THE *IN VITRO* ANTI-MYCOBACTERIAL ACTIVITIES OF THE NOVEL TETRAMETHYLPIPERIDYL-SUBSTITUTED PHENAZINES, B4121 AND B4128

by

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DEDICATED TO MY BELOVED DAUGHTER

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SUMMARY

The intra- and extracellular activities of 2 novel tetramethylpiperidine (TMP)-substituted phenazines, B4121 and B4128 against *Mycobacterium tuberculosis* H37R (ATCC 27294) were determined and compared with those of clofazimine (B663). Clofazimine, together with B4121 and B4128, were also tested for their activities against drug-resistant strains of *M.tuberculosis*. Both B4121 and B4128 were significantly more active than clofazimine against *M.tuberculosis*, including multidrug-resistant clinical strains of this microbial pathogen, demonstrating a lack of cross resistance between the riminophenazines and standard anti-tuberculous drugs. Using *M.tuberculosis*-infected monocyte-derived macrophages both B4121 and B4128 were found to possess intracellular activity, which was superior to that of both clofazimine and rifampicin.

The relationship between antimycobacterial action of the TMP-subsitituted phenazines and clofazimine and the effects of these agents on microbial PLA_2 activity, cation (K^+, Ca^{2+}) fluxes and energy metabolism (ATP) was also investigated. PLA_2 and cation fluxes were measured by radiometric procedures, while microbial ATP was assayed using a luciferin/luciferase chemiluminescence method. All 3 riminophenazines, particularly B4128 caused dose-related enhancement of microbial PLA_2 activity, which was associated with inhibition of K^+-influx and enhancement of uptake of Ca^{2+}. The results of kinetics studies demonstrated that riminophenazine-mediated enhancement of PLA_2 activity and inhibition of K^+ uptake in mycobacteria are rapidly-occurring and probably related events that precede, by several minutes, any detectable effects on microbial ATP concentrations and uptake of Ca^{2+}. Inclusion of the extracellular and intracellular Ca^{2+}-chelating agents EGTA and BAPTA, respectively, individually or in combination, did not prevent the effects of the riminophenazines on mycobacterial PLA_2 (enhancement) or K^+ transport (inhibition), whereas α-tocopherol, which neutralizes PLA_2 primary hydrolysis products, antagonized the inhibitory effects of the riminophenazines on microbial K^+ uptake. These results demonstrated that the riminophenazine-mediated enhancement of PLA_2 is a Ca^{2+}-independent event. The involvement of PLA_2 in the antimicrobial activity of the riminophenazines was supported by the observation that added, exogenous lysophosphotidylcholine (a primary

hydrolysis product of PLA_2 action on membrane phospholipids) also inhibited K^+ transport and growth of mycobacteria.

Enhancement of endogenous PLA_2 as a mechanism of riminophenazine-mediated disruption of cation transport and antimycobacterial activity was further investigated using the conventional calcium-mobilizing stimuli, calcium ionophore A23187 and thapsigargin. Both agents, but A23187 in particular caused in dose-related enhancement of microbial PLA_2 activity, which was associated with inhibition of K^+ influx and growth. Influx of Ca^{2+} into A23187- and thapsigargin-treated mycobacteria was observed using both radiometric and FURA-2-based spectrofluorimetric procedures. Exposure of the mycobacteria to these agents resulted in an immediate increase in uptake of Ca^{2+}, which implies that enhancement of PLA_2 activity in calcium-mobilizing stimuli-treated mycobacteria is Ca^{2+} dependent.

In conclusion, the TMP-substituted phenazines possess antimycobacterial properties which are superior to those of clofazimine, particularly against intraphagocytic *M.tuberculosis* . The superior antimycobacterial properties of these agents is paralleled by their potentiating effects on microbial PLA_2 and consequent inhibitory action on uptake of K^+, particularly in the case of B4128. Mycobacterial PLA_2 and K^+ transporters may therefore represent novel targets for antimicrobial chemotherapy.

Key words: Tuberculosis, antibiotics, bacteria, multidrug-resistance, outer-membrane, enzymes, potassium, calcium and energy metabolism.

SAMEVATTING

Die intra- en ekstrasellulêre aktiwiteit van twee nuwe tetrametielpiperidien (TMP)-vervangde fenasiene, B4121 en B4128, teen *Mycobacterium tuberculosis* H37R (ATCC 27294) is bepaal en vergelyk met dié van klofasimien. Klofasimien, sowel as B4121 en B4128, is ook vir hul aktiwiteit teen geneesmiddelbestande stamme van *M.tuberculosis* ondersoek. Beide B4121 en B4128 was betekenisvol meer aktief as klofasimien teen *M.tuberculosis* sowel as teen die veelvoudige geneesmiddelbestande kliniese stamme van hierdie mikrobiese patogeen wat daarop dui dat die riminofenasiene en standard anti-tuberkulose geneesmiddels nie kruisweerstandbiedendheid toon nie. Deur van *M.tuberculosis* geïnfekteerde, monosietafkomstige makrofage gebruik te maak is vasgestel dat B4121 en B4128 intrasellulêre aktiwiteit besit wat veel beter is as dié van klofasimien en rifampisien.

Die verwantskap tussen die anti-mikobakteriële werking van die TMP-vervangde fenasiene en klofasimien en die uitwerking daarvan op mikrobiese PLA_2 aktiwiteit, katioon (K^+, Ca^{2+}) flukse en energie metabolisme (ATP) is ook ondersoek. PLA_2 en katioonflukse is deur radiometriese metodes bepaal terwyl mikrobiese ATP deur middel van 'n lusiferien/lusiferase chemiluminessensie metode bepaal is. Al drie riminofenasiene, veral B4128, het tot 'n dosisverwante verhoging in mikrobiese PLA_2 aktiwiteit gelei wat met die inhibisie van K^+-influks en verhoging in opname van Ca^{2+} gepaard-gegaan het. Die resultate van kinetiese studies het getoon dat die riminofenasienbemiddelde verhoging van PLA_2-aktiwiteit en inhibisie van K^+ opname in mikobakterieë vinnig plaasvind en moontlik verwant is aan gebeure wat die waarneembare uitwerking op mikrobiese ATP en opname van Ca^{2+} etlike minute voorafgaan. B4128 was die beste agent wat getoets is in terme van die verhoging van PLA_2 en die disregulasie van katioonflukse. Insluiting van die ekstra- en intrasellulêre Ca^{2+}-kelerende agente EGTA en BAPTA, kon beide individueel of in kombinasie, nie die uitwerking van die riminofenasiene op mikobakteriële PLA_2 (verhoging) of K^+ (inhibisie) omkeer nie terwyl alfa-tokoferol, wat die primêre hidrolise produkte van PLA_2 neutraliseer, die inhiberende uitwerking van die riminofenasiene op mikrobiese K^+-

opname kon teenwerk. Hierdie bevindings toon dat die riminofenasienbemiddelde verhoging in PLA_2 'n Ca^{2+}-onafhanklike gebeurtenis is. Die betrokkenheid van PLA_2 in die anti-mikrobiese aktiwiteit van riminophenasiene is verder ondersteun deur die waarneming dat eksogene toegevoegde lisofosfatidielkolien ('n primêre hidrolitiese produk van PLA_2 aktiwiteit op membraan fasfolipiedes) ook die K^+ vervoer en groei van mikrobakterieë geïnhibeer het.

Verhoging van endogene PLA_2 as 'n meganisme van riminofenasienbemiddelde verbreking in katioonvervoer en anti-mikobakteriële aktiwiteit is verder ondersoek deur gebruik te maak van die konvensionele kalsiummobiliserende stimulante, kalsium ionofoor A23187 en thapsiegargin. Beide agente, maar veral A23187 het 'n dosisverwante verhoging van mikrobiese PLA_2 aktiwiteit veroorsaak wat met die inhibisie van K^+-influks en groei, geassosieer kon word. Influks van kalsium in die A23187- en thapsiegargin-behandelde mikobakterieë is waargeneem deur gebruik te maak van beide radiometriese en FURA-2-gebaseerde spektrofluorimetriese metodes. Blootstelling van die mikobakterieë aan hierdie agente het tot 'n onmiddellike vehoging in Ca^{2+}-opname gelei wat daarop dui dat die verhoging van PLA_2 aktiwiteit in mikobakterieë wat met kalsiummobiliserende stimuli behandel word, Ca^{2+} afhanklik is.

Ten slotte, die TMP-vervangde fenasiene besit anti-mikobakteriese eienskappe wat veel beter is as dié van klofasimien, veral ten op sigte van hul aktiwiteit teen intra-fagositiese *M.tuberculosis*. Die voortreflike anti-mikobakteriese eienskappe van hierdie agente word geëwenaar deur hul vermoë om mikrobiese PLA_2 te verhoog wat aanleiding gee tot hul inhiberende werking op K^+-opname, veral in die geval van B4128. Mikobakteriële PLA_2 en K^+ vervoerders mag daarom nuwe teikens bied vir antimikobakteriële chemoterapie.

Sleutelwoorde: Tuberkulose, antibiotika, Bacterieë, veelvoudige geneesmiddel bestande, fagosiete, buitemembraan, ensieme, kalium en energie metabolisme.

LIST OF ABBREVIATIONS

AFB	-	acid fast bacilli
AIDS	-	acquired immunodeficiency syndrome
ATP	-	adenosine triphosphate
ATPase	-	adenosine triphosphatase
BAPTA	-	1,2-bis(2-aminophenoxy) ethane-N,N,N,N'-tetraacetic acid
BCG	-	Bacille Calmette-Guerin
BPI	-	bactericidal/permeability-increasing protein
Ca^{2+}	-	calcium ion
^{45}Ca	-	radioactive calcium
Ca^{2+}-ATPase	-	calcium adenosine triphosphatase
$[Ca^{2+}]_i$	-	intracellular free calcium
$CaCl_2$	-	calcium chloride
CHA	-	calcium/proton antiport
Cl^-	-	chloride ion
CFU	-	colony forming unit
CMI	-	cell-mediated immunity
CR	-	complement receptor
DMP	-	dimycoserosates of phthiocerols
DMT	-	6,6'-dimycoloyl trehalose
DNA	-	deoxyribose nucleic acid
DOT	-	directly observed therapy
dr-PLA	-	detergent-resistant phospholipase A
ds-PLA	-	detergent-sensitive phospholipase A
DTH	-	delayed type hypersensitivity
EGTA	-	ethyleneglycol-bis (β-aminoethylether)-N,N,N',N',-tetraacetic acid
EMB	-	ethambutol
ETZ	-	electron-transparent zone
FMLP	-	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
H^+	-	hydrogen ion

HBSS	-	hanks' balanced salt solution
H_2O_2	-	hydrogen peroxide
HOCl	-	hypochlorous acid
HPTLC	-	high-performance thin-layer chromatography
IFN γ	-	interferon gamma
IL	-	interleukin
INH	-	isoniazid
JNK-c	-	jun N-terminal kinase
K^+	-	potassium ion
^{42}K	-	radioactive potassium
K^+-ATPase	-	potassium adenosine triphosphatase
KHA	-	potassium/proton antiport
KCl	-	potassium chloride
K_2CO_3	-	potassium carbonate
Km	-	affinity constant
KONO buffer	-	buffer without potassium and nitrogen
Kup	-	potassium uptake
LAM	-	lipoarabinomannan
Li^+	-	lithium ion
LPC	-	lysophosphatidylcholine
MDR	-	multi-drug resistant
MHC	-	major histocompatibility complex
MIC	-	minimal inhibitory concentration
MMT	-	6-monomycoloyl trehalose
Mn^{2+}	-	manganese ion
MP	-	mononuclear phagocytes
MR	-	mannose receptor
MRC	-	Medical Research Council
Na^+	-	sodium ion
Na^+,K^+-ATPase		sodium, potassium-adenosine triphosphatase
NADPH	-	nicotinamide adenine dinucleotide phosphate (reduced)

NaOH	-	sodium hydroxide
NEM	-	N-ethylmaleimide
NHA	-	sodium/proton antiport
NH_4Cl	-	ammonium chloride
NK cells	-	natural killer cells
PAS	-	para-aminosalicylic acid
PBS	-	phosphate-buffered saline
PGL	-	phenolic glycolipid
PLA_2	-	phospholipase A_2
PMN	-	polymorphonuclear leukocytes
PZA	-	pyrazinamide
Rb^+	-	rubidium ion
^{86}Rb	-	radioactive rubidium
RIF	-	rifampicin
RNA	-	ribonucleic acid
RNI	-	reactive nitrogen intermediates
ROI	-	reactive oxygen intermediates
SEM	-	standard error of the mean
SA	-	South Africa
Sr^{2+}	-	strontium ion
TAG	-	triacylglycerol
TB	-	tuberculosis
TCA	-	trichloroacetic acid
TGF-β	-	transforming growth factor beta
Th1	-	T helper 1
Th2	-	T helper 2
TMP	-	tetramethylpiperidyl
TNF-α	-	tumour necrosis factor alpha
Trk	-	transport of potassium
TST	-	tuberculin skin test
USA	-	United States of America

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CHAPTER 1

LITERATURE REVIEW

1. INTRODUCTION

Tuberculosis (TB) has affected humans in every part of the world, even today it is a major cause of morbidity and mortality in many countries, despite extensive understanding of its pathogenesis, epidemiology, prevention and therapy. South Africa (SA) is currently facing one of the worst TB epidemics in the world with disease rates of up to 60 times higher than those currently seen in the United States of America (USA) and Western Europe (Fourie, 1997). In 1996, SA had an estimated burden of about 160 000 cases, translating into a rate of 377/100 000 members of the population. This rate is almost double the rate observed in TB hot-spots in other developing countries, where rates are around 200/100 000.

The essential factor in the continued spread of TB in SA has been that treatment programmes have not ensured that infectious patients are cured (Fourie, 1997). Current estimates show that up to 20% of TB patients start TB treatment but do not complete it. Failure to complete the required course of treatment, which normally renders TB completely curable, leads to the increased risk of patients developing multi-drug resistant (MDR) TB. MDR TB is normally a killer, fewer than 30% of patients with this type of TB survive. In 1996 more than 2000 people in SA developed MDR TB, with only 1 in 3 being cured (Fourie, 1997).

The most important factors which have contributed to the resurgence of TB include i) the high rates of population growth in areas with poor hygienic and low socioeconomic resources, ii) the emergence of MDR strains of *Mycobacterium tuberculosis* (*M.tuberculosis*), iii) the human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) onslaught and iv) the lack of a universally efficient vaccine (Brudney & Dobkin, 1991, Colditz *et al*, 1994). In view of this, the discovery and development of new drugs for treatment of TB needs urgent attention. In the search for such novel agents, I have focused on riminophenazine compounds. Clofazimine (also known as B663 or Lamprene), originally described by Barry *et al* in 1957, is the prototype riminophenazine which was tested as an anti-tuberculosis agent, but because of its poor action on experimental TB in monkeys and guinea pigs, the most popular

models at that time, interest in the drug as an anti-tuberculosis agent waned (Barry *et al*, 1957). On the basis of the fact that it concentrates avidly in macrophages and because of the logic of using an intracellular drug for the treatment of intracellular parasites, clofazimine has been used successfully for the treatment of *Mycobacterium avium* (MAC) disease in AIDS patients (Gangadharam *et al* , 1992) and is also widely used in the treatment of leprosy (Atkinson *et al*, 1967). Since TB, especially the more serious disease caused by MDR strains, has re-emerged, I have investigated the anti-tuberculosis potential of two tetramethylpiperidyl (TMP) derivatives of clofazimine B4121 and B4128. The underlying hypotheses tested by my laboratory research are i) that these agents possess anti-mycobacterial properties which are superior to those of clofazimine and ii) that these antimicrobial activities involve phospholipase A_2-mediated alterations in outer membrane cation (K^+ and Ca^{2+}) transport systems.

1.1. *Mycobacterium tuberculosis*

TB, the wasting disease has been known for centuries, but identification of its causative agent as a bacterium did not occur until 1882, when Robert Koch used a special staining method to view *M. tuberculosis* from a pure culture. The tubercle bacillus is characterized as a fastidious, slowly-growing, strictly aerobic, lipid-rich, hydrophobic acid-fast bacterial rod. *M.tuberculosis* is a slender cell that often forms clumps, making it difficult to identify individual microorganisms. The tubercle bacillus is a sinister organism. Although descended from a family of saprophytes, it is a facultative intracellular pathogen that effortlessly invades what should be a hostile and aggressive host cell, the macrophage.

Tubercle bacilli are best isolated from clinical specimens on rich and fairly complex media. Once isolated, *M.tuberculosis* is capable of adapting to growth on an extremely simple source of carbon and nitrogen plus some buffer salts and trace elements. The nutritional requirements of *M.tuberculosis* are simple and the organism can grow on minimal culture media containing a simple source of organic carbon (eg. glycerol), inorganic nitrogen (NH_4^+) , and the usual inorganic elements, no particular growth factor or vitamin is required. However, the nutrition of *M.tuberculosis* is different when it grows

within host tissue, particularly intracellularly, than when it is grown in a culture medium *in vitro*. It prefers to assimilate preassembled molecules such as, amino, nucleic, and fatty acids etc. in order to reduce its synthetic metabolic load and thereby turn off (at the gene level) complex and energy-expensive synthetic machinery, allowing it instead to invest its metabolic energy in crucial virulence determinants required for survival within hostile macrophages. Iron is an essential trace element taken up by the pathogen through a unique capsular compound, mycobactin.

M.tuberculosis and mycobacteria in general appear to be singularly suited to surviving starvation. Although they do not form spores, these organisms can be maintained in distilled water for two years with no loss in viability (Nyka, 1974). It is conceivable that *M.tuberculosis* can never be completely eradicated by antimicrobial agents since dormant, non-replicating organisms are not likely to be susceptible to such agents (Ehlers, 1993). *M.tuberculosis* is considered to be in a constant semi-hibernating state with a minimal flow (or flux) through all metabolic pathways and is therefore well placed to survive the sudden onset of starvation and hypoxia. This, coupled to its unusually thick, waxy cell wall and its facility for entering into a condition of profound dormancy, makes this mycobacterium almost invincible, and it is no surprise that the tubercle bacillus is one of the most formidable pathogens in the long-suffering of mankind.

The tubercle bacillus has a long generation time compared to most commonly studied bacteria. Under optimal conditions, *M.tuberculosis* requires 16 to 18 hours to undergo one cycle of replication (Wayne, 1997). With a generation time in that range, a single bacillus can yield a visible colony on solid medium within two weeks or less after inoculation. The excessively long time of 6 to 10 weeks required for detection of colonies on media planted with some clinical specimens (Krasnow and Wayne, 1969), is probably the result of a need to repair injury of the bacillus in the specimen.

Unlike other bacteria, *M.tuberculosis* is coated with a lipid rich wall which makes it resistant to drying (Chardwick, 1981). Furthermore, cultures maintained at 37°C have been found to be both viable and virulent after storage for 12 years. Organisms from

cultures will die in 2 hours when exposed to direct sunlight, but bacilli contained in sputum require an exposure of 20-30 hours before they are killed. When protected from direct sunlight *M.tuberculosis* can survive for several months in sputum and will normally grow in 14-28 days. However if the patient has been subjected to chemotherapy then the organisms may take up to 8 weeks to grow (Chardwick,1981)

The tubercle bacillus grows most rapidly when aerated and does not appear to multiply under completely anaerobic conditions. *M.tuberculosis* has many of the enzymes required for anaerobic metabolism, and the virulence of this organism appears to be in part a function of its ability to survive and/or grow under the wide range of variation in partial O_2 pressures that may occur in healthy, inflamed or necrotic tissues (Segal, 1984; Wayne, 1994). The tubercle bacillus, unlike all other mycobacteria, is very rich in lipid but unlike some other mycobacterial species, it does not produce a hydrophilic outer sheath. It is indeed hydrophobic and grows as a waxy pellicle in unagitated medium and with extensive clumping in stirred culture unless a detergent is included in the medium. The tendency for pellicle formation reinforced the perception of this organism as a strict aerobe.

1.1.1. Ultrastructure

The cell envelope which surrounds pathogenic mycobacteria appears to be unique among prokaryotes. It is postulated to be a defence barrier against phagocytic cells, and its outermost constituents have a tendency to accumulate in the culture medium (Lemassu & Daffe`, 1994). The unusual property of acid-fast staining place the mycobacteria outside of the broad classification scheme based on Gram-staining. Subsequent chemical analysis revealed it to contain an unusually high proportion of lipids, amounting to over 60% of the weight of the cell wall (Draper, 1982). Knowledge of the architecture of the cell envelopes of mycobacteria is central to our understanding of some unresolved problems of mycobacterial diseases, such as occurrence of the opportunistic mycobaterioses and recrudescence of TB (McNeil & Brennan, 1991).

The cell envelopes of mycobacterial species are composed of two structures, a plasma membrane and a cell wall skeleton. The mycobacterial cell wall skeleton consists of three covalently linked macromolecules, peptidoglycan, arabinogalactan and mycolic acid (Ehlers, 1993). The shape-forming properties of the wall are attributable to the peptidoglycan, the chemical structure of which closely resembles that found in other bacteria. Peptidoglycans are composed of chains of polysaccharides formed from alternating units of glucosamine and muramic acid cross-linked by tetrapeptide side-chains (Ehlers, 1993). The next layer consists of a complex polysaccharides called arabinogalactan, which are covalently linked to muramic acid residues of the peptidoglycan. Mycolic acids are known to be high-molecular weight β-hydroxy fatty acids with a long α-side-chain (the terms α and β refer to the carbons adjacent to the carboxylate function). The main carbon (or alkyl chain) in each mycolic acid contains 50 to 60 carbon atoms, while the α - branched side-chain contains another 24 carbon atoms. The mycolic acids are considered to be responsible for producing a continuous outer waxy coat (Minnikin, 1982).

In addition to the covalently linked macromolecules, the *M.tuberculosis* cell wall contains considerable quantities of non-covalently-associated glycolipids and proteins (Ehlers, 1993). Of these, the most interesting glycolipid is lipoarabinomannan (LAM) which consists of polysaccharides containing arabinose and mannose units, covalently linked to phosphatidylinositol groups which anchor the molecule in the mycobacterial cell membrane. Other loosely bound, complex, free lipids and glycolipids include trehalose-based glycolipids in which long-chain fatty acids, including mycolic acid are esterified to the disaccharide trehalose (the dimycolyltrehaloses are also called 'cord factor'), sulphated acyl trehaloses, also called sulphatides, and true waxes or cerides (eg. phenolic phthiocerol) (Minnikin, 1982). These lipids are thought to interdigitate with the structural mycolic acids covalently bound to the arabinogalactan layer and contribute considerably to the extreme lipophilicity and waxiness of the mycobacterial cell wall.

The most distinctive structural feature of pathogenic mycobacterial species is the presence of an electron-transparent zone, also called a capsule which surrounds each bacterium (Hanks, 1961). This may be part of the defence mechanism permitting these pathogens to resist killing by phagocytic cells (Draper & Rees, 1970). This protective capsule not only controls access of the medium to the inside of the mycobacterial cell but also determines what component comes into contact with host cells and tissues.

It has been found that mycobacteria are not identical in terms of surface exposure of the various classes of lipids, probably reflecting differences in their cell envelope organization (Ortalo-Magne` *et al*, 1996). The first group is composed of *Mycobacterium tuberculosis*, *Mycobacterium kansasii*, and *Mycobacterium gastri* with species-specific lipids such as phenolic glycolipids (PGLs), lipooligosaccharides (LOSs) and dimycoserosates of phthiocerols (DIMs) being exposed on the cell surface whereas ubiquitous lipids such as 6,6`-dimycoloyl trehalose (DMTs), 6-monomycoloyl trehalose (MMTs) and triacylglycerols (TAGs) are not exposed. The second group consists of *Mycobacterium avium* and *Mycobacterium smegmatis*, their species-specific lipids (PGLs) and some of the ubiquitous lipids (MMTs and TAGs) are both exposed on the cell surface. The third group is composed of *Mycobacterium aurum* with all the classes of lipids being exposed at the outermost region of the cell.These classes of specific mycobacterial glycolipids eg. PGLs and glycopeptidolipids (GPLs) may function as virulence factors in the pathogenesis of mycobacterial diseases. PGLs have been shown to inhibit the lymphoproliferative responses (Mehra *et al,* 1984, Fournie *et al*, 1989), to suppress monocyte oxidative responses (Vachula *et al*, 1989) and to scavenge oxygen radicals (Neil & Klebanoff, 1988). Similarly GPLs , have been shown to inhibit both the nonspecific mitogen-induced proliferation of mononuclear cells (Brownback & Barrow, 1988) and mitochondrial oxidative phosphorylation (Sut *et al*,1990).

It has been noted that the hydrophobic mycobacterial envelope constitutes a sophisticated barrier, enabling survival under adverse conditions and obviating the need for specialized survival mechanisms such as sporulation (Minnikin,1982). Thus,

the mycobacterial wall may play a central role in the maintenance of a long-lived dormant state which is responsible for latency and reactivation of TB.

