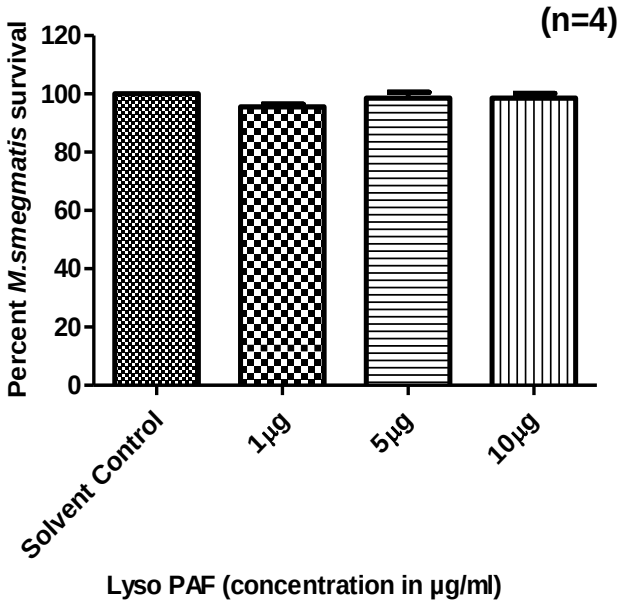


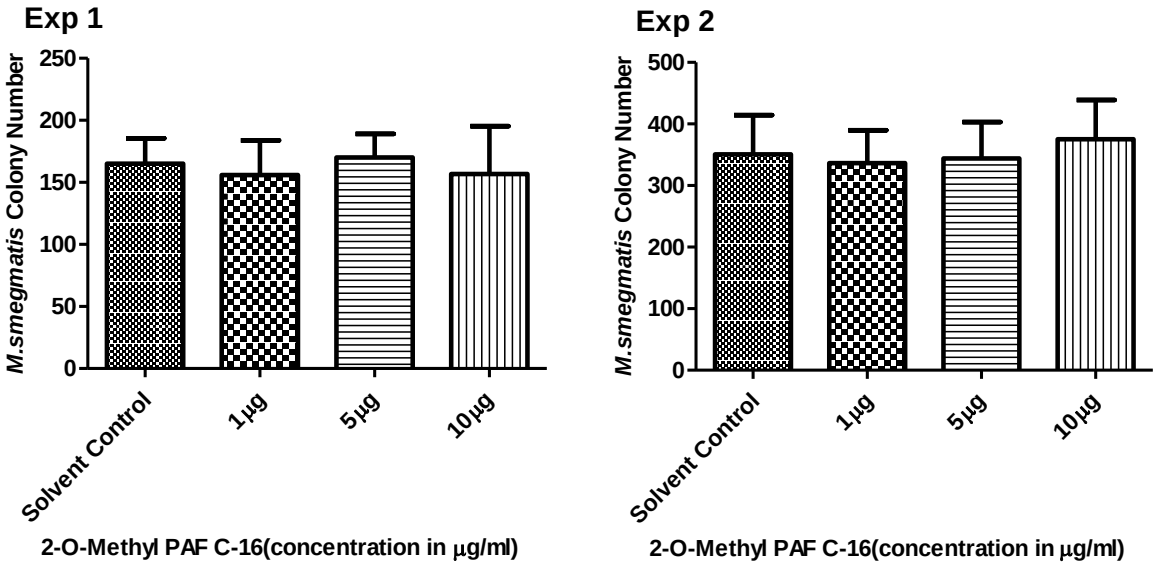
Figure 5.3b: Effect of Lyso PAF on intracellular *M.smegmatis* growth. Each bar represents the average of four individual experiments and the error bars show standard error of means (SEM). The data is expressed in terms of percentage, where solvent control is taken as 100% survival and different Lyso PAF treated test conditions are compared to it. Multiple-comparison of the

data sets using non-parametric Kruskal-Wallis test showed the results to non-significant, p=0.18.

5.4 Effect of 2-O-methyl PAF on *M.smegmatis* growth inside THP-1 cells

2-O-methyl PAF is a synthetic PAF C-16 analogue having a methyl group at the *sn*-2 position instead of an acetyl group as shown in figure 4.2.5a. 2-O-methyl PAF was used to investigate the effect of a methyl group at the *sn*-2 position on the growth inhibition of intracellular *M.smegmatis*.

Four individual experiments (Exp1, 2, 3 and 4) were performed according to the protocol in section 2.7 by treating 2-O-methyl PAF in different concentrations (1µg, 5µg and 10µg/ml of cell suspension) with *M.smegmatis* infected THP-1 cells with for 24 hours. During all four experiments, 2-O-methyl PAF was unable to inhibit the growth of intracellular *M.smegmatis* as indicated by the similar number of CFUs for 2-O-methyl PAF treated test conditions and solvent control (Figure 5.4a and 5.4b). Non-parametric multiple comparison Kruskal-Wallis test was used on ranks to calculate the *p*-value, which was found to be non-significant, p=0.14.



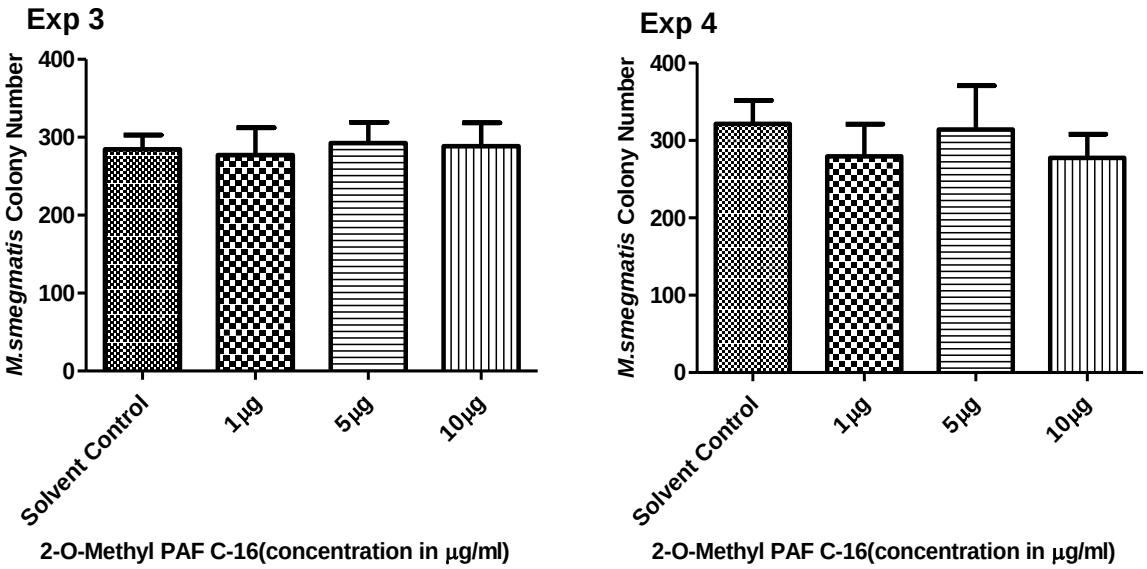


Figure 5.4a: Four individual experiments (Exp1, 2, 3 and 4) for the effect of 2-O-methyl PAF on intracellular *M.smegmatis* growth. Each bar represents the average number of CFUs from triplicate plates and error bars show standard deviation (SD) of the triplicates.

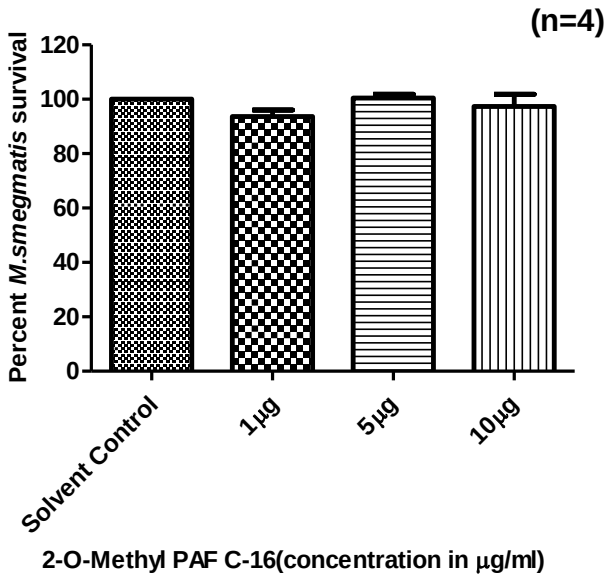


Figure 5.4b: Effect of 2-O-methyl PAF on intracellular *M.smegmatis* growth. Each bar represents the average of four individual experiments and the error bars show standard error of means (SEM). The data is expressed in terms of percentage, where solvent control is taken as 100% survival and different 2-O-methyl PAF treated test conditions are compared to it.

Multiple-comparison of the data sets using non-parametric Kruskal-Wallis test showed the results to non-significant, p=0.14.

5.5 Effect of PAF-C18 on *M.smegmatis* growth inside THP-1 cells

PAF C-18, a structure analogue was used to investigate the effect of an increase in the number of carbon atoms in the aliphatic tail on the growth inhibition of intracellular *M.smegmatis*. The structure analogue PAF C-18 has two additional carbon atoms in the aliphatic carbon tail attached at position *sn*-1 as shown in figure 4.2.3a.

Four individual experiments (Exp1, 2, 3 and 4) were performed *in vitro* according to the protocol in chapter 2.7 to investigate the effect of PAF C-18 treatment in different concentrations (1µg, 5µg and 10µg/ml of cell suspension) on the growth of intracellular *M.smegmatis*. It was observed that PAF C-18 was able to inhibit the growth of intracellular *M.smegmatis* during all four experiments. Furthermore, PAF C-18 at a concentration of 1µg/ml was more potent in inhibiting the growth of intracellular *M.smegmatis* as compared to 5µg and 10µg/ml (Figure 5.5a). On average, treatment of *M.smegmatis* infected THP-1 cells with PAF C-18 at concentrations of 1µg, 5µg and 10µg/ml resulted in 49.9%, 9.2% and 7.2% reduction in the growth of intracellular *M.smegmatis* respectively as indicated by comparing the number of CFUs with solvent control (Figure 5.5b). The level of significance was determined by applying non-parametric multiple comparison Kruskal Wallis test on ranks and the p-value was found to be significant, p=0.01. Individual data sets were compared by applying post hoc Dunn's multiple comparison test and 1µg/ml PAF C-18 vs Solvent control was found to be significant, $p \leq 0.01$.

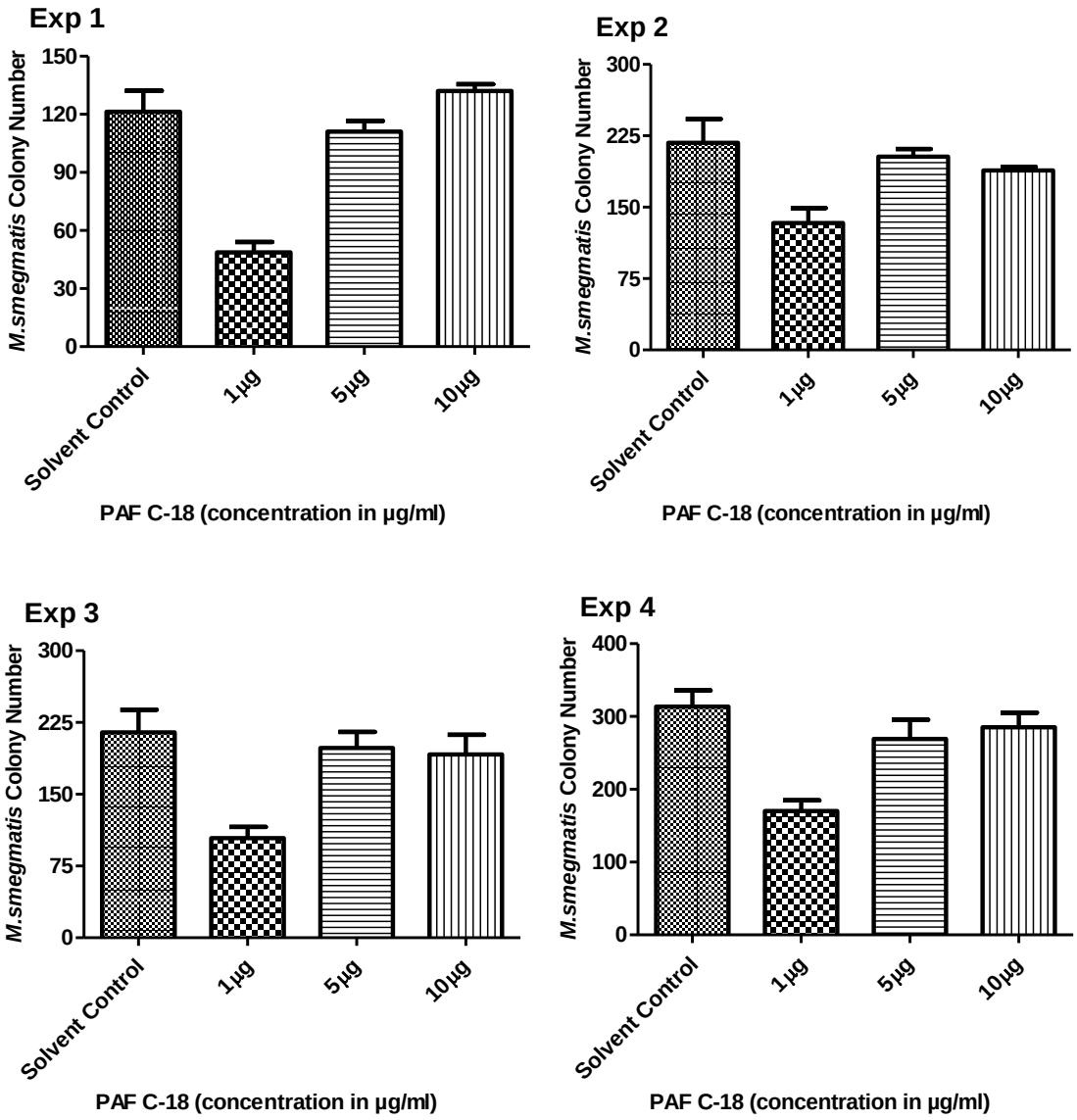


Figure 5.5a: Four individual experiments (Exp1, 2, 3 and 4) for the effect of PAF C-18 on intracellular *M.smegmatis* growth. Each bar represents the average number of *M.smegmatis* CFUs for triplicate plates and error bars show standard deviation (SD) of the triplicates.

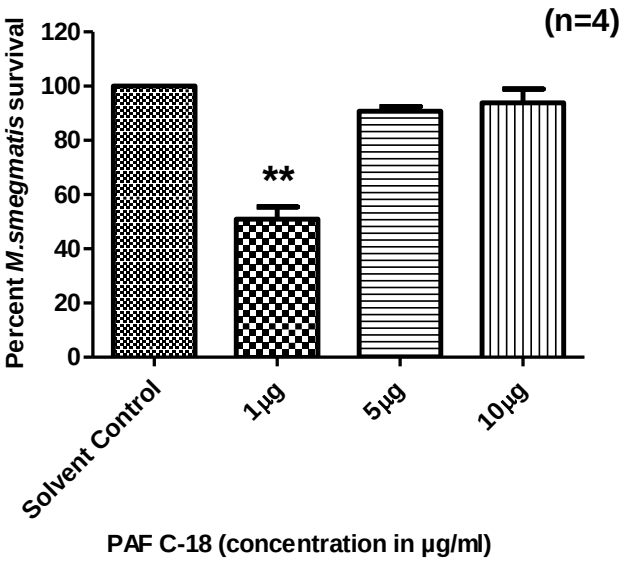


Figure 5.5b: Effect of PAF C-18 on intracellular *M.smegmatis* growth. Each bar represents the average of four individual experiments and the error bars show standard error of means (SEM). The data is expressed in terms of percentage, where solvent control is taken as 100% survival and different test conditions are compared to it. Level of significance was calculated by applying non-parametric multiple-comparison Kruskal-Wallis test on ranks, p=0.01 and individual data sets were compared through post hoc Dunn's multiple comparison test where PAF C-18 (1µg/ml) vs solvent control was found to be significant, **($p \leq 0.01$).

5.6 Effect of Hexanolamino PAF on *M.smegmatis* growth inside THP-1 cells

The structure analogue Hexanolamino PAF was used to investigate the effect of a change in position of the terminal amino group of PAF C-16 on the growth inhibition of intracellular *M.smegmatis*. In Hexanolamino PAF, the terminal amino group is attached the phosphate group via an additional 4-carbon atoms chain as shown in figure 4.2.4a.

Four individual experiments (Exp1, 2, 3 and 4) were performed to investigate *in vitro* the effect of Hexanolamino PAF treatment in different concentrations (1µg, 5µg and 10µg/ml of cell suspension) on the growth of intracellular *M.smegmatis* according to the protocol in chapter 2.7. It was observed that Hexanolamino PAF was able to inhibit the growth of intracellular *M.smegmatis* during all four experiments. Hexanolamino PAF at a concentration of 5µg/ml was more potent in inhibiting the growth of intracellular *M.smegmatis* as compared to 1µg and 10µg/ml (Figure 5.6a). On average, treatment of *M.smegmatis* infected THP-1 cells with Hexanolamino PAF at concentrations of 1µg, 5µg and 10µg/ml resulted in 6.1%, 42.6% and

9.6% reduction in the growth of intracellular *M.smegmatis* respectively as indicated by comparing the number of test CFUs to solvent control (Figure 5.6b). The level of significance was determined by applying non-parametric multiple comparison Kruskal Wallis test on ranks and the *p*-value was found to be significant, $p=0.01$. Individual data sets were compared by applying post hoc Dunn's multiple comparison test and 5µg/ml Hexanolamino PAF vs Solvent control was found to be significant, $p \leq 0.01$.

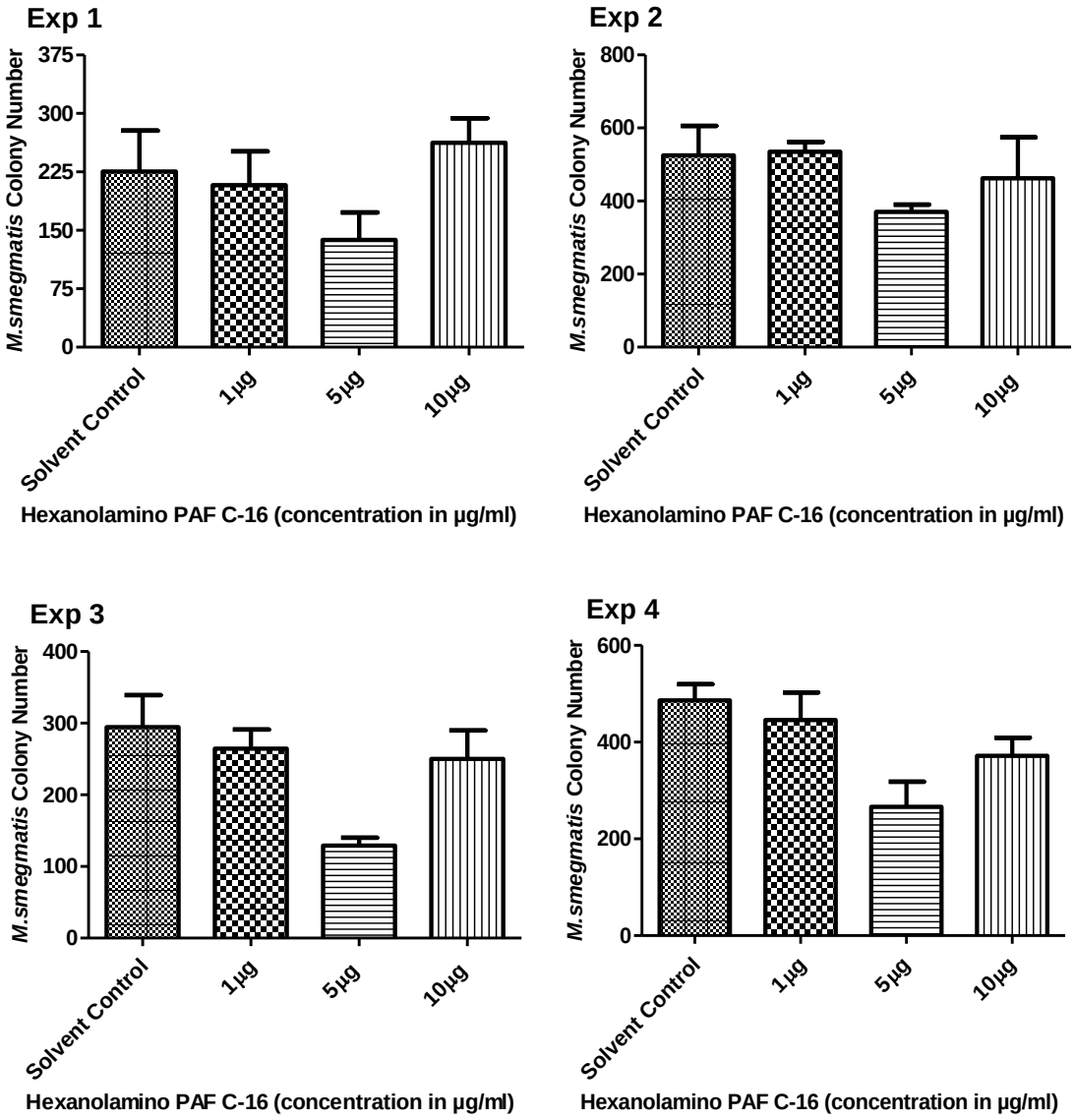


Figure 5.6a: Four individual experiments (Exp1, 2, 3 and 4) showing the effect of Hexanolamino PAF on the growth of *M.smegmatis*. Each bar represents the average number of *M.smegmatis* CFUs for triplicate plates and error bars show standard deviation (SD) of the triplicates.