1.1.2. Pathogenesis

Humans remain the main reservoir of the tubercle bacilli in nature with the majority of the infected individuals carrying their infection without developing any clinical illness (Schluger & Rom, 1998). Some will develop active disease in the context of some impairment of their immune system such as that caused by infection with HIV, malnutrition or advanced malignancy (Young, 1993), although most cases of active disease occur in persons with no obvious defect in host immunity. TB can infect many organs of the body. This infection in many people progresses into the disease and these individuals become transmitters of *M.tuberculosis* to the non-infected (Rastogi & David, 1988). The transmission of the bacilli from the diseased to the non-infected can be stopped by diagnosing and treating the person who has TB, while the means to eliminate tubercle bacilli from healthy infected individuals does not exist. Therefore, it is clear that elimination is not in the realm of immediate possibilities and it appears that a better understanding of the components of the host-parasite relationship in TB infection will result in better and novel approach to prevention and therapy of this disease.

Interest in the immunophathogenesis of *M.tuberculosis* infection stems not only from its importance as a pathogen, but, also from the hope that such work would reveal the factor(s) responsible for its ability to multiply within human tissues, and, at the same time, for its capacity to withstand host defence mechanisms. This is thought to be linked to the unusual physio-chemical properties of the mycobacterial surface (Brennan, 1989). Several chemical compounds such as cord factor, the sulpholipids and mycosides have been implicated in the mechanisms of pathogenicity of mycobacterium.

Acquired resistance against TB depends on cell-mediated immune mechanisms, with major factors being mononuclear phagocytes (MP) which act as the principal effectors

and T-lymphocytes which serve as the predominant inducers of protection. However, MP play a dual role in TB, promoting not only protection against the disease but also survival of the pathogen. Similarly , T-cells not only are indispensable for protective immunity but also contribute to pathogenesis.

The route of entry of the *Mycobacterium tuberculosis* bacillus into the body is via the respiratory tract through the inhalation of respiratory droplet nuclei, which are small in size (1 to 2 µm or less) and allow passage into the lower respiratory tract (Riley *et al*, 1959). Once inhaled, fewer than 10% of the tubercle bacilli will reach the respiratory bronchioles and alveoli but most will settle in the upper respiratory epithelium where they are likely to be expelled by the mucociliary escalator (Nardell, 1993). The bacilli that survive in the deep lung are phagocytosed by alveolar macrophages and are either killed, or else survive to initiate an infection (Dannenberg, 1994).

Once the tubercle bacilli have made their way into the lung, they have four potential fates,(1) the initial host response can be completely effective and kill all bacilli, such that the patient has no chance of developing TB at any time in the future, (2) the organisms can begin to multiply and grow immediately after infection, causing clinical disease known as primary TB, (3) bacilli may become dormant and never cause disease at all, such that the patient has what is referred to as latent infection, manifest only by a positive tuberculin skin test (TST), (4) the latent organisms can eventually begin to grow, with resultant clinical disease, known as reactivation TB (Dannenberg, 1994). On the other hand, *M.tuberculosis* has also developed several strategies to:(1) enhance its entry into the mononuclear phagocytes, (2) circumvent potential toxic host cellular responses during and after entry of the phagocyte, and (3) modulate phagocyte effector function during cellular immune response (Schlesinger,1996b).

Macrophages have the following important functions in TB infection : (1) first, they produce proteolytic enzymes and other metabolites which exhibit mycobatericidal effects, (2) macrophages process and present mycobacterial antigens to T-lymphocytes, including CD4+ and CD8+ T-lymphocytes, which are central to acquired

resistance to *Mycobacterium tuberculosis* (3) lastly, the macrophages produce a characteristic pattern of soluble mediators (cytokines) in response to the tubercle bacillus which not only have important immunoregulatory effects, but also mediate many of the clinical manifestations.

The initial defence against infection with *M.tuberculosis* once it reaches the lower respiratory tract, is the alveolar macrophage. Generally, phagocytosis usually begins with phagocytic cell engulfing the invading microbe in a membrane-bound tight vacuole, which is created when pseudopods surround the bacterium and fuse distally (Schlesinger, 1996a). Substantial experimental evidence exists that in the *M.tuberculosis*/mononuclear phagocyte interaction, the creation of the vacuole, or phagosome is accompanied by binding of the bacilli to the phagocyte through complement receptors, CR1, CR3 and CR4 (Schlesinger,1990) as well as mannose receptors (MR) (Schlesinger, 1993) and other receptors such as scavenger receptors, Fc receptors and the β-glucan receptor. The latter two do not seem to be of major importance in mediating binding of *M.tuberculosis* to alveolar macrophages (Schlesinger, 1990; Czop & Kay, 1991). Schlesinger (1990) has shown that addition of non-immune serum to monocyte derived macrophages enhances binding of organisms to phagocytic cells, indicating the importance of complement in this system (Schlesinger, 1990). Kang and Schlesinger (1998) have reported that the interaction between mannose receptors on phagocytic cells and mycobacterium seems to be mediated through the mycobacterial surface glycoprotein lipoarabinomannan, which is present on the cell wall of the *M.tuberculosis*, and is capped by mannose residues.

After pathogenic mycobacteria are engulfed into phagosomes they are subject to killing via a variety of mechanisms, including phagosome-lysosome fusion, generation of reactive oxygen intermediates (ROI), and the generation of reactive nitrogen intermediates (RNI), particularly nitric oxide (Rich *et al*, 1997). Phagosome-lysosome fusion has been extensively studied with regard to mycobacteria , but the exact role of this cellular process in host defence against *M.tuberculosis* remains somewhat unclear (Allen *et al*, 1965; Carrol *et al*, 1979; Mor, 1983). Studies by Gordon and coworkers

(1980) demonstrated that mycobacteria are capable of producing ammonia, which could both inhibit phagosome-lysosome fusion and, by alkalinizing the intra-lysosomal contents, diminish the potency of the fusion complex. Similarly sulphatides (derivatives of trehalose 2-sulphate, a glycolipid produced by *M.tuberculosis*) also inhibit phagosome-lysosome fusion (Goren *et al*, 1974 & 1979). Ferrari and coworkers (1999) have recently identified a host protein, termed tryptophane aspartate-containing coat protein (TACO) that is recruited to and retained on phagosome by living mycobacterium. Active retention of TACO protein on phagosome by living mycobacteria prevents their delivery to lysosomes, thus causing survival of the mycobacteria within macrophages.

Once inside the macrophages, there is evidence that *M.tuberculosis* can be killed by several different mechanisms through a host of complicated interactions mediated by cytokines and involving T- lymphocytes and phagocytes (Schluger & Rom, 1998). CD4+ T-cells express the α/β T-cell receptor, and they are involved in recognition of antigens that have been processed in the phagosome and presented as small peptide fragments in the context of major histocompatibility complex (MHC) class II molecules on the surface of antigen presenting cells such as monocytes, macrophages, or dendritic cells (Boom, 1996). Type 1 CD4+ T-lymphocytes (Th 1) and natural killer cells (NK cells) secrete interferon gamma (IFNγ) which activates alveolar macrophages to produce a variety of mediators, including reactive oxygen and nitrogen species, which are involved in growth inhibition and killing of mycobacteria (Schluger & Rom, 1998). The production of nitric oxide by IFNγ-treated alveolar macrophages increases until several days after infection (Vanham *et al*, 1997).

Another interesting cytokine that is produced during mycobacterial infection is interleukin 12 (IL-12). Its main source during infection are the macrophages which are stimulated by the phagocytic event (Fulton *et al*, 1996) and the presence of tumor necrosis factor alpha (TNF-α) and IFN-γ in the local environment (Flesch *et al*, 1995). Interleukin-12 (IL-12) is produced by the macrophages within a few hours of infection. This cytokine in turn then induces the production of IFN-γ from T-lymphocytes and

natural killer cells (Trichieri, 1998). IFN-γ has a powerful enhancing effect on the ability of phagocytic cells to produce IL-12, probably by potentiating IL-12 production within inflammatory tissue. Thus, IL-12-induced IFN-γ acts as a positive feedback mechanism in inflammation by enhancing IL-12 production. Flesch and coworkers (1995) assume that IFN-γ primes macrophages for TNF-α production and that both cytokines then induce IL-12 synthesis in response to mycobacterial infection. IL-12 produced during the early phase of infection and inflammation, sets the stage for the ensuing antigen-specific immune response, favouring differentiation and function of T helper 1 (Th 1) cells while inhibiting the differentiation of T helper 2 (Th 2) cells (Trinchieri, 1998). Furthermore, the enhancing effect of IFN-γ on IL-12 production may represent a mechanism by which Th1 responses are maintained *in vivo*.

The importance of IL-12 has also been demonstrated in murine studies by Flynn and colleagues (1995) who showed that when Balb/c mice, a strain highly susceptible to virulent *M.tuberculosis* infection, were given IL-12 at the initiation of infection with *M.tuberculosis*, their mean survival time doubled from 58 to 112 days. Furthermore, IL-12 treatment also delayed pathology in Balb/c mice. Children genetically deficient in IL-12 mediated immunity, due to either IL-12p40 or IL-12Rβ1 defects, have impaired IFN-γ production by NK cells and T lymphocytes *in vitro* (Altare *et al*, 1998). Moreover, IFN-γ therapy appears to be beneficial *in vivo*, as attested to by the marked symptomatic improvement after cytokine therapy was commenced in IL-12p40- and IL-12Rβ1-deficient patients.

Great attention has been focused on the role of the cytokines IFN-γ and transforming growth factor beta (TGF-β), in terms of their ability to activate and deactivate the macrophage's ability to inhibit mycobacterial growth. Condos and coworkers (1997) have administered IFN-γ to several patients with multidrug-resistant tuberculosis who had previously failed to respond to therapy (as evidenced by persistently positive smears). They have demonstrated improvement in several clinical parameters: patients became sputum-smear-negative, they gained weight, cavitary lesions seen on chest CT

scans improved, and the time it took to isolate *M.tuberculosis* from sputum increased, a finding suggestive of a decreasing bacterial burden.

TGF-β on the other hand is a macrophage inactivator produced in an autocrine fashion by monocytes and macrophages (Schluger & Rom, 1998). Although it has some proinflammatory effects such as enhancement of monocyte chemotaxis and augmented expression of Fc receptors, TGF-β also has important anti-inflammatory effects, including deactivation of macrophage production of reactive oxygen and nitrogen intermediates, inhibition of T-cell proliferation, interference with natural killer and cytotoxic T-lymphocyte function, and down regulation of IFN-γ, TNF-α and interleukin 1 release (Ruscetti, *et al*, 1993).

On the other hand, the role of CD8+ lymphocytes in host defence against *M. tuberculosis* is to recognize antigens that have been processed in the cytosol and are represented in the context of MHC class 1 molecules on the cell surface (Schluger and Rom, 1998). These cells may participate in the lysis of infected cells and induction of apoptosis, but evidence also exists that they are capable of secreting cytokines such as IFN-γ and IL-4, and thus may play a role in regulating the balance of Th1 and Th2 cells in the lungs of patients with pulmonary TB. CD8+ T cell lines and clones lyse *M. tuberculosis*-primed macrophages in an antigen-specific manner and restrict the growth of *M. tuberculosis* in macrophages (Kaufmann and Flesch, 1988). Flynn and coworkers, (1993) demonstrated the importance of CD8+ cells on cellular immunity in β2-microglobulin knockout mice with resultant failure to express functional MHC class 1 molecules and a paucity of CD8+ T cells. Infection with *M.tuberculosis* resulted in death of 70% of β2-microglobulin-deficient mice, whereas all the control mice survived. In β2-microglobulin-deficient mice, granuloma formation was intact, but there were tenfold more acid-fast bacilli in the infected tissues compared to controls. In general, CD4+ cells help to amplify the host immune response by activating effector cells and recruiting additional immune cells to the site of disease, while CD8+ cells are more likely to be directly cytotoxic to target cells. The exact functions of CD8+ cells in

tuberculosis are unclear, as are those of "double-negative" T-cells, as well as those cells which recognise mycobacterial lipids via CD 1 (Schluger & Rom, 1998).

Surviving mycobacteria multiply and kill their host macrophages; this is followed by mycobacterial release and subsequent infection of additional host cells (Fenton & Vermeulen, 1996). Furthermore, the early exudate contains chemotactic factors that attract circulating monocytes, lymphocytes, and neutrophils, none of which kills the bacteria very efficiently. Granulomatous focal lesions which are composed of macrophage-derived epithelioid giant cells and lymphocytes, begin to form. Generally, the process of granuloma formation serves as an effective means for containing pathogens, preventing their continued growth and dissemination. Its success depends on both the number of macrophages at the site of infection and on the number of mycobacteria present. While granuloma formation is quite an effective defence mechanism, even contained *M.tuberculosis* organisms are not always completely eradicated.

Granuloma formation and destruction of mycobacterium by macrophages are not antigen-specific events (Youmans & Youmans, 1964) and this observation contrasts with both delayed-type hypersensitivity (DTH) and cell-mediated immunity (CMI). In DTH, antigen-specific T-cell immune responses are evoked, and in CMI live mycobacteria are required for the development of protective immunity (Orme *et al*, 1993). The pathogenesis of TB can be considered to be an interplay between tissue-damaging immune responses which are produced during delayed-type hypersensitivity (DTH) reactions and macrophage-activating immune responses which are produced by cell-mediated immune reactions (Dannenberg & Rook 1991; Dannenberg, 1994).

In the first few days following infection, a strong granulomatous response is vital (Fenton & Vermeulen, 1996). However, after about three weeks, antigen-specific defences develop and contribute greatly to the resolution of infection. With the emergence of DTH responses, infected macrophages in the interior of each granuloma are killed as the periphery becomes fibrotic and caseated (Fenton & Vermeulen, 1996).

As infection progresses, the granulomas enlarge as the individual foci expand and coalesce (Nardell, 1993). This results in relatively large areas of necrotic debris, each surrounded by a layer of epithelioid histiocytes and multinucleated giant cells. These granulomas, or tubercles, are surrounded by a cellular zone of fibroblasts, lymphocytes, and blood-derived monocytes. Although *M. tuberculosis* bacilli are unable to multiply within this caseous tissue, due to its acidic pH, low availibity of oxygen, and the presence of toxic fatty acids, some pathogens may remain dormant there for decades (Fenton & Vermeulen, 1996). The strength of the host's CMI responses determines whether an infection is arrested here or progresses to the next stages.

In individuals who have good CMI, the infection may be arrested permanently at this point. The granulomas subsequently heal, leaving small fibrous and calcified lesions (Nardell, 1993). Where CMI is inadequate, the host's DTH responses battle the ever-multiplying *M.tuberculosis* bacilli, but simultaneously, lung tissue is destroyed, leading to both pulmonary damage and the spread of the organisms via the lymphatics and the blood (Fenton & Vermeulen, 1996). As the disease progresses further, the semisolid caseous centre of the granuloma begins to soften and liquefy, providing a rich and oxygenated environment for extracellular mycobacterial replication (Dannenberg, 1982). Enlarged lymph nodes can rupture into adjacent airways, releasing liquefied necrotic material and causing tuberculous bronchopneumonia (Fenton & Vermeulen, 1996).

M.tuberculosis organisms can persist for decades in a dormant state inside a granuloma. A decline in immunity, which may be permanent or transient, is believed to be responsible for reactivation TB. Factors which may lead to this include, diabetes mellitus, malnutrition, alcohol abuse, increasing age, viral infection and immune suppression, particularly due to HIV (Maartens, 1997). However, in many instances the cause of the decrease in immunity is never identified. There are two routes to a repeat episode of TB (post-primary pulmonary TB): either by inhalation of additional *M.tuberculosis* bacilli or by reactivation of a dormant primary lesion (Fenton & Vermeulen, 1996).

In reinfection TB, a hypersensitivity reaction is the characteristic response, accompanied by tissue necrosis and caseation (Fenton & Vermeulen, 1996). In an attempt to seal off the necrotic site, lymphocytes and other cells converge upon the site and direct the formation of a wall of fibrous tissue. Then caseated granulomas heal over time, shrinking as they become fibrotic and calcified. However, if healing is impaired, the growing lesions may erode adjacent bronchi, resulting in the formation of cavities. *M.tuberculosis* bacilli multiply freely in these cavities, leading to huge numbers of bacilli (estimated at greater than 10^8) (Nardell, 1993). An open cavitated lesion can leak infectious material directly into the bronchus, resulting in the continuous discharge of bacilli into the sputum (Fenton & Vermeulen, 1996). Leaked *M.tuberculosis* bacilli can also be inhaled into other lesions of the host's lungs, resulting in tuberculous bronchopneumonia. If the growing granulomatous lesion erodes the wall of a vein, organisms can spread in the circulating blood, resulting in miliary disease (Hopewell, 1994). Reactivation TB that progresses to the cavitary stage favors the propagation of virulent and drug resistant strains, as the increased oxygen concentration allows multiplication of the bacilli, and large numbers are thought to be necessary for the evolution of drug resistance mutants (Nardell, 1993).

Finally, it is likely that virulent *M.tuberculosis*, like certain other intracellular pathogens, including rickettsiae (Winkler, 1990) ,listeriae (Bielecki *et al*, 1990), and shigellae (Sansonetti *et al*, 1986) evade macrophage killing by escaping from phagocytic vacuoles into the cytoplasm (Falkov *et al*, 1992). Falkov *et al* (1992) also reported that lytic potential resulting in lysis of vacuolar membranes is the possible common virulent determinant that enables successful parasitization of the cytoplasm.

1.1.3. Prevention and chemotherapy

Bacille Calmette-Guèrin, (BCG) vaccination has been employed for decades to prevent TB. It converts tuberculin-negative individuals to positive status which is believed to confer immunity against tuberculosis bacilli, thereby preventing the development of progressive pulmonary tuberculosis meningitis, and endogenous reactivation. However, this BCG vaccine is of dubious efficacy. United Kingdom trials have shown it to be

efficient and it is still routinely administered to teenagers. However, studies conducted in other countries have not been successful and it is not recommended in many contries including the United State of America (USA) and Netherlands.

Appropriate use of chemotherapeutic agents in the treatment of TB is important for achieving a successful outcome. In planning chemotherapy of TB, three aspects of disease must be recognized: (1) tubercle bacilli, (2) TB lesions, and (3) action of antituberculosis drugs on the tubercle bacilli. Long before the advent of modern anti-tuberculous chemotherapy, garlic was used, evidently with some success, as a treatment of TB (Bolton *et al*, 1982; Watt, 1986). It was given as an oral medication, by inhalation, or applied topically as an ointment or in the form of a compress.

The era of modern tuberculosis chemotherapy began in 1944 with the discovery of streptomycin which rendered TB a disease that could be cured (Schaltz *et al*, 1944). Soon thereafter, it became evident that streptomycin monotherapy resulted in treatment failure that was associated with *in vitro* resistance of the drug (Canetti, 1965). Addition of para-aminosalicylic acid (PAS) in 1946 [British Medical Research Council (MRC), 1950] delayed the development of resistant strains. Although isoniazid (INH) has been known since 1912, its anti-tuberculosis activity was only discovered 40 years later in 1952 (Robitzek & Selikoff, 1952). The disadvantage of INH is the rapidity with which turbecle bacilli develop resistance to it. For this reason it is never used alone, but invariably in combination with other drugs such as PAS, ethambutol, pyrazinamide, rifampicin and streptomycin given over a period of 6 months.

Treatment was highly successful because it was carried out in hospitals, where compliance could be assured, and therefore acquired resistance to the drug was uncommon. However, in the late 1960s therapy was shifted to the outpatient setting, because patients with TB were not thought to be public health hazard when receiving chemotherapy. Unfortunately, this resulted in reduced compliance and led to rising rates of treatment failure, relapse and acquired drug resistance. Later, in the 1960s ethambutol and rifampicin were discovered (Bates, 1995). It soon became clear that

effective treatment of TB was being achieved. Chemotherapy soon replaced treatment such as prolonged bed rest, surgery, and nutritional supplements, which became largely irrelevant. Treatment of TB for the past three decades has undergone a revolution with the availability of more potent drugs which can cure this disease within 6 months or less of therapy (Riley, 1993) if the proper drugs are used.

Presently, among alternative experimental agents, the fluoroquinolone agent sparfloxacin, is a promising anti-tuberculous agent since it is active against the tubercle bacillus in vitro, but it is not yet apparent whether the dosage required to achieve bactericidal effects is achievable or tolerable in humans (Skinner et al, 1995). Another promising drug for TB is the macrolide agent clarithromycin and its analogue 14-hydroclarithromycin. Cavalieri and coworkers (1995) found that these agents enhance the anti-mycobacterial activities of INH, ethambutol and rifampicin in vitro.

Since the 1970s, chemotherapy has been based on the combination of effective bactericidal drugs (Grosset, 1978; Fox,1981). The first line drugs in common use are streptomycin (SM), isoniazid, rifampicin (RIF) and pyrazinamide (PZA). Ethambutol (EMB) is bactericidal if given initially in dose of a 25mg/kg per day. The second line drugs include ethionamide, cycloserine, para-aminosalicylic acid, kanamycin, and capreomycin. In areas with a high prevalence of drug-resistant disease and HIV infection, it is necessary to start therapy with five to seven drugs, including second line drugs, until susceptibility results are known.

In the treatment of multi-drug resistant TB (i.e., resistance to two or more drugs), some basic principles must be followed: (1) a single drug must not be added to a failing regimen; (2) at least the new drugs to which the patient has not been exposed should replace the existing regimen until the drug-susceptibility results are available; (3) duration of therapy is prolonged to 24 months or more; (4) an injectable drug should be included in the regimen to improve compliance and generally may be discontinued 2 months after bacteriologic conversion to negative; (5) directly observed therapy (DOT)

should be used to ensure compliance, and lastly the susceptibility test should be repeated if the culture remains positive more than 2 months after the start of therapy.

1.2. RIMINOPHENAZINES

A chemotherapy unit was established by the Irish Medical Research Council in the late 1940s with the aim of finding an anti-tuberculosis agent. One result of this work was the discovery of the anti-mycobacterial riminophenazine agent clofazimine, or Lamprene (B663), in 1957, which was found to be a disappointing agent in the treatment of animal models of tuberculosis (Barry et al, 1957). Barry and coworkers continued with the development of analogues of clofazimine with improved activity against M. tuberculosis in vitro (Barry et al, 1957; 1970).

Riminophenazines are lipophilic, relatively non-toxic, non-carcinogenic, and non-myelosuppressive agents (Van Rensburg et al, 1994). It has been found that clofazimine, the riminophenazine agent which has been most extensively studied, causes hyper-pigmentation of the skin, dry skin, conjunctival pigmentation, abdominal pain, anorexia and weight loss (Atkinson et al, 1967; Moore, 1983; Fleerksen & Seydel, 1992). It was found that these side-effects may in some cases limit the prolonged use of the drug in high doses, but are fully reversible on discontinuation. The complete disappearance of the hyper-pigmentation side-effects, in most of cases, takes about six months or more after stopping therapy.

1.2.1. Structures and Pharmacology

The riminophenazine compound, clofazimine and its analogues are synthesized from a prototype anilinoaposafranin (2-anilino-3:5-dihydro-3-imino-5-phenylphenazine) and its isomer 2-amino-3:5-dihydro-3-phenylphenazine . Each phenazine consists of a three ring phenazine nucleus, with variation occurring in substituent side chains at positions 2, 3 and 10 of the phenazine core. Oxidation of the *o*-phenylenediamine derivative with certain ketones results in a new phenazine type, glyoxalino-phenazine which, upon further catalytic hydrogenation, yields imino-substituted compounds, riminophenazines (Barry *et al,* 1957). The prototype riminophenazine, clofazimine is obtained by oxidizing

derivatives of *o*-phenylenediamine with *p*-benzoquinone. Various 2,2,6,6- tetramethyl-piperidyl (TMP) substituted phenazine analogues have been developed from clofazimine (Franzblau & O'Sullivan, 1988; Franzblau *et al*, 1989, Van Rensburg *et al*, 1997b). These TMP-substituted phenazines differ from clofazimine in containing a basic nitrogen attached to the imino-nitrogen and in some cases, in halogenation profile of the phenazine nucleus or at the para position of the aniline and phenyl rings. The molecular structures of the 2,2,6,6-tetramethylpiperidyl phenazines, B4121 and B4128 as well as that of clofazimine (B663) are shown in figure 2, page 42.

Clofazimine and the few analogues that have been pharmacologically tested, concentrate primarily in lipid rich tissues and in the cells of the mononuclear phagocyte system, with high concentrations also found in the breast, liver and intestine (Garrelts, 1991). Clofazimine also crosses the placenta (Holdiness,1985), as well as the blood-brain barrier, although in very small amounts (O' Connor *et al*, 1995). Studies performed on mice by Barry and Conalty (1965) showed that clofazimine concentrations in the spleen, lungs, fat and plasma of mice treated with 25 mg/kg of this agent were approximately 4000, 800, 80 and 3 µg/ml respectively. Twenty one days after cessation of administration of clofazimine the corresponding values were reduced by 98, 3, 56 and 63%. Its high degree of lipid solubility ensures that the drug is slowly eliminated, with an elimination half life of 70 days.

In contrast to clofazimine most of the TMP-substituted phenazine derivatives tested to date are less soluble in fat, do not form crystals in macrophages and are excreted more rapidly (Van Landingham *et al*, 1993). They also cause less pigmentation and they lack direct toxicity in experimental animals.