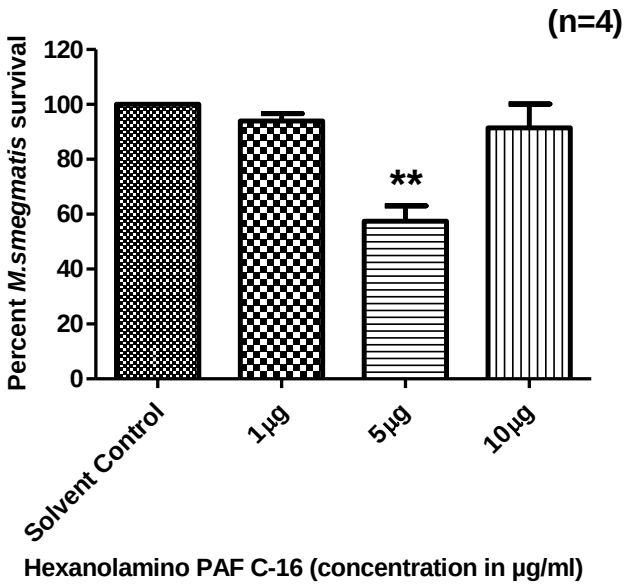


Figure 5.6b: Effect of Hexanolamino PAF on intracellular *M.smegmatis* growth. Each bar represents the average of four individual experiments and the error bars show standard error of means (SEM). The data is expressed in terms of percentage, where solvent control is taken as 100% survival and different test conditions are compared to it. Level of significance was determined by applying non-parametric multiple-comparison Kruskal-Wallis test on ranks, p=0.01 and individual data sets were compared through post hoc Dunn's multiple comparison test where Hexanolamino PAF (5µg/ml) vs solvent control was found to be significant, **($p \leq 0.01$).

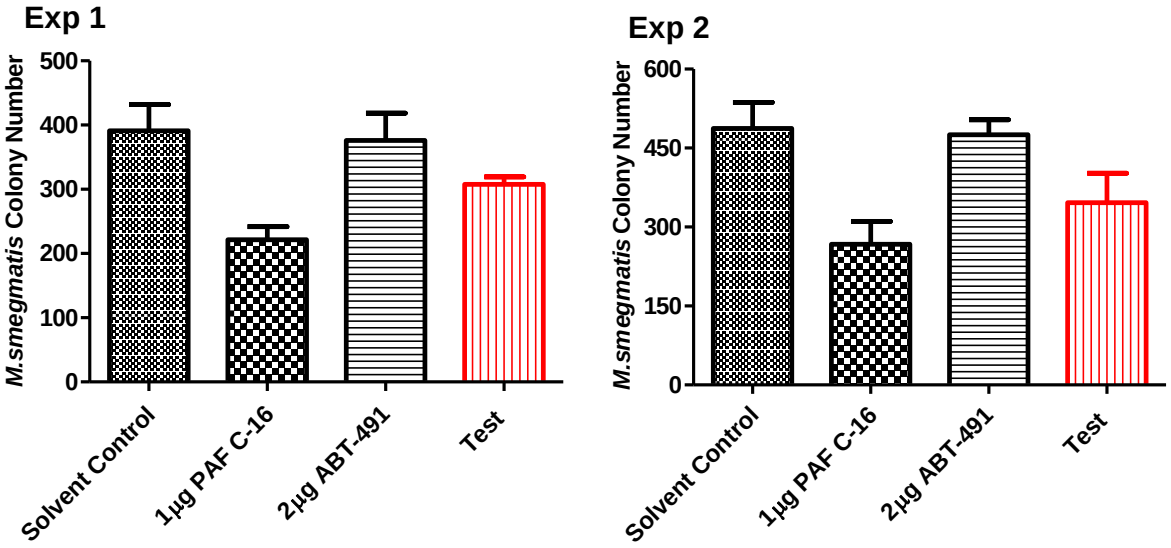
5.7 PAF receptor antagonists partially reverse PAF C-16 induced growth inhibition of intracellular *M.smegmatis*

PAF C-16 activates different signalling pathways in target cells by binding to specific G-protein coupled receptors known as PAF receptor (PAFR) (Honda *et al*., 2002). A number of naturally occurring and synthetic compounds known as PAFR antagonists can bind to PAFR with high affinity and prevent the biological effects of PAF C-16 due to blocking of PAFR (Singh *et al*., 2013).

Two synthetic PAFR antagonists, ABT-491 and WEB-2086 were used to investigate the effect of prior treatment of these PAFR antagonists on PAF C-16 induced growth inhibition of intracellular *M.smegmatis* inside THP-1 cells.

5.7.1 Effect of ABT-491 on PAF C-16 induced intracellular *M.smegmatis* growth inhibition

Four individual experiments (Exp1, 2, 3 and 4) were performed with PAFR antagonist ABT-491 according to the protocol in chapter 2.10. *M.smegmatis* infected THP-1 cells were treated with ABT-491 at a concentration of 2µg/ml for 1 hour prior to treatment with 1µg/ml PAF C-16 for 24 hours. All the four experiments showed that prior treatment of *M.smegmatis* infected THP-1 cells with ABT-491 partially mitigated the intracellular growth inhibition of *M.smegmatis* due to PAF C-16 as indicated by an increase in the number CFUs when compared to 1µg/ml PAF C-16 only (Figure 5.7.1a). Furthermore, 2µg/ml ABT-491 on its own had no inhibitory effect on the growth of intracellular *M.smegmatis* (Figure 5.7.1a). On average, treatment of *M.smegmatis* infected THP-1 cells with ABT-491 prior to PAF C-16 increased the number of intracellular *M.smegmatis* by 24.5% as indicated by the number of CFUs when compared to the number of CFUs from 1µg/ml PAF C-16 only treated THP-1 cells (Figure 5.7.1b). Non-parametric Mann Whitney test was used to determine the level of significance for "Test" (ABT-491 2µg/ml and PAF C-16 1µg/ml) versus 1µg/ml PAF C-16 only conditions and the *p*-value was found to be significant, $p=0.02$.



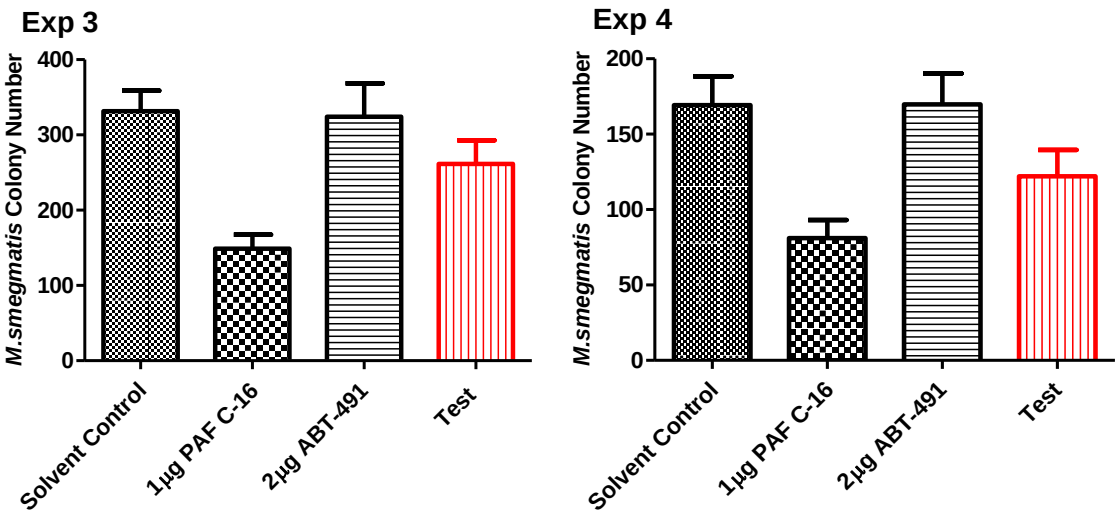


Figure 5.7.1a: Four individual experiments (Exp1, 2, 3 and 4) for the effect of ABT-491 on PAF C-16 induced intracellular *M.smegmatis* growth inhibition. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bars show standard deviation (SD) of the triplicates. **Test** consists of 2µg/ml ABT-491 and 1µg/ml PAF C-16.

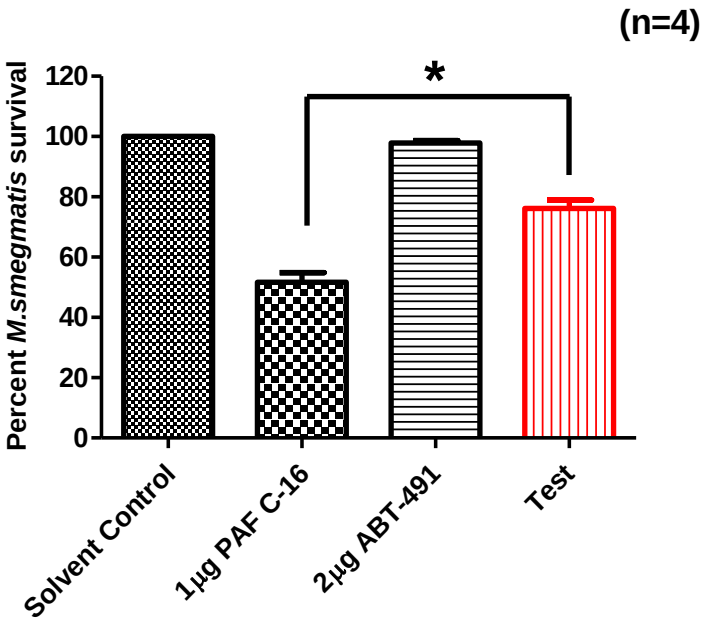
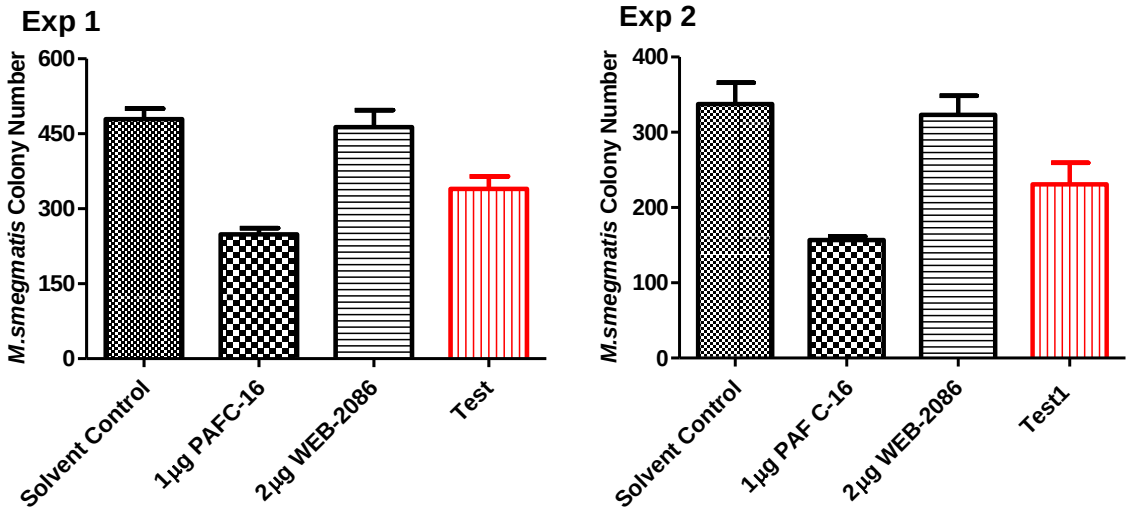


Figure 5.7.1b: Effect of ABT-491 prior treatment on PAF C-16 induced intracellular *M.smegmatis* growth inhibition. Each bar represents the average of four independent experiments and the error bars show standard error of means (SEM). **Test** consists of 2µg/ml ABT-491 and 1µg/ml PAF C-16. The data is expressed in terms of percentage, where solvent control is taken as 100% survival and test conditions are compared to it. By applying Mann

Whitney test, the *p*-value was found to be significant for Test (2µg/ml ABT-491 and 1µg/ml PAF C-16) vs 1µg/ml PAF C-16, *(*p*=0.02).

5.7.2 Effect of WEB-2086 on PAF C-16 induced intracellular *M.smegmatis* growth inhibition

Four individual experiments (Exp1, 2, 3 and 4) were performed by treating *M.smegmatis* infected THP-1 cells with WEB-2086 at a concentration of 2µg/ml for 1 hour prior to treatment with 1µg/ml PAF C-16 for 24 hours. It was observed that prior treatment with WEB-2086 partially mitigated the PAF C-16 induced growth inhibition of intracellular *M.smegmatis* during all four experiments as indicated by an increase in the number CFUs when compared to 1µg/ml PAF C-16 only (Figure 5.7.2a). Furthermore, 2µg/ml WEB-2086 on its own showed no inhibitory effect on the growth of intracellular *M.smegmatis*. On average, WEB-2086 treatment of *M.smegmatis* infected THP-1 cells with WEB-2086 prior to PAF C-16 increased the number of intracellular *M.smegmatis* by 20% when compared to the number of CFUs from 1µg/ml PAF C-16 only treated cells (Figure 5.7.2b). Non-parametric Mann Whitney test was used to determine the level of significance for "Test" (WEB-2086 2µg/ml and PAF C-16 1µg/ml) versus 1µg/ml PAF C-16 only conditions and the *p*-value was found to be significant, *p*=0.02.



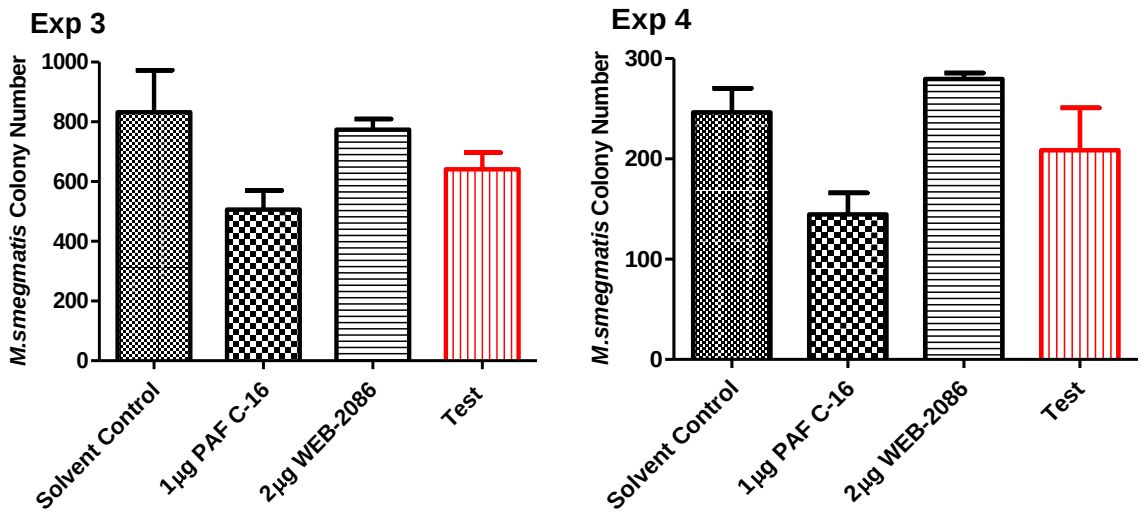


Figure 5.7.2a: Four individual experiments (Exp1, 2, 3 and 4) for the effect of WEB-2086 on PAF C-16 induced intracellular *M.smegmatis* growth inhibition. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of the triplicates. **Test** consists of 2µg/ml WEB-2086 and 1µg/ml PAF C-16.

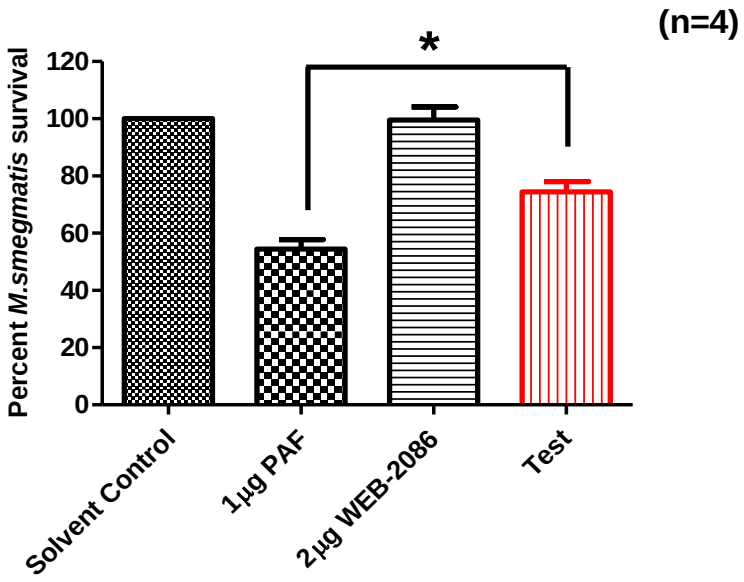


Figure 5.7.2b: Effect of WEB-2086 prior treatment on PAF C-16 induced intracellular *M.smegmatis* growth inhibition. Each bar represents the average of four independent experiments and the error bars show standard error of means (SEM). **Test** consists of 2µg/ml WEB-2086 and 1µg/ml PAF C-16. The data is expressed in terms of percentage, where solvent control is taken as 100% survival and test conditions are compared to it. *p*-value was calculated

by applying Mann Whitney test for Test (2µg/ml WEB-2086 and 1µg/ml PAF C-16) vs 1µg/ml PAF C-16 and was found to be significant, *(p=0.02).

5.8 Investigating the role PAF C-16 intracellular signalling pathway components in the PAF C-16 indeed intracellular growth inhibition of *M.smegmatis*

Binding of PAF C-16 to its receptor on the target cells results in the activation of intracellular signalling components such as phosphoinositide phospholipase C (PLC) and cytosolic phospholipase A_2 ($cPLA_2$) enzymes. The activated PLC further causes the metabolism of phosphatidylinositol 4,5-bisphosphate (PIP_2) and leads to the production of second messengers such as diacylglycerol (DAG) and inositol trisphosphate (IP_3). The activated $cPLA_2$ leads to the production of intracellular arachidonic acid (AA) that is used for the production of prostaglandins and the leukotrienes (Montrucchio *et al*., 2000).

Potent PLC and $cPLA_2$ inhibitors were used to block these enzymes and investigate their role in PAF C-16 induced intracellular growth inhibition of *M.smegmatis*. In addition, signalling pathway components such as DAG and AA were also investigated *in vitro* for their direct effect on the growth of *M.smegmatis*.

5.8.1 Effect of PLC inhibitor "U-73122" on PAF C-16 induced intracellular *M.smegmatis* growth inhibition

PLC inhibitor, U-73122 was used to investigate the role of PLC in PAF C-16 induced growth inhibition of intracellular *M.smegmatis*.

Four individual experiments (Exp1, 2, 3 and 4) were performed with U-73122 according to the protocol in chapter 2.10. *M.smegmatis* infected THP-1 cells were treated with U-73122 at a concentration of 2µM for 30min prior to treatment with 1µg/ml PAF C-16 for 24 hours. It was observed that U-73122 at a concentration of 2µM was partially effective in mitigating the inhibitory effect PAF C-16 on the growth of intracellular *M.smegmatis* during all four experiments (Figure 5.8.1). On average, treatment of *M.smegmatis* infected THP-1 cells with U-73122 prior to PAF C-16 increased the number of intracellular *M.smegmatis* by 25.2% when compared to the number of CFUs from 1µg/ml PAF C-16 only treated cells (Figure 5.8.2a). Non-parametric Mann Whitney test was used to determine the level of significance for "Test"

(2µM U-73122 and PAF C-16 1µg/ml) versus 1µg/ml PAF C-16 only and the *p*-value was found to be significant, *p*=0.02.

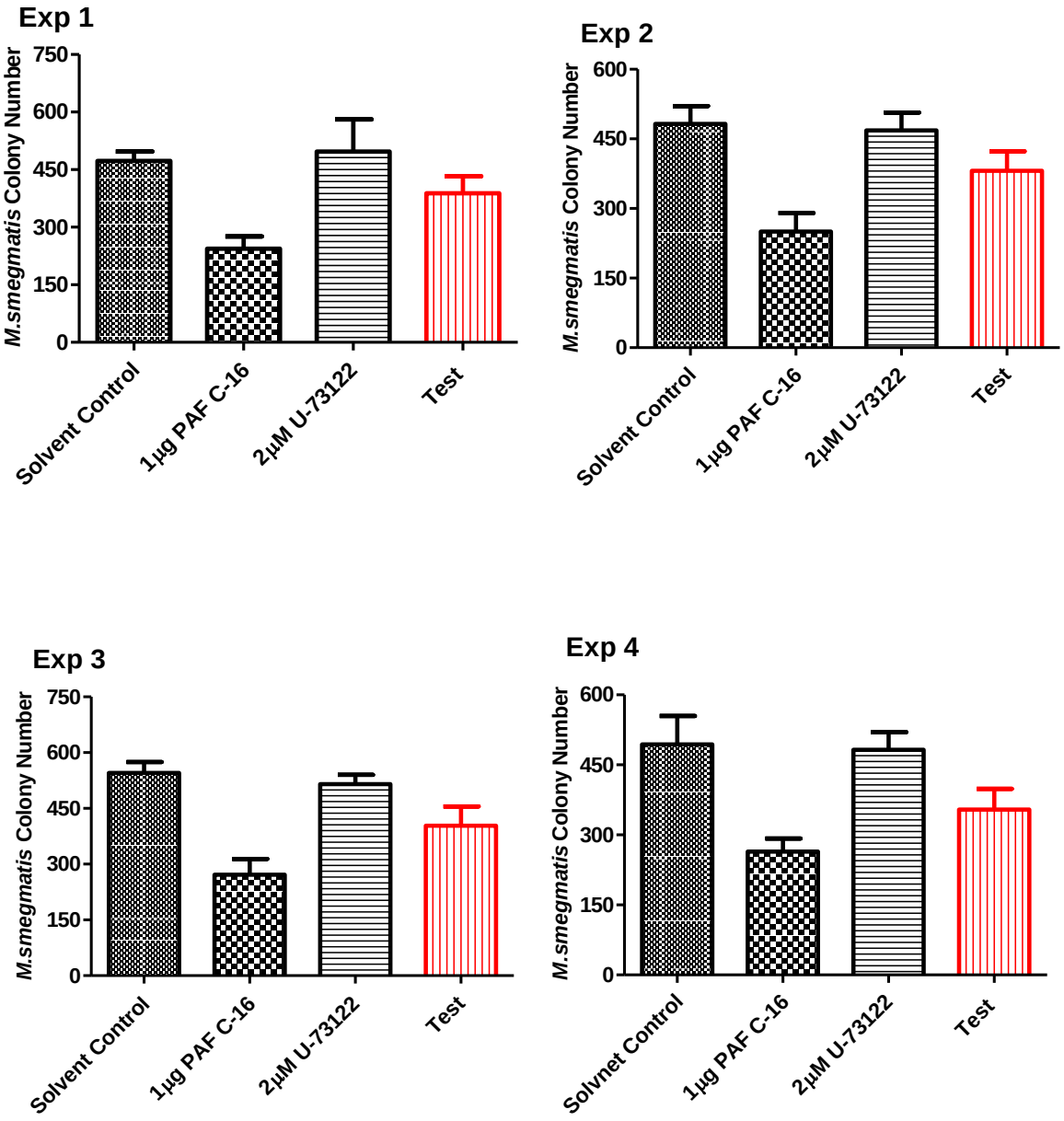


Figure 5.8.1a: Four individual experiments (Exp1, 2, 3 and 4) for the effect of U-73122 on PAF C-16 induced intracellular *M.smegmatis* growth inhibition. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of the triplicates. **Test** consists of 2µM U-73122 and PAF C-16 1µg/ml.

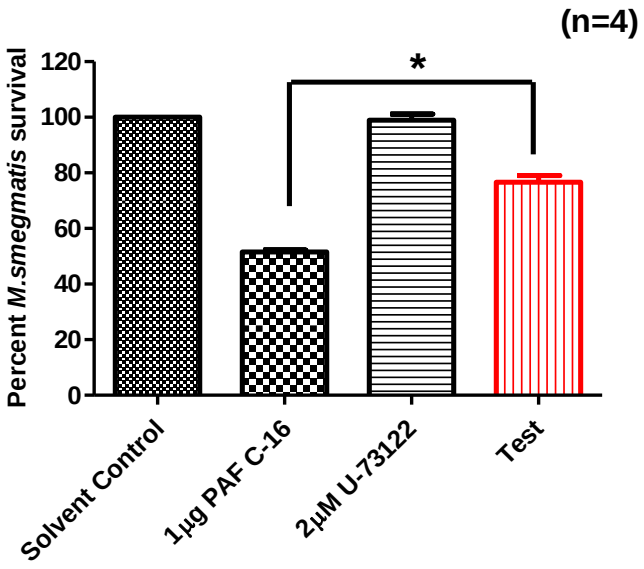


Figure 5.8.1b: Effect of U-73122 on PAF C-16 induced intracellular *M.smegmatis* growth inhibition. Each bar represents the average of four independent experiments and the error bars show standard error of means (SEM). Test consists of 2µM U-73122 and 1µg/ml PAF C-16. The data is expressed in terms of percentage, where solvent control is taken as 100% survival and test conditions are compared to it. *P* value was calculated by applying Mann Whitney test for "Test" (2µM U-73122 and 1µg/ml PAF C-16) vs 1µg/ml PAF C-16 and was found to be significant, *(p=0.02).

5.8.2 Effect of $cPLA_2$ inhibitor "Benzenesulfonamide" on PAF C-16 induced intracellular *M.smegmatis* growth inhibition

PAF C-16 also causes the activation of cytosolic phospholipase A_2 ($cPLA_2$). This activated $cPLA_2$ further leads to the intracellular production of arachidonic acid (AA) and lysophosphatidylcholine. Benzenesulfonamide, a $cPLA_2$ inhibitor, was used to investigate the role of $cPLA_2$ in PAF C-16 induced growth inhibition of intracellular *M.smegmatis*.

Four individual experiments (Exp1, 2, 3 and 4) were performed with benzenesulfonamide according to the protocol in chapter 2.10. *M.smegmatis* infected THP-1 cells were treated with benzenesulfonamide at a concentration of 23nM (IC_{50}) or 56nM ($2IC_{50}$) for 30min prior to treatment with 1µg/ml PAF C-16 for 24 hours. It was observed that benzenesulfonamide at concentrations of 23nM and 56nM partially mitigated the inhibitory effect PAF C-16 on the growth of intracellular *M.smegmatis* during all four experiments (Figure 5.8.2a). On average, treatment of *M.smegmatis* infected THP-1 cells with benzenesulfonamide at concentrations

23nM (Test 1) or 56nM (Test 2) prior to 1µg/ml PAF C-16 treatment increased the number of intracellular *M.smegmatis* by 25.5% and 30.1% respectively when compared to the number of CFUs from 1µg/ml PAF C-16 only treated cells (Figure 5.8.2b). Non-parametric Mann Whitney test was used to determine the level of significance for "Test 1 vs 1µg/ml PAF C-16 only" and "Test 2 vs 1µg/ml PAF C-16 only" conditions and the *p*-value was found to be significant, p=0.02.

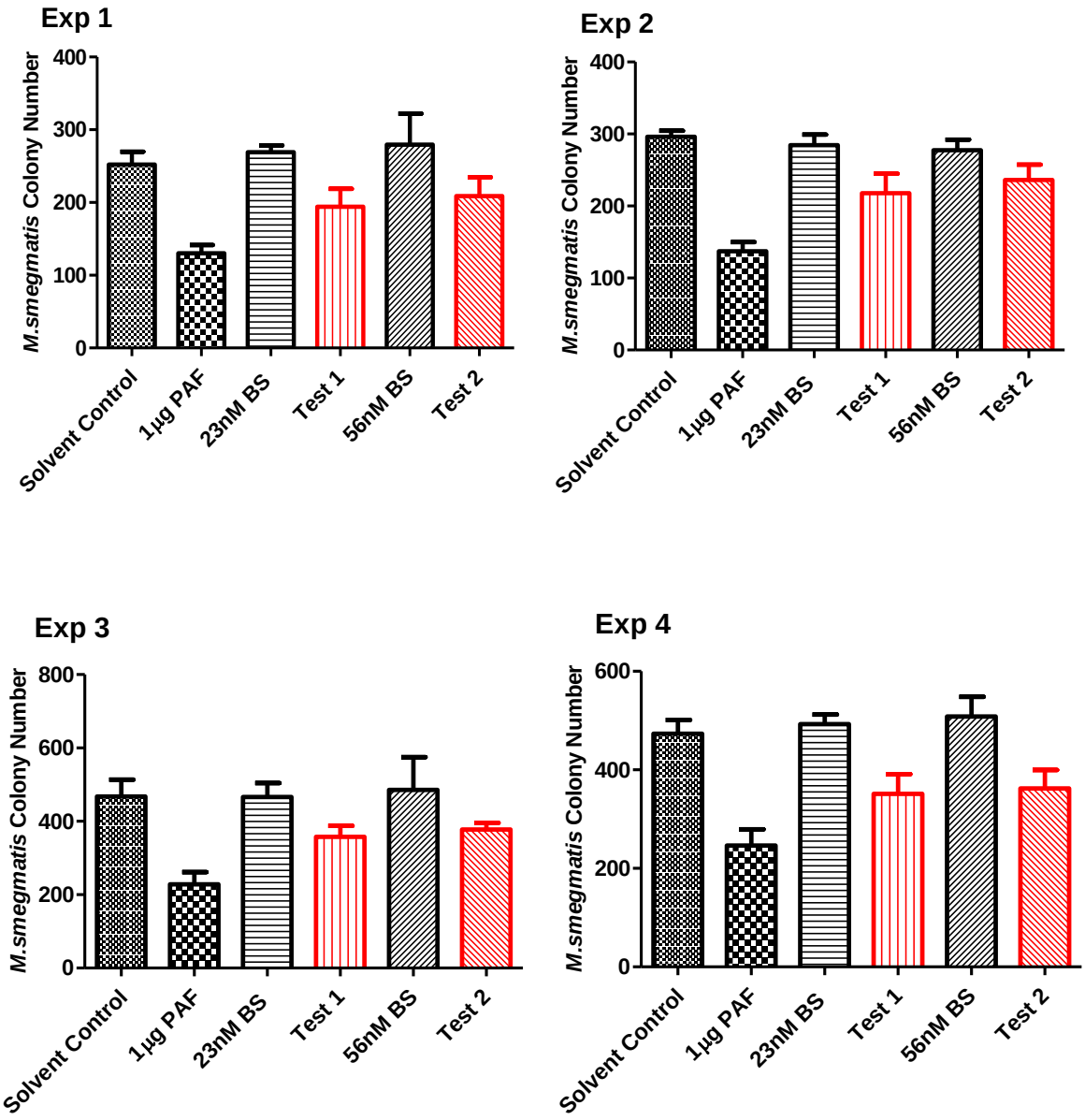


Figure 5.8.2a: Four individual experiments (Exp1, 2, 3 and 4) for the effect of benzenesulfonamide on PAF C-16 induced intracellular *M.smegmatis* growth inhibition. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar

shows standard deviation (SD) of the triplicates. **Test 1** consists of 23nM benzenesulfonamide and 1µg/ml PAF C-16, **Test 2** consists of 56nM benzenesulfonamide and 1µg/ml PAF C-16.

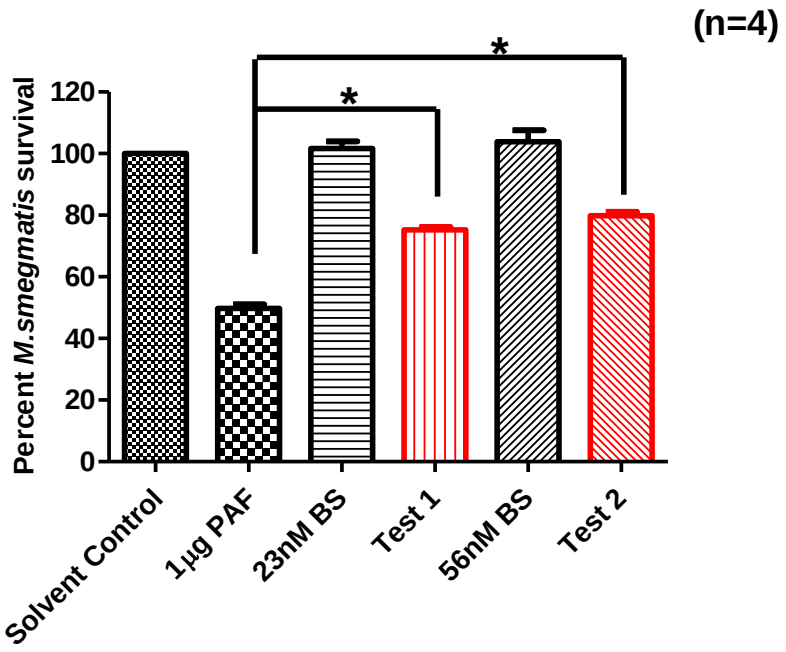


Figure 5. 8.2b: Effect of $cPLA_2$ inhibitor benzenesulfonamide on PAF C-16 induced growth inhibition of intracellular *M.smegmatis*. Each bar represents the average of four independent experiments and the error bars show standard error of means (SEM). The data is expressed in terms of percentage, where solvent control is taken as 100% survival and test conditions are compared to it. **Test 1** consists of 23nM benzenesulfonamide and 1µg/ml PAF C-16, **Test 2** consists of 56nM benzenesulfonamide and 1µg/ml PAF C-16. *P*-value was calculated by applying Mann Whitney test was found to be significant for Test 1 vs 1µg/ml PAF C-16 and Test 2 vs 1µg/ml PAF C-16 only, *(p=0.02).

5.8.3 Direct effect of Diacylglycerol (DAG) on *M.smegmatis* growth *in vitro*

Diacylglycerol (DAG) is a second messenger produced during PAF C-16 signalling pathway. Chemically DAG consists of two fatty acid chains attached to a glycerol backbone via ester bonds as shown in the figure 5.8.3a.

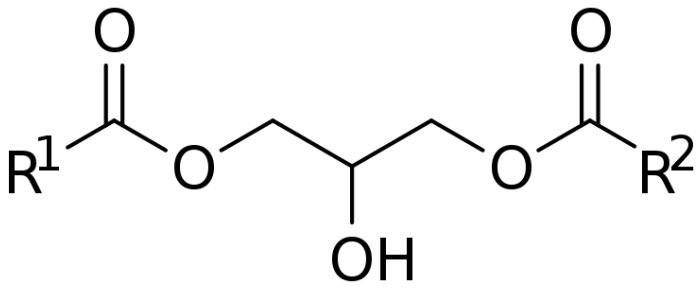
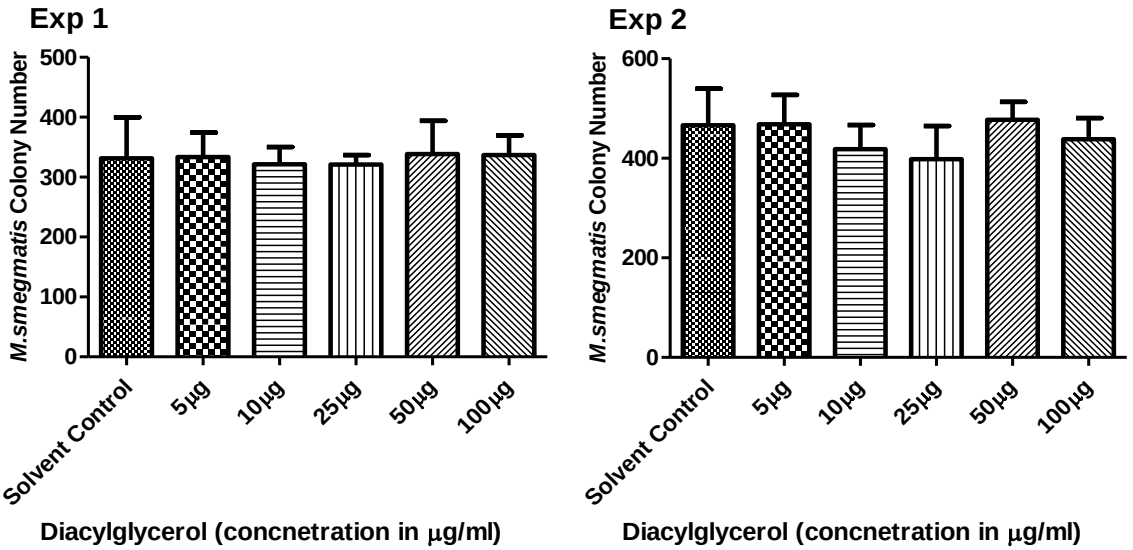


Figure 5.8.3a: Chemical structure of diacylglycerol. Two fatty acid chains (R^1 and R^2) are attached via ester bonds to the glycerol backbone.

Three individual experiments (Exp1, 2 and 3) were performed to investigate the direct effect of DAG in different concentrations (5µg, 10µg, 25µg, 50µg and 100µg/ml of bacterial suspension) on the growth of *M.smegmatis* according to the protocol in section 2.2. The results showed that *in vitro* DAG in concentrations of up to 100µg/ml had no direct inhibitory effect on the growth of *M.smegmatis* as indicated by the similar number of CFUs for DAG treated and solvent control *M.smegmatis* (Figure 5.8.3b and 5.8.3c). The level of significance was determined by applying non-parametric Kruskal-Wallis test on ranks and the *p*-value was found to be non-significant, p=0.23.



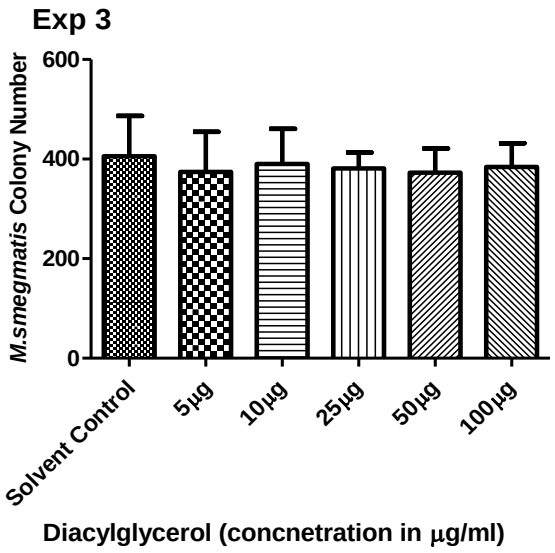


Figure 5.8.3b: Three individual experiments (Exp1, 2 and 3) for the direct effect of DAG on the growth of *M.smegmatis*. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicates.

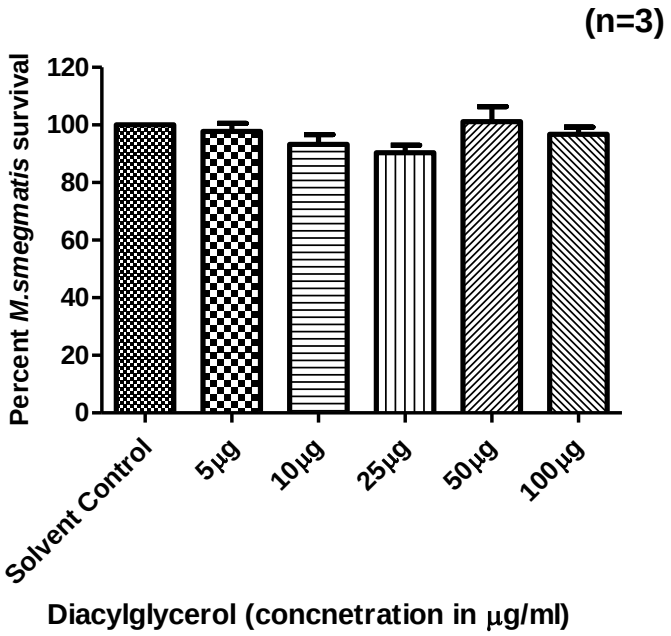


Figure 5.8.3c: Direct effect of DAG on *M.smegmatis* growth. Each bar represents the average of three individual experiments and the error bars show standard error of means (SEM). The data is expressed in terms of percentage, where solvent control is taken as 100% survival and

different test conditions are compared to it. Non-parametric Kruskal-Wallis test was used to determine the *p*-value, which was found to be non-significant, p=0.23.

5.8.4 Direct effect of arachidonic acid (AA) on *M.smegmatis* growth *in vitro*

Arachidonic acid (AA) is produced by activated cPLA$_2$ from intracellular phospholipids during PAF C-16 signalling pathway. Chemically, AA is a carboxylic acid containing 20 carbon atoms chain with four double bonds as shown in figure 5.8.4a.

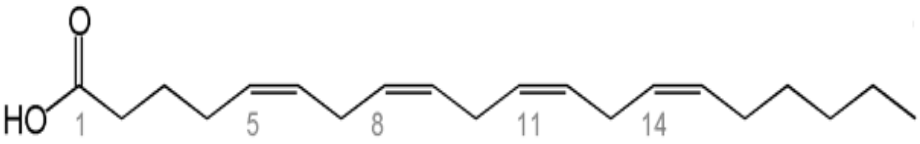


Figure 5.8.4a: Chemical structure of arachidonic acid. The compound contains a 20 carbon atoms chain with 4 double bonds at positions 5, 8, 11 and 14.

Three individual experiments (Exp1, 2 and 3) were performed to investigate the direct effect of AA in different concentrations (5µg, 10µg, 25µg, 50µg and 100µg/ml of bacterial suspension) on the growth of *M.smegmatis* according to the protocol in section 2.2. AA inhibited the growth of *M.smegmatis* in all three experiments (Figure 5.8.4b). On average, more than 98% *M.smegmatis* growth inhibition was observed at all concentrations (Figure 5.8.4c). The level of significance was determined by applying non-parametric multiple comparison Kruskal Wallis test on ranks and the *p*-value was found to be significant, p=0.007.

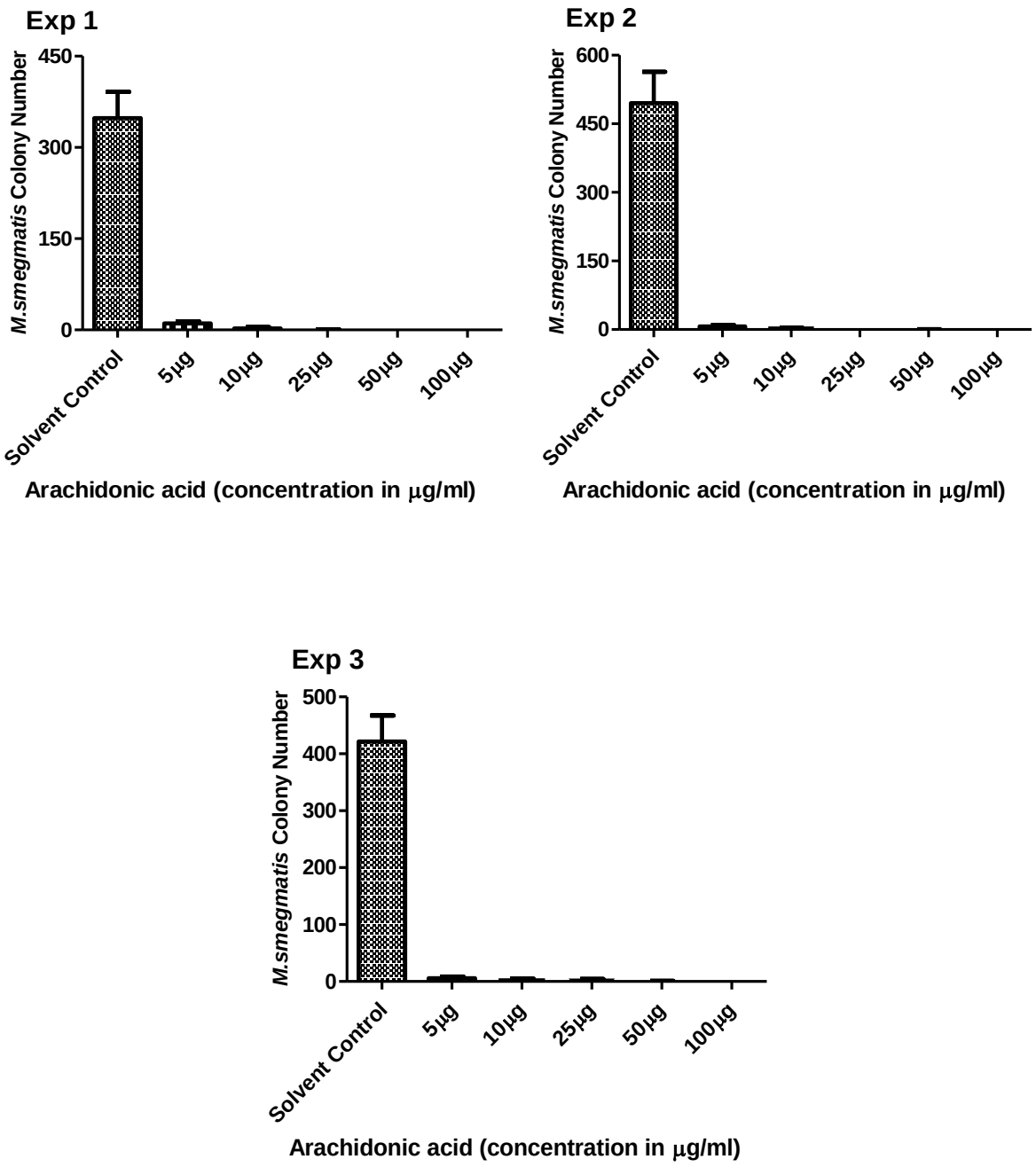


Figure 5.8.4b: Three individual experiments (Exp1, 2 and 3) for the direct effect of AA on *M.smegmatis* growth. Each bar represents the average of triplicate values and error bar shows standard deviation (SD) of the triplicates from the average.