1.2.2. Immunomodulatory Actions

Clofazimine potentiates the anti-microbial function of human phagocytes, an activity which may also contribute to the therapeutic effects of this agent. A major constituent of phagocytes (neutrophils and monocytes) is the enzyme myeloperoxidase, which exerts bactericidal properties by catalysing the oxidation of chloride ions (Cl^-) by

hydrogen peroxide (H_2O_2) to hypochlorous acid (HOCl), a powerful, antimicrobial chlorinating oxidant (Weiss & Peppin, 1986). Van Zyl and coworkers (1991) found that clofazimine increases the production of HOCl by activated phagocytes, probably as a result of the stimulatory effects of this agent on the production of superoxide and H_2O_2 by these cells, as well as by enhancement of release of myeloperoxidase. Wadee and coworkers (1995) have found that clofazimine was capable of enhancing the spontaneous production of hydrogen peroxide and the intracellular killing by phagocytic cells, but had no effects on lysozyme release by resting phagocytes. Importantly, Edwards and coworkers (1986) showed that hydrogen peroxide together with superoxide anions are the oxygen metabolites required for the efficient killing of mycobacteria. The susceptibility to H_2O_2 appears inversely related to mycobacterial virulence. Wadee *et al* (1995) have reported that a 25kDa glycolipoprotein derived from *M.tuberculosis* markedly inhibits the intracellular killing ability of phagocytic cells. Riminophenazines antagonize the immunosuppressive activity of this mycobacterial glycolipoprotein.

Clofazimine has also been found to stimulate oxygen consumption and superoxide generation by neutrophils. The increase in superoxide generation by clofazimine-treated neutrophils was studied by Krajewska and Anderson (1993), who found that this riminophenazine and its analogue B669 increase the activity of phospholipase A_2 (PLA_2) in neutrophils, resulting in increased release of lysophosphatidylcholine and arachidonic acid from neutrophil membrane phosphatidylcholine. Both of these bioactive lipids formed during exposure of human neutrophils to riminophenazines potentiate the activity of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (Krajewska & Anderson, 1993). Clofazimine treatment of macrophages, also induces an increase in the lysosomal enzyme levels of these cells which results in the increased killing of intracellular parasites (Sarracent & Findlay, 1982).

Anderson and Smit (1993) have demonstrated that co-incubation of human mononuclear leucocytes with riminophenazines is also associated with increased activity of PLA_2 leading to increased generation of anti-proliferative lysophospholipids

eg. lysophosphatidylcholine (LPC). It was found that the activity of purified PLA_2 was unaffected by either clofazimine or B669, leading to the conclusion that these highly lipophilic riminophenazines may disrupt lymphocyte cell membrane architecture making the integral phospholipids more susceptible to attack by PLA_2. Furthermore, it was found that the intra-membrane accumulation of LPC during exposure of lymphocytes to riminophenazines was associated with a decrease in the activity of the essential membrane-associated enzyme, Na^+, K^+ -adenosine triphosphatase (Na^+,K^+-ATPase). The addition of alpha-tocopherol (vitamin E), a lysophospholipid complex-forming agent, to clofazimine- or B669- treated lymphocytes was found to prevent the inhibitory effects of riminophenazines on Na^+, K^+- ATPase activity and lymphocyte proliferation.

1.2.3. Anti-tumour actions

Recent research into the anti-tumour potential, as well as into the molecular/ biochemical mechanism and cellular site of action of the riminophenazine group of compounds, has identified these agents as being prime contenders for intensive evaluation as novel anti-cancer drugs with the potential to circumvent or modulate multidrug resistance mechanisms (VanRensburg *et al*, 1997a). Unlike presently used, standard anti-cancer drugs which primarily affect cytoplasmic or nuclear targets, the riminophenazines mediate their anti-neoplastic effects at the level of the plasma membrane by a unique dual mechanism of anti-tumour activity (Van Rensburg *et al,* 1993). Again, the primary, molecular target of the riminophenazines is Na^+, K^+-ATPase, the membrane associated enzyme, which is essential for tumour cell proliferation (Van Rensburg *et al*, 1994).

Van Rensburg *et al*, (1993), found that clofazimine and B669 inhibit the proliferation of various cancer cell lines *in vitro* by a dual action, involving phospholipase A_2-dependent oxidative and non-oxidative mechanisms. The pro-oxidative mechanism is secondary and indirect, and involves activation of PLA_2 by the riminophenazines in phagocytic cells (Krajewska & Anderson, 1993). Addition of neutrophils to tumour cells in the presence of riminophenazines greatly increased the cytotoxic effect of these drugs, by an oxidant-mediated, catalase-inhibitable mechanism (Van Rensburg *et al,* 1993).

Furthermore, exposure of the various tumour cell lines to clofazimine and B669 in the absence of phagocytes is also accompanied by inhibition of the proliferation of these cells by the aforementioned direct mechanism involving inhibition of Na^+, K^+-ATPase.

Recently, Van Rensburg and coworkers (1997b) demonstrated the anti tumour activity of TMP-substituted phenazine molecules. Unchlorinated TMP-phenazines were found to be more cytotoxic than their chlorinated counterparts, while the halogenated molecules, especially those with chlorine atoms at position 3 on the aniline and phenyl rings, were less cytotoxic, but more effective as chemosensitizing, P-glycoprotein (P-gp)-neutralizing agents. One of the TMP phenazines, B4121, increased the sensitivity of Pg-expressing leukaemia cell lines to vinblastine, an anti-tumour drug, by 100-fold.

1.2.4. Antibacterial Actions

Clofazimine possesses well documented anti-mycobacterial properties which make this drug useful in the treatment of leprosy (Browne & Hogerzeil, 1962). O'Connor and coworkers (1995) reported that, in lepromatous cases, clofazimine produces improvement between the first and the third months of treatment and is useful in controlling erythema nodosum leprosum (ENL) reactions occurring in multi-bacillary forms of leprosy. In contrast to other leprosy drugs such as dapsone, rifampicin and ethionamide, clofazimine has been exceptional with regard to the incidence of resistance with only one documented report (Warndorff-Van, 1982).

The TMP-phenazines were tested *in vitro* against *Mycobacterium leprae* in the late 1980s. Franzblau and O'Sullivan (1988) found that the TMP-substituted phenazines have higher anti-*M. leprae* activity than clofazimine. In a subsequent study with five additional TMP-phenazines, Franzblau and coworkers (1989) found that the anti-*M. leprae* activity increased in the TMP derivatives with substitution of the hydrogens on the phenyl and aniline groups (R_2) by fluorines, ethoxy groups, methyl groups, chlorines and bromines in that order. A trichlorinated derivative (B4090) demonstrated even higher activity than the other TMPs.

Although riminophenazines have been in clinical use for decades, the biochemical basis of their antibacterial activity has not been clearly defined. It has been postulated that the riminophenazine agent clofazimine binds to guanine residues of microbial DNA, thereby blocking the template function of the DNA leading to inhibition of growth (Morrison & Marley, 1976). The increase in the guanine and cytosine levels of microbial DNA relative to that of human DNA may explain the selective inhibitory effects of clofazimine on the growth of microbial cells (Barry *et al*, 1957).

Van Rensburg and van Straten (1994) have recently reported that the riminophenazines are broadly active against Gram-positive bacteria, including multi-drug-resistant *Enterococcus* species, as well as mycobacteria, while Gram-negative microorganisms are uniformly resistant (Van Rensburg *et al*, 1992). They found that exposure of Gram-positive bacteria to the highly lipophilic riminophenazines, clofazimine and B669, may alter membrane structure, making the integral phospholipids more susceptible to attack by phospholipase A_2. This enzyme increases the level of lysophospholipids, which in turn selectively inhibit the growth of mycobacteria and other Gram-positive bacteria. The efficiency of the riminophenazines, clofazimine and B669 against Gram-positive and Gram-negative bacteria was intensively studied by De Bruyn and colleagues (1996) who found that treatment of Gram-positive bacteria with these agents is associated with dose related inhibition of K^+-uptake, using radiolabelled potassium (^{42}K) and rubidium (^{86}Rb) as tracers.

Clofazimine is also a component of the multiple drug regimen used in the treatment of infections caused by *Mycobacterium avium* complex, a frequent pathogen in individuals with human immunodeficiency virus (HIV) infections (Woods & Washington, 1987; Horsburgh *et al*, 1991; Young, 1993). Clinical efficiency has also been found with clofazimine included in multidrug combinations in the treatment of other mycobacterial infections (*i.e. Mycobacterium kansasii, Mycobacterium flavenscens,* and *Mycobacterium haemophilum* (Dautzenberg *et al,* 1991; Yarrish *et al*, 1992).

1.3. BACTERIAL POTASSIUM TRANSPORT SYSTEMS

Bacterial K^+ transport systems have been well characterized in *Escherichia coli (E. coli)* and *Enterococcus hirae* , but this is generally not the case for other Gram-negative and Gram-positive bacteria, including mycobacteria.

In bacteria as in other cells and organisms, potassium (K^+) is the major intracellular cation, which is actively accumulated in cells to achieve higher intracellular concentrations relative to those in the extracellular environment. It is a major cytoplasmic cation in growing bacterial cells in which it plays a role in the activation of cytoplasmic enzymes (Steinbach,1962), as well as in DNA, RNA and protein synthesis and in the maintenance of turgor pressure (Reed & Walsby, 1985), and possibly in the regulation of cytoplasmic pH (Brey *et al*, 1978; Plack & Rosen, 1980). The K^+ concentration is determined by the sum of the K^+ transport systems located in the cell membrane. It is reported that *E. coli* (Bakker *et al*, 1987; Dosch *et al,*1991) and probably also all other bacteria possess several K^+ uptake and K^+ efflux systems

The work of Epstein and Schultz (1965) showed that the size of the K^+ pool is determined by the osmotic pressure of the growth medium. In *E.coli,* this regulation of K^+ is believed to occur at the level of control of both the induction and activity of the cation uptake systems, as well as the activity of the corresponding efflux systems (Rhoads & Epstein,1978; Meury & Kepes,1981; Meury *et al*, 1985). It is reported that all cells extrude Na^+ and accumulate K^+. The reason for the preference for K^+ in the cytoplasm is due to the somewhat large ionic radius of the cation. Accordingly, the interaction of K^+ with the water molecules of the cytoplasm and with the anionic groups of the cellular macromolecules enables enzymes to possess native conformation and optimum activity (Wiggins,1990). Accumulation of K^+ by cells also enables them to maintain a negative turgor pressure.

Cell-associated K^+ cations can be detected by atomic absorption spectroscopy, flame photometry, or alternatively, by measuring the amount of ^{42}K in cells loaded with this

isotope. ^{42}K has a half-life of only 12.4 hours however, and because of this ^{86}Rb has become widely adopted as a convenient substitute for the measurement of K^+ fluxes in cells since its half-life is 40 times greater than that of ^{42}K (i.e 19.5 days vs 12.4 hours, Rhoads *et al*, 1977).The validity of using ^{86}Rb as a radioactive tracer for K^+, or the ability of chemical Rb^+ to satisfy a cell's K^+ requirement, has been demonstrated for *E.coli* (Rhoads *et al*, 1977), and *Neurospora crava* (Slayman & Tatum, 1965).

Rhoads and coworkers (1977) demonstrated that although a high concentration of Rb^+ can stimulate growth of *E. coli,* the growth achieved is only a fraction of that achieved at low concentrations of K^+, with the molar growth yield of *E.coli* with Rb^+ being 5% of that with K^+. Furthermore, they acknowledge the fact that although ^{86}Rb is less efficient when compared to ^{42}K it can serve a function similar to that served by ^{42}K. Slayman and Tatum (1965) reported that the K^+ flux is characterized with respect to three basic properties, namely energy dependence, dependence upon the extracellular K^+ concentration, and competitive inhibition.

1.3.1. Potassium Uptake Systems

K^+-uptake systems are electrogenic systems, which are greatly facilitated by the internal negative membrane potential present in most bacteria. These systems are stimulated by low turgor pressure (Rhoads & Epstein, 1978; Meury & Kepes, 1981), while the osmolarity of the solutes determines the extent of K^+ uptake. The cell turgor pressure is believed to bring the cell into a swollen state which is essential for growth (Epstein,1986).

Epstein (1986) further reported that K^+ uptake by bacteria is mediated both by constitutive systems with relatively low affinities for this cation and by inducible high affinity systems. *E.coli* possesses several constitutively operative K^+ transporters known as Trk G/H, Trk F and Kup. Trk is the abbreviation for transport of potassium, while Kup is the abbreviation for potassium uptake. The Trk G/H system consists of several subunits. In the case of TrkG, these are the integral membrane protein, TrkG, as well as a peripheral protein, Trk A, while the Trk E gene product increases the affinity of the

system for K^+ (Dosch *et al*, 1991). These systems are unusual since they are not completely ATPases or secondary porters coupling energy from transmembrane movement of Na^+ or H^+, but appear rather to combine both properties *ie.* a requirement for cellular ATP and high transmembrane potential (Rhoads & Epstein,1977). Attempts to combine the cloned genes into artificial operons have been unsuccessful because: i) the genes have been cloned separately ii) expression levels are low and iii) the Trk systems consists of more than one subunit.

The Trk G/H system is activated by low turgor pressure, has a moderate-to-low affinity for $K^+$and is active at K^+ concentrations of > 1mM. It is the most active K^+ transporter during normal culture conditions. Although it has been speculated that ATP is necessary for the regulation of cation transport by Trk G/H and that uptake of K^+ is driven by proton motive force (K^+/H^+ symport), the exact mechanism by which this is achieved have not been elucidated.

The Kup system of *E. coli* consists of a single hydrophobic protein of molecular weight 45 kDa (Bakker,1993). Like Trk G/H, it is a constitutive, low affinity system and is probably a K^+/H^+ symporter which utilizes proton motive force. The Kup system however, promotes K^+ transport at a much slower rate (about x 10 slower) than Trk G/H.

The third constitutive system of *E. coli,* Trk F, has been less well characterized. This system has a very low affinity for K^+ and promotes the growth of *E.coli* (in strains deficient in Trk G/H, Kup and Kdp) at potassium concentrations above 15 mM (Epstein & Kim, 1971). No gene product has been identified and it is therefore unclear whether a protein or proteins are involved, or if this system simply represents a membrane-potential-driven, non-specific permeation of K^+.

Kdp is an inducible K^+ transporter of *E.coli.* It is a high affinity K^+-ATPase with a Km for K^+ of 2 µM (Rhoads *et al,* 1976). This K^+ transporter is activated at low turgor pressure when K^+ concentrations are limiting. It consists of 5 polypeptides (A, B, C, D and E)

arranged into a transporter complex consisting of 3 inner membrane proteins (Kdp A, B and C) and a sensor unit, Kdp D and E (Waldenhaug *et al*, 1992).

As is the case with Gram-negative bacteria, relatively little is known about K^+ transporters operative in Gram-positive bacteria. These have been best characterized in *Enterococcus hirae* in which two K^+ uptake systems have been described *viz.* Ktr 1 and Ktr II (Ktr is the abbreviation for potassium transport) (Kakinuma & Harold, 1985). Ktr I is very similar to the Trk G/H system of *E. coli.* It also appears to be a secondary porter, requiring both ATP and proton motive force, which functions constitutively with a moderate affinity for K^+ (Km of 2 mM) and a pH optimum of 7. It is the major potassium uptake system under most conditions. The second system known as Krt II is inducible with a Km value of 0.2 mM. It is a sodium-activated ATPase which exchanges Na^+ for K^+ and has a pH optimum of 9, which makes it of dubious physiologic significance (Kakinuma & Igarashi, 1989).

Although these various K^+ transport systems have only been well described in *E.coli* and *Enterococcus hirae*, it has been proposed that they may be broadly operative in other Gram-negative and Gram-positive bacteria. Recently, the genes that are involved in potassium transport of *M.tuberculosis* H37Rv have been identified, *viz.* the Rv2691 and Rv2692 genes which are homologues of the trkA and trkB genes (http://www.pateur.fr/Biot/TubercuList/). Rv2691 is reported to be similar to *Streptomyces coelicor* TrkA, and also to the N-terminal half of the trk system potassium uptake protein (eg. P39448). Rv2692 is trkA2, a putative potassium uptake protein that is similar to both the N- and C-terminal halves of trk system potassium proteins and especially to *S.coelicor* trkA (eg. G1120706) and to certain *Bacillus subtilis* proteins (eg. P39760). At present however, the relative physiological significance of these K^+ transportes, as well as mechanisms of activation and de-regulation, have not been established.

1.3.2. Potassium Efflux Systems

K^+ efflux is electroneutral occurring via antiporters (Brey *et al*, 1978). There are at least three antiporter systems in bacterial cells, namely:

I) the potassium/proton antiport (KHA) system for K^+, Rb^+, and Na^+

ii) the sodium/proton antiport (NHA) system for Na^+, and Li^+, and

iii) the calcium/proton antiport (CHA) system for Ca^{2+}, Mn^{2+} and Sr^{2+}

The first two antiport systems exchange protons for monovalent cations and the third system functions with divalent cations. The turning on or off of the efflux pathways in response to a decrease of external osmolarity is also immediate.

K^+ efflux is stimulated by high turgor pressure or high cytoplasmic pH (Brey *et* al, 1978). Meury and coworkers (1985) demonstrated that decreasing the external osmolarity results in a sudden increase in the turgor pressure, resulting in the switch-on of the efflux pathway which causes a loss of potassium and a decrease of internal pressure until pressure returns to its normal value. K^+ efflux is then reduced or terminated.

The loss of cations is subject to the usual restrictions of electroneutrality. There is significant retention of K^+ which is in balance with the fixed anions of the cells and this can only be altered by exchange with another anion (Epstein, 1986). K^+ efflux systems can be elicited by a variety of cell treatments including addition of the cytotoxic, lipophilic sulphydryl-reactive agent, N-ethylmaleimide (NEM), alkalinization of the cytoplasm or by increased turgor pressure (Meury *et al,* 1980; Meury *et al*, 1985). Efflux does not involve reversal in function of a K^+ uptake system, because efflux is unaffected in strains defective in uptake (Bakker *et al,* 1987).

E.coli possesses at least three K^+/H^+ antiporters of which two are encoded by the KefB and KefC genes and are activated by glutathione (Meury & Kepes, 1982; Elmore *et al*, 1990). The Kef B and KefC systems are considered to be involved in the homeostatic process of pH and turgor-pressure regulation but are thought to act as emergency system when glutathione in the cell reacts with sulphydryl reactive agents (Meury & Kepes, 1982). Recently, genetic analysis of *M.tuberculosis* H37Rv has revealed a

membrane protein of unknown physiological function, Rv3236c which is thought to have some similarity to microbial glutathione-regulated K^+ efflux systems. To date, very little is known about the efflux system of *M.tuberculosis*.

1.4. CALCIUM TRANSPORT SYSTEMS

The maintenance of a low cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is required not only to protect the cell from the toxic effects of Ca^{2+}, but also to permit the use of Ca^{2+} as a second messenger (Smith, 1995). Cytosolic free calcium concentrations are maintained at sub- micromolar levels in living cells by energy-dependent calcium efflux (Gambel *et al*, 1992).This takes place by either secondary transport systems or by a primary active transport system, *i.e.*, a transport system where chemical energy is coupled directly to Ca^{2+} translocation. Eukaryotic cells utilize both of these mechanisms for calcium homeostasis, but bacteria appear to favour calcium efflux via a secondary transporter (Rosen,1987; Gambel *et al*, 1992). An increase in the local free $[Ca^{2+}]_i$ in a cell may occur either by an influx of external Ca^{2+} through the Ca^{2+} channels in the outer membrane of the cell or by release from an endogenous Ca^{2+} sequestering compartment through similar channels (Smith, 1995).

The specific subcellular localization of the cytosolic free calcium concentration elevation depends on the source of Ca^{2+} rise (*i.e.* Ca^{2+} influx vs Ca^{2+} release) (Favre *et al*, 1996). Thus Ca^{2+} influx preferentially raises the submembranous cytosolic free calcium concentration and thereby preferentially activates Ca^{2+}-dependent cellular functions that occur in the submembranous space. There are multiple types of Ca^{2+} influx mechanisms with Ca^{2+} influx through the voltage-operated channels being the best known. However, voltage-gated Ca^{2+} channels are found only in restricted number of cell types, mainly in excitable tissues such as nerve and muscle (Favre *et al*, 1996). Favre and coworkers (1996) further report that the activation of Ca^{2+} influx after store depletion is caused by the decrease in calcium concentration within intracellular stores and is not caused by the increase in cytosolic free calcium concentrations. This type of store-operated influx of Ca^{2+} is operative in most eukaryotic cells.

Several techniques can be used to detect store-operated Ca^{2+} influx such as Ca^{2+} sensitive fluorescent dyes, ^{45}Ca fluxes, and electrophysiologic measurement of Ca^{2+} currents or Ca^{2+}-activated currents (Favre *et al*, 1996). Any procedure that depletes intracellular Ca^{2+} stores leads to an opening of plasma membrane Ca^{2+} channels which results in the store-operated Ca^{2+} influx. In addition to the emptying of Ca^{2+} stores by the receptor agonist and second messengers, store-operated influx of Ca^{2+} can be activated by the following: (i) inhibitors of the Ca^{2+} -ATPase of intracellular Ca^{2+} stores such as thapsigargin, cyclopiazonic acid, and di-tert-butyl-hydro-quinone (Mason *et al*, 1991); (ii) Ca^{2+} ionophores (Hoth & Penner, 1992; Morgan & Jacob, 1994); (iii) introduction of high concentrations of a Ca^{2+} chelator into the cytosol (Hoth & Penner, 1992; Hoth & Penner, 1993); (iv) prolonged incubation of cells in a medium with a very low Ca^{2+} concentration (Alvarez *et al*, 1991; Randriamampita & Tsien, 1993).

Several studies have attempted to measure the time necessary for activation of Ca^{2+} influx by store depletion and have reported a delay of 4 to 20 seconds between the onset of Ca^{2+} release and the increase in plasma membrane Ca^{2+} permeability (Demaurex *et al*, 1992; Hoth & Penner, 1993). Studies in different cell types (Gamberucci, 1994; Marriott & Mason, 1995) have investigated the effect of ATP depletion on the activation of Ca^{2+} influx. These researchers found that a decrease in the cytosolic ATP concentration inhibited store-operated Ca^{2+} influx without inhibiting Ca^{2+} release from intracellular stores.

Calcium Transport Systems in Eukaryotic Cells

Calcium ion (Ca^{2+}) in mammalian cells is moved between four different pools: extracellular milieu, cytoplasm, mitochondria, and a non-mitochondrial pool (endoplasmic reticulum) and sarcoplasmic reticulum (SR) (Carafoli,1987). The presence of voltage-gated, mechanosensitive Ca^{2+} channels in the membranes of eukaryotic cells is well established. There is, however, little direct evidence for similar channels in bacterial membranes even though other bacterial ion channels have been detected and investigated. True Ca^{2+} -transport is carried out by membranous proteins

such as channels, pumps (Ca^{2+} -ATPases), exchangers, and electrophoretic uniport. The maintenance of a low cytosolic free-Ca^{2+} concentration ($[Ca^{2+}]_i$ is a common feature of all eukaryotic cells.

Ca^{2+} is maintained in the cytoplasm of mammalian cells at a concentration that is 10 000-fold lower than that of the extracellular milieu (Pietrobon *et al*, 1990). This is achieved by means of homeostatic mechanisms which involve the natural impermeability of phospholipid bilayers to charged species. However, permeability of Ca^{2+} is significant even across a pure lipid bilayer and even more so across the plasma membrane of living cells that also possess specific pathways (channels) for Ca^{2+} influx. These pathways can be opened by ligands or by depolarization and allow passive diffusion of calcium ions. Furthermore, calcium ions can also be released from stores by intracellular messengers. Pietrobon (1990) suggested that opening of plasma membrane channels or release of Ca^{2+} from intracellular pools leads to elevation of $[Ca^{2+}]_i$ and as a result, Ca^{2+} binds to cytosolic proteins which translate the changes in $[Ca^{2+}]_i$ into activation of a number of key functions many of which involve activation and translocation of cytosolic nuclear transcription factors including NFκB, c-Jun N-terminal kinase (JNK) and NFAT (Dolmetsch *et al*,1997).

In all eukaryotes, the primary barrier to calcium ion inflow into the cytoplasm is represented by the plasma membrane, in which the ultimate long-term regulators of $[Ca^{2+}]_i$ homeostasis, *i.e.* the Ca^{2+}-efflux mechanisms, are located (Pietrobon, 1990). This Ca^{2+} extrusion is accomplished by means of membranous Ca^{2+}-binding proteins that reversibly complex and translocate Ca^{2+} outside the cytosol. Two main types of Ca^{2+}-extruding systems are known to exist in the plasma membrane involving (a) Ca^{2+} -ATPases and (b) a Na^{+}/ Ca^{2+} exchanger which differ in their Ca^{2+} affinity and maximum velocity of Ca^{2+} transport. The most well characterized Ca^{2+}-ATPase is the one situated in the plasma membrane of erythrocytes. Ca^{2+} -ATPase is a polypeptide that transports Ca^{2+} with high affinity and low capacity (Caroni & Carafoli, 1981).