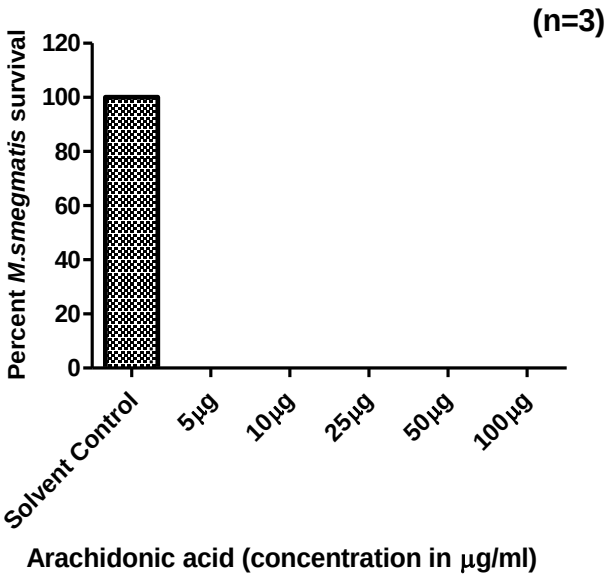


Figure 5.8.4c: Direct effect of AA on *M.smegmatis* growth. Each bar represents the average of three individual experiments and the error bars show standard error of means (SEM). The data is expressed in terms of percentage, where control is taken as 100% survival and different test conditions are compared to it. Level of significance was determined using non-parametric Kruskal-Wallis test and the *p*-value was found to be significant *** (P=0.007).

Furthermore, lower concentrations of AA were also investigated for their direct growth inhibitory effect against *M.smegmatis*. Three independent experiments (Exp1, 2 and 3) were performed using lower concentrations of AA (0.1µg, 0.5µg and 1µg and 2.5µg/ml bacterial suspension) and a solvent control for AA (2.5µl ethanol/ml bacterial suspension) according to the protocol in section 2.2. The results showed dose-dependent growth inhibition of *M.smegmatis* during all the experiments (Figure 5.8.4d). AA at concentrations of 2.5µg/ml and 1µg/ml on average caused a reduction of 86.4% and 36% respectively in the number of *M.smegmatis* CFUs as compared to solvent control (Figure 5.8.4e). The level of significance was determined by applying non-parametric multiple comparison Kruskal Wallis test on ranks and the *p*-value was found to be significant, $p=0.008$. By applying post hoc Dunn's multiple comparison test only 2.5µg/ml AA versus control was found to be significant, $p \leq 0.01$.

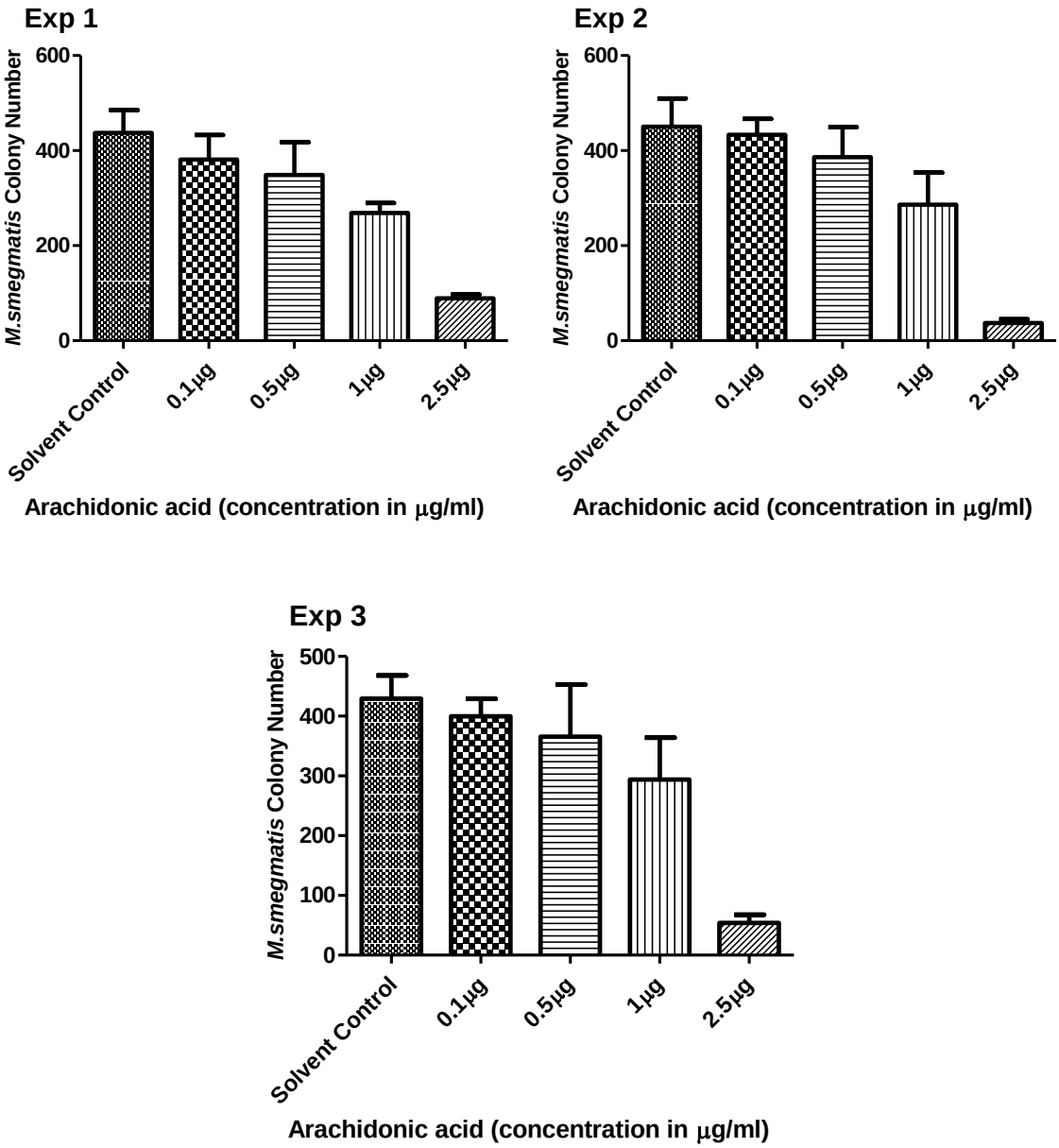


Figure 5.8.4d: Three individual experiments (Exp1, 2 and 3) for the direct effect of AA (lower concentrations) on *M.smegmatis* growth. Each bar represents the average of triplicate values and error bar shows standard deviation (SD) of the triplicates from the average.

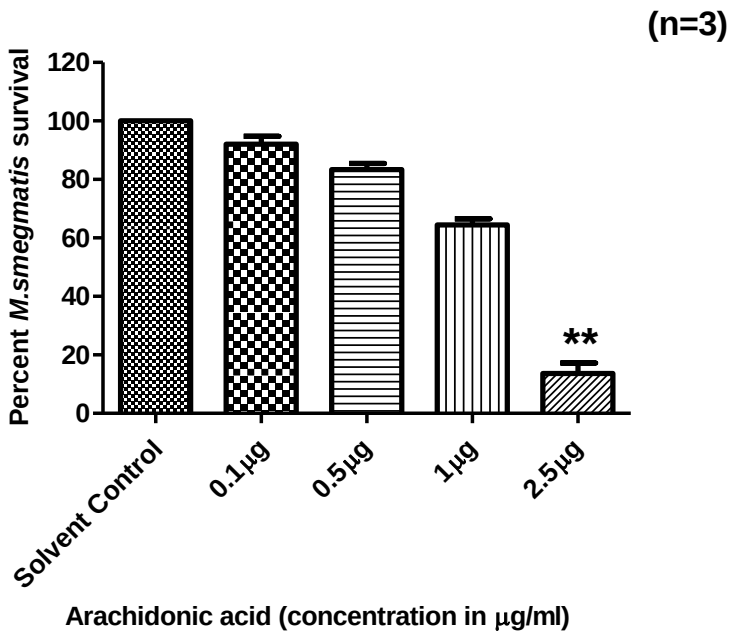


Figure 5.8.4e: Direct effect of AA (lower concentrations) on *M.smegmatis* growth. Each bar represents the average of three individual experiments and the error bars show standard error of means (SEM). The data expressed in terms of percentage, where control is taken as 100% survival and different test conditions are compared to it. Non-parametric Kruskal-Wallis test was applied on ranks to determine the *p*-value, which was found to be significant (p=0.008). Individual data sets were compared using Dunn's multiple comparison test and the results were significant for 2.5µg/ml AA vs solvent control, **($p \leq 0.01$).

5.9 Investigating the mechanism of PAF C-16 induced growth inhibition of intracellular *M.smegmatis*

Immune cells such as macrophages can inhibit the growth intracellular *mycobacteria* by various mechanisms including apoptosis and production of reactive nitrogen species (Navarre and Zychlinsky, 2000; MacMicking *et al*., 1997). Therefore, these growth inhibitory mechanisms were also investigated for their role in PAF C-16 induced growth inhibition of intracellular *M.smegmatis*.

5.9.1 Investigating PAF C-16 induced apoptosis in THP-1 cells

PAF C-16 has previously been shown to induce apoptosis in neuronal cells, epithelial cells and keratocytes (Brewer *et al*., 2002; Chandrasekher *et al*., 2002; Barber *et al*., 1998). Therefore, PAF C-16 treated THP-1 cells were also analyzed by flow cytometry to detect apoptosis.

Three independent experiments were performed to detect PAF C-16 induced apoptosis in THP-1 cells using fluorescence dyes; Annexin-V conjugated FITC and PI. THP-1 cells treated with PAF C-16 at a concentration of 1µg/ml for 24 hours were stained with apoptosis detection dyes and analyzed by performing flow cytometry. A solvent control (2µl ethanol/ml) and a positive control for apoptosis (2µM staurosporine) were also included in the experiment. The data was collected by acquiring 20,000 cells for each experimental condition and visualized on a density plot using NovoExpress® software.

The figure 5.9.1a shows the density plots for solvent control (2µl ethanol/ml) (slide A), positive control (2µM staurosporine) (slide B) and PAF C-16 (1µg/ml) (slide C) treated THP-1 cells. The results showed no appreciable increase in the percentage of Annexin-V conjugated FITC positive cells in Q5-4 representing cells in early apoptotic stages or the percentage of Annexin-V conjugated FITC and PI double positive cells in Q5-2 indicating late apoptosis after treatment with 1µg/ml PAF C-16 (slide C) when compared with the solvent control for PAF C-16 (2µl ethanol/ml) (slide A) used as a negative control.

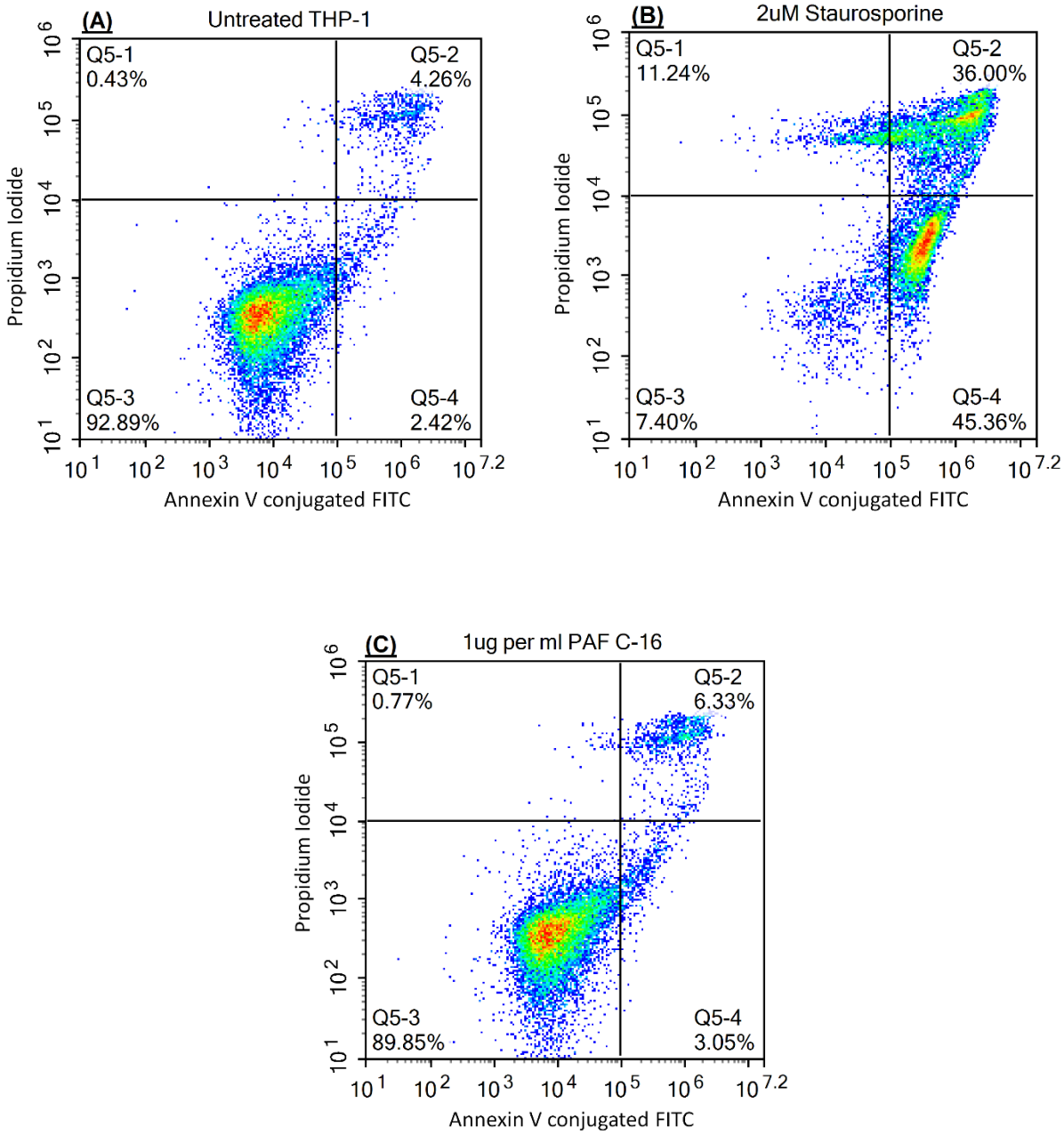


Figure 5.9.1a: Flow cytometry analysis for detection of apoptosis in PAF C-16 treated THP-1 cells. The data is presented on a density plot where X-axis shows the fluorescence intensity for Annexin-V conjugated FITC and Y-axis represents the fluorescence intensity of PI. Slide A is the negative control (THP-1 cells treated with the solvent for PAF C-16, 2µl ethanol/ml), slide B is the positive control (THP-1 cells treated with 2µM staurosporine) and slides C is the Test (THP-1 cells treated with 1µg/ml PAF C-16). Live cells (Q5-3) are negative for both Annexin-V conjugated FITC and PI. Early apoptotic cells (Q5-4) are Annexin-V conjugated FITC positive and PI negative. Late apoptotic/necrotic cells (Q5-2) are double positive and show

staining for both Annexin-V conjugated FITC and PI whereas necrotic cells (Q5-1) are only PI positive.

5.9.2 Investigating PAF C-16 induced apoptosis in *M.smegmatis* infected THP-1 cells

Although there was no evident apoptosis in PAF C-16 treated THP-1 cells, however, further investigation was done for detection of PAF C-16 induced apoptosis in *M.smegmatis* infected THP-1 treated with PAF C-16.

Three independent experiments were performed by treating *M.smegmatis* infected THP-1 cells with 1µg/ml PAF C-16 for 24 hours. The cells were analyzed by flow cytometry using apoptosis detection dyes; Annexin-V conjugated FITC and PI. Appropriate controls including two solvent controls (THP-1 cells only and *M.smegmatis* infected THP-1 cells treated with 2µl ethanol/ml) and a positive control for apoptosis (THP-1 cells treated with 2µM staurosporine) were also included in the experiment. The data was collected by acquiring 20,000 events for each experimental condition and visualized on a density plot using NovoExpress® software.

The figure 5.9.2a shows the density plots for solvent control THP-1 cells only (2µl ethanol/ml) (slide A), positive control THP-1 cells (2µM staurosporine) (slide B), solvent control THP-1 cells infected with *M.smegmatis* (2µl ethanol/ml) (slide C) and test THP-1 cells having intracellular *M.smegmatis* treated with 1µg/ml PAF C-16 (Slide D).

The density plots in figure 5.9.2a showed that phagocytosis of *M.smegmatis* by THP-1 cells (slide C) induced apoptosis in these cells as indicated by the increase in the percentage of Annexin-V conjugated FITC positive cells in Q5-4 and Annexin V conjugated FITC and PI double positive cells in Q5-2 when compared with solvent control THP-1 cells without any intracellular *M.smegmatis* (Slide A). Furthermore, treatment of PAF C-16 (1µg/ml) with *M.smegmatis* infected THP-1 cells with for 24 hours did not induce or enhance the apoptosis as there was no increase in the percentage of Annexin-V conjugated FITC positive THP-1 cells (Q5-4) representing early apoptosis or Annexin-V conjugated FITC and PI double positive cells (Q5-2) indicating late apoptosis (Slide D) when compared to solvent control *M.smegmatis* infected THP-1 cells (Slide C) (Figure 5.9.2a).

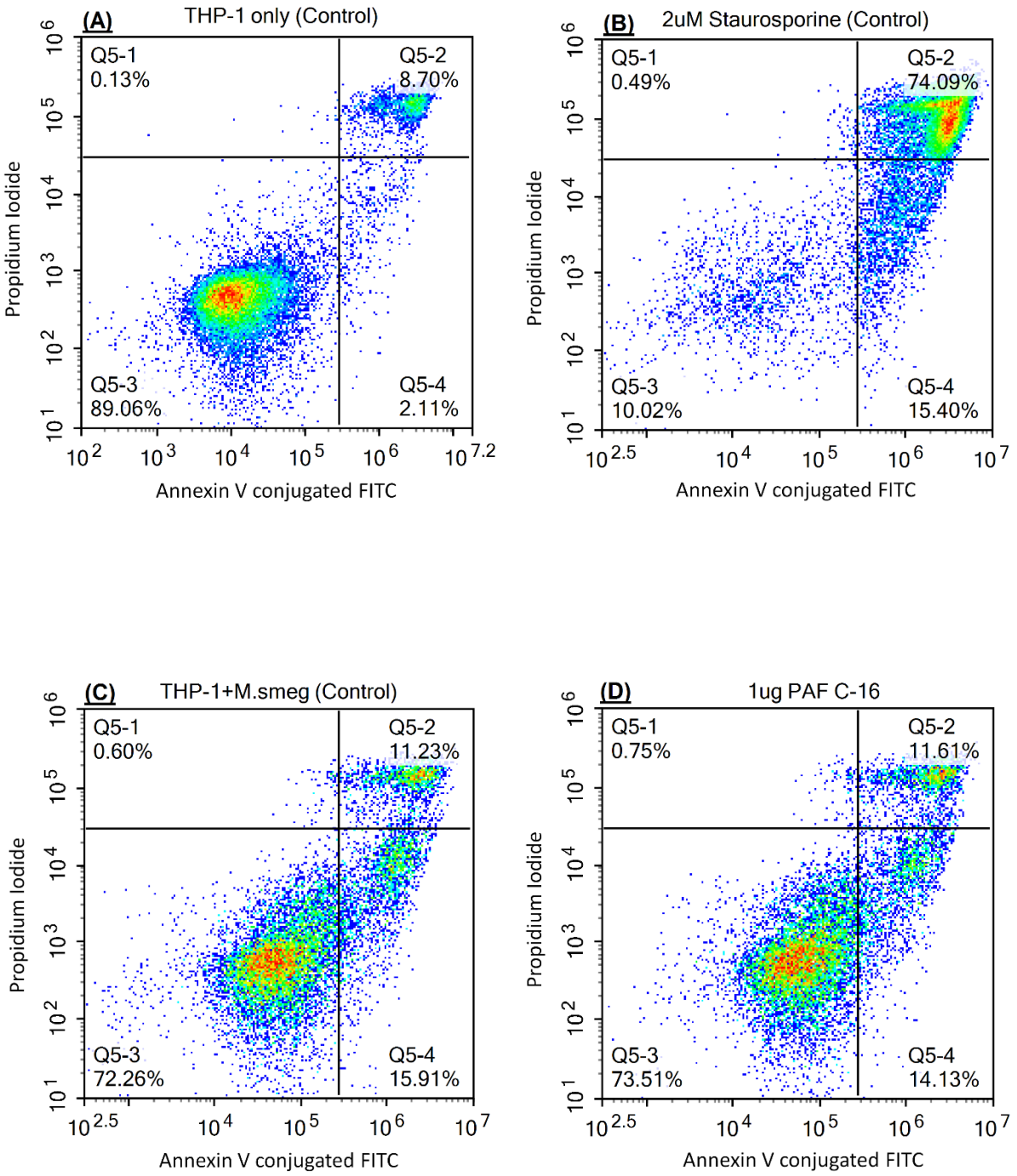


Figure 5.9.2a: Flow cytometry analysis for detection of PAF C-16 induced apoptosis in *M.smegmatis* infected THP-1 cells. The data is presented on a density plot where X-axis shows the fluorescence intensity for Annexin-V conjugated FITC and Y-axis represents the fluorescence intensity of PI. Slide A shows solvent control THP-1 cells only (THP-1 cells without *M.smegmatis* treated with 2µl ethanol/ml), slide B is the positive control (THP-1 cells treated with 2µM Staurosporine), slides C shows solvent control *M.smegmatis* infected THP-1

cells treated with 2µl ethanol/ml and slide D shows *M.smegmatis* infected THP-1 cells treated with 1µg/ml PAF C-16 for 24 hours. Live cells (Q5-3) are negative for both Annexin V conjugated FITC and PI. Early apoptotic cells (Q5-4) are Annexin-V conjugated FITC positive and PI negative. Late apoptotic/necrotic cells (Q5-2) are double positive and show staining for both Annexin-V conjugated FITC and PI whereas, necrotic cells (Q5-1) are only PI positive.