Exposure of neutrophils to receptor-linked stimuli such as N-formyl-*L*-leucyl-*L*-phenylalanine (FMLP) leads to mobilization of Ca^{2+} from intracellular stores (Anderson & Goolam Mahomed, 1997). Anderson and Goolam Mahomed (1997) demonstrated that the combination of fura-2 fluorescence and a radiometrc procedure using ^{45}Ca revealed that exposure of neutrophils to the chemotactic tripeptide FMLP, results in a rapid efflux of Ca^{2+}, which coincides with release of the cation from intracellular stores. Furthermore, the radiometric procedure, unlike the fura-2 procedure was able to distinguish between net efflux and influx of Ca^{2+} following activation of neutrophils with fMLP.

The Ca^{2+}-ATPase of plasma membranes is one of the so-called E_1 E_2 transport ATPases, which are postulated to exist in two different conformational states E_1 and E_2 in different moments of the reaction mechanism, and which conserve the ATP energy intramolecularly in the form of an acyl phosphate, most likely an aspartyl phosphate (Carafoli, 1987; Schatzmann,1989). Ca^{2+}-ATPase possesses two binding sites for ATP (Carafoli,1991), whereas the Na^{+}/Ca^{2+} exchanger is not energized by ATP, but it appears that the exchanger is phosphorylated under normal intracellular concentrations of ATP (Caroni & Carafoli,1983)

1.4.2. Calcium Transport Systems in Prokaryotic Cells

The role of calcium ions in bacterial cells includes heat shock, pathogenicity, chemotaxis, differentiation, and the cell cycle (Norris *et al*, 1996). A large fraction of the Ca^{2+} associated with the prokaryotic cell is found in the region of the cell wall and the cell membrane. By using X-ray analysis, Chang and coworkers (1986) found that 10nm-wide regions, which included the cell envelope contain an average of 17 times more Ca^{2+} than the corresponding cytoplasmic region of exponential phase cells of *E.coli*.

Labelling studies with ^{45}Ca have also shown that a large fraction of Ca^{2+}associated with prokaryotic cells is bound externally and may be removed by washing procedures (Gangola and Rosen,1987) or treatment with Ca^{2+} chelating agents such as ethyleneglycol-bis- (β-aminoethylether)-N,N,N',N',-tretraacetic acid (EGTA) (Smith *et*

al, 1987). The introduction of chelating agents further reduced the availability of Ca^{2+} in media (Youatt, 1993). In addition to occurring in the lipopolysaccharide layer, Ca^{2+} is also located in the regular outer surface layer, known as S-layers that occur in most bacteria (Smith, 1995).

Some bacterial phospholipases have been found to require Ca^{2+}. Concentrations of Ca^{2+} exceeding 1nM are required to maintain the activity of phopholipase A_1 which resides on the outer membrane of *E.coli* (Elbasch *et al*, 1985). The work of Cullis and de Kruijff (1979) and Zachowski *et al*, (1987) indicate that Ca^{2+} has major effects on the diversity of membrane structure. Norris (1989a, 1989b & 1992) has proposed a mechanism for the regulation of DNA replication and cell division in prokaryotes that brings together the effects of Ca^{2+} on membrane structure and the proposed Ca^{2+}/calmodulin-mediated regulation of phospholipid synthesis. It is suggested that a sudden increase in cytoplasmic Ca^{2+} may trigger a major translocation of phospholipid from the inner to the outer monolayer of the bilayered cell membrane. Since the translocation may be expected to disrupt momentarily the integrity of the membrane, depolarization of the membrane and a substantial influx of Ca^{2+} might occur (Smith, 1995).

The immediate driving force for calcium efflux is a proton electrochemical gradient or a sodium electrochemical gradient for *Halobacterium halobium* (*H.halobium*) and *Bacillus SpA*-007 respectively (Gambel *et al*, 1992). Calcium extrusion in *H.halobium* occurs by a Ca^{2+}/Na^{+} exchange mechanism, but in other bacteria Ca^{2+} efflux seems to be powered by an electrochemical proton gradient, with a Ca^{2+}/H^{+} antiport mechanism best explaining the data (Heefner, 1982). In these bacteria Ca^{2+} efflux is a secondary process, *i.e.*, Ca^{2+} translocation is coupled to previously established electrochemical proton gradient.

1.5. Phospholipase A_2

Phospholipase A_2 (PLA_2) belongs to a class of enzymes that catalyse the hydrolysis of membrane phospholipids to release free fatty acids. Much of the interest in

phospholipid metabolism is due to the recognition that enzymatic reactions involving phospholipases participate in many vital cellular functions, including signal transduction (Mukherjee *et al*, 1994). Mukherjee and colleagues (1994) reported that phospholipid-metabolizing enzymes *i.e.* phospholipases are involved in the pathogenesis of many devastating disease processes and/or contribute to aggravating existing pathological conditions. The nomenclature of phospholipases is a function of their specificity as indicated in figure1.

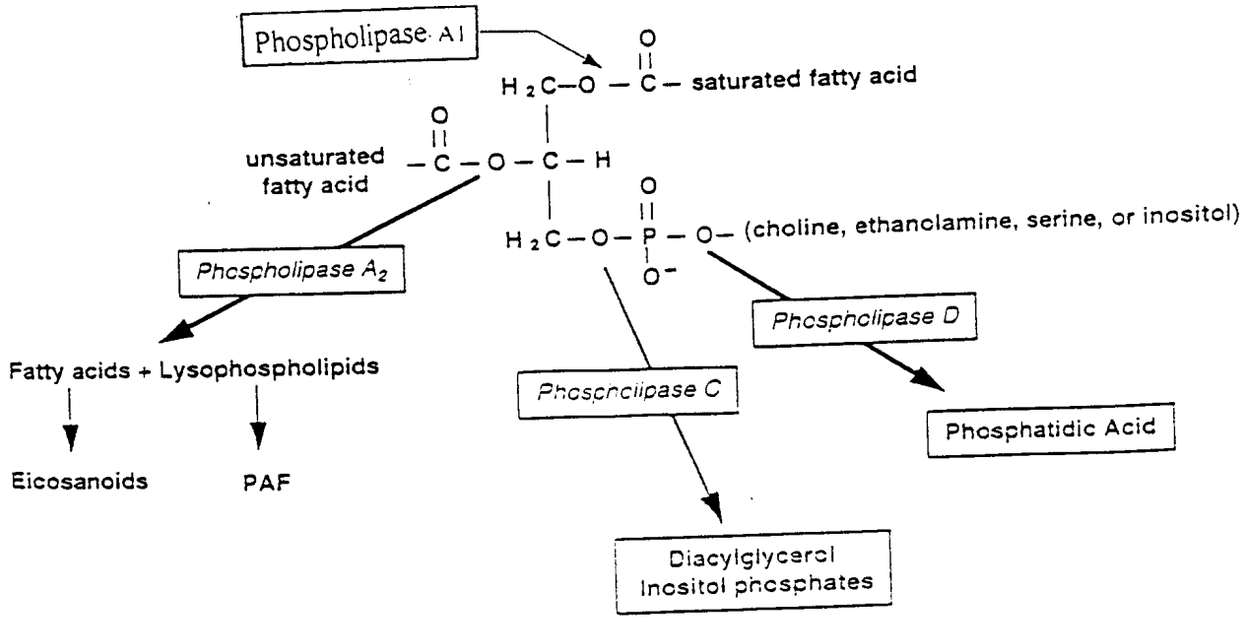


Figure 1: The nomenclature of Phospholipases

The acyl ester bond at position 1 of 3-sn-phosphoglycerides is attacked by phospholipase A_1 (PLA_1) and 2-sn acyl ester bond by phospholipase A_2 (PLA_2) . PLA_2 attack results in the production of equimolar amounts of lysophospholipids and free fatty acids. Phospholipase B displays both activities of type A_1 and A_2, and it also hydrolyses lysophospholipids. Another two phospholipase species attack the phosphodiester linkage, resulting in the release of diacyglycerol by the action of phospholipase C or phosphatidic acid by phospholipase D.

1.5.1. General Features of Phospholipase A_2

Phospholipase A_2 (EC 3.1.1.4; phosphatidylcholine 2-acyl-hydrolase) is an esterase that hydrolyses the sn-2 ester bond in phosphoglyceride molecules releasing a free fatty acid and a lysophospholipid. PLA_2s have attracted a lot of attention due to their ability to produce substrates for the generation of inflammatory lipid mediators. This is the predominant (but not the only) pathway for the production of arachidonic acid from 2-arachidonyl glycerophospholipids. Inhibition of PLA_2 activity offers an attractive therapeutic approach to the design of novel drugs for the treatment of inflammation and tissue injury (Chang *et al*, 1987).

PLA_2s are located both inside and outside the cell. The intracellular enzymes are found in low concentrations in almost every mammalian cell (Van den Bosch, 1980), where they play an important role in membrane metabolism and in the release of arachidonic acid. Certain intracellular PLA_2s also have a digestive role in the breakdown of phagocytosed material (Elsbach & Weiss, 1988). The extracellular PLA_2s are abundant in mammalian pancreatic juice and in snake and bee venom (Verheij & Dijkstra, 1994). Irrespective of the source , all extracellular PLA_2s are small, water soluble proteins (13-14 kDa) (Verheij *et al*, 1981). These enzymes are able to hydrolyse monomeric substrate molecules, but their full activity only becomes evident in the presence of certain lipid-water interfaces.

PLA_2 enzymes are widespread, being found in nearly all cell types including bacteria and protozoa. These enzymes contain a high degree of disulphide cross linkages and are extremely stable to heat and acid treatment. In fact solubilization of membrane-bound enzymes can easily be achieved through mineral acid extraction (Elsbach *et al*, 1979). PLA_2 has a higher affinity for aggregated than non-aggregated phospholipids and can exist in either the inactivated or activated state (Chang *et al*, 1987). The enzymatic activity of PLA_2 can be measured by the disappearance of substrate or by the appearance of products (Verheij *et al*, 1981). PLA_2 enzymes share some sequence homology, especially in their active site regions which bind calcium. The enzyme is sometimes dependent on Ca^{2+} and is also highly stereospecific, since only the natural

occurring L-isomers of phospholipid molecules (3-sn-phosphoglycerides) can be degraded.

Several extracellular PLA_2 enzymes have been isolated from various snakes and bee venoms as well as from mammalian pancreas. These are classified into three main groups, I, II, and III and several subgroups (Dennis, 1994). Group I, II, and III PLA_2 are secreted enzymes which have a high disulfide bond content, low molecular weight and they require Ca^{2+} for catalysis. Group IV PLA_2 is a distinctly different Ca^{2+} - dependent high molecular weight intracellular enzyme that is specific for arachidonic acid. This enzyme has been identified in a variety of cells, human U937/platelets, raw 264.7 and rat kidney (Dennis, 1994, 1997).

Genetic and bioinformatic analysis has led to the identification of four phospholipase C genes, namely, Rv2351c, Rv2350c, Rv2349c and Rv1755c in the *M.tuberculosis* H37Rv genome sequence (http://www.pasteur.fr /Bio/TubercuList). To date, however, gene(s) encoding for PLA_2 in *M.tuberculosis* have not been identified.

1.5.2. Activation of Phospholipase A_2

PLA_2 is activated by various physiological stimuli such as angiotensin II, bradykinin, prolactin and thrombin resulting in release of arachidonic acid when added to responsive cells (McGiff *et al*, 1972; Vargaftig & Hai, 1972; Bills *et al*, 1977; Rillema & Wild, 1977) . Once activated, PLA_2 can mediate a variety of pathological reactions either through a direct action, or through subsequent transformation of its products (lysophospholipid and arachidonic acid) to several potent biologically active substances such as prostaglandins and leukotrienes.

Lysophospholipids, the co-products of PLA_2 activation are cytotoxic substances and membrane fusogens (Chang *et al*, 1987) and have been implicated in several human inflammatory conditions(Secchi *et al*, 1979). Acetylation of lysophospholipids at the 2-hydroxy group gives rise to another lipid mediator, platelet activating factor (PAF), a potent platelet aggregating substance and inducer of various inflammatory reactions

such as erythema, chemotaxis and increased vascular permeability (Chilton *et al*, 1982).

The regulation of PLA_2 activity is complex and several factors modulate its activity. One prime regulator of PLA_2 is calcium. A calcium binding site has been identified on PLA_2, and addition of calcium increases enzyme activity, whereas addition of Quin 2, a calcium chelator, inhibits enzyme activity (Van Scharrenburg *et al*, 1985; Simon *et al*, 1986). Furthermore, the calcium ionophore A23187 potentiates arachidonic acid release from intact cells. Although such studies demonstrate that PLA_2 requires calcium for activity, its exact role is uncertain.

1.5.3. Bacterial Phospholipase A_2

Studies on enzymes involved in catabolism of phospholipids in *E. coli* have identified the presence of PLA_1 and PLA_2 in the wall of the bacterium (Proulx & Fung, 1969; Albright *et al*, 1973). The enzymatic activities of both PLA_1 and PLA_2 manifest similar properties and requirements for optimal activity although PLA_2 specific activity was found to be much lower than that of PLA_1 (Albright *et al*, 1973). Both PLAs are heat stable and have the same Ca^{2+} and detergent requirement. Songer (1997) reported that virulence of many bacterial pathogens is based on the action of phospholipases. PLA_1 and PLA_2 as well as lysophospholipase has also been detected in both *Mycobacterium microti* and *Mycobacterium avium* (Wheeler & Ratledge,1992). However, the phospholipase activities of *M.avium* were cryptic while phospholipase activities of *M.microti* were located on the bacterial surface.

Isolated bactericidal agents such as the bactericidal/permeability-increasing protein (BPI) and the antibiotic polymyxin B, trigger bacterial phospholipid degradation by both endogenous and exogenous PLAs (Weiss *et al*, 1979; Elsbasch *et al*, 1985). The phospholipids in intact biological membranes of *E. coli* are resistant to the action of both exogenous and endogenous PLA_2 (Duckworth *et al*, 1974). However, bactericidal concentrations of the membrane-active cationic peptide polymyxin B render *E. coli* susceptible to the action of purified exogenous PLA_2 as well as endogenous bacterial

phospholipase(s) (Weiss *et al*, 1979). This hydrolysis occurs equally well with or without added Ca^{2+}.

Bacterial phospholipases also play the dominant role in the hydrolysis of phospholipids of ingested *E. coli* (Wright *et al* , 1990a). A comparison of three isogenic *E. coli* strains established that among the several phospholipases that have been identified in *E. coli,* it is the pldA gene product that is activated during phagocytosis (Wright *et al*, 1990a), while the others encode a detergent-sensitive PLA (ds-PLA) and a detergent-resistant PLA (dr-PLA) (Doi & Nojima, 1975; 1976). An *E. coli* mutant (pldA-) that lacks the gene for drPLA demonstrates no hydrolysis when exposed to bactericidal agents such as BPI and polymyxin B. It follows that the drPLA is the enzyme that is activated under adverse conditions (Wright *et al*, 1990a).

1.5.4. Antimicrobial activity of PLA_2

Human and other vertebrate leukocytes contain PLA_2s which are specifically involved in the breaking down of bacterial phospholipids (Ganz & Weiss, 1997). Ganz and Weiss (1997) further report that the granules of polymorphonuclear leukocytes (PMN) contain a 16 kDa PLA_2 that selectively hydrolyses phospholipids in bacterial membranes. The enzyme is a member of a large family of "secretory" (s)PLA_2 (Dennis, 1997) produced by both invertebrates and vertebrates, that share several highly conserved features including size, high disulphide content (> six disulphides, six of which are invariant), a Ca^{2+}-binding loop, and closely similar catalytic machinery and secondary and tertiary structure.

In mammals, such as man, two isoforms (type I and II) of $sPLA_2$ have been extensively characterized (Cupillard *et al*, 1997; Dennis, 1997). Type I PLA_2 is exemplified by mammalian pancreatic enzymes, whereas type II enzymes include intestinal and splenic PLA_2 (Heinrikson *et al*, 1977; Johnson *et al*, 1990). In addition to the granules of phagocytes, type II PLA_2 is present in granules of platelets, mast cells and paneth cells, keratinocytes, and lacrimal glands (Dennis, 1997). Bacterial killing by PLA_2 depends on binding to the bacterial surface (Ca^{2+}-independent, presumably to the sites

in the cell wall), penetration of the cell wall and Ca^{2+}-dependent degradation of membrane phospholipids (Dennis, 1997).

The intracellular killing and overall destruction of the Gram-negative bacteria of *E.coli* ingested by PMN involves degradation of bacteria by PLA_2 and the extent of intracellular destruction of these bacteria is closely linked to the magnitude of phospholipase action (Elsbach & Weiss, 1988; Wright *et al*, 1990 b). PLAs contributing to digestion of ingested Gram-negative bacteria include bacterial outer envelope PLA and a granule associated PLA_2 of the PMN (Elsbach & Weiss, 1988; Wright *et al*, 1990 b). In addition, an extracellular inflammatory fluid PLA_2 added either as part of the whole inflammatory fluid or as purified protein can also participate in intracellular bacterial digestion apparently by associating with the surface of bacteria and/or PMN before phagocytosis and acting after co-internalization with ingested bacteria (Wright *et al*, 1990 b). Madsen and coworkers (1996) demonstrated significant amplification of bacterial phospholipid degradation concomitant with increased intracellular bacterial killing after the pretreatment of *E.coli* with nonlethal doses of serum prior to phagocytosis. This phospholipase-mediated degradation of *E.coli* is due to host PLA_2 mobilized to the phagolysosome during phagocytosis.

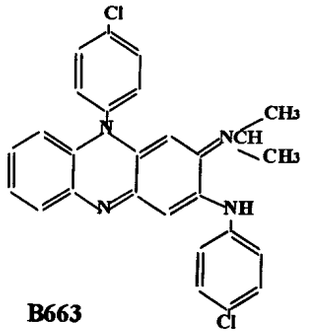
Salmonella typhimurium normally infects its hosts via the gastrointestinal tract, where it encounters type II PLA_2 molecules at least twice, first in Paneth cell secretion and next in the lysosomal apparatus of the macrophages (Harwig *et al*, 1995). The phoP mutant *S.typhimurium* 7953S is much more susceptible to intestinal PLA_2 than its isogenic parent *S.typhimurium* 14028S. PhoP is the regulatory component of a bacterial transcriptional activator or sensory kinase regulon which modulates the production of a cohort of proteins, some of which are virulence-determining (Miller *et al*, 1989). PhoP mutants were found to show an impaired ability to survive in murine macrophages (Fields *et al*, 1986; Buchmeier & Heffron, 1989). Consequently, phoP-regulated resistance to type II PLA_2 may contribute to the virulence of this group of enteric pathogens (Harwig *et al*, 1995).

Wheeler and Ratledge(1992), demonstrated that phospholipase activity for releasing fatty acids from phospholipids is induced in pathogenic mycobacteria by exogenous phospholipids. In nature, these pathogens are only likely to encounter phospholipids when they are inside a host. The observation of relatively high phospholipase activity in mycobacteria harvested from host tissue is consistent with the view that the role of their phospholipases is to release fatty acyl moieties from host phospholipids (Wheeler & Ratledge, 1992).

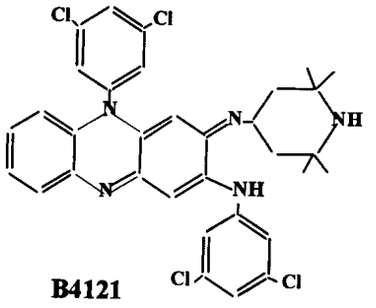
1.6. Aims and Objectives

In my effort to develop new drugs for the treatment of TB, especially that caused by multi-drug resistant strains, my laboratory research was directed at describing the *in vitro* and intracellular anti-mycobacterial activities of the prototype riminophenazine, clofazimine and two novel tetramethylpiperidyl (TMP) derivatives, B4121 and B4128 which showed considerable promise in preliminary screening of a series of TMP-substituted phenazines. The following were my major objectives:

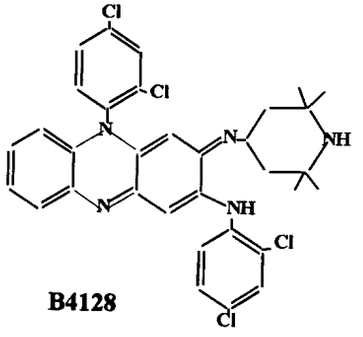
1. To investigate the antimycobacterial activity of the novel TMP-substituted phenazines, B4121 and B4128 against a series of sensitive and multi-drug resistant strains of *M.tuberculosis*.
2. To measure the uptake of riminophenazines by human macrophages and to assess their antimicrobial activity against intracellular *M. tuberculosis*.
3. To characterize the biochemical mechanisms by which riminophenazines modulate potassium (K^+) transport in mycobacteria with emphasis on microbial PLA_2, Ca^{2+} fluxes and energy metabolism.
4. To investigate the antimycobacterial potential of conventional calcium-mobilizing stimuli (the calcium ionophore A23187 and thapsigargin, a specific inhibitor of the endomembrane Ca^{2+} - ATPase of eukaryotic cells) and to compare these with the TMP-substituted phenazines.



B663



B4121



B4128

Figure 2: Molecular structures of the riminophenazine agents

CHAPTER 2

AN *in vitro* INVESTIGATION OF THE EXTRACELLULAR AND INTRACELLULAR ANTIMYCOBACTERIAL ACTIVITIES OF CLOFAZIMINE AND THE TMP-SUBSTITUTED PHENAZINES B4121 AND B4128 AGAINST A SERIES OF SENSITIVE AND MULTI-DRUG RESISTANT STRAINS OF *M. tuberculosis* (H37Rv).

2.1. INTRODUCTION

The simplest *in vitro* model for determining the effects of chemotherapy against strains of *Mycobacterium tuberculosis* or other mycobacterial infections is the use of *in vitro* susceptibility testing, in which a suspension of bacteria is mixed with various concentrations of the drug under test and the capacity of the inoculum to grow is determined (Orme *et al*, 1994). Since *M. tuberculosis* is an intracellular pathogen, the development of *ex vivo* human macrophage models of experimental *M. tuberculosis* infection has been highly instructive in expanding our knowledge of the activity of chemotherapeutic agents against virulent intracellular turbercle bacilli. Reddy *et al*, (1996) using *ex vivo* human macrophage models found that isoniazid, one of the first-line antituberculosis drugs, possesses impressive activity in human macrophages as compared to clofazimine and its analogue B4157.

The major objective of the studies presented in this chapter was to compare the intra- and extracellular activities of the two novel TMP-substituted phenazines (B4121 and B4128) against *M. tuberculosis* H37Rv (ATCC 27294) with those of clofazimine and rifampicin. The *in vitro* activity of these two agents (B4121 and B4128), together with clofazimine, was also determined against a susceptible and five drug-resistant strains of *M. tuberculosis*, using conventional *in vitro* antibiotic sensivity testing procedure.

2.2. MATERIALS AND METHODS

2.2.1. ANTIMICROBIAL AGENTS.

All the riminophenazine compounds were synthesized by Dr J F O'Sullivan, Department of Chemistry, University College Belfield, Dublin, Republic of Ireland, and their molecular structures are shown in Figure 2, page 42. These agents were dissolved in ethanol containing 10 mM acetic acid, while rifampicin was dissolved in 50 mM NaOH, to give stock concentrations of 2 and 5 mg/ml respectively. Dilutions were made in RPMI 1640 supplemented with 10% autologous plasma for measurement of intracellular activity, or in Middlebrook 7H12 medium for standard susceptibility testing.
Appropriate solvent controls were included.

2.2.2. CHEMICALS AND REAGENTS

Unless otherwise indicated, all chemicals used were obtained from the Sigma Chemical Company, St Louis, MO, USA.

2.2.3. MYCOBACTERIAL STRAINS

H37Rv (ATCC 27294), 5 drug-resistant clinical isolates of *M.tuberculosis*, as well as a susceptible clinical isolate were provided by the Medical Research Council, Tuberculosis Research Institute, Pretoria, South Africa.

2.2.4. DETERMINATION OF MINIMAL INHIBITORY CONCENTRATIONS (MICs) BY AGAR DILUTION METHOD

The MICs of the new riminophenazine compound B4121 relative to those of clofazimine (B663) were initially determined against five drug-resistant clinical isolates of *M. tuberculosis* and a susceptible reference strain H37Rv (ATCC 27294) by the agar proportion method (Lee & Heifets, 1987). Serial 2-fold dilutions of the two drugs, ranging from 0.075 to 20 µg/ml, were incorporated into Middlebrook 7H10 agar medium. Inoculates were from a log phase culture in Middlebrook 7H9 broth with an optical density of a McFarland number 1 standard. Aliquots of the cultures were then inoculated, in duplicate, into drug-containing and drug-free control plates to yield final concentrations of approximately 2×10^4 cfu/ml. The plates were sealed in plastic bags and incubated at 37°C for 3 weeks before final readings were made. The lowest drug concentration that inhibited more than 99% of the bacterial population was considered to be the MIC.