5.10 Effect of nitric oxide synthase inhibitor on PAF C-16 induced growth inhibition of intracellular *M.smegmatis*

PAF C-16 has previously been shown to activate iNOS expression and inhibit the growth of intracellular parasites such as Leishmania and Trypanosoma via production of reactive nitrogen species (Lonardoni *et al*., 2000; Aliberti *et al*., 1999). Since, reactive nitrogen species are also effective in eliminating intracellular *mycobacteria*, an iNOS inhibitor, Aminoguanidine hemisulfate (AG), was used to check whether PAF C-16 was inhibiting the growth of intracellular *M.smegmatis* by causing the production of reactive nitrogen species in THP-1 cells.

Four individual experiments (Exp1, 2, 3 and 4) were performed to investigate the effect of iNOS inhibitor, Aminoguanidine hemisulfate (AG) on PAF C-16 induced intracellular *M.smegmatis* growth inhibition according to the protocol in section 2.10. During all four experiments, treatment of *M.smegmatis* infected THP-1 cells with 1mM AG for 30min prior to treatment with 1µg/ml PAF C-16 partially reversed PAF C-16 induced growth inhibition of intracellular *M.smegmatis* as indicated by an increase in the number CFUs when compared to 1µg/ml PAF C-16 only (Figure 5.10a). On average, prior treatment with 1mM AG increased the growth of intracellular *M.smegmatis* by 23.4% when compared to 1µg/ml PAF C-16 only as indicated by a comparison of the number of CFUs (Figure 5.10b). The level of significance for "Test" (1mM AG and 1µg/ml PAF C-16) versus 1µg/ml PAF C-16 only was determined using non-parametric Mann Whitney test and the *p*-value was found to be significant, p=0.02.

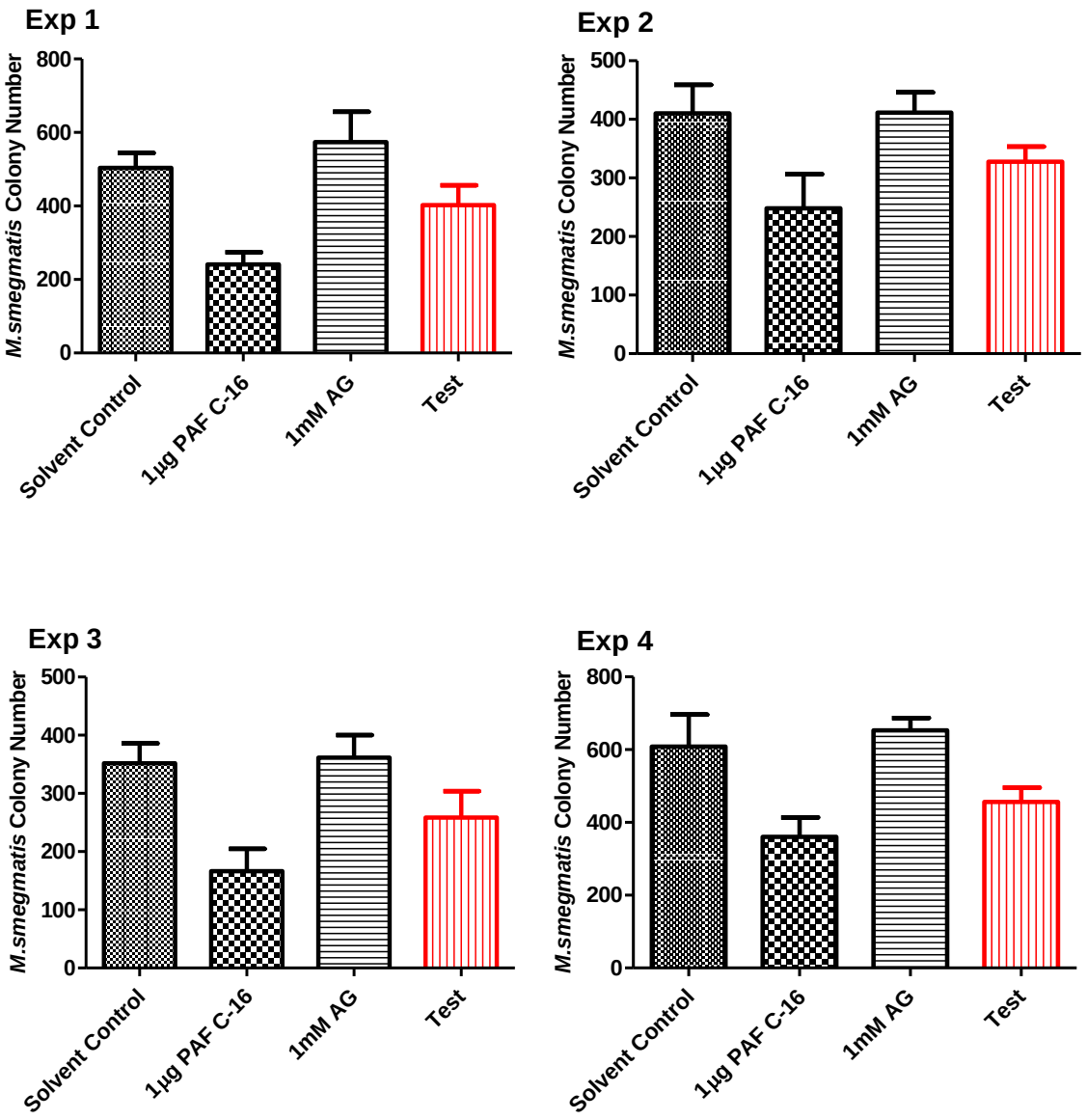


Figure 5.10a: Four individual experiments (Exp1, 2, 3 and 4) for the effect of Aminoguanidine hemisulfate on PAF C-16 induced intracellular *M.smegmatis* growth inhibition. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicate. **Test** consists of THP-1 cells treated with 1mM AG and 1µg/ml PAF C-16.

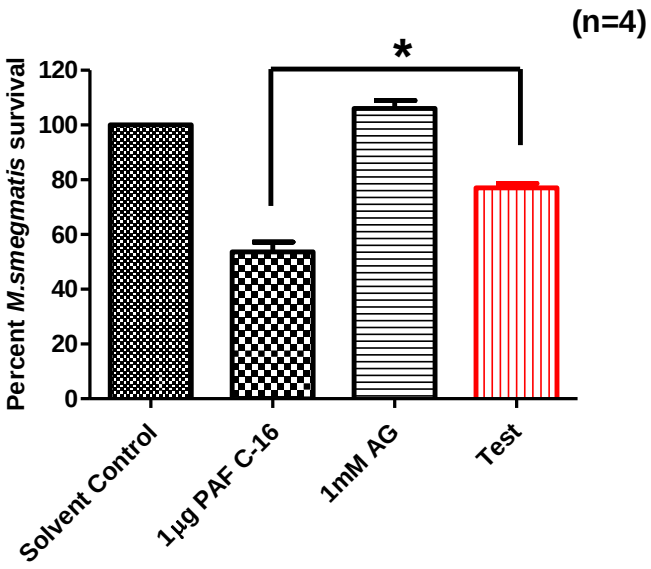


Figure 5.10b: Effect of Aminoguanidine hemisulfate prior treatment on PAF C-16 induced intracellular *M.smegmatis* growth inhibition. Each bar represents the average of four individual experiments and the error bars show standard error of means (SEM). The data is expressed in terms of percentage, where solvent control is considered as 100% survival and test conditions are compared to it. **Test** consists of THP-1 cells treated with 1mM AG and 1µg/ml PAF C-16. The *p*-value for Test (1mM AG and 1µg/ml PAF C-16) vs 1µg/ml PAF C-16 was calculated using Mann Whitey test and was significant, *(p=0.02).

5.11 Effect of TNF-α, IL-6, and IL-10 neutralization on PAF C-16 induced intracellular *M.smegmatis* growth inhibition

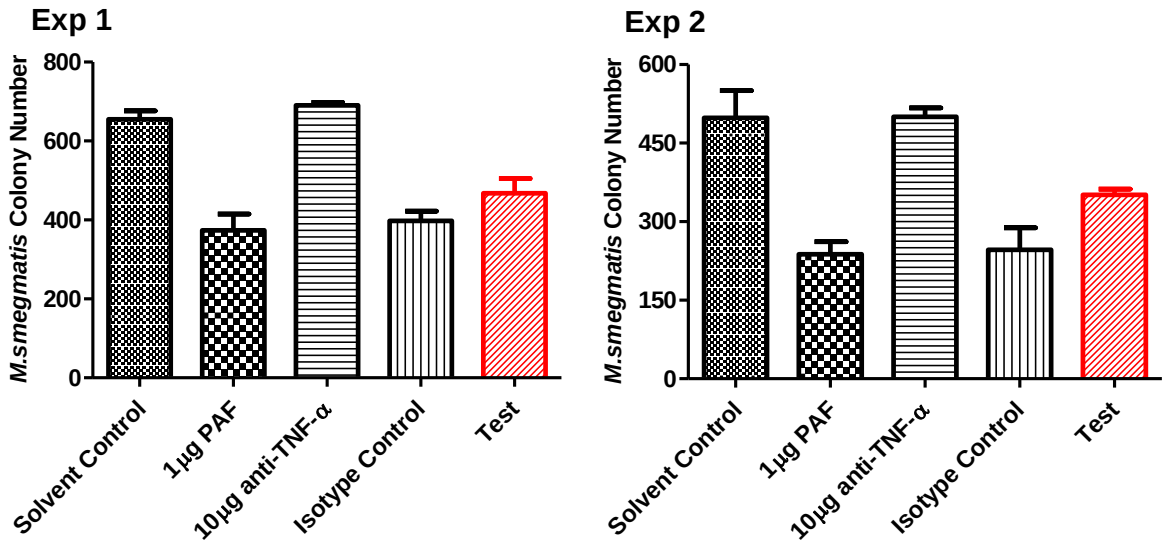
PAF C-16 can cause the production of cytokines such as IL-1, TNF-α etc. (Thivierge and Rola-Pleszczynski, 1992; Poubelle *et al*., 1991). Different cytokines have previously been shown to play important roles in facilitating the elimination of intracellular *mycobacteria*. Therefore, the role of a few cytokines in PAF C-16 induced growth inhibition of intracellular *M.smegmatis* was investigated by blocking them with neutralizing antibodies.

5.11.1 Effect of anti-TNF-α on PAF C-16 induced intracellular *M.smegmatis* growth inhibition

Four individual experiments (Exp1, 2, 3 and 4) were performed according to the protocol in chapter 2.10. *M.smegmatis* infected THP-1 cells were treated with 10µg/ml mouse anti-human TNF-α antibody and 1µg/ml PAF C-16 for 24 hours. Appropriate controls comprising of

M.smegmatis infected THP-1 cells treated with 2µl ethanol/ml, 1µl PAF C-16/ml, 10µg/ml mouse anti-human TNF-α antibody and isotype control (10µg/ml mouse IgG + 1µg/ml PAF C-16) were also included in the experiment.

All four experiments showed that treatment of 10µg/ml mouse anti-human TNF-α (neutralizing antibody) partially mitigated the PAF C-16 induced growth inhibition of intracellular *M.smegmatis* as indicated by an increase in the number CFUs compared to isotype control (10µg/ml mouse IgG + 1µg/ml PAF C-16) and control treated with 1µg/ml PAF C-16 only (Figure 5.11.1a). On average, intracellular *M.smegmatis* growth increased by 17% in the presence of 10µg/ml anti-TNF-α neutralizing antibody as compared to the isotype control condition and by 18.7% as compared to 1µg/ml PAF C-16 only condition (Figure 5.11.1b). The level of significance was determined using non-parametric Mann Whitney test, and was found significant, *p=0.02* for "Test" (10µg/ml anti-TNF-α and 1µg/ml PAF C-16) vs Isotype control (10µg/ml mouse IgG + 1µg/ml PAF C-16) and "Test" (10µg/ml anti-TNF-α and 1µg/ml PAF C-16) vs 1µg/ml PAF C-16 only.



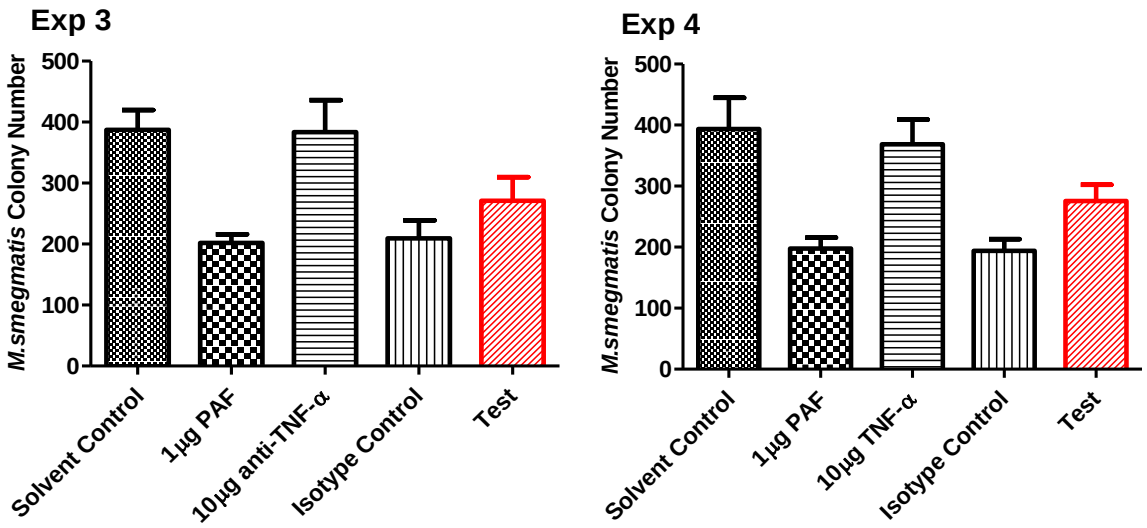


Figure 5.11.1a: Four individual experiments (Exp1, 2, 3 and 4) for the effect anti-TNF-α neutralizing antibody on PAF C-16 induced intracellular *M.smegmatis* growth inhibition. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicates. **Test** consists of 10µg/ml anti-TNF-α and 1µg/ml PAF C-16 and **Isotype control** consists of 10µg/ml mouse IgG + 1µg/ml PAF C-16.

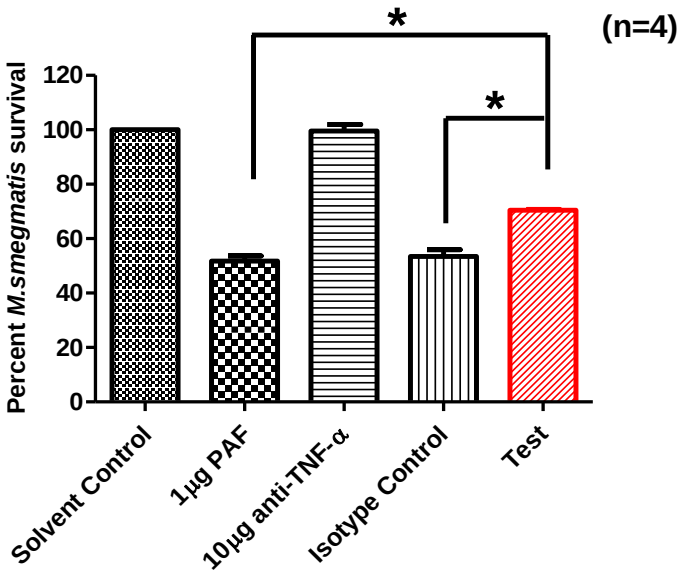


Figure 5.11.1b: Effect of anti-TNF-α neutralizing antibody on PAF C-16 induced intracellular *M.smegmatis* growth inhibition. Each bar represents the average of four individual experiments and the error bars show standard error of means (SEM). The data is expressed in terms of percentage, where solvent control is considered as 100% survival and test conditions are

compared to it. **Test** consists of 10µg/ml anti-TNF-α and 1µg/ml PAF C-16 and **Isotype control** consists of 10µg/ml anti-IgG + 1µg/ml PAF C-16. The level of significance for Test (10µg/ml anti-TNF-α and 1µg/ml PAF C-16) vs Isotype control (10µg/ml mouse IgG + 1µg/ml PAF C-16) and Test (10µg/ml anti-TNF-α and 1µg/ml PAF C-16) vs 1µg/ml PAF C-16 only was calculated by non-parametric Mann Whitney test, and was found significant, *(*p=0.02*).

5.11.2 Effect of anti-IL-6 on PAF C-16 induced intracellular *M.smegmatis* growth inhibition

Four individual experiments (Exp1, 2, 3 and 4) were performed according to the protocol in chapter 2.10. *M.smegmatis* infected THP-1 cells were treated with 10µg/ml rat anti-human IL-6 neutralizing antibody and 1µg/ml PAF C-16 for 24 hours. Appropriate controls comprising of *M.smegmatis* infected THP-1 cells treated with 2µl ethanol/ml, 1µl PAF C-16/ml, 10µg/ml rat anti-human IL-6 antibody and isotype control (10µg/ml rat IgG + 1µg/ml PAF C-16) were also included in the experimental design.

All four experiments showed that treatment of 10µg/ml mouse anti-human IL-6 (neutralizing antibody) showed no effect on PAF C-16 induced growth inhibition of intracellular *M.smegmatis* as indicated by a similar number CFUs compared to isotype control (10µg/ml rat IgG + 1µg/ml PAF C-16) and control treated with 1µg/ml PAF C-16 only (Figure 5.11.2a and Figure 5.11.2b). The level of significance was determined using non-parametric Mann Whitney test, and was found non-significant, p=0.68 and p=0.88 for "Test" (10µg/ml anti-IL-6 and 1µg/ml PAF C-16) vs Isotype control (10µg/ml rat IgG + 1µg/ml PAF C-16) and "Test" (10µg/ml anti-IL-6 and 1µg/ml PAF C-16) vs 1µg/ml PAF C-16 only.

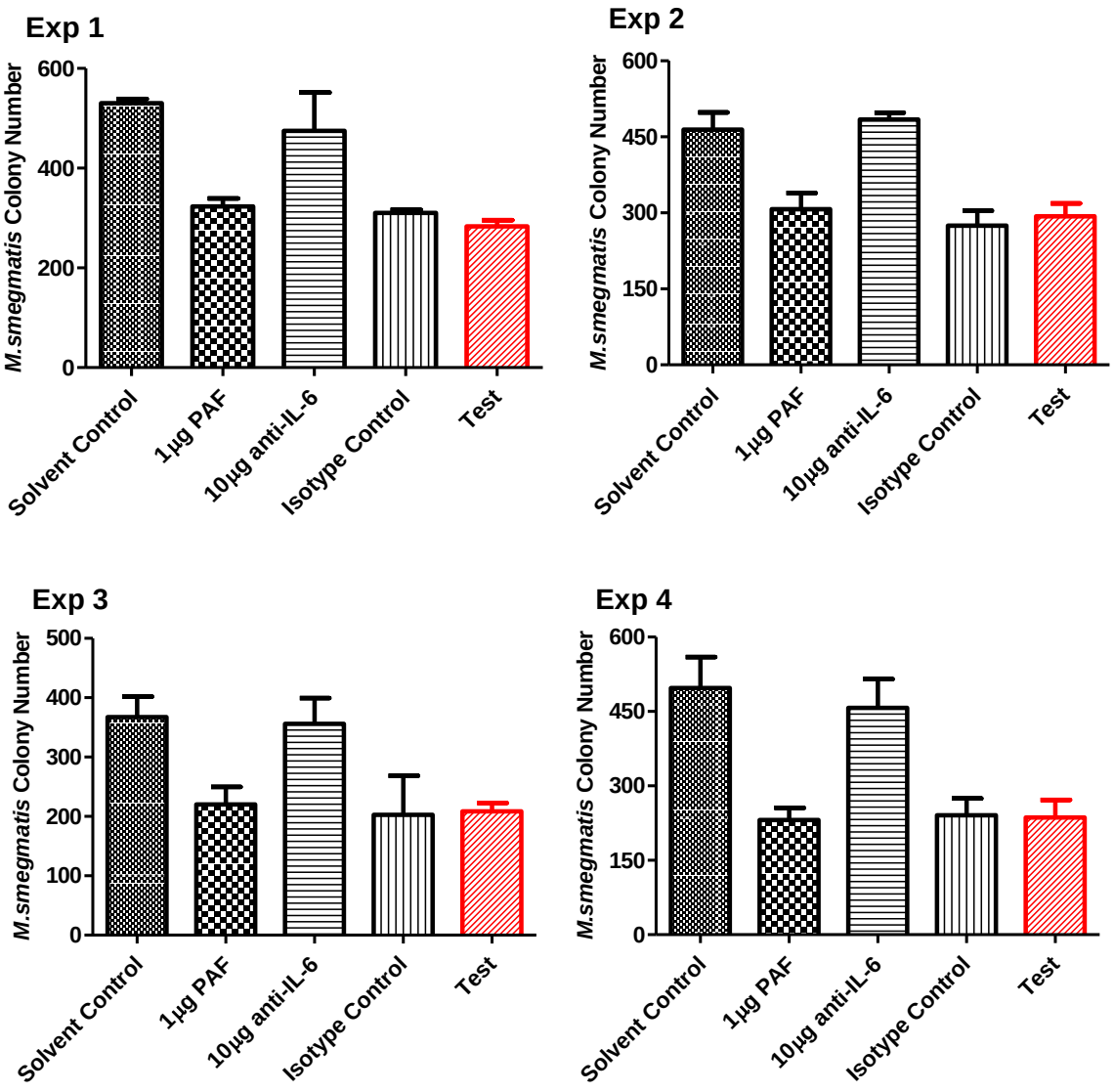


Figure 5.11.2a: Four individual experiments (Exp1, 2, 3 and 4) for the effect anti-IL-6 neutralizing antibody on PAF C-16 induced intracellular *M.smegmatis* growth inhibition. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicates. **Test** consists of 10µg/ml anti-IL-6 and 1µg/ml PAF C-16 v and **Isotype control** of 10µg/ml rat IgG + 1µg/ml PAF C-16.