2.2.5. DETERMINATION OF MINIMAL INHIBITORY CONCENTRATIONS (MICs) BY THE RADIOMETRIC METHOD

The radiometric method (Lee & Heifets, 1987) was used to estimate the MICs of clofazimine and the TMP-substituted phenazines, B4121 and B4128, against *M. tuberculosis* H37Rv (ATCC 27294). The *in vitro* activities of these two promising compounds (B4121 and B4128) together with that of B663, were subsequently measured against six clinical isolates of *M. tuberculosis*. One of the isolates was fully

susceptible to the conventional antituberculosis drugs, but the remaining five were resistant to at least one of the drugs. Precultures were prepared by transferring 0.1 ml of log phase organisms, grown in Middlebrook 7H9 medium, to BACTEC 12B vials. The vials were then incubated at 37°C until the growth index reached 500 to 600. Aliquots (0.1 ml) were then inoculated into 12B vials with or without appropriate dilutions of the test drugs or solvent controls.

Prior to inoculation, 0.1 ml of the antimicrobial agents at doubling concentrations, ranging from 0.015 to 10 µg/ml, were injected into BACTEC 12B vials. The 1% drug-free control vials received 0.1 ml of a 100-fold dilution of the preculture. Solvent controls containing 0.05% ethanol and 5 µM acetic acid (final solvent concentrations) were also included. Vials were incubated at 37°C and the growth index (GI) readings were recorded daily in a BACTEC 460 TB instrument until the 1:100 controls reached a GI of ≥30. The MIC was defined as the lowest concentration of the drug that caused an increase in GI equal to or less than that of the 1% control.

2.2.6. INTRACELLULAR ACTIVITY OF THE ANTIMICROBIAL AGENTS

Mononuclear leukocytes were prepared from heparin-treated (5 units of preservative-free heparin/ml) venous blood of healthy, adult human volunteers and separated from erythrocytes and polymorphonuclear leukocytes by centrifugation on Histopaque-1077 (Sigma Chemical Co.) cushions at 950 rpm for 25 min at room temperature. The cells were aliquoted into two tubes and sterile ammonium chloride (NH_4Cl) solution was added to the tubes which were held on ice for 5-10 minutes. NH_4Cl solution is used to lyse the remaining red blood cells. The tubes were then centrifuged for 10 min at 950 rpm and the supernatant was removed and the pellets resuspended in 5 ml RPMI. The cell preparation containing monocytes was counted microscopically and adjusted to 1 x 10^6 cells/ml RPMI supplemented with 10% autologous serum. Two millilitres of cell suspension were then incubated in a glass test tube at 37°C for 60 min after which the non-adherent cells were removed by washing. The cultures were then incubated in 2 ml RPMI supplemented with 10% autologous serum for two weeks, washed and infected with *M. tuberculosis* (H37Rv) for 45 min at 37°C in a shaking water bath at a

concentration of approximately 2 organisms/phagocyte, washed once and exposed for 2 days in 2 ml of culture medium to the antimicrobial agents (0.001-0.5 µg/ml). The intracellular bacteria were released by resuspending the cells in BACTEC diluting fluid and the clumps were dispersed with a tuberculin syringe. BACTEC 12B vials were inoculated with the bacterial suspensions and incubated at 37°C for up to 7 days. The GI was recorded daily until the vials containing the untreated control suspension reached ≥ 600 cpm (Van Rensburg, *et al*, 1995).

2.3. RESULTS

In vitro MICs of the riminophenazines

The MIC results, determined by both the agar and radiometric dilution methods, are summarised in Tables 1, 2 and 3 (pages, 50, 51 and 52). These results show that the MICs on 7H10 agar medium (Table 1) were 10-fold higher than those determined by the radiometric method (7H12 broth) (Tables 2 and 3). An MIC value of 0.03 µg/ml was obtained in 7H12 broth for B4121 and B4128 against the reference strain, *M. tuberculosis* H37Rv (ATCC 27294), as opposed to an MIC of 0.06 µg/ml for B663, while the corresponding MIC values on 7H10 agar for clofazimine (B663) and B4121 against H37Rv and five clinical isolates were 0.63 and 0.31, respectively. Radiometrically determined MICs for B663, B4121 and B4128 against H37Rv and various clinical isolates of *M.tuberculosis* were also in a narrow range of 0.03 to 0.12 µg/ml, irrespective of the susceptibilities of the organisms to standard antituberculosis drugs. The latter results imply that there is no cross-resistance between the riminophenazines and the conventional agents normally used for tuberculosis therapy. In control systems which contained the solvent (0.05% ethanol and 5 µM acetic acid), the growth of *M. tuberculosis* was unaffected.

Effects of the test antimicrobial agents on the intracellular growth of *M. tuburculosis* (H37Rv).

The effects of the test agents on the intracellular growth of *M. tuberculosis* (H37Rv) are shown in Figure 3 (page 53). The riminophenazines were significantly more active

intracellularly at all the concentrations tested (0.001-0.5 µg/ml) than rifampicin, with clofazimine being the least active. In the case of B4121 and B4128 intracellular activity (between 45 and 60% inhibition of growth) against *M.tuberculosis* was observed at a concentration of 0.001 µg/ml.

TABLE 1: The minimal inhibitory concentrations (MICs in µg/ml) of clofazimine (B663) and the tetramethylpiperidyl-substituted phenazine B4121 for the sensitive strain (H37Rv) and five resistant strains of *M. tuberculosis* determined on Middlebrook 7H10 agar medium.

***M. tuberculosis* strains**	**Drug Resistance profile***	**Clofazimine (µg/ml)**	**B4121 (µg/ml)**
H37Rv ATCC 27294	Susceptible	0.63	0.31
TBRP 46	INH; RPM, SM; EMB	0.63	0.31
TBRP 171	INH; RMP	0.63	0.31
TBRP 178	INH; RMP; SM	0.63	0.31
TBRP 218	INH	0.63	0.31
TBRP 4591	RMP	0.63	0.31

*INH, isoniazid; RMP, rifampicin; SM, streptomycin; EMB, ethambutol

TABLE 2: The minimal inhibitory concentrations (MICs) of clofazimine (B663) and two tetramethylpiperidyl substituted phenazines, B4121 and B4128, for *M. tuberculosis* H37Rv (ATCC 27294), determined by the standard BACTEC radiometric method.

Riminophenazine	**MIC (µg/ml)**
Clofazimine	0.06
B4121	0.03
B4128	0.03

TABLE 3: The minimal inhibitory concentrations (MICs) of clofazimine, B4121 and B4128 for a sensitive and five multidrug-resistant clinical isolates of *M. tuberculosis* determined by the BACTEC method.

***M. tuberculosis* strains**	**Drug resistance profile***	**Clofazimine (µg/ml)**	**B4121 (µg/ml)**	**B4128 (µg/ml)**
TBRP 17	sensitive	0.12	0.06	0.06
TBRP 171	INH; RMP	0.12	0.03	0.03
TBRP 204	INH; RMP, SM	0.06	0.03	0.03
TBRP 229	INH; RMP; SM	0.06	0.03	0.03
TBRP 274	INH; SM	0.06	0.03	0.03
TBRP 321	INH; RMP	0.12	0.03	0.06

* INH, isoniazid; RMP, rifampicin; SM, streptomycin; EMB, ethambutol

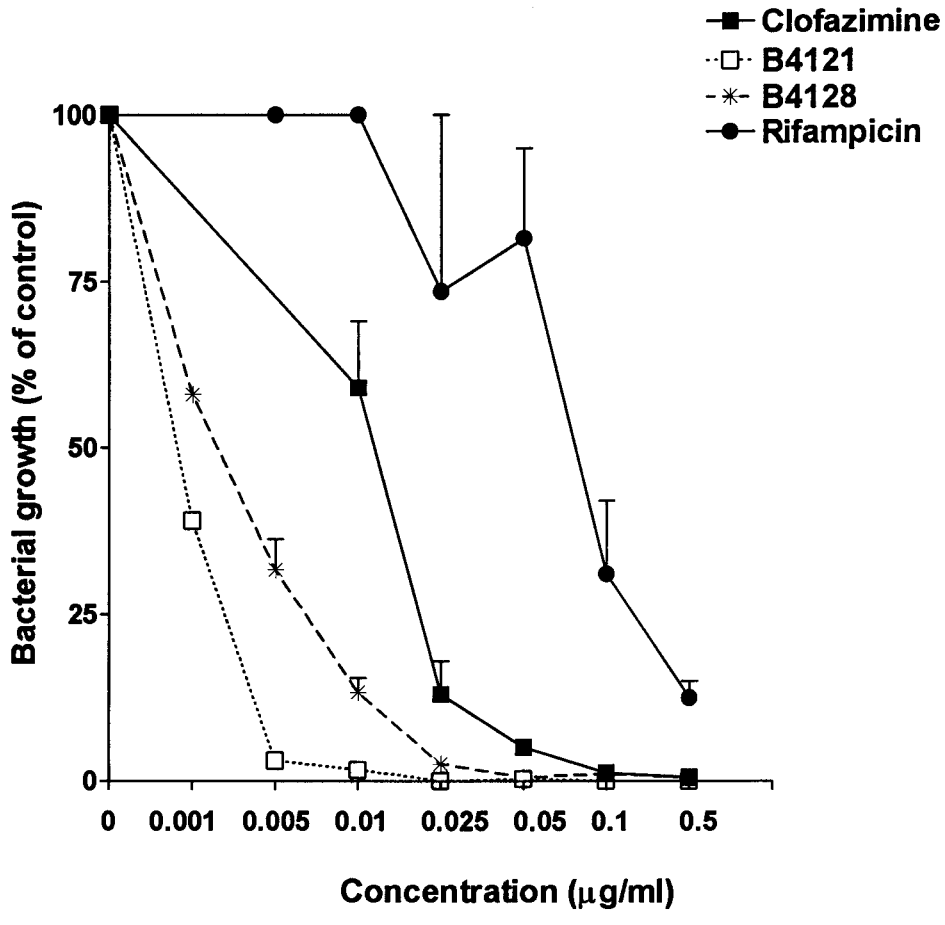


Figure 3: The intracellular activity of clofazimine, B4121, B4128 and rifampicin against *M. tuberculosis* (H37Rv) in human monocytes/macrophages. Data from 1 to 3 different experiments are presented as the mean percentages of the drug-free control systems ± SEMs.

2.4. Discussion

While re-evaluating the antituberculosis activity of clofazimine, I have screened several new analogues of this prototype riminophenazine and have identified several agents with antimycobacterial activity superior to that of clofazimine and rifampicin. The latter is a potent first-line antituberculosis drug that possesses impressive intracellular antimycobacterial activity in human macrophages. The TMP-substituted phenazines have previously been reported to be more active than clofazimne, against *M.leprae in vitro* (Franzblau & O'Sullivan, 1988; Franzblau *et al*, 1989) as well as *in vivo* (Van Landingham *et al*, 1993). Unlike clofazimine, these agents do not crystallize inside eukaryotic cells, are less soluble in fat, are excreted more rapidly and cause less pigmentation (Van Landingham *et al*, 1993).

In this study, the most important finding with regard to the *in vitro* activity of clofazimine and its TMP-substituted derivatives, B4121 and B4128 was that none of the *M.tuberculosis* strains tested, including the multidrug resistant strains, showed resistance to these drugs. These findings may suggest that clofazimine and its TMP-substituted derivatives inhibit rifampicin (RMP)-, isoniazid (INH) -, streptomycin (SM)- or ethambutol (EMB)-resistant *M.tuberculosis* by a mechanism other than the inhibition of RNA synthesis, mycolic acid synthesis, protein synthesis or arabinan biosynthesis. The *in vitro* activity of the TMP-substituted phenazines, B4121 and B4128 was at least double that of clofazimine when the radiometric method was used. Based on the MICs obtained with the radiometric method, *M.tuberculosis* is highly sensitive to the TMP-substituted phenazines. Although the MICs determined with the agar method were 10-fold higher than those observed with the radiometric method, this discrepancy may be explained by increased availability of the compouds in broth compared to that of agar. There was no cross-resistance between the riminophenazines and the standard antituberculosis drugs, which is a potentially important observation since there is a worldwide concern about the emergence of multidrug resistant tuberculosis, and thus an urgent need for new antituberculosis drugs which are active against these resistant strains.

The two TMP-substituted phenazines, B4121 and B4128 as well as clofazimine, were more active than rifampicin against intracellular *M.tuberculosis* in an infected macrophage model of intracellular antimicrobial activity. The experimental compounds inhibited the intracellular growth of *M.tuberculosis* at concentrations which were approximately 10-fold less than the corresponding MIC values obtained using conventional *in vitro* sensitivity testing procedures. These observations suggest that the riminophenazines, which are highly lipophilic, concentrate in macrophages and that their intracellular potency is underestimated by routine MIC determinations. Previously, it was reported that exposure of Gram-positive bacteria to the highly lipophilic riminophenazines, clofazimine and B669 may alter the membrane structure, making the intergral phospholipids more susceptible to attack by PLA_2 (Van Rensburg *et al.*, 1992). Elsbasch and Weiss (1988) reported that the engulfment of microoganisms by phagocytic cells is accompanied by activation of phospholipid degrading-enzymes of both the ingesting and ingested cells, stimulating the turnover of phospholipids in the membrane of both partners in the interaction. The impressive intraphagocytic activity of the TMP-substituted phenazines may be due to the fact that these riminophenazines increase PLA_2 activity in the membrane of both the ingesting and ingested cells. Further studies on the intracellular distribution of the TMP-substituted phenazines and the site of actual interaction between the drugs and the mycobacteria residing in macrophages are necessary.

In conclusion, the TMP-substituted phenazines B4121 and B4128 were found to possess extra- and intracellular antimycobactrial activity which was superior to that of the prototype riminophenazine, clofazimine. Furthermore, the finding that all multidrug-resistant strains were susceptible to clofazimine and its analogues (B4121 and B4128) warrants examination of their usefulness for the treatment of tuberculosis caused by MDR strains. The effectiveness of these agents against multidrug-resistant strains of *M.tuberculosis* together with their impressive intracellular activity against this persistent microbial pathogen, makes them potential agents for intensive evaluation in murine models of experimental anti-tuberculosis chemotherapy.

CHAPTER 3

EFFECTS OF B663 (CLOFAZIMINE) AND THE TWO NOVEL TMP-SUBSTITUTED PHENAZINES B4121 AND B4128 ON CATION TRANSPORT AND PHOSPHOLIPASE A_2 ACTIVITY IN *M. aurum* A^+ AND IN THE AVIRULENT AND VIRULENT STRAINS OF *M. tuberculosis* (H37R)

3.1. INTRODUCTON

Recent research into the antimicrobial activity of the riminophenazines has focused on identifying the mechanisms by which these agents inhibit the growth of Gram-positive bacteria and mycobacteria. This has led to the discovery that K^+ transport systems are the possible targets of riminophenazines (De Bruyn *et al*, 1996). The inhibitory effects of the riminophenazines, B663 (clofazimine) and B669 on the growth of Gram-positive bacteria result from riminophenazine-mediated increased activity of microbial PLA_2 (Van Rensburg *et al*, 1992; De Bruyn *et al*, 1996). This leads to the release of the primary hydrolysis products, lysophospholipids and unsaturated fatty acids, from membrane phospholipids. Lysophospholipids are potent antimicrobial agents which function at the level of the cell membrane by inhibiting K^+ uptake, at least in Gram-positive bacteria. K^+ is critically involved in the activation of microbial cytoplasmic enzymes (Steinbach, 1962), as well as in DNA, RNA and protein synthesis and in the maintenance of turgor pressure (Reed & Walsby, 1985), and possibly in the regulation of cytoplasmic pH (Brey *et al*, 1978; Plack & Rosen, 1980).

In my M.Sc studies I have identified two novel TMP-substituted phenazines B4121 and B4128, with antimycobacterial properties which are superior to those of the prototype riminophenazine, clofazimine, and have suggested a mechanistic relationship between inhibition of K^+- transport and decreased bacterial growth, which was strengthened, but not proven, by the observation that α-tocopherol neutralizes the inhibitory effects of the TMP-substituted phenazines, as well as those of clofazimine, on mycobacterial K^+-transport and growth.

The relationship between riminophenazine-mediated enhancement of PLA_2 activity and inhibition of K^+-transport and growth has been demonstrated in Gram-positive bacteria (Van Rensburg *et al*, 1992). In the experiments described in the present chapter, I have addressed important, unresolved aspects of the antimycobacterial mechanism of action of the TMP-substituted phenazines, using non-pathogenic and pathogenic mycobacteria, which are the primary chemotherapeutic targets of these agents. Most importantly, I have investigated the biochemical mechanisms by which TMP-

phenazines modulate potassium (K^+) transport in mycobacteria with emphasis on microbial PLA_2, Ca^{2+} fluxes and ATP metabolism. The involvement of extracellular and intracellular Ca^{2+} in riminophenazine-mediated enhancement of microbial PLA_2 activity was investigated using the Ca^{2+}-chelating agents, EGTA and BAPTA.

3.2. MATERIALS AND METHODS

3.2.1. RIMINOPHENAZINES

The molecular structures of B663 and its analogues B4121 and B4128 are shown in Figure 2, page 43. The agents were dissolved in 100% ethanol containing 10 mM acetic acid to a stock concentration of 2 mg/ml. Subsequent dilutions were made in absolute ethanol and B663, B4121 and B4128 were used at final concentrations ranging from 0.15-1.25 µg/ml for potassium transport assays and 0.15-10 µg/ml for calcium transport assays. B663 and B4128 were used at final concentrations ranging from 0.15-2.5 µg/ml for PLA_2 activity assays while B4121 was used at final concentrations ranging from 2.5-10 µg/ml. Appropriate solvent controls were included in the assays. The final ethanol concentration in the assay systems was 0.2%.

3.2.2. CHEMICALS AND REAGENTS

Unless otherwise indicated, all chemicals used were obtained from the Sigma Chemical Co., St Louis, MO, USA.

3.2.3. MYCOBACTERIAL STRAINS

The rapidly proliferating, non-pathogenic mycobacterium, *M.aurum* A^+, was obtained from the Intitut Pasteur, Paris, France. The virulent and avirulent H37R strains of *M. tuberculosis* were obtained from the Medical Research Council, Tuberculosis Research Institute, Pretoria, South Africa. Only a limited number of confirmatory experiments were performed using the *M.tuberculosis* H37Rv strain.

3.2.4. ^{86}Rb-UPTAKE STUDIES

^{86}Rb (Rubidium-86 chloride, specific activity (3.56 mCi/mg), Du Pont-NEN Research

Products, Boston, MA, USA) was used as a tracer for measuring K^+ -uptake by mycobacteria. ^{86}Rb has been described in several previous studies as being a useful tracer for the measurement of microbial transport of K^+ (Abrams & Smith, 1971; Rhoads *et al*, 1977; Bakker & Harold *et al*, 1980, De Bruyn *et al*, 1996). The validity of using ^{86}Rb as a tracer for K^+ -uptake by mycobacteria was established by my colleague Dr H.C. Steel who demonstrated i) that the uptake kinetics of ^{86}Rb and ^{42}K by *M.aurum* A^+ and by the avirulent and virulent strains of *M.tuberculosis* (H37R) are comparable and ii) that the uptake of ^{86}Rb by these mycobacteria is inhibited in a dose-dependent manner by the addition of increasing concentrations (0.01-10 mM) of cold KCl (personal communication).

M. aurum A^+ and the *M.tuberculosis* H37R strains were cultured for 4 days and 14 days respectively before being harvested,washed and resuspended in glucose- and K^+ -free buffer (KONO buffer) to a concentration of 0.5×10^7 cfu/ml for *M.aurum* A^+ and 1×10^7 cfu/ml for the *M.tuberculosis* H37R strains. However, the final buffer contains approximately 20µM of K^+ due to contamination of the sodium salts. Suspension in KONO buffer stimulates net K^+ -uptake by the mycobacterial cells. After 15 minutes in this minimal media the mycobacteria were treated with the riminophenazines (0.15-1.25 µg/ml) at 37°C for 30 min. Appropriate solvent controls were included. The mycobacteria were then concentrated by centrifugation and resuspended in 2 ml KONO buffer supplemented with 22 mM glucose and ^{86}Rb (2 µCi/ml). Uptake of the cation was measured using fixed incubation times of 45 min and 90 min for *M.aurum* A^+ and the *M.tuberculosis* H37R strains respectively at 37°C, which were predetermined in kinetic studies as described below. After incubation, the mycobacteria were washed twice with cold phosphate-buffered saline (PBS) and the pellets finally disrupted by adding 0.4 ml pre-heated 5% trichloroacetic acid (TCA). Radioactivity was assayed for in a liquid scintillation spectrometer and the net uptake was taken as the difference in uptake of ^{86}Rb in the tubes incubated at 37°C and the controls kept on ice. The effects of pretreatment with a fixed concentration (25 µg/ml) of α-tocopherol (vitamin E) on the uptake of ^{86}Rb by control and riminophenazine-treated mycobacteria were also investigated. In these experiments α-tocopherol was added 1 min before the

riminophenazine agents (0.6 µg/ml for both B663 and B4121 and 0.3 µg/ml for B4128).

The effect of ouabain (0.1-2 mM), a potent inhibitor of the Na^+ K^+-ATPase of eukaryotic cells (Skou, 1988), on the uptake of ^{86}Rb by the mycobacteria was also investigated.

3.2.5. KINETICS OF K^+ -INFLUX AND EFFLUX

Since apparent inhibition of K^+ -transport may be due to decreased uptake and/or accelerated efflux of the cation, the effects of B663 (0.3 µg/ml), B4121 and B4128 (both at 0.15 µg/ml) on the kinetics of influx and efflux of K^+ were investigated in *M.aurum* A^+ using ^{42}K (K_2CO_3, specific activity (37 MBq), South African Atomic Energy Corporation, Pretoria, South Africa) as a tracer. For uptake studies the mycobacteria were exposed to B663, B4121 and B4128 for 30 min at 37°C as described above, followed by addition of glucose and ^{42}K (3 µCi/ml) and uptake of the cation was measured after 0, 5, 10, 15, 30, 45 and 60 min incubation at 37°C. After incubation, the mycobacteria were washed twice with PBS and the pellets finally disrupted by adding 0.4 ml pre-heated TCA.

In an additional set of influx kinetics experiments the effect of B663, B4121 and B4128 (0.6 µg/ml and 2.5 µg/ml) on the uptake of K^+ by *M. aurum* A^+ was measured using ^{86}Rb as a tracer after short time intervals (0, and 30 seconds, and 1, 3 and 5 minutes).

For efflux studies the *M. aurum* A^+ was prepared as above and resuspended in KONO buffer supplemented with 22 mM glucose. ^{42}K (3 µCi/ml) was then added and the tubes were incubated for 2 hrs at 37°C to promote uptake of ^{42}K. The mycobacteria were then washed twice in ice-cold KONO buffer supplemented with 100 mM cold KCl to minimize efflux of bacterial ^{42}K. The ^{42}K-loaded cells (0.5×10^7 cfu/ml) were then resuspended in 10 ml of KCl (100 mM)-supplemented KONO buffer and exposed to B663 (0.3 µg/ml), B4121 and B4128 (both at 0.15 µg/ml). The kinetics of efflux of ^{42}K from control and riminophenazine-treated mycobacteria were then determined after 0, 5, 10, 15 and 30 minutes at 37°C by measuring the amount of bacteria-associated ^{42}K.

3.2.6. BACTERIAL GROWTH STUDIES

The effects of B663, B4121 and B4128 on the growth of the mycobacteria were presented in the previous chapter. In the present chapter only the effect of ouabain on bacterial growth has been studied. The effect of ouabain on the growth of the test mycobacterial strains was investigated using the BACTEC 460 TB system (Becton Dickinson Diagnostic Instrument Systems, Towson, MD, USA). Bacterial growth is measured as the extent of bacterial metabolic activity according to the amount of $^{14}CO_2$ liberated during the decarboxylation of ^{14}C-labelled substrates present in the medium (Deland *et al*, 1969).

Following the culture of *M. aurum* A^+ for 4 days and the *M. tuberculosis* H37R strains for 14 days in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA), the bacterial cells were resuspended in 0.15 M PBS, pH 7.4, to obtain a McFarland Number 1 opacity density (3x 10^8 cfu/ml). These suspensions were diluted 10-fold and 5-fold in the case of *M. aurum* A^+ and the *M. tuberculosis* H37R strains respectively. One hundred microlitres of the diluted suspensions were inoculated into the vials containing 12B TB medium (Becton Dickinson and Company, Cockeysville, MD, USA) with or without ouabain (0.1-2mM). The vials were incubated at 37°C for 48 hrs and bacterial growth measured using the BACTEC 460 TB system.