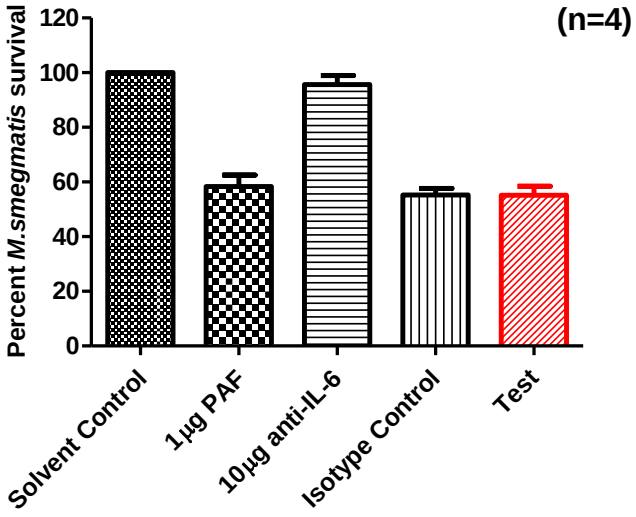


Figure 5.11.2b: Effect of anti-IL-6 neutralizing antibody on PAF C-16 induced intracellular *M.smegmatis* growth inhibition. Each bar represents the average of four individual experiments and the error bars show standard error of means (SEM). The data is expressed in terms of percentage, where solvent control is considered as 100% survival and test conditions are compared to it. **Test** consists of 10µg/ml anti-IL-6 and 1µg/ml PAF C-16 v and **Isotype control** of 10µg/ml rat IgG + 1µg/ml PAF C-16. The level of significance for Test (10µg/ml anti-IL-6 and 1µg/ml PAF C-16) vs Isotype control (10µg/ml rat IgG + 1µg/ml PAF C-16) and Test (10µg/ml anti-IL-6 and 1µg/ml PAF C-16) vs 1µg/ml PAF C-16 only was calculated by non-parametric Mann Whitney test, and was found non-significant, p=0.68 and p=0.88.

5.11.3 Effect of anti-IL-10 on PAF C-16 induced intracellular *M.smegmatis* growth inhibition

Four individual experiments (Exp1, 2, 3 and 4) were performed according to the protocol in chapter 2.10. *M.smegmatis* infected THP-1 cells were treated with 10µg/ml rat anti-human IL-10 neutralizing antibody and 1µg/ml PAF C-16 for 24 hours. Appropriate controls comprising of *M.smegmatis* infected THP-1 cells treated with 2µl ethanol/ml, 1µl PAF C-16/ml, 10µg/ml rat anti-human IL-10 antibody and isotype control (10µg/ml rat IgG + 1µg/ml PAF C-16) were also included in the experimental design.

All four experiments showed that treatment of 10µg/ml rat anti-human IL-10 (neutralizing antibody) showed no effect on PAF C-16 induced growth inhibition of intracellular *M.smegmatis* as indicated by a similar number CFUs compared to isotype control (10µg/ml rat

IgG + 1µg/ml PAF C-16) and control treated with 1µg/ml PAF C-16 only (Figure 5.11.3a and Figure 5.11.3b). The level of significance was determined using non-parametric Mann Whitney test, and was found non-significant, p=0.66 and p=0.73 for "Test" (10µg/ml anti-IL-10 and 1µg/ml PAF C-16) vs Isotype control (10µg/ml rat IgG + 1µg/ml PAF C-16) and "Test" (10µg/ml anti-IL-10 and 1µg/ml PAF C-16) vs 1µg/ml PAF C-16 only.

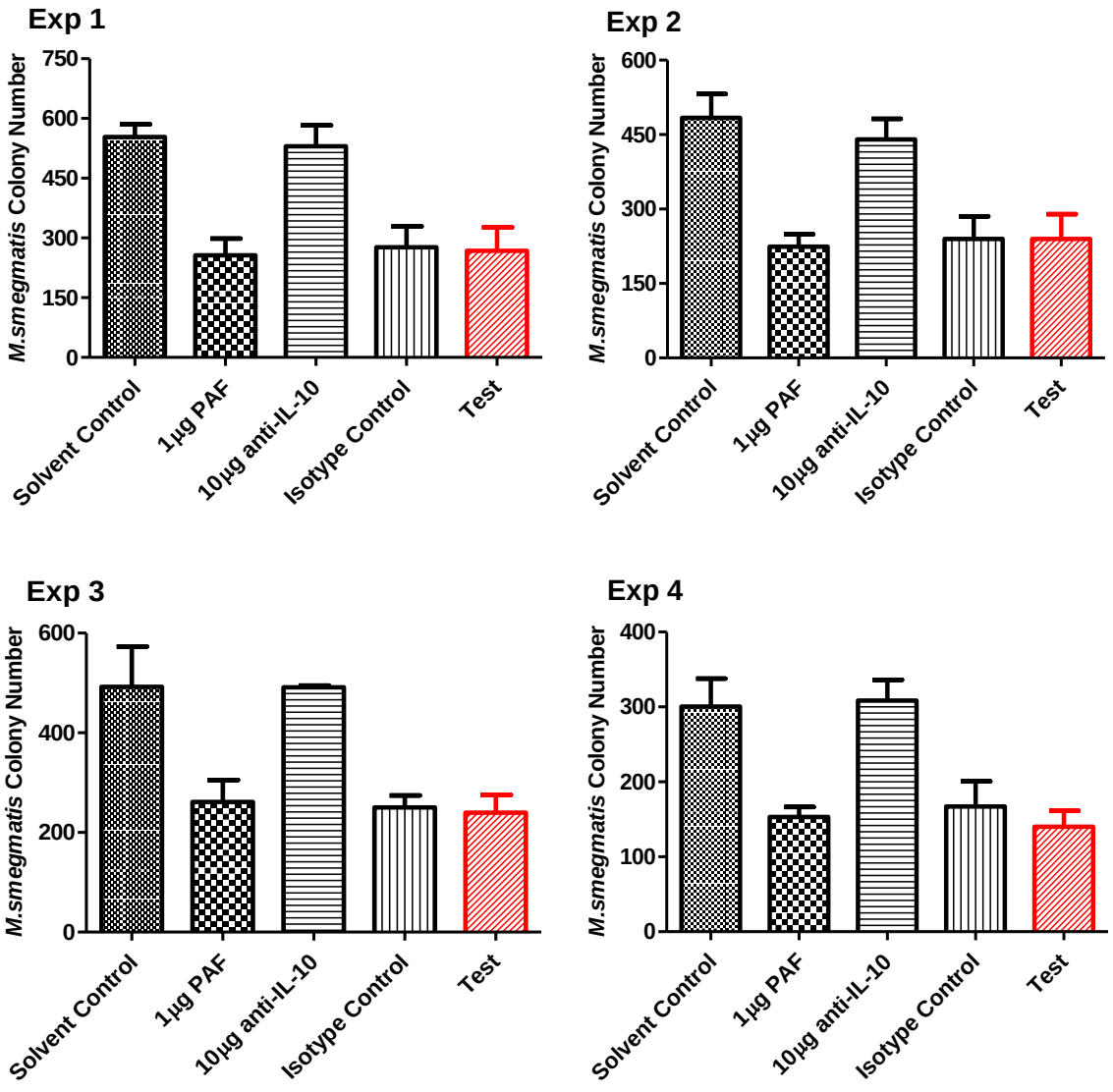


Figure 5.11.3a: Four individual experiments (Exp1, 2, 3 and 4) for the effect anti-IL-10 neutralizing antibody on PAF C-16 induced intracellular *M.smegmatis* growth inhibition. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bar shows standard deviation (SD) of triplicates. **Test** consists of 10µg/ml anti-IL-10 and 1µg/ml PAF C-16 and **Isotype control** consists of 10µg/ml rat IgG + 1µg/ml PAF C-16.

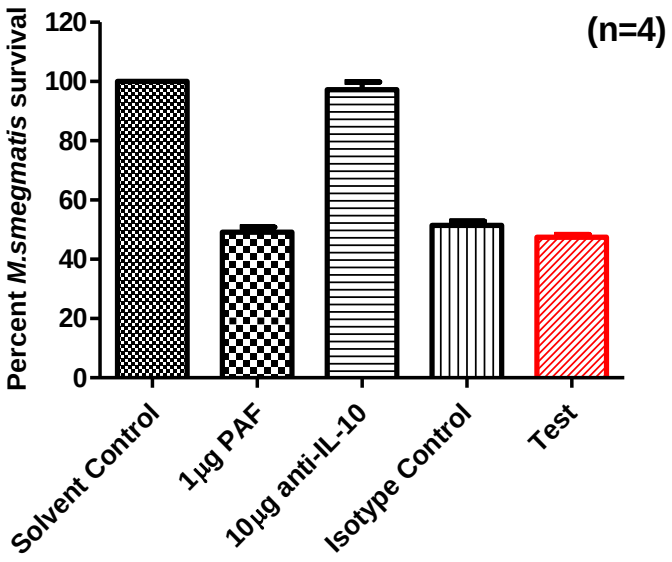


Figure 5.11.3b: Effect of anti-IL-10 neutralizing antibody on PAF C-16 induced growth inhibition of intracellular *M.smegmatis*. Each bar represents the average of four individual experiments and the error bars show standard error of means (SEM). The data is expressed in terms of percentage, where solvent control is considered as 100% survival and test conditions are compared to it. **Test** consists of 10µg/ml anti-IL-10 and 1µg/ml PAF C-16 and **Isotype control** consists of 10µg/ml anti-IgG + 1µg/ml PAF C-16. The level of significance for "Test" (10µg/ml anti-IL-10 and 1µg/ml PAF C-16) vs Isotype control (10µg/ml anti-IgG + 1µg/ml PAF C-16) and "Test" (10µg/ml anti-IL-10 and 1µg/ml PAF C-16) vs 1µg/ml PAF C-16 only was calculated by non-parametric Mann Whitney test, and was found to be non-significant, p=0.66 and p=0.73.

5.12 Effect of PAF C-16 on *M. bovis BCG* growth inside THP-1 cells

The effect of PAF C-16 on the growth of intracellular *M.bovis BCG* was investigated according to the protocol in chapter 2.7. Four individual experiments (Exp1, 2, 3 and 4) were performed by treating *M.bovis BCG* infected THP-1 cells with PAF C-16 in different concentrations (0.01µg, 0.1µg, 1µg, 5µg and 10µg/ml of cell suspension) for 24 hours. The effect of PAF C-16 treatment on the growth of intracellular *M.bovis BCG* was determined by comparing the number of CFUs from different PAF C-16 treated test conditions to the solvent control for PAF C-16 (2µl ethanol /ml cell suspension).

However, the results obtained were inconsistent and no particular trend of growth inhibition was observed during all four experiments performed (Figure 5.12a and Figure 5.12b). Statistical analysis of the results by applying non-parametric multiple comparison Kruskal Wallis test on ranks showed that the results were non-significant, p=0.57.

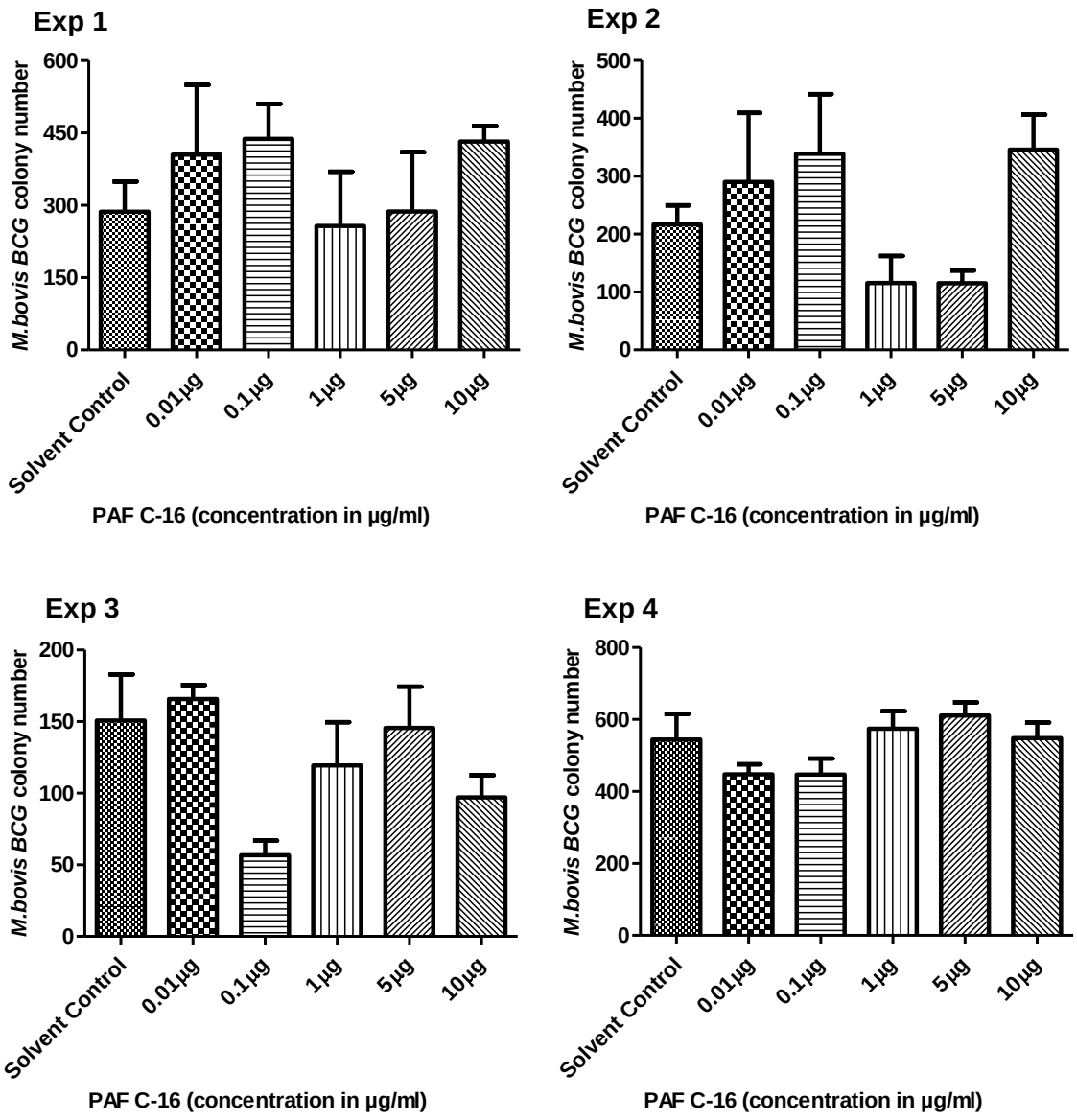


Figure 5.12a: Four individual experiments (Exp1, 2, 3 and 4) for the effect of PAF C-16 on intracellular *M.bovis BCG* growth. Each bar represents the average CFUs for triplicate plates and error bars show standard deviation (SD) of the triplicates.

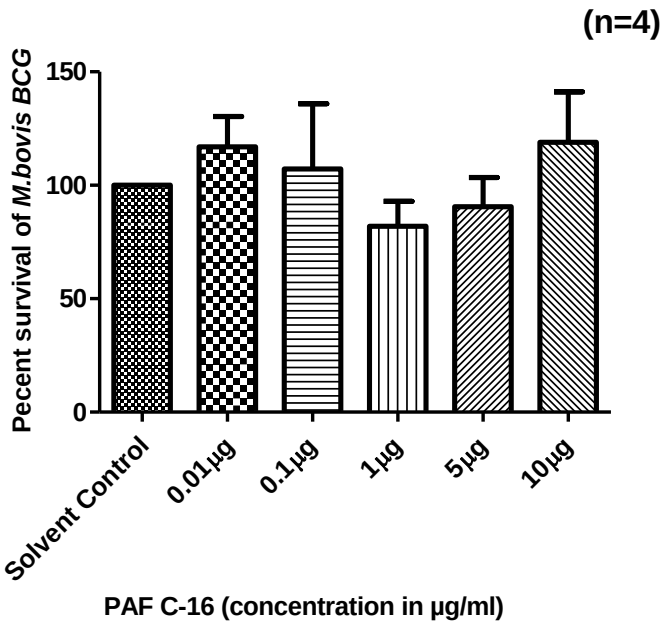


Figure 5.12b: Effect of PAF C-16 on intracellular *M.bovis BCG* growth. Each bar represents the average of four individual experiments and the error bars show standard error of means (SEM). The data is expressed in terms of percentage, where solvent control is taken as 100% survival and different PAF C-16 treated test conditions are compared to it. Level of significance was calculated by applying multiple comparison non-parametric Kruskal-Wallis test on ranks and was found to be non-significant, p=0.57.

5.13 Effect of PAF C-16 treatment with THP-1 on the phagocytosis of *M.smegmatis*

The effect of exogenous PAF C-16 on the phagocytosis of *M.smegmatis* by THP-1 cells was investigated according to the protocol in chapter 2.11. Four individual experiments (Exp1, 2, 3 and 4) were performed by treating THP-1 cells with different concentrations of PAF C-16 0.01µg, 0.1µg, 1µg and 5µg/ml for 2 hours. The PAF C-16 treated THP-1 cells were then incubated with *M.smegmatis* for 2 hour to allow phagocytosis of bacteria. Finally the effect of PAF C-16 treatment on phagocytosis of *M.smegmatis* was determined by lysing the THP-1 cells with intracellular *M.smegmatis* and comparing the number of CFUs from different PAF C-16 treated test conditions to the solvent control for PAF C-16 (1µl ethanol /ml cell suspension).

It was observed that PAF C-16 treatment of THP-1 cells decreased the phagocytosis of *M.smegmatis* during all four experiments (Figure 5.13a). On average, PAF C-16 treatment of

THP-1 cells at concentrations 0.01µg, 0.1µg, 1µg and 5µg/ml resulted in 78.1%, 79.3%, 80.5% and 74.4% decrease in the phagocytosis of *M.smegmatis* respectively as indicated by the number of CFUs when compared to the solvent control (Figure 5.13b). The level of significance was determined by applying non-parametric multiple comparison Kruskal Wallis test on ranks and the *p*-value was found to be significant, $p=0.036$. Individual data sets were compared by applying post hoc Dunn's multiple comparison test and 0.01µg, 0.1µg and 1µg/ml PAF C-16 vs Solvent control were found to be significant, $p \leq 0.05$.

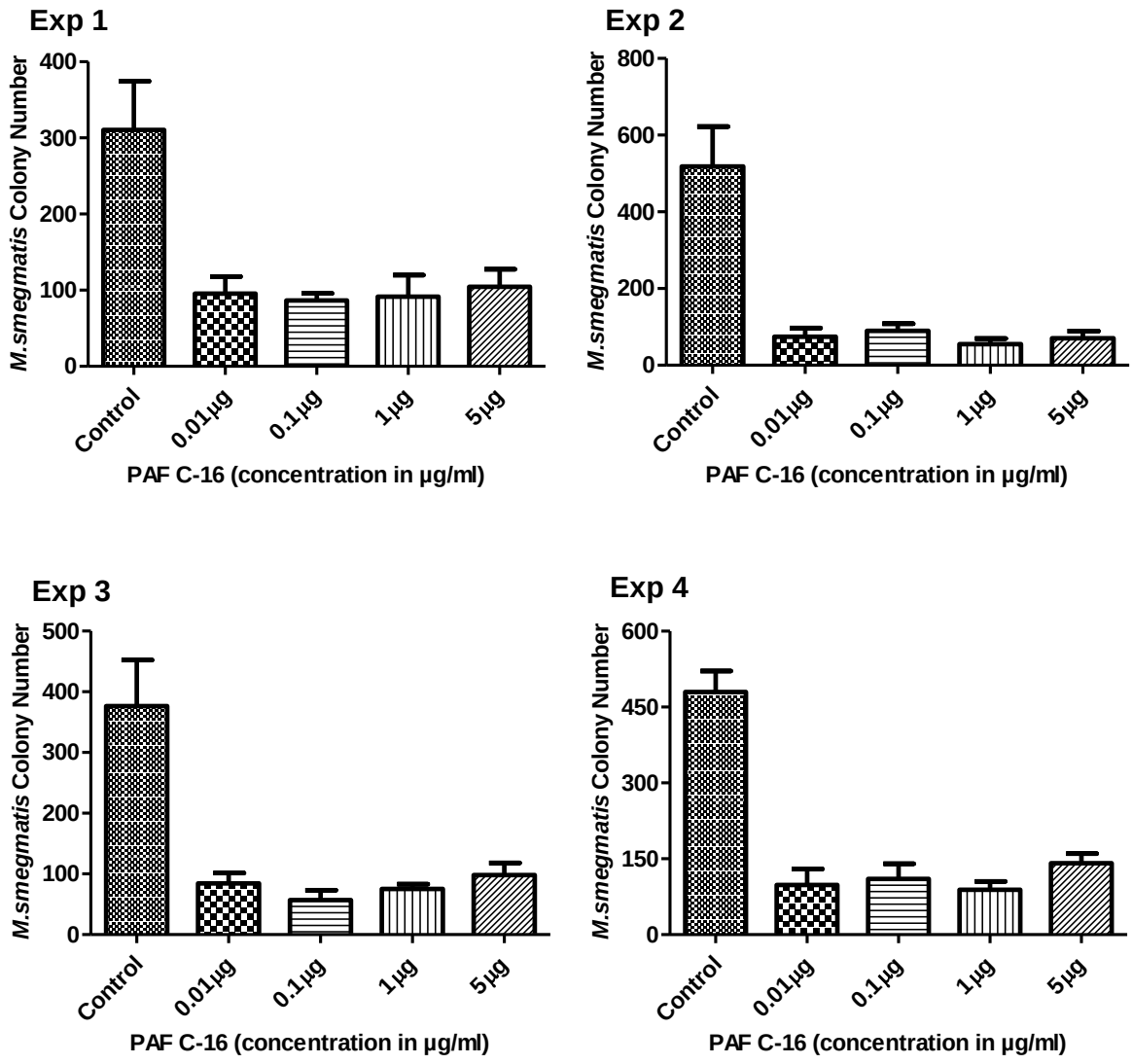


Figure 5.13a: Four individual experiments (Exp1, 2, 3, and 4) for the effect of PAF C-16 on THP-1 cells phagocytosis of *M.smegmatis*. Each bar represents the average number of *M.smegmatis* CFUs from triplicate plates and error bars show standard deviation (SD) of the triplicates.