3.2.7. Ca^{2+} -UPTAKE STUDIES

M. aurum A^+ and *M. tuberculosis* (H37Ra) were cultured for 4 days and 14 days, respectively, before being harvested, washed and resuspended in Ca^{2+}-free Hanks balanced salt solution (the concentration of contaminating Ca^{2+} in the Ca^{2+}-free HBSS is $\leq$ 1µM according to Highveld Biological (Pty) Ltd, Kelvin, South Africa) to a concentration of 1x10^7 cfu/ml for *M. aurum* A^+ and 2x10^7 cfu/ml for the *M. tuberculosis* H37Ra strain. The bacteria were then exposed to 4 µCi/ml ^{45}Ca (Calcium-45 chloride specific activity (28.84 mCi/mg), Du Pont-NEN Research Products, Boston, MA, USA) containing 20 µM cold, carrier calcium chloride ($CaCl_2$) for 15 minutes at Following the addition of B663 or B4121 or B4128 (0.15-10 µg/ml), the mycobacteria were incubated for a further 15 minutes at 37°C, after which Ca^{2+} uptake was terminated by the

addition of ice-cold PBS. The cells were then washed with PBS and assayed for incorporated ^{45}Ca following disruption of the pellets by addition of 0.4ml of pre-heated 5% TCA. The incorporated ^{45}Ca was assayed for in a liquid scintillation spectrometer. To eliminate the complicating effects of non-specific binding of the radiolabelled cation to the bacteria, net uptake of ^{45}Ca was taken as the difference in uptake of ^{45}Ca in the tubes at 37°C and the controls kept on ice. In kinetics studies, the time-course of Ca^{2+}-uptake by control and B663- or B4121- or B4128 (0.6 and 2.5 µg/ml) -treated bacteria was measured at 0, and 30 seconds, and 1, 3 and 5 minutes after addition of the riminophenazines.

The effect of α-tocopherol (25µg/ml) on the uptake of ^{45}Ca by control and riminophenazine-treated *M. aurum* A^+ was also investigated. In these experiments, α-tocopherol was added either 5 minutes before or 15 minutes after B663 (5µg/ml) and the mycobacteria were incubated for a further 15 minutes before being assayed for incorporated ^{45}Ca.

3.2.8. PHOSPHOLIPASE A_2 ACTIVITY

PLA_2 (EC 3.1.1.4; phosphatidylcholine 2-acyl-hydrolase) activity in the mycobacteria was measured according to the release of [^{14}C]-arachidonate from the C-2 position of added phosphatidylcholine. Release of [^{14}C]-arachidonate was measured by a high-perfomance thin-layer chromatography (HPTLC) method (Bradova *et al*, 1990). The mycobacteria were resuspended in HBSS, with 1.25 mM calcium chloride to concentrations of 1 x 10^7 and 2 x 10^7 cfu/ml for the *M. aurum* A^+ and the *M. tuberculosis* H37R strains respectively. Phosphatidylcholine, 0.5 µCi/ml (Phosphatidylcholine, L-α-1-palmitoyl-2-arachidonyl, [arachidonyl-1-^{14}C], specific activity (180-240 Ci/mmol), Du Pont-NEN Research Products, Boston, MA, USA) was added to the bacterial suspensions which were then incubated for 30 minutes at 37°C after which 0.25 ml aliquots were added to tubes containing 0.75 ml HBSS, and the appropriate concentrations of the riminophenazines (0.15-2.5 µg/ml for B663 and B4128, and 2.5-10 µg/ml for B4121). After incubation for a further 15 minutes, the reactions were terminated by the addition of 2 ml chloroform:methanol (2:1 [vol/vol]) and the fatty acid-

containing lower phase was removed and evaporated to dryness under nitrogen. The samples were stored at -20°C until assayed.

The evaporates were reconstituted with 40 µl of chloroform:methanol (2:1 [vol/vol]) containing 0.01 mM arachidonate standard and spotted onto silica gel 60-precoated HPTLC plates (Merck, Germany). The plates were developed in chloroform:acetone (96:4 [vol/vol]). After exposure to iodine vapours, the arachidonate spots were localised and the silica was removed and assayed for radioactivity in a liquid scintillation spectrometer.

In kinetics experiments, the effects of B663 or B4121 or B4128 (0.6 and 2.5 µg/ml) on PLA_2 activity in *M. aurum* A^+ at early time intervals (0, and 30 seconds and 1, 3 and 5 minutes) were investigated.

3.2.9. Ca^{2+}-CHELATING AGENTS

The effects of Ca^{2+} chelation on B663 (0.6 µg/ml and 1.25 µg/ml)- or B4121 (0.6 µg/ml and 2.5 µg/ml) -mediated alterations in K^+ transport and PLA_2 activity were investigated in *M.aurum* A^+ by preincubating the bacteria with the extracellular Ca^{2+}-chelating agent, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and the intracellular Ca^{2+}-chelating agent 1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetra-acetic acid (BAPTA) (Calbiochem, La Jolla, CA, USA) for 15 minutes at 37°C prior to addition of the riminophenazines. EGTA and BAPTA were used at final concentration ranges of 5-20 mg/ml and 5-20 µg/ml respectively.

3.2.10. ATP LEVELS

Microbial ATP levels were determined using a sensitive luciferin-luciferase chemiluminescence method (Holmsen *et al*, 1966, Holmsen *et al*, 1972). After injection of firefly lantern extract into the solution containing ATP, maximum light intensity is reached after 2 seconds. The light intensity of the initial flash is found to be directly proportional to the amount of ATP present, up to a final concentration of 2 µM. Bacterial cells (0.01 mg protein per 10 ml KONO buffer) were coincubated for 30 minutes at 37°C

with or without the riminophenazines (0.15-5 µg/ml), after which the cells were concentrated by centrifugation, lysed with a nucleotide releasing agent (Lumac, Landgraaf, The Netherlands) and assayed for ATP, over a 10 second period using a chemiluminometer (Biocounter M2010 Multijet, Lumac Systems Inc., Titusville, FL, USA).

3.2.11. EXPRESSION AND STATISTICAL ANALYSIS OF RESULTS

The results are expressed as the mean value ± the standard error of the mean (SEM) for each series of experiments, usually as percentage of the corresponding drug-free control system. Levels of statistical significance were calculated using the Students paired t-test (paired t statistic).

3.3. RESULTS

Effects of B663, B4121 and B4128 on K^+-uptake by *M.aurum* A^+ and the *M.tuberculosis* (H37R) strains using ^{86}Rb as a tracer

Inspection of the results obtained at concentrations of 0.15-1.25 µg/ml of the riminophenazines indicated that B4121 and B4128 were the most potent inhibitors of uptake of ^{86}Rb by *M.aurum* A^+, while B663 was the least active agent (Figure 4; page 68). The mean percentage uptake of ^{86}Rb, for B663 at a concentration of 1.25 µg/ml relative to the corresponding drug free control system was 31 ± 3 while those of B4121 and B4128 were 0 (complete inhibition) at the same concentration (Table 4; page 93). Treatment of the avirulent strain of *M.tuberculosis* with riminophenazines (0.15-1.25 µg/ml) caused a dose-related inhibition of uptake of ^{86}Rb with no differences observed among the three drugs tested (Figure 5; page 69). B4128 was the most potent inhibitor of uptake of ^{86}Rb by the virulent strain of *M.tuberculosis* while B663 was the least active agent (Figure 6; page 70).

The effects of α-tocopherol (25 µg/ml) on K^+ -uptake by *M.aurum* A^+ coincubated with B663 (0.6 µg/ml) or B4121 (0.3 µg/ml) or B4128 (0.3 µg/ml) are shown in Figure 7 (page 71). Alpha-tocopherol protected *M.aurum* A^+ against the inhibitory effects of the

riminophenazines on the uptake of ^{86}Rb. The protection afforded by α-tocopherol was statistically significant for each riminophenazine (B663-$p<0.01$; B4121-$p<0.0001$; B4128-$p<0.01$).

Kinetics of K^+ -influx and -efflux by *M. aurum* A^+

The kinetics of K^+ -influx into and -efflux out of *M. aurum* A^+ using ^{42}K as tracer in the presence and absence of 0.3 µg/ml B663 and 0.15 µg/ml B4121 and B4128 are shown in Figures 8, 9,10,11, 12 and 13 (pages 72, 73, 74, 75, 76 and 77). B663 and B4128 inhibited the influx of ^{42}K, while no accelerated efflux of the cation was noted throughout the entire time-course (0-30 min). B4121 inhibited uptake of ^{42}K while efflux was slightly increased throughout the later stages of the time course.

Ouabain did not inhibit the uptake of ^{86}Rb in any of the mycobacteria tested (Figures 14, 15 and 16; pages 78, 79 and 80).

In experiments designed to determine the involvement of the extracellular and intracellular Ca^{2+} -chelating agents, EGTA and BAPTA on riminophenazine-mediated dysregulation of uptake of K^+, I have found that both chelating agents, either alone or in combination with B663 or B4121 had no effects on the uptake of ^{86}Rb (Tables 6 and 7; pages 95 and 96).

Effects of ouabain on the growth of *M. aurum* A^+ and the *M. tuberculosis* (H37R) strains

Ouabain at concentrations of 0.1-2 mM did not inhibit, but rather potentiated the growth of the test microorganisms (Figures 14, 15 and 16; pages 78, 79 and 80).

Effects of B663, B4121 and B4128 on ^{45}Ca-uptake by *M. aurum* A^+ and the avirulent strain of *M. tuberculosis* (H37R).

The effects of B663, B4121 and B4128 on Ca^{2+}-uptake by *M. aurum* A^+ and the andavirulent strain of *M. tuberculosis* (H37R) are shown in Figures 17, 18, 19, 20, 21 and 22 (pages 81, 82, 83, 84, 85 and 86). All 3 agents, particularly B4128 caused

dose-related influx of ^{45}Ca into all of the test mycobacteria. B663, B4121 and B4128 at concentrations of 2.5-10 µg/ml were found to cause a significant dose-related increase in the influx of Ca^{2+} by the H37Ra strain of *M. tuberculosis* (B663- $p \leq 0.006$-$p < 0.0001$, B4121-$p \leq 0.006$ - $P \leq 0.0006$ and B4128-$p \leq 0.03$ - $p \leq 0.002$).

Exposure of *M. aurum* A^+ to α-tocopherol, prior to treatment with B663 (5 µg/ml), inhibited the riminophenazine-mediated influx of ^{45}Ca into the mycobacteria. The mean percentage of ^{45}Ca-uptake in the bacteria exposed to B663 in the absence of α-tocopherol was 290 ± 11 relative to the untreated controls. When *M. aurum* A^+ was pretreated with α-tocopherol, the mean percentage uptake was 151 ± 4 for B663. Delayed addition of α-tocopherol, 15 minutes after exposure to B663 and 1 minute prior to termination of the experiment, resulted in efflux of ^{45}Ca from the mycobacteria. The mean percentages uptake of ^{45}Ca by *M.aurum* A^+ treated with B663 alone or in combination with α-tocopherol were 290 ± 11 and 174 ± 9 respectively.

Effects of B663, B4121 and B4128 on PLA_2 activity in *M. aurum* A^+ and the *M. tuberculosis* (H37R) strains.

The effects of B663, B4121 and B4128 on mycobacterial PLA_2 activity are shown in Figures 17, 18, 19, 20, 21 and 22 (pages 81, 82, 83, 84, 85 and 86) as well as in Table 5 (page 94). Exposure of *M. aurum* A^+ and the *M. tuberculosis* (H37R) strains to either B663 or B4128 (0.15-2.5 µg/ml) or B4121 (2.5-10 µg/ml) resulted in a dose-related enhancement of PLA_2 activity according to increased release of [^{14}C]-arachidonate from riminophenazine-treated bacteria. In the case of B4121 enhanced release of radiolabelled arachidonate was significant on exposure of *M. aurum* A^+ to 5 µg/ml ($p = 0.0029$) and 10 µg/ml ($p = 0.0018$). Exposure of the *M. tuberculosis* H37Rv strain to B663 at concentrations of 0.6 µg/ml and higher, resulted in a significant increase of PLA_2 activity ($p \leq 0.005$- $p < 0.0004$), whereas B4128 caused a significant increase in PLA_2 activity at concentrations of 1.25 µg/ml ($p = 0.0140$) and 2.5 µg/ml ($p = 0.0067$), Table 5 (page 94).

Incubation of *M. aurum* A^+ with EGTA and BAPTA before the addition of B663 (0.6

µg/ml) or B4121 (2.5 ug/ml), did not prevent the riminophenazine-mediated enhancement of PLA_2 activity (Tables 8 and 9; pages 97 and 98), indicating that enhancement of PLA_2 activity is not calcium dependent. In fact B663-mediated enhancement of PLA_2 activity was potentiated by the combination of the drug with EGTA and BAPTA (Table 9; page 98)

Time-course of altered uptake of ^{86}Rb and ^{45}Ca by, and PLA_2 activity in B663-, B4121- and B4128-treated *M. aurum* A^+

The effects of B663, B4121 and B4128 (0.6 µg/ml and 2.5 µg/ml) on the kinetics of uptake of ^{86}Rb and ^{45}Ca by, and PLA_2 activity in *M. aurum* A^+ are shown in Figures 23, 24, 25, 26, 27 and 28 (pages 87, 88, 89, 90, 91 and 92). Exposure to B663 and B4128 caused an immediate dose-related increase in PLA_2 activity and inhibition of the uptake of ^{86}Rb, while uptake of ^{45}Ca was observed only after 3 minutes of exposure of the mycobacteria to 2.5 µg/ml of the antimicrobial agents. Unlike B663 and B4128, exposure to 0.6 µg/ml of B4121 caused an immediate inhibition of the uptake of ^{86}Rb with no increase in uptake of ^{45}Ca and PLA_2 activity throughout the entire time course of the experiment, whereas exposure to 2.5 µg/ml of B4121 caused an immediate dose-related enhancement of PLA_2 activity and inhibition of the uptake of ^{86}Rb while uptake of ^{45}Ca was minimally affected throughout the entire time course.

Effects of B663 , B4121 and B4128 on microbial ATP levels

Exposure of the mycobacteria to B663 or B4121 at concentrations of 0.15-2.5 µg/ml did not signicantly affect bacterial ATP levels while at a concentration of 5µg/ml ATP levels were decreased (Tables 10 and 11; pages 99 and 100). B4128 also did not significantly affect bacterial ATP levels at concentrations of 0.15 - 0.6 µg/ml, but caused significant reductions in mycobacterial ATP levels at higher concentrations (Table 12; page 101).

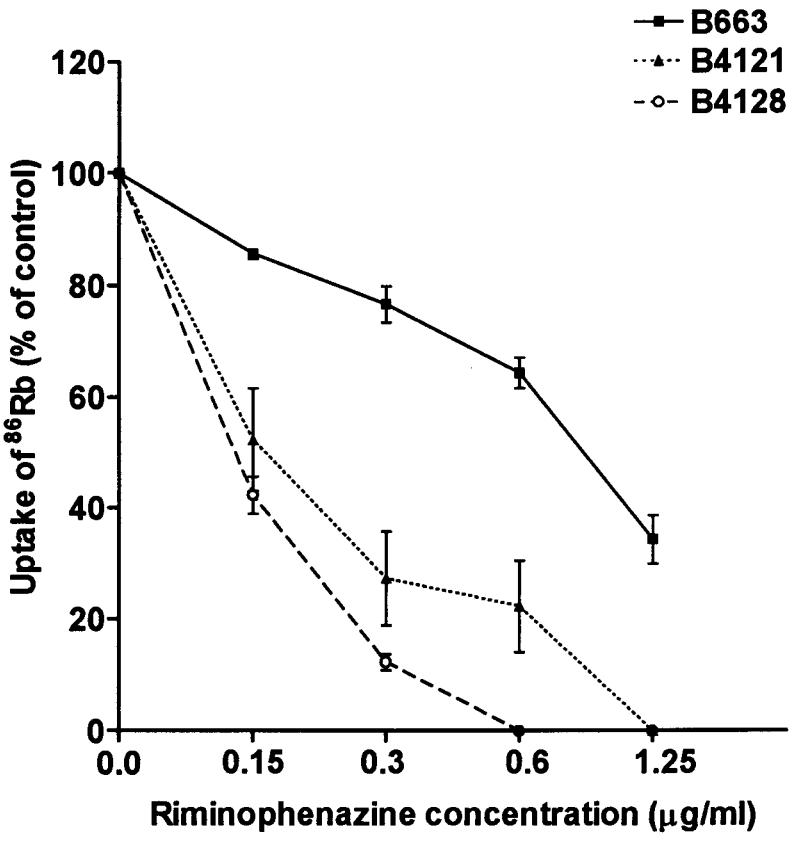


Figure 4: Effects of the riminophenazines, B663, B4121 and B4128 on K^+ -uptake by *M.aurum* A^+ using ^{86}Rb as a tracer. The results of 3-6 experiments, each with triplicate determinations are expressed as the mean percentage of the untreated control systems ± SEMs. The absolute values for uptake of ^{86}Rb by the drug-free control systems were 14 575 ± 926, 18 026 ± 698 and 16544 ± 896 for B663, B4121 and B4128 respectively.

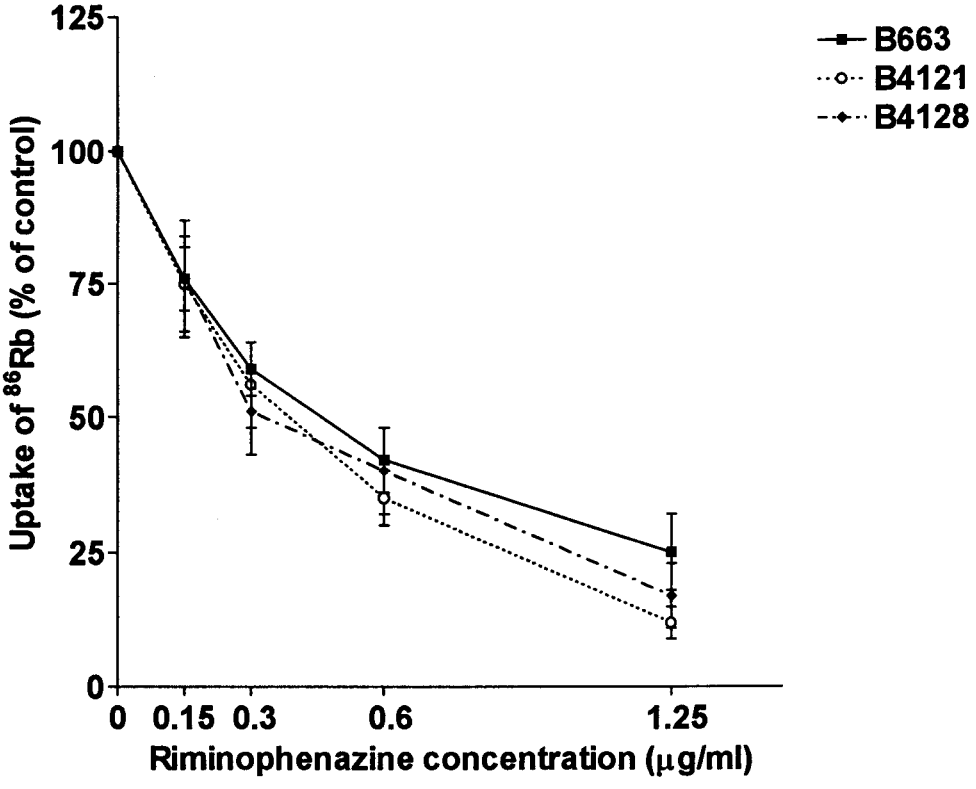


Figure 5: Effects of the riminophenazines, B663, B4121 and B4128 on K^+-uptake by the avirulent strain of *M.tuberculosis* H37R using ^{86}Rb as a tracer. The results of a single experiment with quintuplicate determinations for each concentration are expressed as the mean percentage of the untreated control systems ± SEMs. The absolute values for uptake of ^{86}Rb by the drug-free control systems were 46920 ± 14060, 46260 ± 11169 and 48801 ± 13158 cpm for B663, B4121 and B4128 respectively.

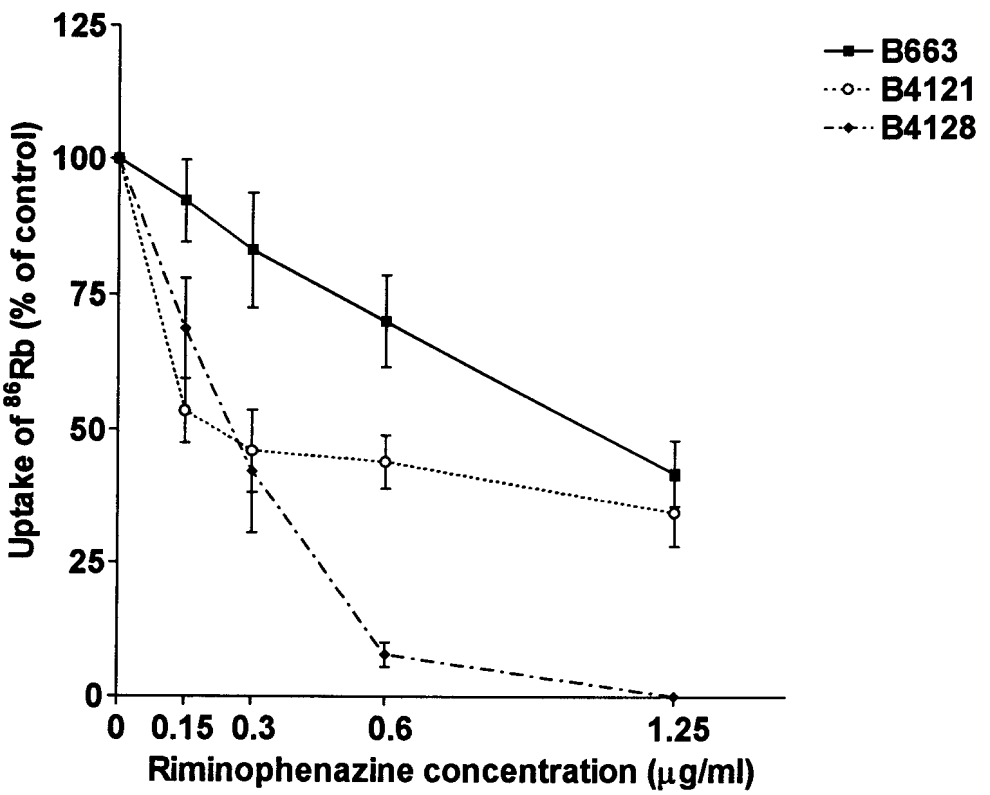


Figure 6: Effects of the riminophenazines, B663, B4121 and B4128 on K^+-uptake by the virulent strain of *M.tuberculosis* H37R using ^{86}Rb as a tracer. The results of a single experiment with quintuplicate determinations for each concentration are expressed as the mean percentages of the untreated control systems ± SEMs. The absolute values for uptake of ^{86}Rb by the drug-free control systems were 159228 ± 7939, 87665 ± 11599 and 94908 ± 14068 cpm for B663, B4121 and B4128 respectively.

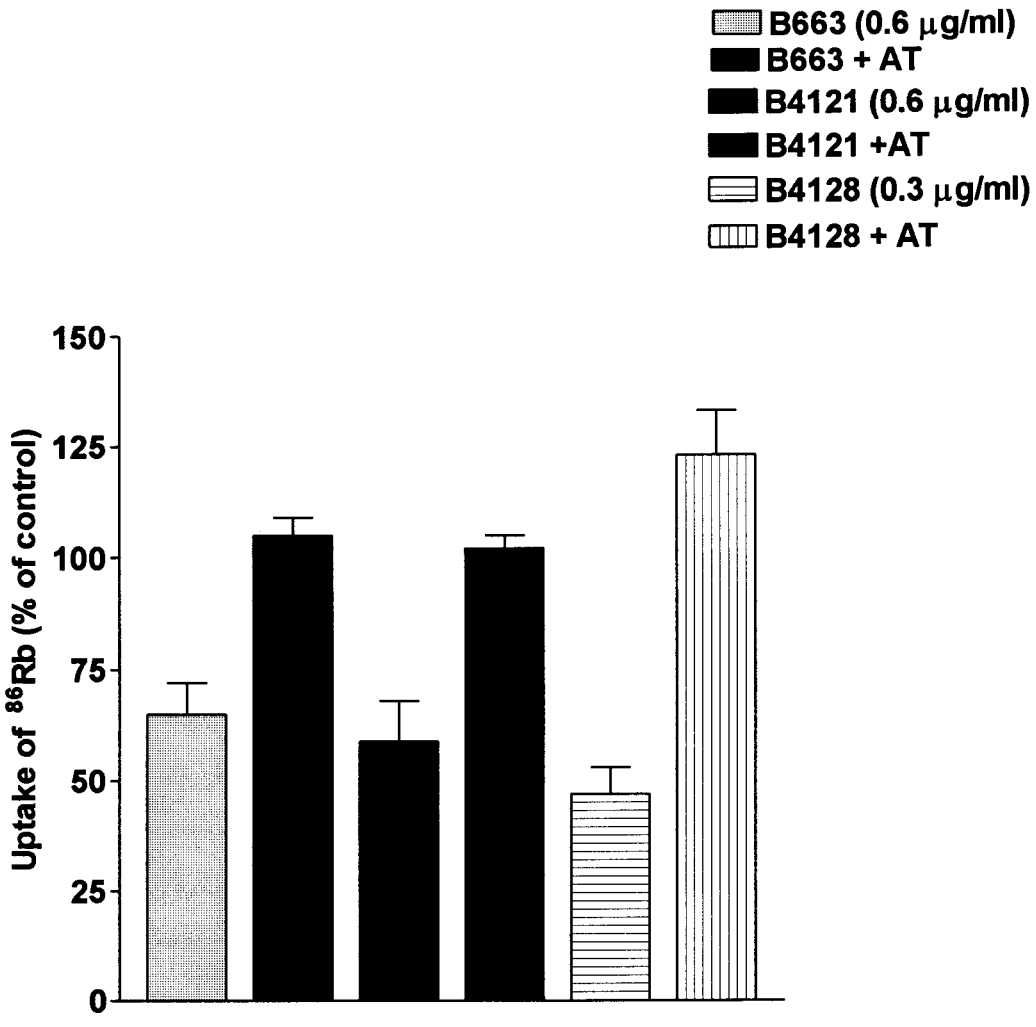


Figure 7: Effects of B663, B4121 and B4128 with or without alpha-tocopherol (25 µg/ml) on the uptake of ^{86}Rb by *M.aurum* A^+. The results of 2-3 experiments are expressed as the mean percentages of the drug-free control systems ± SEMs. The absolute values for uptake of ^{86}Rb by the drug-free control systems with α-tocopherol added were 19111 ± 72, 19695 ± 98 and 19941 ± 50 cpm for B663, B4121 and B4128 respectively.

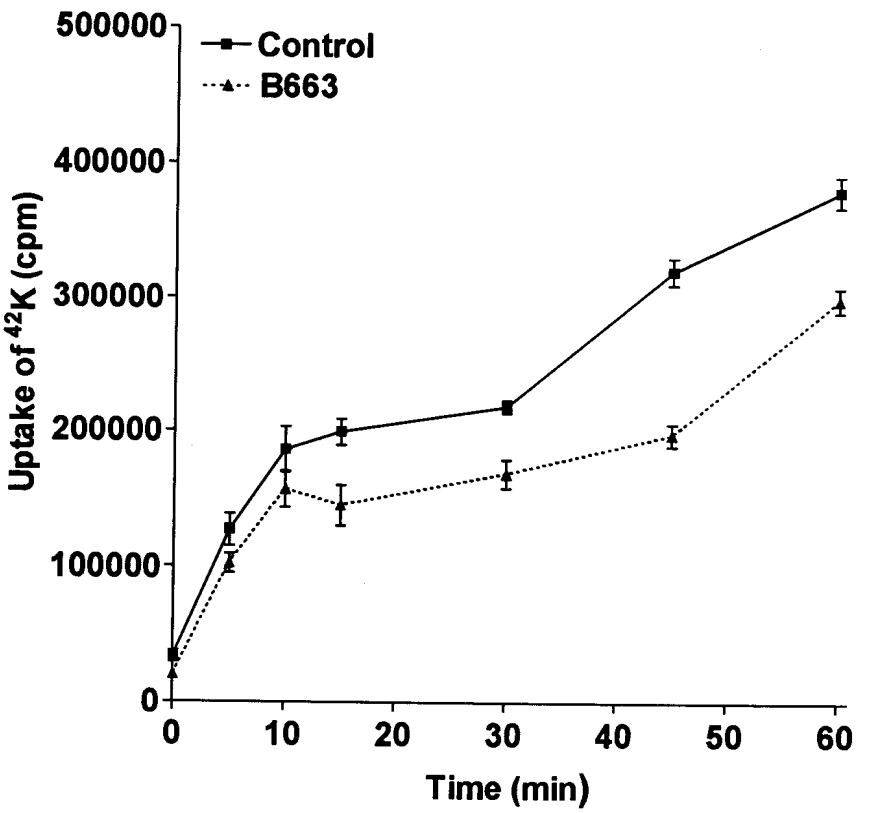


Figure 8: The kinetics of influx of K^+ into *M. aurum* A^+ using ^{42}K as a tracer, in the presence or absence of B663 (clofazimine) at a fixed concentration of 0.3 µg/ml. The results of a single experiment with quintuplicate determinations for each time point are expressed as the mean actual counts (cpm) ± SEMs.

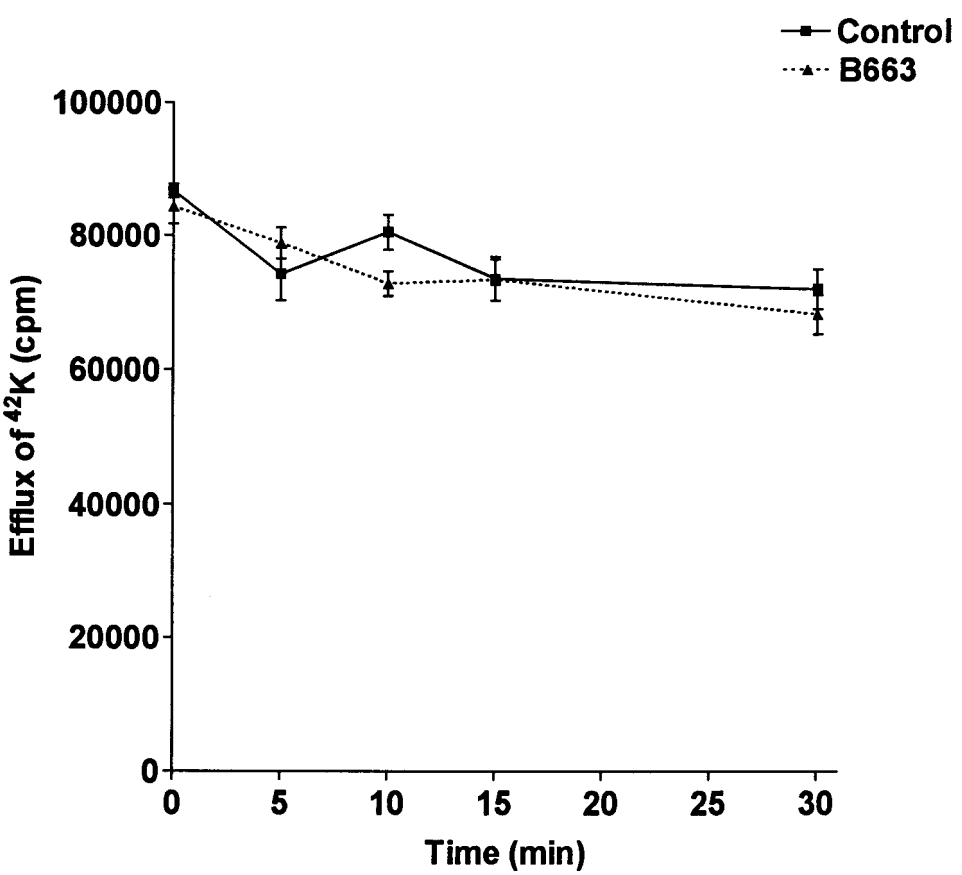


Figure 9: The kinetics of K^+-efflux out of *M. aurum* A^+ using ^{42}K as a tracer, in the presence or absence of B663 at a fixed concentration of 0.3 µg/ml. The results of a single experiment with quintuplicate determinations for each time point are expressed as the mean actual counts (cpm) ± SEMs.

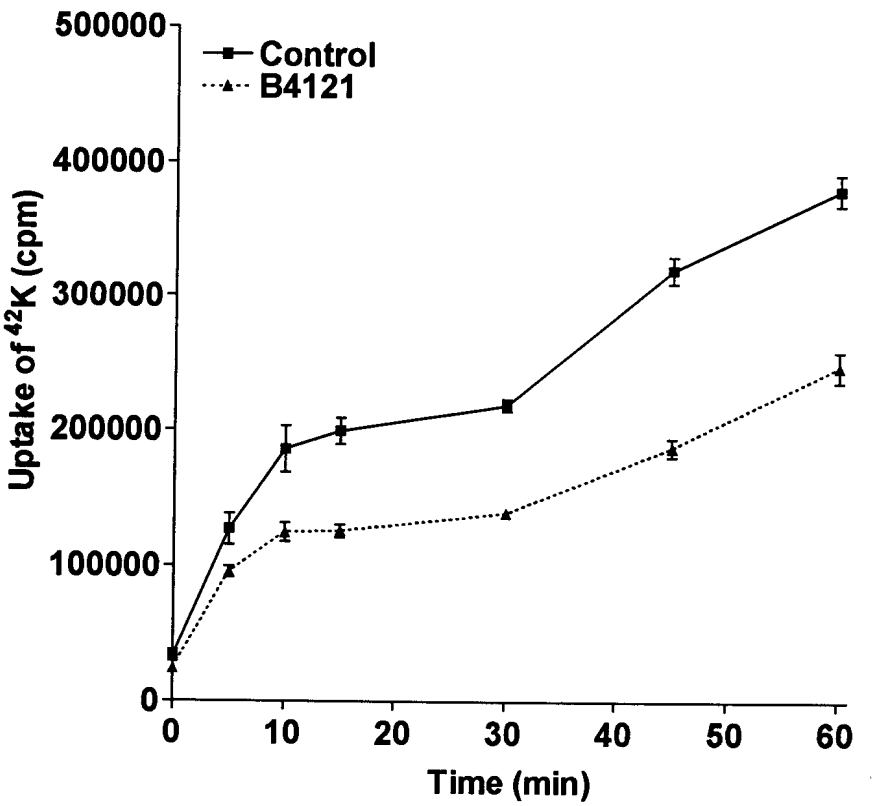


Figure 10: The kinetics of influx of K^+ into *M. aurum* A^+ using ^{42}K as a tracer, in the presence or absence of B4121 at a fixed concentration of 0.15 µg/ml. The results of a single experiment with quintuplicate determinations for each time point are expressed as the mean actual counts (cpm) ± SEMs.

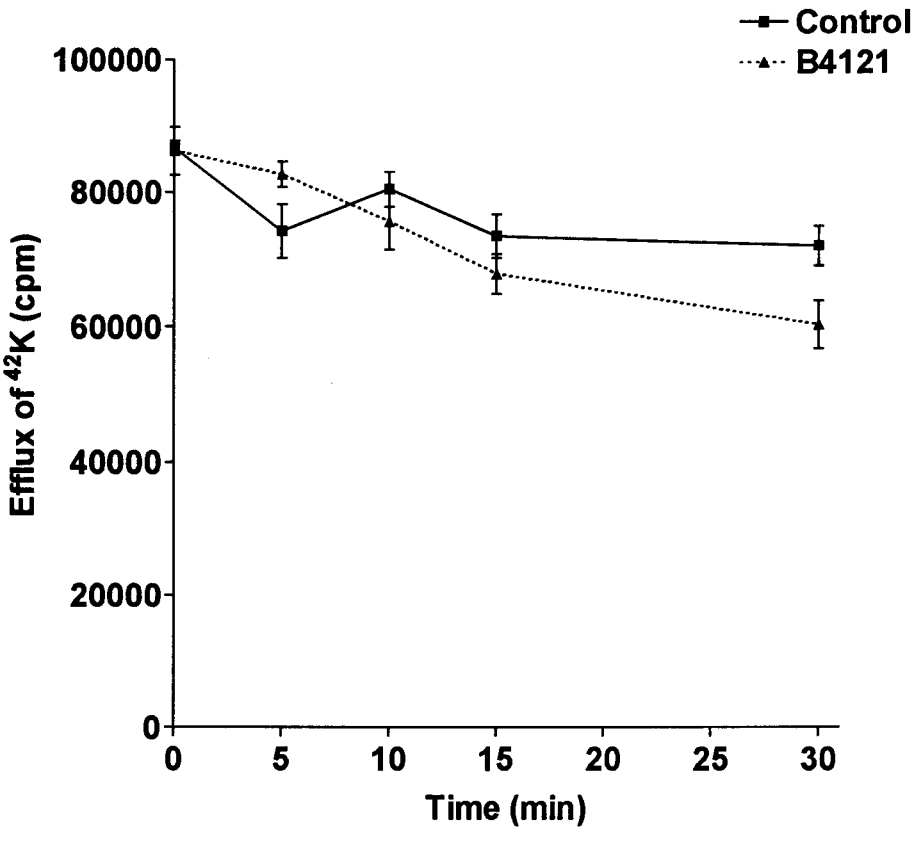


Figure 11: The kinetics of K^+-efflux out of *M. aurum* A^+ using ^{42}K as a tracer, in the presence or absence of B4121 at a fixed concentration of 0.15 µg/ml. The results of a single experiment with quintuplicate determinations for each time point are expressed as the mean actual counts (cpm) ± SEMs.

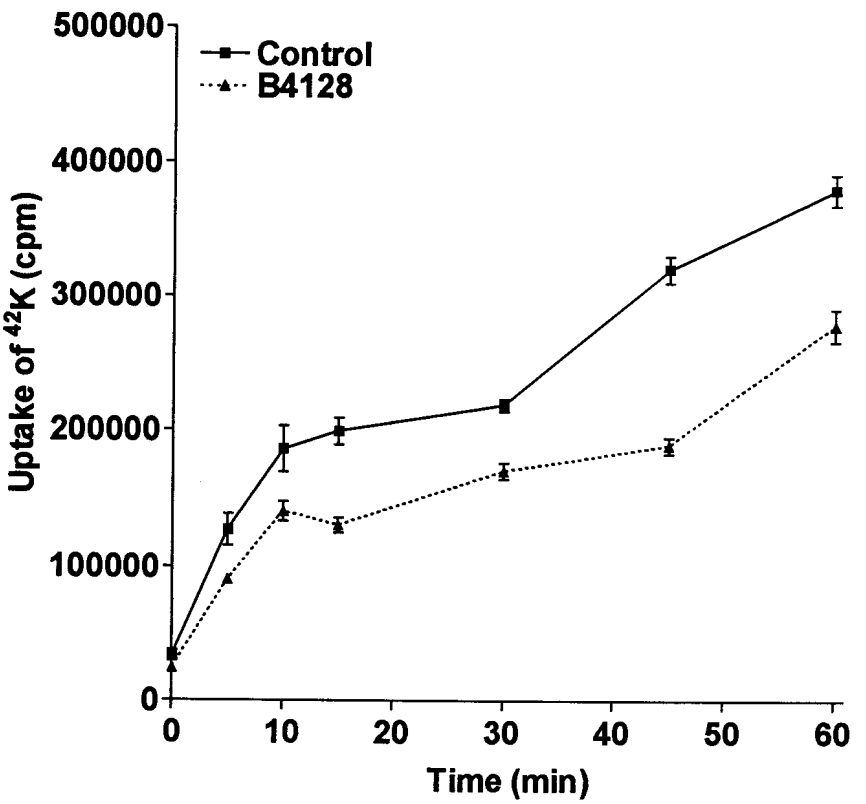


Figure 12: The kinetics of influx of K^+ into *M. aurum* A^+ using ^{42}K as a tracer, in the presence or absence of B4128 at a fixed concentration of 0.15 µg/ml. The results of a single experiment with quintuplicate determinations for each time point are expressed as the mean actual counts (cpm) ± SEMs.

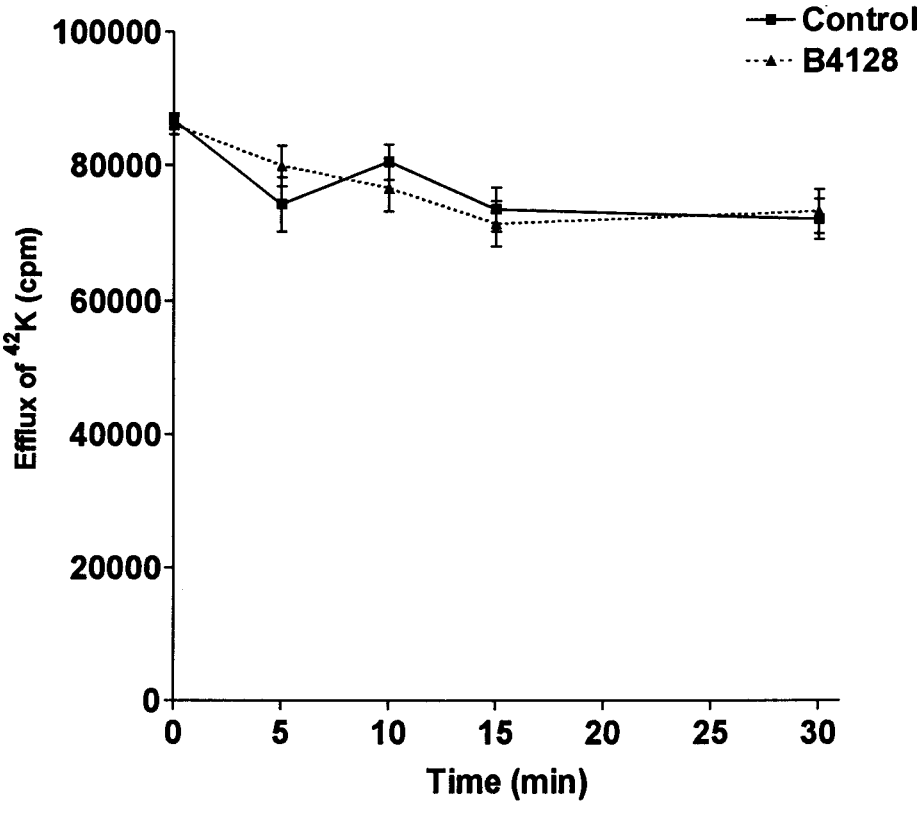


Figure 13: The kinetics of K^+-efflux out of *M. aurum* A^+ using ^{42}K as a tracer, in the presence or absence of B4128 at a fixed concentration of 0.15 µg/ml. The results of a single experiment with quintuplicate determinations for each time point are expressed as the mean actual counts (cpm) ± SEMs.

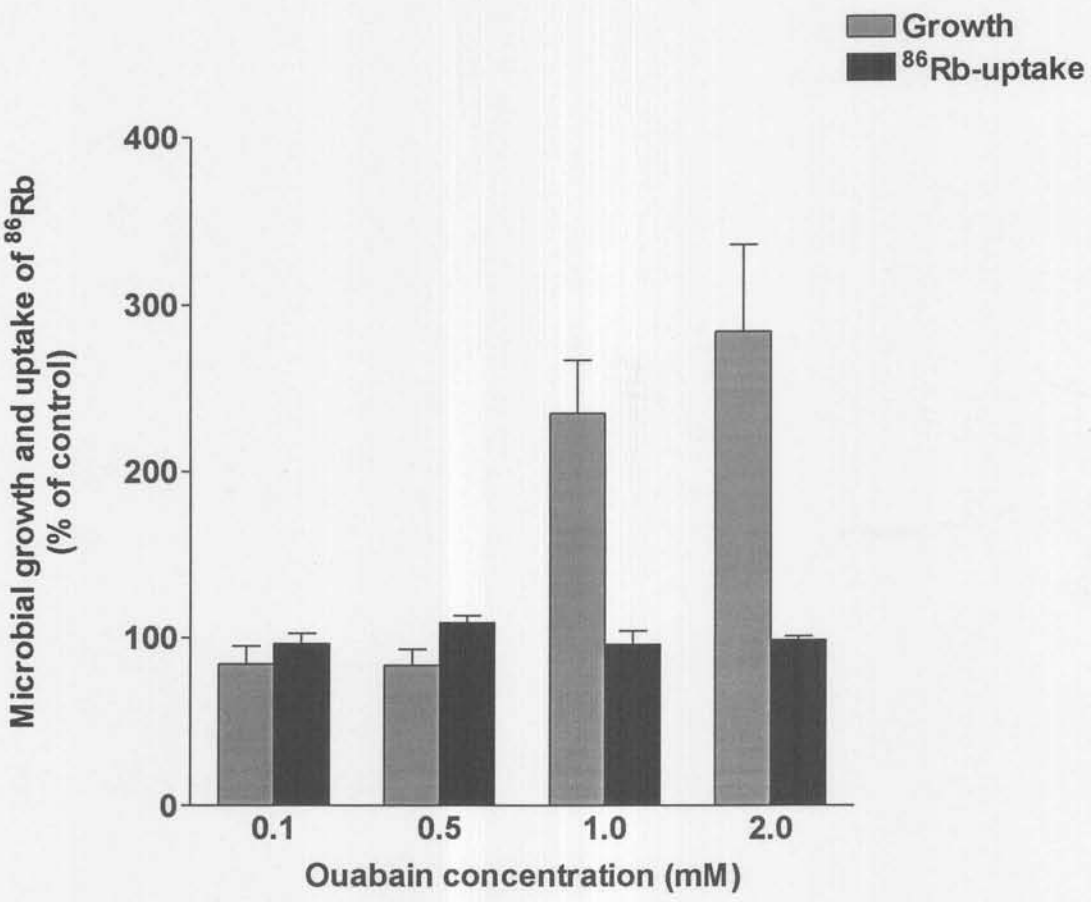


Figure 14: Effects of ouabain on ^{86}Rb-uptake by, and growth of *M. aurum* A^{+}. The results of a single experiment with quintuplicate determinations for uptake of ^{86}Rb assay and triplicate determinations for the growth assay are presented as the mean percentage of the untreated control systems ± SEMs. The absolute values for uptake of ^{86}Rb and growth by the drug-free control systems were 215530 ± 8753 and 333 ± 18 cpm respectively.

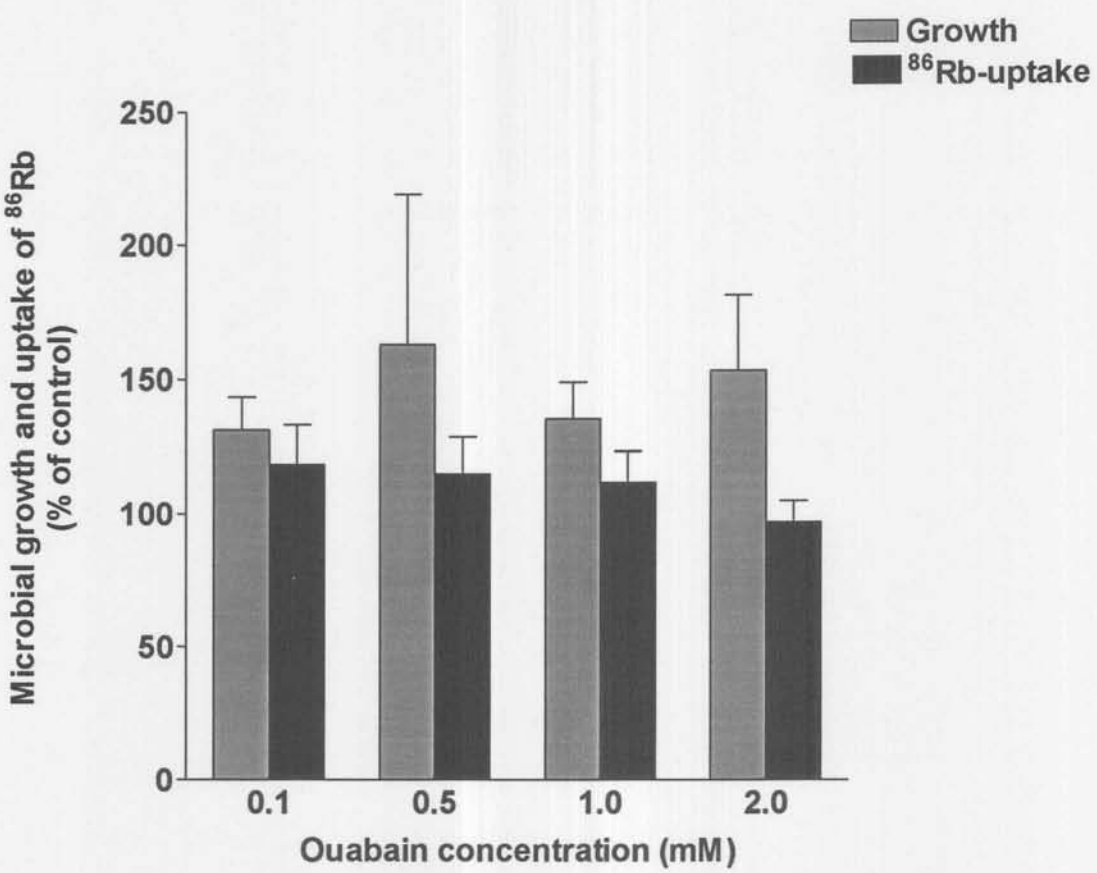


Figure 15: Effects of ouabain on ^{86}Rb-uptake by, and growth of the avirulent strain of *M. tuberculosis* H37R. The results of a single experiment with quintuplicate determinations for the ^{86}Rb-uptake assay and triplicate determinations for the growth assay are presented as the mean percentages of the untreated control systems ± SEMs. The absolute values for uptake of ^{86}Rb and growth by the drug-free control systems were 34745 ± 1244 and 362 ± 27 cpm respectively.