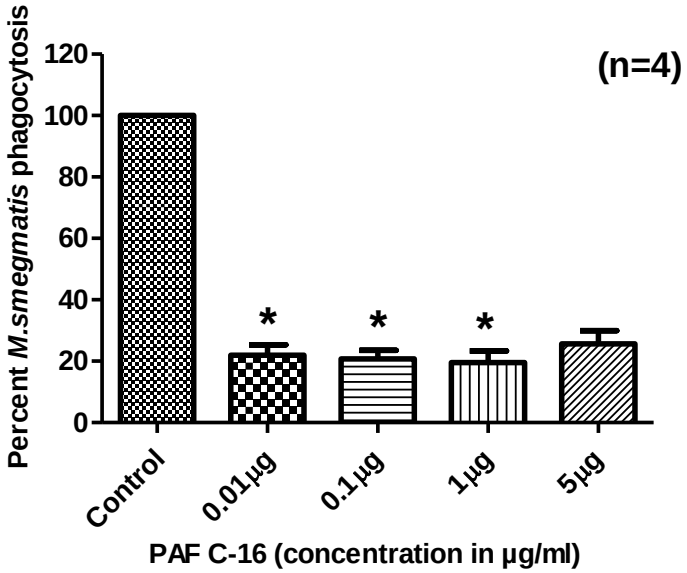


Figure 5.13b: Effect of PAF C-16 on THP-1 cells phagocytosis of *M.smegmatis*. Each bar represents the average of four individual experiments and the error bars show standard error of means (SEM). The data is expressed in terms of percentage, where solvent control is taken as 100% phagocytosis and different PAF C-16 treated test conditions are compared to it. Level of significance was calculated by applying multiple-comparison non-parametric Kruskal-Wallis test on ranks, p=0.036 and individual data sets were compared through post hoc Dunn's multiple comparison test where 0.01µg, 0.1µg and 1µg/ml PAF C-16 vs solvent control were significant, *($p \leq 0.05$)

5.14 Discussion

In this chapter, the effect of exogenous PAF C-16 on the growth of intracellular *mycobacteria* was investigated *in vitro*. Phagocytic THP-1 cells infected with *mycobacteria* were used as a model to investigate the growth inhibitory effect of PAF C-16. THP-1 cells are human leukaemia derived monocytic cells that mimic blood-derived monocytes and are a valuable tool for research because of their homogenous genetic background (Chanput *et al*., 2014). THP-1 cells have previously been used to study intracellular *mycobacteria* (Iona *et al*., 2012; Fontán *et al*., 2008; Rohan *et al*., 2008; Rajavelu and Das, 2007). THP-1 cells used during most of the previous studies were treated with phorbol 12-myristate 13-acetate (PMA) in order to differentiate them into macrophages. However, PMA treatment of THP-1 cells has been shown to cause the rearrangement of macrophage-specific kinome that leads to the activation of various genes such as IL-1β, IL-8 and TNF-α and hence make these THP-1 cells biased towards a more pro-inflammatory type of response (Richter *et al*., 2016). In this study, unmanipulated THP-1 cells were used to investigate the effect of PAF C-16 on the growth of intracellular *mycobacteria*. In our lab, we have previously shown that THP-1 cells without PMA stimulation can inhibit mycobacterial growth (Shawayat, 2017).

PAF C-16 can activate monocytes/macrophages through interaction with specific cell membrane receptors known as PAFR (Simon *et al*., 1994) and has been shown to stimulate the production of different cytokines such as TNF-α, IL-1β, reactive oxygen species and reactive nitrogen species in these cells (Muehlmann *et al*., 2012; Poubelle *et al*., 1991; Bonavida *et al*., 1989; Hartung *et al*., 1983) suggesting that PAF C-16 may play a protective role during pathogen infection. Therefore, we investigated the effect of exogenous PAF C-16 in different concentrations on the growth of *M.smegmatis* inside THP-1 cells. PAF C-16 was able to inhibit the growth of intracellular *M.smegmatis* only at a concentration of 1µg/ml. On average, 1µg/ml PAF C-16 treatment for 24 hours caused ~50% reduction ($p \leq 0.001$) in the growth of intracellular *M.smegmatis* as indicated by the number of surviving *M.smegmatis* CFUs when compared with the solvent control. PAF C-16 treatment at higher concentrations (5µg/ml and 10µg/ml) and lower concentrations (0.001µg, 0.01µg and 0.1µg/ml) failed to show any significant inhibition of intracellular *M.smegmatis* growth. An important observation from these experiments was the inability of higher doses of exogenous PAF C-16 to inhibit the growth of intracellular *M.smegmatis*, indicating that PAF C-16 acts in concentration-specific manner. These results are in line with some of the previous studies performed with PAF C-16. It was shown that human macrophages when treated with higher concentration (10^{-6} M) of

exogenous PAF C-16 showed reduced phagocytic activity as compared to those treated with lower concentrations (10^{-8} and 10^{-10} M) of PAF C-16 (Borges *et al*., 2017). Similarly, human monocytes showed maximum production of ROIs when stimulated with PAF C-16 at a particular concentration ($2x10^{-6}$M) and increasing the concentration of PAF C-16 ($5x10^{-6}$M) caused a decrease in ROIs production (Pustynnikov *et al*., 1991). In our study, trypan blue staining showed increased cell death in THP-1 cells after treatment with higher concentrations of PAF C-16, especially at 10µg/ml PAF C-16. These results indicated a negative effect for higher doses of PAF C-16 on the survival of THP-1 cell and can be a possible explanation for the lack of growth inhibitory effect of higher doses of PAF C-16. Four independent experiments were performed to investigate the effect of PAF C-16 on the growth of intracellular *M.bovis BCG*. However, no consistent trend of growth inhibition was observed during these experiments and the results were statistically non-significant. Several previous studies have shown that exogenous PAF C-16 can inhibit the growth of intracellular pathogens including protozoans such as *Leishmania donovani* (Lonardoni *et al*., 2000), *Leishmania braziliensis* (Borges *et al*., 2017), *Trypanosoma cruzi* (Aliberti *et al*., 1999) and fungi such as *Candida albicans* (Kim *et al*., 2008; Im *et al*., 1997) both *in vivo* and *in vitro*. PAF C-16 induced growth inhibition of these intracellular pathogens was shown to be associated with the enhanced production of ROIs, RNIs and cytokines such as TNF-α. There is very limited information regarding the effect of PAF C-16 on the growth of intracellular *mycobacteria*. A single previous study to investigate the role of endogenous PAF C-16 during *M.tb* infection in mice showed that there was no significant difference in mortality between PAFR deficient ($PAFR^{-/-}$) and wild-type control mice when infected with *M.tb* and both $PAFR^{-/-}$ and control mice showed similar *M.tb* loads in lungs and liver (Weijer *et al*., 2003). However, to the best of our knowledge, there is currently no information about the effect of exogenous PAF C-16 on the growth inhibition of intracellular *mycobacteria*.

The biological activity of PAF C-16 in eukaryotic cells can be affected by small modifications in its structure (Marathe *et al*., 2014). In chapter 4, it was shown that small changes in the structure of PAF C-16 such as changes in functional groups and increase in carbon chain length had no effect on the direct growth inhibition activity of PAF C-16 against *mycobacteria*. Therefore, the effect of small changes in the structure of PAF C-16 was also investigated on the growth inhibition of intracellular *M.smegmatis* using different PAF C-16 structure analogues.

Lyso-PAF is the precursor form of PAF C-16 that contains a hydroxyl group in place of the acetyl group at *sn*-2 position of the glycerol backbone. The enzyme PAF-AH tightly regulates the level of active PAF C-16 in the body and converts excess PAF C-16 into Lyso PAF (McIntyre *et al*., 2009). Due to the inability of Lyso PAF to perform most the biological functions associated with PAF C-16, this precursor analogue is mostly used as a control in experiments performed with PAF C-16 (Montrucchio *et al*., 2000). In this study, it was observed that exogenous Lyso PAF at concentrations 1µg, 5µg and 10µg/ml failed to inhibit the growth of intracellular *M.smegmatis* suggesting that the acetyl group at position *sn*-2 was important for the intracellular growth inhibitory effect of PAF C-16. These results were further confirmed by testing another synthetic PAF C-16 analogue, 2-O-methyl PAF for its ability to inhibit the growth of intracellular *M.smegmatis*. It was observed that 2-O-methyl PAF at concentrations 1µg, 5µg and 10µg/ml also did not show any inhibitory effect on the growth of intracellular *M.smegmatis*. In 2-O-methyl PAF the acetyl group at position *sn*-2 is replaced by a methyl group. These results further confirmed that the presence of acetyl group at the *sn*-2 position was important for the growth inhibition of intracellular *M.smegmatis*. Structure analogues with changes in functional group at *sn*-2position have previously been shown to lack different PAF C-16 associated activities such as aggregation of platelets (McManus *et al*., 1981) NO production from endothelial cells (Kikuchi *et al*., 2008), excitation of synaptic transmission in neuronal cells (Clark *et al*., 1992) and bronchial hyper responsiveness (Cuss *et al*., 1986) etc.

PAF C-18, a naturally occurring PAF C-16 analogue was also included in the study to investigate the effect of increase in the number of carbon atoms in the aliphatic carbon tail on the intracellular *M.smegmatis* growth inhibition. PAF C-18 has two additional carbon atoms in the aliphatic carbon tail attached at position *sn*-1 as compared to PAF C-16. PAF C-18 was able to inhibit the growth of intracellular *M.smegmatis*. PAF C-18 at a concentration of 1µg/ml was most potent and on average caused ~50% reduction in the growth of intracellular *M.smegmatis* when compared with the solvent control. Higher concentrations of PAF C-18 including 5µg/ml and 10µg/ml failed to show any significant inhibitory effect on the growth of intracellular *M.smegmatis*. Previous studies show that PAF C-18 is less potent in activation of platelet aggregation but has similar potency to PAF C-16 in activating guinea pig macrophages (Stewart and Grigoriadis, 1991). Our results showed that increase in the length of aliphatic carbon tail of PAF C-16 did not affect its intracellular growth inhibition potential as PAF C-18

showed similar level and pattern of intracellular *M.smegmatis* growth inhibition when compared to PAF C-16.

Hexanolamino PAF differs from PAF C-16 in the position of the terminal amino group which is linked by an additional 4-carbon atoms chain to the phosphate group. Hexanolamino PAF was tested for its ability to inhibit the growth of intracellular *M.smegmatis* and the results showed that this structure analogue inhibited the growth of intracellular *M.smegmatis* at a concentration of 5µg/ml. On average, Hexanolamino PAF treatment at 5µg/ml resulted in 42.6% reduction in the growth of intracellular *M.smegmatis*, whereas the other two concentrations of Hexanolamino PAF (1µg/ml and 10µg/ml) failed to show any significant inhibition of intracellular *M.smegmatis*. Hexanolamino PAF has previously been shown to act as both PAF C-16 antagonist and agonist. Hexanolamino PAF inhibited PAF C-16 stimulated production of ROS by human macrophage (Rouis *et al*., 1988) and acted as a partial PAF C-16 agonist in guinea pig macrophages (Stewart and Grigoriadis, 1991). Our results with Hexanolamino PAF showed that changing the position of terminal amino group of PAF C-16, reduced the growth inhibition potency due to which an increased concentration was required for growth inhibition of intracellular *M.smegmatis*.

PAF C-16 performs most of its functions by binding to G-protein coupled receptors, known as PAF receptor (PAFR) present on the cell membrane of target cells in mammals (Nakamura *et al*., 1991). To investigate the role of PAF C-16 signalling through PAFR during the intracellular growth inhibition of *M.smegmatis*, PAFR antagonists were used. PAFR antagonists are compounds that can bind to PAFR and reduce PAF C-16 activity by blocking PAFR (Singh *et al*., 2012). Treatment of *M.smegmatis* infected THP-1 cells with highly potent PAFR antagonists, ABT-491 or WEB-2086 prior to PAF C-16 treatment, increased the growth of intracellular *M.smegmatis* by 20-24% on average when compared with the *M.smegmatis* infected THP-1 cells treated with 1µg/ml PAF C-16 only, suggesting that PAF C-16 induced growth inhibition of intracellular *M.smegmatis* was due to signalling through its receptor, PAFR. PAF receptor antagonist ABT-491 has been shown to be highly effective in suppressing PAF C-16 induced platelets degranulation and other PAF C-16 mediated pathological conditions such as inflammation, hypotension and lethal effects in rat and guinea pig models (Albert *et al*., 1997). WEB-2086 is also a potent PAF receptor antagonist and has been shown to inhibit PAF C-16 induced important activities like platelet aggregation, hypotension and vascular permeability in rats (Clavijo *et al*., 2001).

Binding of PAF C-16 to PAFR results in the activation of intracellular signalling components. Therefore, we further investigated the role of PAF C-16 intracellular signalling pathway components such as PI-PLC, $cPLA_2$ and different second messengers in PAF C-16 induced growth inhibition of intracellular *M.smegmatis*. Binding of PAF C-16 to its receptor on the target cell results in the activation of a membrane bound enzyme, Phosphoinositide Phospholipase C (PI-PLC) through the associated G-proteins (Shukla, 1992). Treatment of *M.smegmatis* infected THP-1 cells with PLC inhibitor, U-73122 partially mitigated the inhibitory effect of PAF C-16 on the growth of intracellular *M.smegmatis*. On average, U-73122 treatment at a concentration of 2µM increased the number of intracellular *M.smegmatis* by 25.2 % as compared to *M.smegmatis* infected THP-1 cells treated with 1µg/ml PAF C-16 only. During PAF C-16 signalling pathway, the activated PLC causes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and further leads to the production of second messengers Diacylglycerol (DAG) and Inositol triphosphate (IP_3) (Ishii and Shimizu, 2000). Since these second messengers are produced inside the cell and may come in contact with the intracellular *M.smegmatis*, therefore we investigated one of the second messenger, DAG for its direct inhibitory effect on the growth of *M.smegmatis*. However, DAG did not show any direct inhibitory effect on the growth of *M.smegmatis* in different concentrations (10µg, 25µg, 50µg and 100µg/ml). DAG consists of two carbon chains attached to a glycerol backbone via ester bonds and was included in the study to check whether compounds with two carbon chains attached via ester bond can directly inhibit the growth of *mycobacteria*.

PAF C-16 binding to its receptor also causes the activation of cytosolic phospholipase A_2 ($cPLA_2$) due to increased levels of intracellular Ca^{+2} (Ishii and Shimizu, 2000). Treatment of *M.smegmatis* infected THP-1 cells with $cPLA_2$ inhibitor, Benzenesulfonamide at concentrations of 23nM (IC_{50}) and 56nM ($2IC_{50}$) partially mitigated the growth inhibitory effect of PAF C-16 and increased the survival of intracellular *M.smegmatis* by 25.5% and 30.1% respectively when compared to the *M.smegmatis* infected THP-1 cells treated with 1µg/ml PAF C-16 only. Activated $cPLA_2$ causes the production of arachidonic acid from phospholipids inside the cell (Nakashima *et al*., 1989). In our study, arachidonic acid at a concentration as low as 2.5µg/ml showed direct inhibitory effect on the growth of *M.smegmatis*. These results showed that PAF C-16 signalling via PAFR leads to the production of an intermediate intracellular molecule, arachidonic acid, which possess direct growth inhibition capacity and might be involved in the growth inhibition of intracellular *M.smegmatis*.

Apoptosis is one of the host protective mechanisms against intracellular pathogens that involves programmed cell death resulting in the elimination of the pathogen (Behar *et al*., 2011). It has been shown that intracellular *mycobacteria* can be eliminated by macrophages through apoptosis (Lee *et al*., 2006). Flow cytometry was performed to detect PAF C-16 induced apoptosis in *M.smegmatis* infected THP-1 cells. However, the results showed that PAF C-16 was unable to induce apoptosis in THP-1 cells containing intracellular *M.smegmatis* and that apoptosis had no role in the PAF C-16 induced growth inhibition of intracellular *M.smegmatis*. Previously PAF C-16 has been shown to cause apoptosis in different cells such as human epidermal cells (Barber *et al*., 1998), enterocytes (Lu *et al*., 2004) and neuronal cells (Ryan *et al*., 2007).

NO and RNIs such as peroxynitrite have a damaging effect on *mycobacteria* (Jamaati *et al*., 2017; Long *et al*., 1999). The role of reactive nitrogen species in PAF C-16 induced intracellular growth inhibition of *M.smegmatis* was investigated by using a potent iNOS inhibitor, Aminoguanidine hemisulfate (AG). Treatment of *M.smegmatis* infected THP-1 cells with 1mM AG along with 1µg/ml PAF C-16 increased the survival of intracellular *M.smegmatis* by 23.4% on average as compared to 1µg/ml PAF C-16 only treated *M.smegmatis* infected THP-1 cells. PAFC-16 has previously been shown to cause the production of NO and RNIs in murine cells including monocytes, macrophages and Kupper cells through activation of inducible nitric oxide synthase (iNOS) enzyme (Mustafa *et al*., 1996; Szabo *et al*., 1993). It was shown that PAF C-16 treatment of *T.cruzi* infected mouse macrophages resulted in the inhibition of intracellular parasite due PAF C-16 induced NO production in these cells (Aliberti *et al*., 1999).

We also investigated the role of different cytokines (TNF-α, IL-6 and IL-10) in the intracellular growth inhibition of *M.smegmatis* by using neutralizing antibodies against these cytokines. It was observed that the addition of 10µg/ml TNF-α neutralizing antibody (mouse anti human-TNF-α) to *M.smegmatis* infected THP-1 cells partially mitigated the growth inhibitory effect of PAF C-16 on intracellular *M.smegmatis* as indicated by increase in the number of CFUs when compared to 1µg/ml PAF C-16 only treated control. It has been shown that PAF C-16 can stimulate macrophages to produce TNF-α (Ruis *et al*., 1991; Dubois *et al*., 1989). The protective role of TNF-α during *M.tb* infection is well established (Keane *et al*., 2001; Mohan *et al*., 2001; Bean *et al*., 1999; Flynn *et al*., 1995). Several studies have shown that TNF-α helps in the induction of NO by activation of iNOS genes in macrophages and causes the elimination of intracellular pathogens such as *L.major* (Fonseca *et al*., 2003) and *T.cruzi* (Silva *et al*., 1995).

Additionally, two more cytokines, IL-6 and IL-10 were also investigated for their role in PAF C-16 induced intracellular *M.smegmatis* growth inhibition by using neutralizing antibodies (rat anti human IL-6 and anti human IL-10) against these cytokines. However, these neutralizing antibodies showed no effect on the PAF C-16 induced growth inhibition of intracellular *M.smegmatis*, suggesting that IL-6 and IL-10 are not involved in the PAF C-16 induced growth inhibition of *M.smegmatis* inside THP-1 cells. PAF C-16 enhances the production of IL-6 in mouse macrophages (Thivierge and Rola-Pleszczynski, 1992) and IL-6 has been shown to have a protective role during *M.tb* infection in mice models (Ladel *et al*., 1997). However, *in vitro* IL-6 was also shown to promote the growth of *M.avium* by inhibiting the production of IL-1 and TNF-α (Schindler *et al*., 1990). IL-10 is an anti-inflammatory cytokine that supresses the release of different pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 etc. from activated immune cells such as macrophages (Moore *et al*., 1993). Interestingly, IL-10 has been shown to induce the synthesis of PAF C-16 in human monocytes (Bussolati *et al*, 1997) and upregulates the expression of PAFR in monocytes and neutrophils (Thivierge *et al*., 1999).

Finally, the effect of exogenous PAF C-16 on the phagocytic activity of THP-1 cells was investigated. It was observed that THP-1 cells treated with PAF C-16 in different concentrations showed decreased phagocytosis of *M.smegmatis* when compared with the solvent control. On average PAF C-16 treatment at concentrations 0.01µg, 0.1µg, 1µg and 5µg/ml of THP-1 cells for 2 hours reduced the phagocytosis of *M.smegmatis* by ≥70% on average. These results are contradictory to some of the previous studies where exogenous PAF C-16 treatment enhanced the phagocytic activity of immune cell such as macrophages and neutrophils (Borges *et al*., 2017; Muehlmann *et al*., 2012; Ichinose *et al*., 1994).

In conclusion, this study showed that exogenous PAF C-16 was able to inhibit the growth of intracellular *M.smegmatis* in phagocytic THP-1 cells at a specific concentration of 1µg/ml. The presence of acetyl group at *sn*-2 position of the glycerol backbone was shown to be important for the growth inhibition of intracellular *M.smegmatis*. The use of PAF receptor antagonists and blocking PAF C-16 intracellular signalling pathway components suggested that the growth inhibition of intracellular *M.smegmatis* was mediated by PAF C-16/PAFR signalling. Arachidonic acid a product of the PAF C-16 signalling pathway showed direct inhibitory effect on the growth of *M.smegmatis*. Neutralization of iNOS and TNF-α partially mitigated the growth inhibitory effect of PAF C-16, suggesting their involvement in PAF C-16 induced growth inhibition of intracellular *M.smegmatis*. Finally, treatment of THP-1 cells with PAF C-16 resulted in reduced phagocytosis of *M.smegmatis*. The data from PAF C-16 and its structure

analogs for their inhibitory effect on the growth of intracellular *M.smegmatis* is summarized in table 5.1.

Table 5.1: Summary for inhibitory effect of PAF C-16 and its analogs on the growth of *M.smegmatis* inside THP-1 cells.

Compound	**Results from our study**	**Results from other groups**
	Inhibits Intracellular *M.smegmatis*	**Inhibits other intracellular pathogens**
PAF C-16	**+**	**T.cruzi & *L.donovani* (Aliberti *et al*., 1999; Lonardoni *et al*., 2000)**
Lyso PAF	**–**	**Not investigated**
2-O-methyl PAF	**–**	**Not investigated**
PAF C-18	**+**	**Not investigated**
Hexanolamino PAF	**+**	**Not investigated**

"+" indicates intracellular growth inhibition and "–" indicates lack of intracellular growth inhibition.

Chapter 6: Conclusions and future perspectives

6.1 Concluding remarks and future perspectives

In this study, different endogenous host factors (proteins and non-proteins) namely, transferrin, lactoferrin, fibrinogen, C-reactive protein (CRP), vitronectin, plasminogen, α-2-macroglobulin (α2M), low-density lipoprotein (LDL), high-density lipoprotein (HDL), serotonin, L-alpha dipalmitoylphosphatidylcholine (DPPC) and platelet activating factor C-16 (PAF C-16) were investigated *in vitro* for their inhibitory effect on the growth of *mycobacteria*. Non-pathogenic mycobacterial species, *M.smegmatis* and *M.bovis BCG* were used as model organisms for *M.tb*.

As a result of this screening, a phospholipid compound, PAF C-16, was identified, which inhibited the growth of both *M.smegmatis* and *M.bovis BCG* in a dose and time-dependent manner. The growth inhibitory effect of PAF C-16 seemed to be due to its damaging effect on the mycobacterial cell membrane, as indicated by microscopy and flow cytometry. The growth inhibitory effect of PAF C-16 was partially mitigated by membrane stabilizing compounds such as α-tocopherol, tween-80 and tween-20. The bacterial membrane damaging results presented in this thesis can be further validated by performing electron microscopy with PAF C-16 treated *M.smegmatis* and *M.bovis BCG* in order to physically visualize the mycobacterial cell membrane and observe the nature of PAFC-16 induced damage.

Structurally, PAF C-16 consists of a glycerol backbone with a 16 carbon atoms tail attached at position *sn*-1 via an ether bond, an acetyl group at position *sn*-2 and a phosphocholine group attached to the *sn*-3 position. In this study, the attachment of carbon tail to the glycerol backbone via ether bond was shown to be important for the direct growth inhibitory effect of PAF C-16 on *mycobacteria*, as suggested by the use of compound 1-O-hexadecyl-*sn*-glycerol. Two additional compounds, hexadecyl lactate and miltefosine, having similar carbon tails as PAF C-16 but attached via ester bond, were also able to directly inhibit the growth of *M.smegmatis*. To the best of our knowledge, these compounds have previously not been investigated for their direct effect on the growth of *mycobacteria*. Miltefosine is currently used for the treatment of Leishmaniasis and has a good safety profile in humans (Jha *et al*., 1999). Similarly, hexadecyl lactate has also been shown to be safe in humans and is used as an additive in food, medicines and personal care products (Zhang *et al*., 2010; Clary *et al*., 1998). Therefore, these compounds need further investigation both for their growth inhibitory effect on *M.tb* directly as well as on the growth of intracellular *M.tb* and *in vivo* using animal models. The proposed mechanism for the direct M.smegmatis growth inhibition by PAF C-16 is given in figure 6.1.

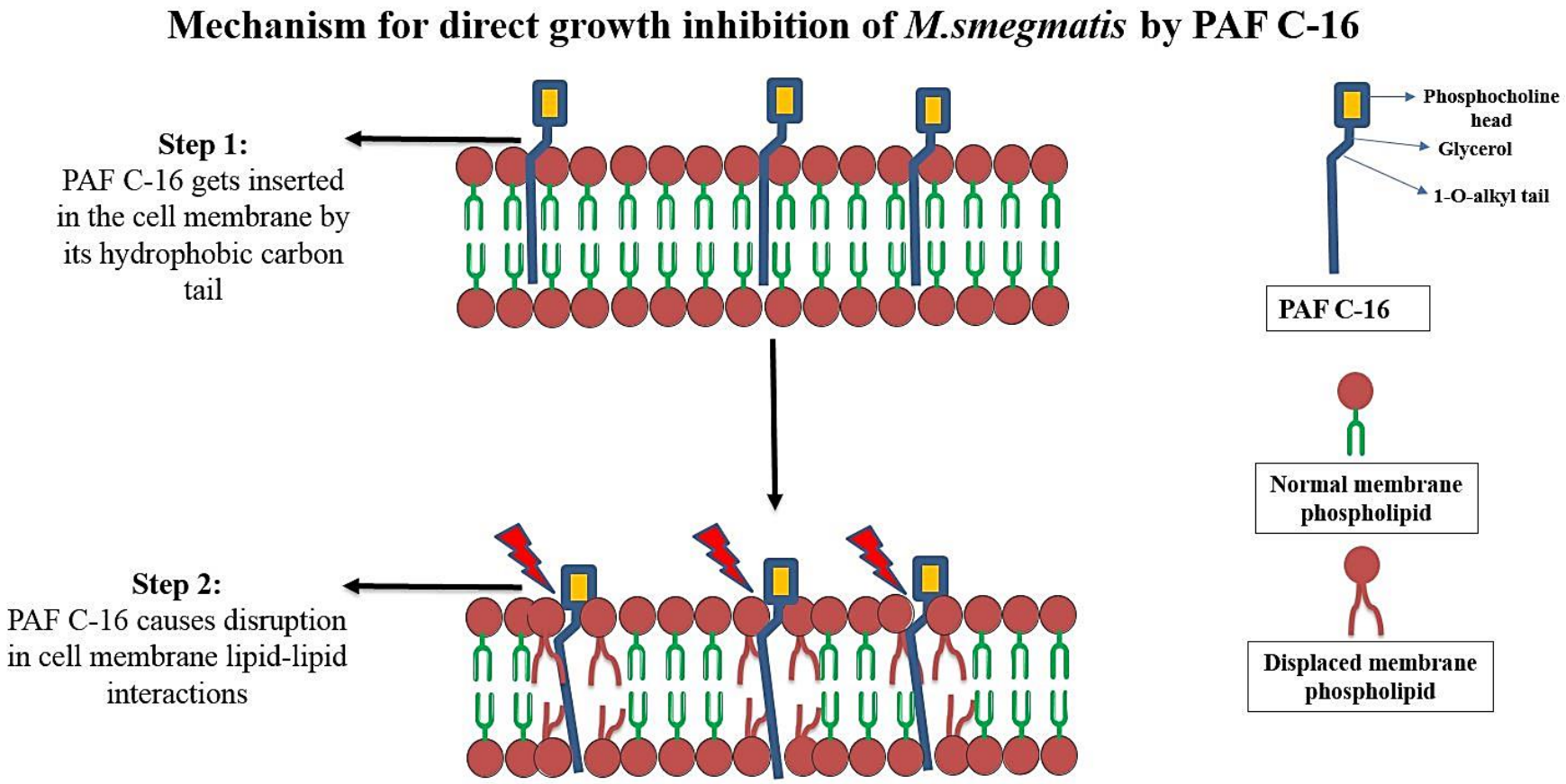


Figure 6.1: Proposed mechanism for direct *M.smegmatis* growth inhibition by PAF C-16. PAF C-16 interact with the bacterial cell membrane via its hydrophobic carbon tail and gets inserted in the lipid membrane. It is hypothesized that the oxygen atom at the sn-2 position produces a kink in the carbon tail of PAF C-16. This kink carbon tail of PAF C-16 pushes the phospholipid molecule apart after insertion and disrupts the lipid-lipid interaction of the bacterial cell membrane resulting in the formation of pores. This pore formation causes to the efflux of cytoplasmic content and changes in osmotic pressure inside the bacteria which ultimately leads to bacterial death.

Different PAF C-16 structure analogues including Lyso PAF, PAF C-18, Hexanolamino PAF, 2-O-methyl PAF and Pyrrolidino PAF were also investigated *in vitro* to ascertain if small changes in the structure of PAF C-16 modulated its growth inhibitory potency against *mycobacteria*. Previously, it has been shown that the biological activity of PAF C-16 in mammalian systems can be affected to varying degree by small modifications in its structure (Marathe *et al*., 2014; Prescott *et al*., 2000). However, in the current study, PAF C-16 structure analogues including Lyso PAF, PAF C-18, Hexanolamino PAF, 2-O-methyl PAF and Pyrrolidino PAF were able to directly inhibit the growth of both *M.smegmatis* and *M.bovis BCG* in culture at level comparable to PAF C-16. Among the PAF C-16 structure analogues, the naturally produced precursor form Lyso PAF is of particular importance since it is considered to be biologically inactive and lacks most of the pathological functions associated with PAF C-16. Therefore, Lyso PAF can be investigated further for its direct growth inhibitory effect against *M.tb* in culture and *in vivo* using animal models. However, there could be certain limitations associated with testing Lyso PAF as a therapeutic agent for treating mycobacterial infections *in vivo*, as there is a possibility of its conversion into the active form PAF C-16 by host enzyme PAF acetyltransferase. Colard *et al*. showed that mast cells from mice were able to convert exogenous Lyso PAF added to the cell culture to active form, PAF C-16 (Colard *et al*., 1993). Similarly, other PAF C-16 structure analogues such as PAF C-18 and Hexanolamino PAF also need *in vitro* and *in vivo* testing for their growth inhibitory effect on *M.tb.*

In our investigation, PAF C-16 was also found to inhibit the growth of intracellular *M.smegmatis* in THP-1 cells at a specific concentration of 1µg/ml. PAF receptor antagonists (ABT-491 and WEB-2086) partly mitigated the inhibitory effect of PAF C-16 on the growth of intracellular *M.smegmatis*, suggesting that the growth inhibitory effect of PAF C-16 was due to its binding and intracellular signalling via its receptor, PAFR on THP-1 cells. It seemed that the acetyl group at the *sn*-2 position of glycerol backbone of PAF C-16 is important for growth inhibition of *M.smegmatis* inside phagocytic cells. This study showed that changing the acetyl functional group at position *sn*-2 in PAF C-16 could lead to the loss of its ability to inhibit the growth of intracellular *M.smegmatis*. For example, the precursor form Lyso PAF and a synthetic PAF C-16 structure analogue, 2-O-methyl PAF lacking the acetyl group at position *sn*-2 were unable to inhibit the growth of intracellular *M.smegmatis*. These results are in agreement with previous studies which show that the presence of acetyl group at position *sn*-2 is important for most of the biological activities of PAF C-16 (Kikuchi *et al*., 2008; Clark *et al*., 1992; Cuss *et al*., 1986; McManus *et al*., 1981).

Binding of PAF C-16 to its G protein-coupled receptors (PAFR) on the target cells activate intracellular signalling pathways (Honda *et al*., 2002; Ishii *et al*., 2002). PAF C-16 intracellular signalling pathway components, phospholipase C (PLC) and phospholipase A_2 (PLA_2), were shown to be important for the growth inhibition of *M.smegmatis* inside phagocytic cells in this study, as blocking these components resulted in increased survival of the intracellular *M.smegmatis*. Arachidonic acid, a product of the PAF C-16 signalling pathway was shown to be able to directly inhibit the growth of *M.smegmatis* in culture, indicating its potential role in the growth inhibition of intracellular *M.smegmatis*. In addition, inhibition of iNOS enzyme and antibody neutralisation of the pro-inflammatory cytokine, TNF-α, suggested that these factors were also associated with PAF C-16 induced mycobacterial growth inhibition inside phagocytic cells. Both iNOS activation and TNF-α have previously been shown to play an important role in the elimination of intracellular *mycobacteria* (Jamaati *et al*., 2017; Keane *et al*., 2001; Mohan *et al*., 2001; Chan *et al*., 1992). Our results support previous studies where exogenous PAF C-16 has been shown to inhibit the growth of different pathogens such as Gram-positive bacteria directly in culture (Steel *et al*., 2002), *Leishmania donovani* inside human macrophages (Lonardoni *et al*., 2000), and *Trypanosoma cruzi* (Aliberti *et al*., 1999) and *Candida albicans* in mice models (Im *et al*., 1997) indicating the potential protective role of PAF C-16 during the host infection with pathogens.

The proposed mechanism for PAF C-16 induced growth inhibition of intracellular *M.smegmatis* is given in figure 6.2

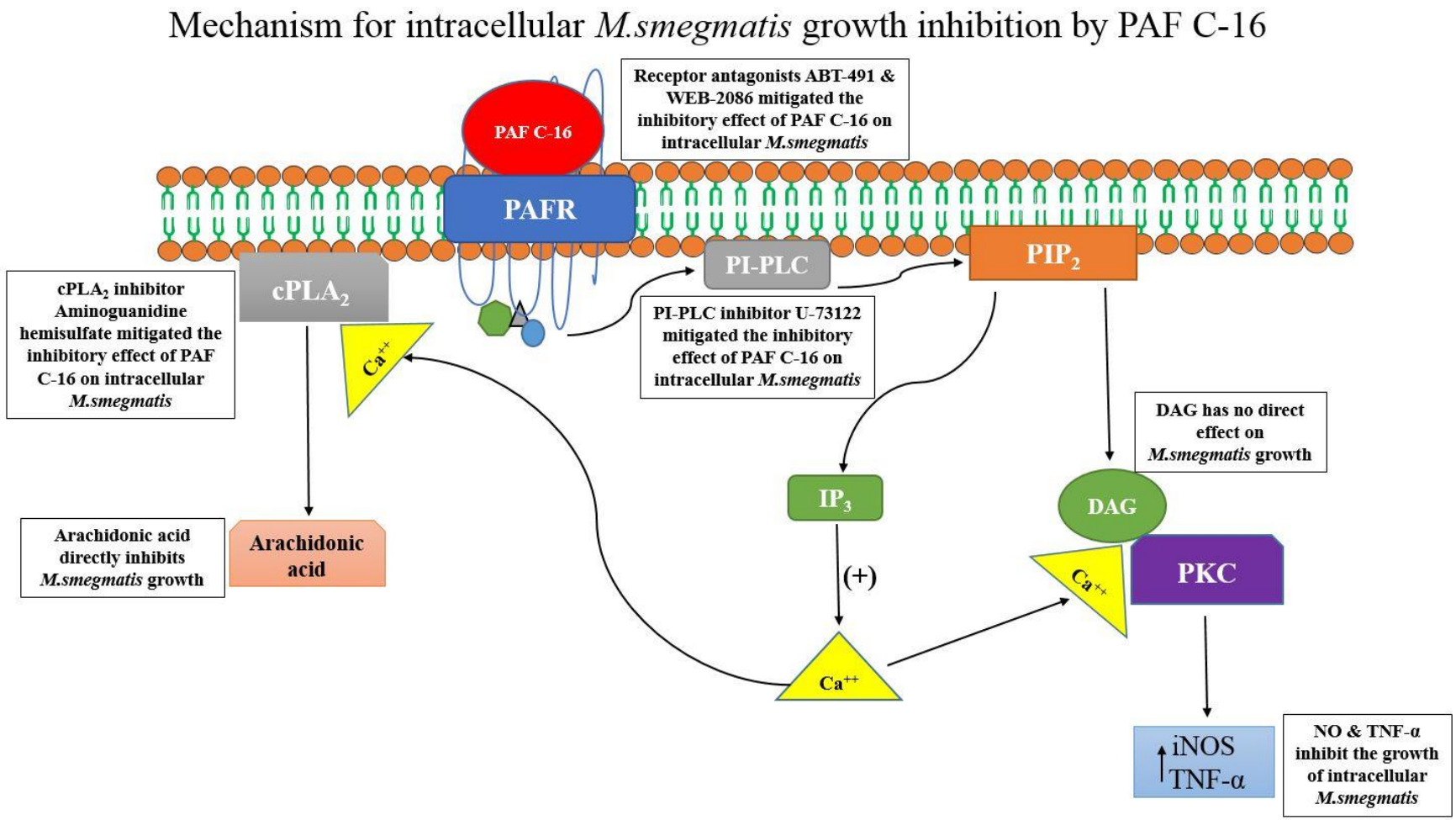


Figure 6.2: Proposed mechanism for PAF C-16 induced growth inhibition of intracellular *M.smegmatis*. Binding of PAF C-16 to its receptor PAFR on the target cell activates phosphatidylinositol specific phospholipase C (PI-PLC) enzyme which causes the production of second messengers IP_3 and DAG inside the cell. The second messenger IP_3 then causes the mobilization of intracellular Ca^{++}. DAG along with Ca^{++} further leads to the

activation of phosphokinase C (PKC) enzyme that upregulates the production of reactive nitrogen intermediates and TNF-α, which can inhibit the growth of intracellular *M.smegmatis*. In addition, the elevated level of intracellular Ca^{++} also causes the activation of cytosolic phospholipase A_2 ($cPLA_2$) enzyme that hydrolyses phospholipids and results in the production of arachidonic acid inside the cell. This arachidonic acid has also the potential to inhibit the growth of *M.smegmatis*.

Intestinal epithelial cells were shown *in vitro* to produce increased quantities of PAF C-16 in response to enteric pathogen *Salmonella enteritidis* as compared to uninfected control cells (Egea *et al*., 2008). Similarly, elevated plasma levels of PAF C-16 were reported in human patients during HCV (Caini *et al*., 2007) and dengue virus infections (Jeewandara *et al*., 2015). We consider that the increased production of PAF C-16 during inflammation and pathogen infection might be a part of the host protective immune response to the invading pathogens. This argument is supported by a study performed in mice model by Choi *et al* during which endogenous PAF C-16 was shown to be important in conferring protection to the host against *Candida albicans* infection. The protective effect of endogenous PAF C-16 was associated with the early activation of NF-κB and upregulation of TNF-α in the infected mice (Choi *et al*., 2001). Therefore, it will be interesting to determine levels of PAF C-16 in the blood and at the site of infection in animal models infected with *M.tb* and to compare it with healthy control to determine if there are differences in the levels of endogenously produced PAF C-16. In humans, it will be interesting to measure PAF C-16 levels at disease sites such as in pleural, ascitic and cerebrospinal fluids. Pleural fluid can result due to pleural involvement in different disease situations such as infections (tuberculosis and bacterial pneumonia), cancer (lung cancer), autoimmune disorders (such as lupus or rheumatoid arthritis) etc. PAF C-16 levels in these situations may prove as a diagnostic or prognostic indicator for disease. In TB pleurisy, PAF C-16 in pleural fluid may correlate with severity of the disease.

Granuloma is the hallmark of *M.tb* infection, where *M.tb* infection is contained. Granuloma is made up of collection macrophages with engulfed bacteria that is surrounded by a collar of lymphocytes and is a sign of chronic inflammation (Gonzalez-Juarrero *et al*., 2001). Immune cells such as macrophages (Shindou *et al*., 2005) and neutrophils (Owen *et al*., 2005) at the site of inflammation have been shown to be produce PAF C-16, which must be interacting with surrounding immune cells in autocrine or paracrine fashion. This interaction may be important for the integrity of granuloma itself and/or containment of infection within it. Production of PAF C-16 can be detected in *M.tb* infected tissue biopsy by immunohistochemistry using PAF C-16 specific antibodies. *In vitro* granuloma formation technology is now available (Kapoor *et al*., 2013), which will help in understanding the role of PAF C-16 in its formation, modulation of its functions such as cytokine production profile and thus help in understanding the containment of *M.tb* infection inside these granuloma.

The results obtained with PAF C16, PAF C-16 structure analogues and other compounds (1-O-hexadecyl-*sn*-glycerol, miltefosine and hexadecyl lactate) need to be validated by repeating

the experiments using *M.tb*. If these compounds are found to be effective in inhibiting the growth of *M.tb in vitro*, then further investigations need to be carried out in animal models for their abilities to control *M.tb* and to monitor their side effects *in vivo*. Those compounds showing potent *M.tb* inhibition and minimum or no side effects need further investigation for finding the optimum dosage in animal models. The commonly used animal models for studying *M.tb* infections include mice, guinea pigs, cattle and macaques. Each of these animal models has its advantages and disadvantages. Mice models are good for initial testing because these animals are genetically well characterized, cost-effective and due to the availability of a large number of reagents for immunologic analysis. On the other hand, the guinea pig is a very sensitive model for *M.tb*, however scarcity of well-characterised immunological reagents is an issue. Macaque model of tuberculosis is considered a surrogate model for human tuberculosis, but they are expensive to maintain and there are ethical issues in using these animals.

Once drugs show efficacy and minimum or no side effects in preclinical trials in animal models, these can be tested further in humans, initially in phase I, later Phase II, and finally in Phase III clinical trials, before they can be awarded license to be used in humans. It is less likely that PAF C-16 will pass the stage of preclinical testing, because of its side effects. Increased levels of PAF C-16 in humans have been shown to be associated with a number pathological conditions such as asthma, increased vascular permeability, thrombocytopenia, tissue injury and different inflammatory, gastrointestinal, cardiac and renal disorders (Stafforini *et al*., 2003).

Overall, this study has identified a number of novel compounds such as PAF C-16, Lyso-PAF and 1-O-hexadecyl-*sn*-glycerol, miltefosine and hexadecyl lactate with anti-mycobacterial activity. Further investigations are needed to demonstrate their effectiveness both *in vitro* and in animal models to see their effectiveness as anti-TB drugs. Furthermore, this study has thrown light on the possible mechanisms of PAF C-16 induced mycobacterial growth inhibition both inside and outside phagocytic cells.

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