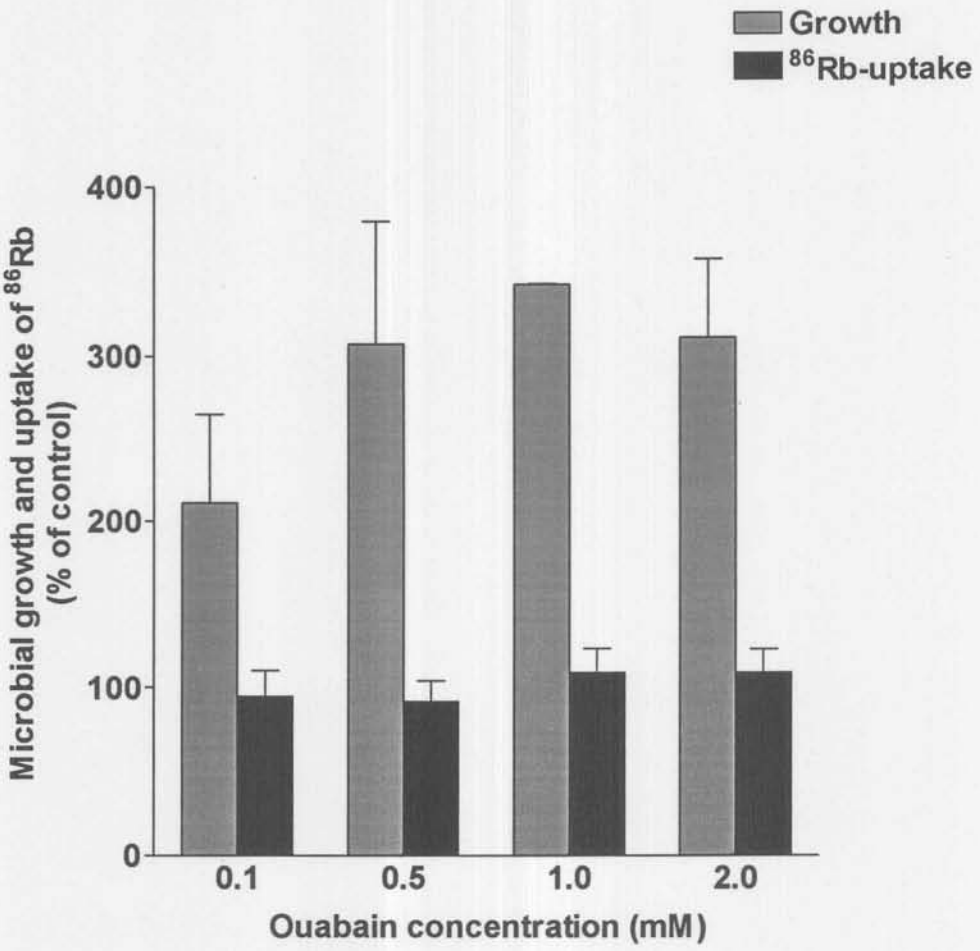


Figure 16: Effects of ouabain on ^{86}Rb-uptake by, and growth of the virulent strain of *M. tuberculosis* H37R. The results of a single experiment with quintuplicate determinations for the ^{86}Rb-uptake assay and triplicate determinations for the growth assay are presented as the mean percentages of the untreated control systems ± SEMs. The absolute values for uptake of ^{86}Rb and growth by the drug-free control systems were 37721 ± 2490 and 270 ± 12 cpm respectively.

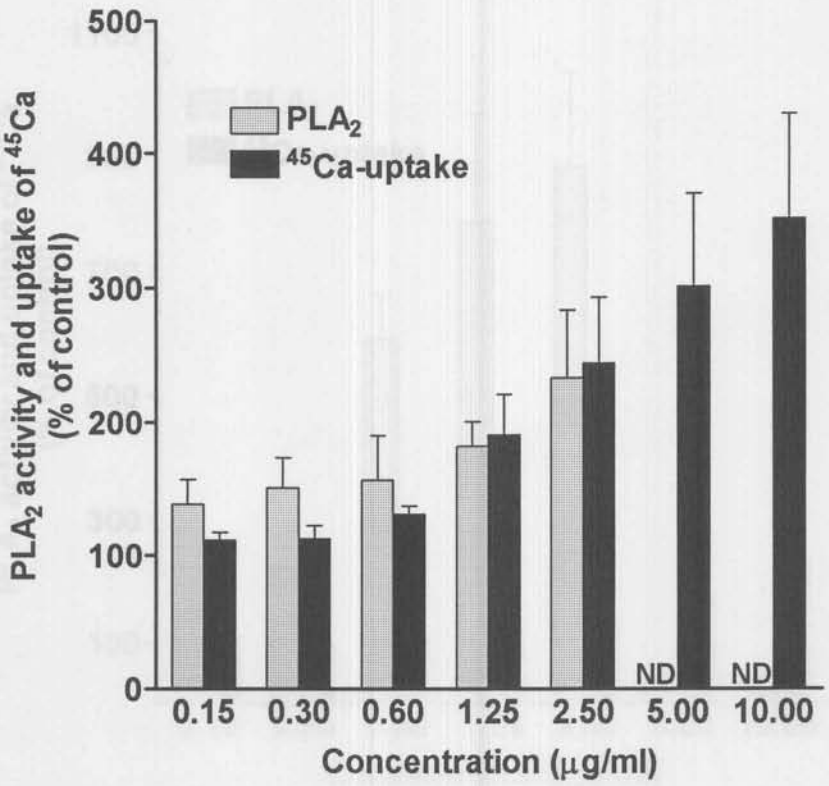


Figure 17: Effects of B663 on ^{45}Ca-uptake by, and PLA_2 activity of *M. aurum* A^+. The results of 3 experiments, each with triplicate determinations are expressed as the mean percentages of the untreated control systems ± SEMs. The absolute values for uptake of ^{45}Ca and PLA_2 activity by the drug-free control systems were 6016 ± 282 and 252 ± 16 cpm respectively.

ND = not done

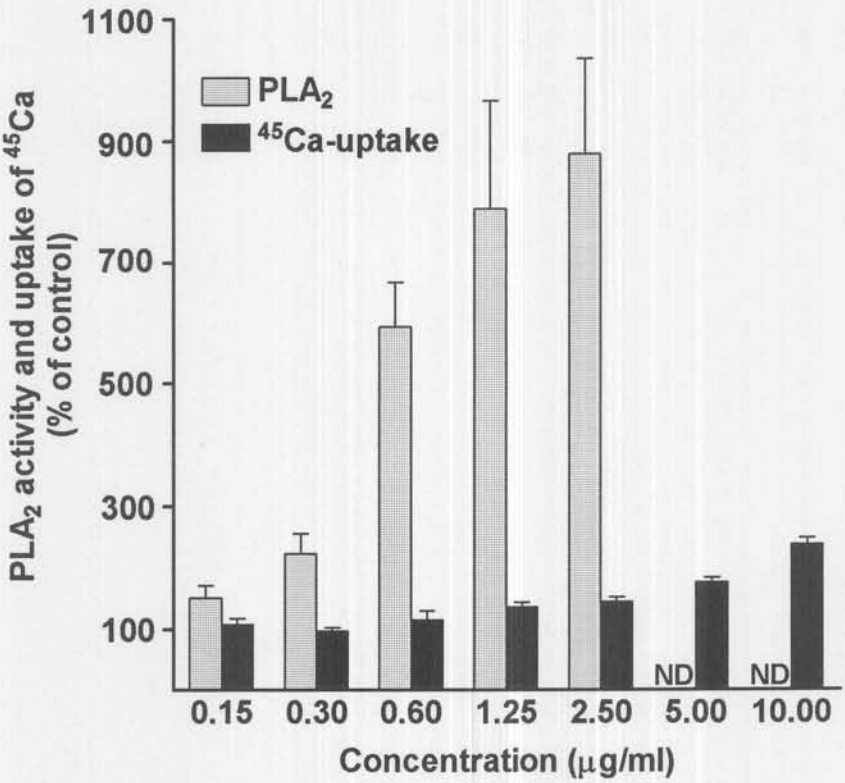


Figure 18: The effects of B663 on ^{45}Ca-uptake by, and PLA_2 activity of the avirulent strain of *M. tuberculosis* H37R. The results of a single experiment with quintuplicate determinations are presented as the mean percentages of the untreated control systems ± SEMs. The absolute values for uptake of ^{45}Ca and PLA_2 activity by the drug free control systems were 5193 ± 233 and 286 ± 37 cpm respectively.

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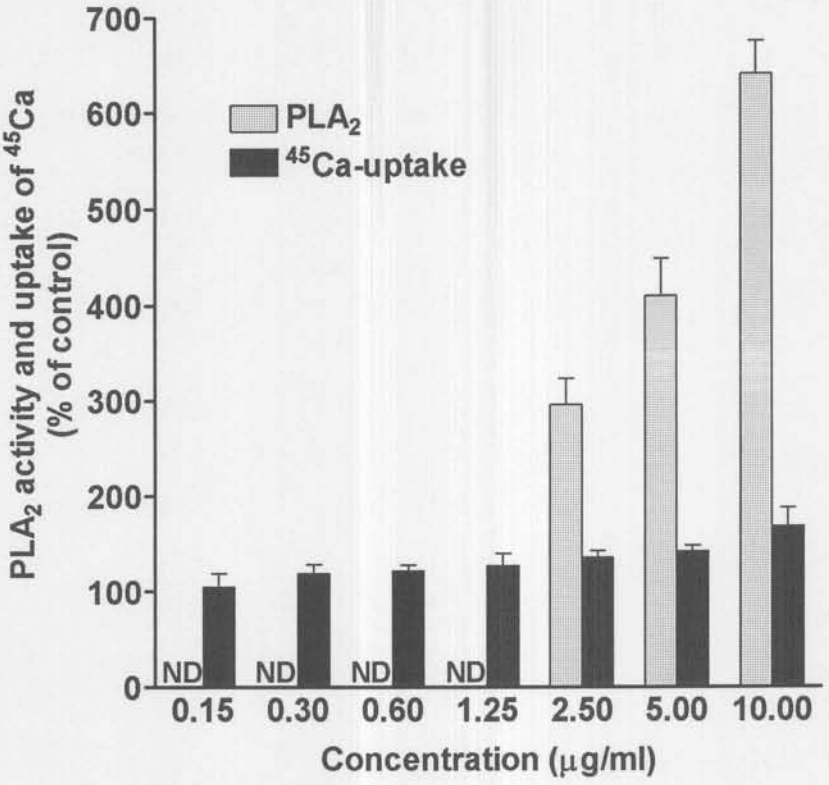

Figure 19: Effects of B4121 on ^{45}Ca-uptake by, and PLA_2 activity of *M. aurum* A^+. The results of 3 experiments each with triplicate determinations , are presented as the mean percentages of the untreated control systems ± SEMs. The absolute values for uptake of ^{45}Ca and PLA_2 activity by the drug-free control systems were 3769 ± 137 and 191± 7 cpm respectively.

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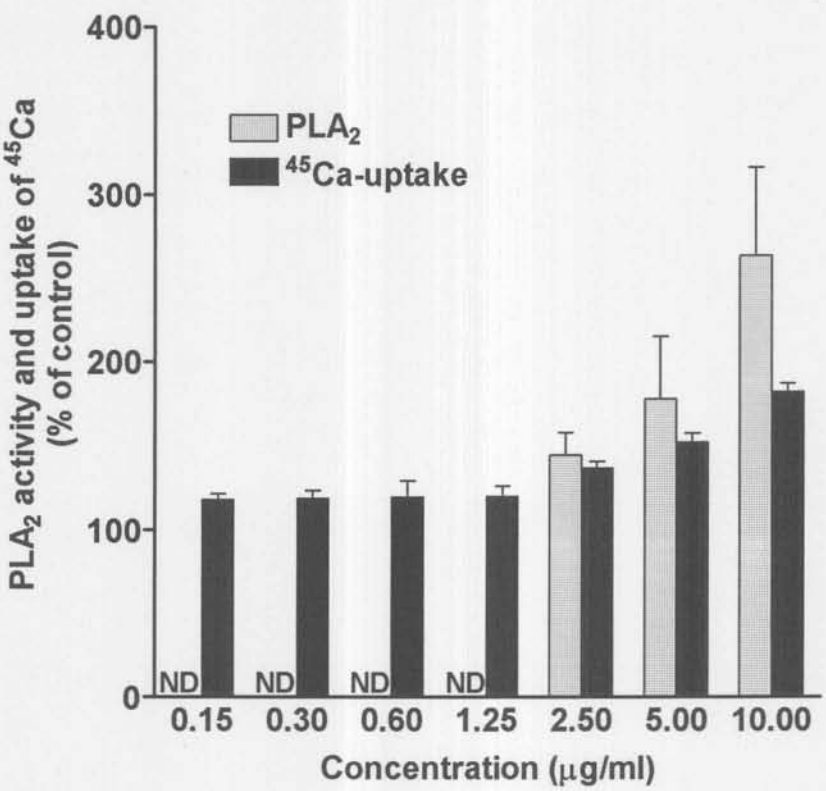

Figure 20: Effects of B4121 on ^{45}Ca-uptake by, and PLA_2 activity of the avirulent strain of *M. tuberculosis* H37R. The results of 2-3 experiments each with triplicate determinations are expressed as the mean percentages of the untreated control systems ± SEMs. The absolute values for uptake of ^{45}Ca and PLA_2 activity by the drug-free control systems were 10143 ± 347 and 204 ± 38 cpm respectively.

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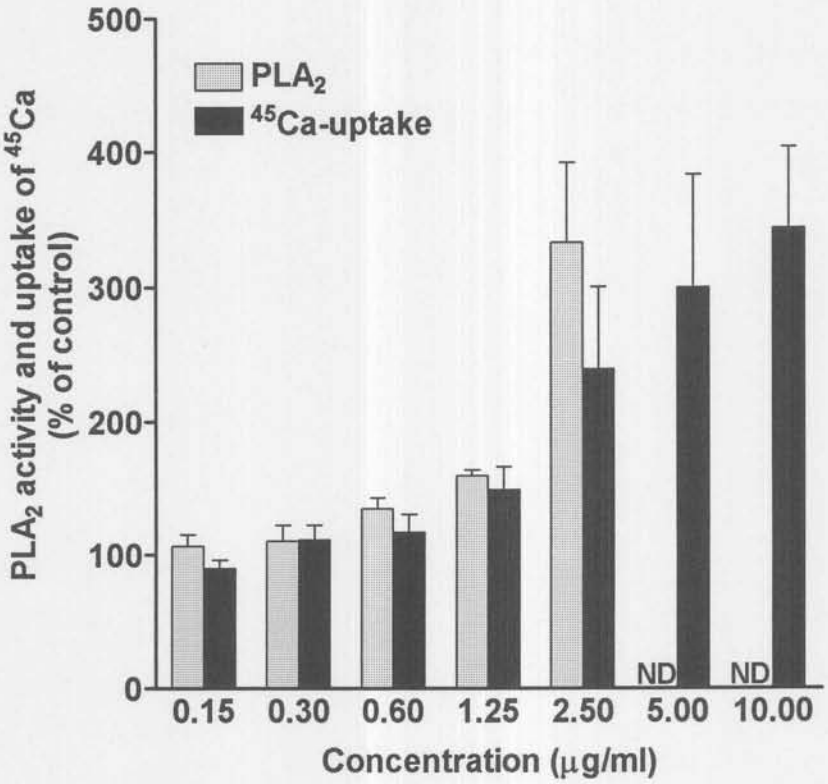

Figure 21: Effects of B4128 on ^{45}Ca-uptake by, and PLA_2 activity of *M. aurum* A^+. The results of 3 experiments each with triplicate determinations, are expressed as the mean percentages of the untreated control systems ± SEMs. The absolute values for uptake of ^{45}Ca and PLA_2 activity by the drug-free control systems were 6016 ± 282 and 438 ± 55 cpm respectively.

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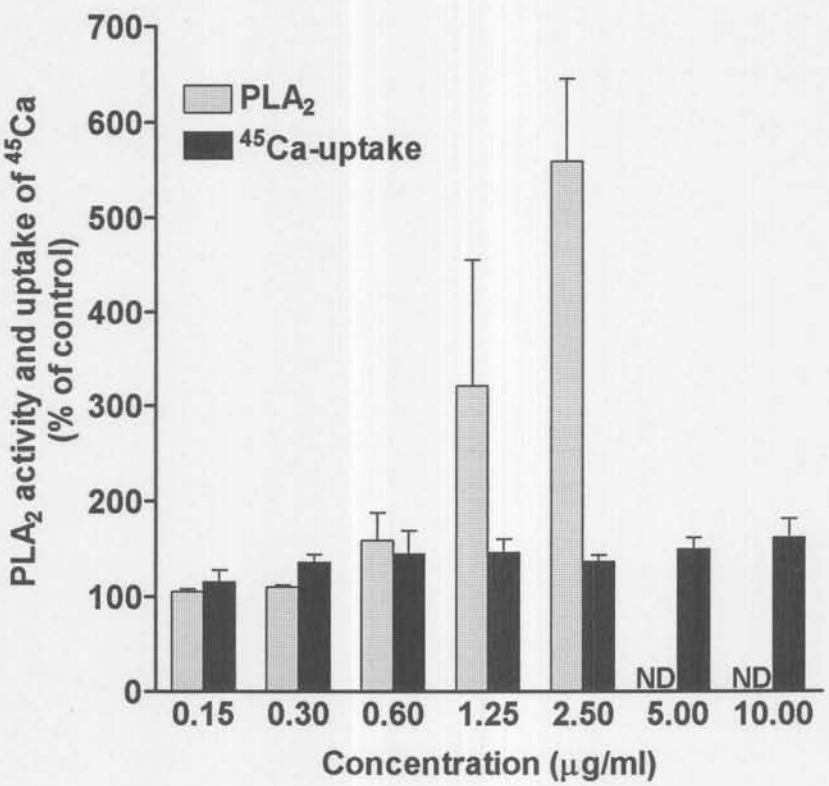


Figure 22: Effects of B4128 on ^{45}Ca-uptake by, and PLA_2 activity of the avirulent strain of *M. tuberculosis* H37R. The results of a single experiment with quintuplicate determinations for Ca^{2+}-uptake assays and of three experiments each with triplicate determinations for PLA_2 assays, are expressed as the mean percentages of the untreated control systems ± SEMs. The absolute values for uptake of ^{45}Ca and PLA_2 activity by the drug-free control systems were 5193 ± 254 and 286 ± 37 cpm respectively.

ND = not done

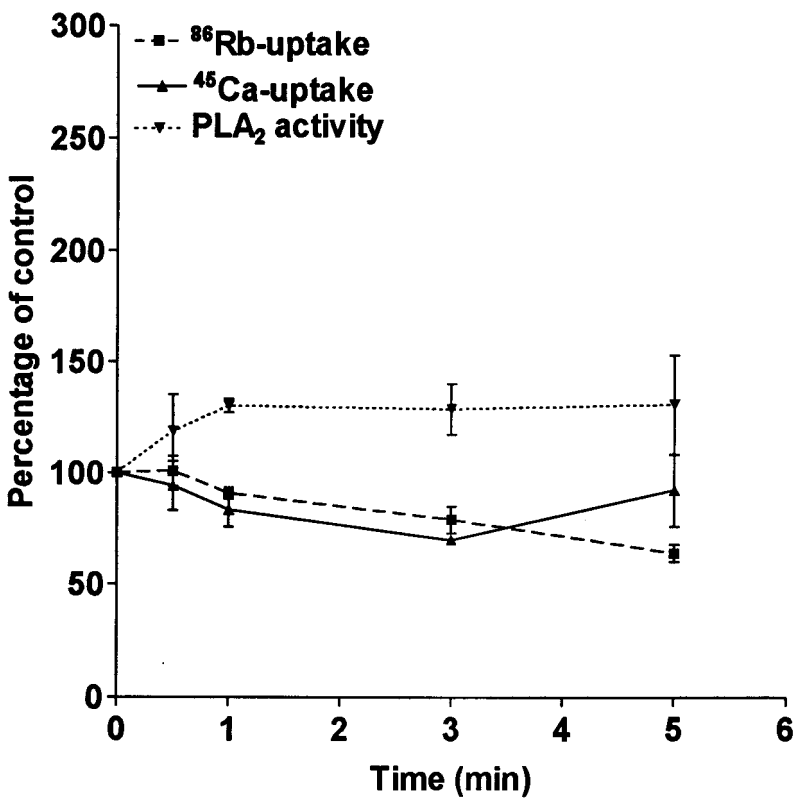


Figure 23: The time-course of altered uptake of ^{86}Rb and ^{45}Ca by, and PLA_2 activity in B663 (0.6 µg/ml) - treated *M. aurum* A^+. The results of 3-4 experiments are expressed as the mean percentages of untreated control systems ± SEMs. The absolute values for uptake of ^{86}Rb and ^{45}Ca, and PLA_2 activity by the drug-free control systems at time zero were 1090 ± 67, 2469 ± 87 and 278 ± 17 cpm respectively.

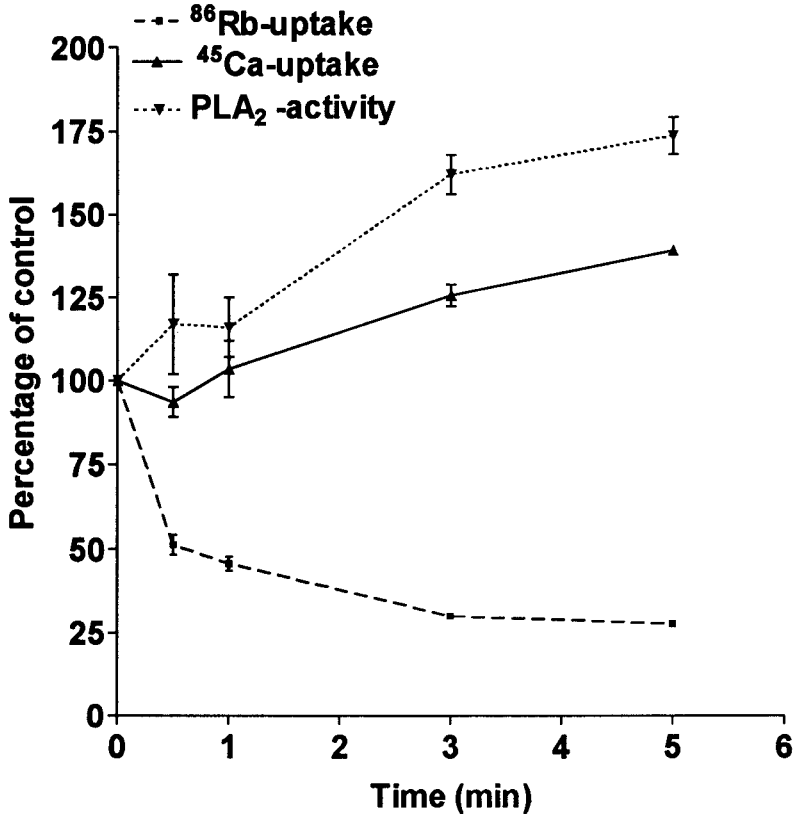


Figure 24: The time-course of altered uptake of ^{86}Rb and ^{45}Ca by, and PLA_2 activity in B663 (2.5 µg/ml)-treated *M.aurum* A^+. The results of a single experiment with quintuplicate determinations for ^{86}Rb uptake assays and 3-4 experiments for ^{45}Ca uptake and PLA_2 activity assays are expressed as the mean percentages of untreated control systems ± SEMs. The absolute values for uptake of ^{86}Rb and ^{45}Ca, and PLA_2 activity by the drug-free control systems at time zero were 1236 ± 16, 2469 ± 87 and 278 ± 18 cpm respectively.

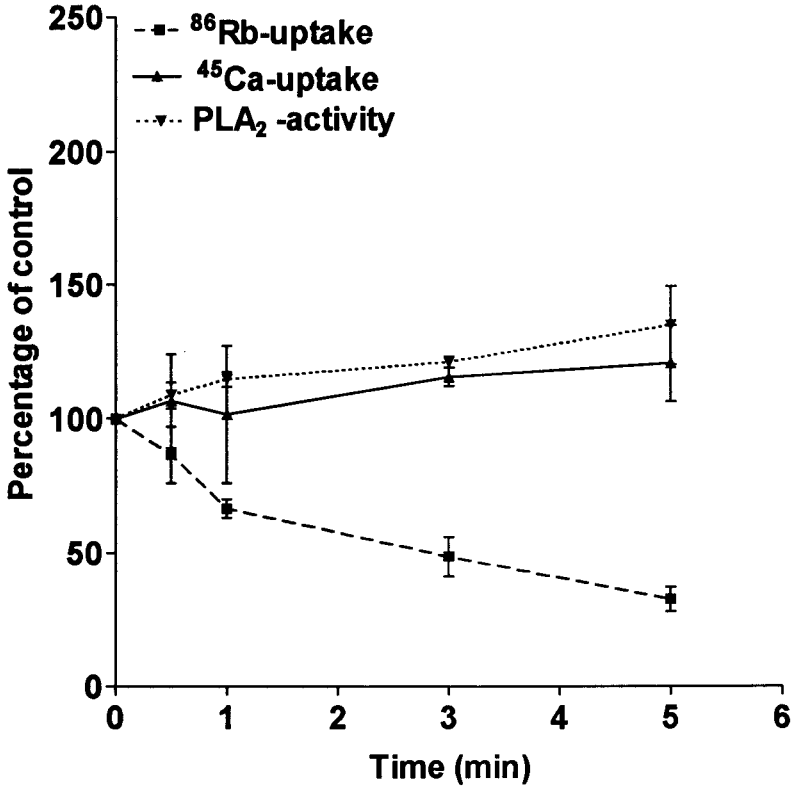


Figure 25: The time-course of altered uptake of ^{86}Rb and ^{45}Ca by, and PLA_2 activity in B4121 (0.6 µg/ml)-treated *M. aurum* A^+. The results of 3-4 experiments are expressed as the mean percentages of untreated control systems ± SEMs. The absolute values for uptake of ^{86}Rb and ^{45}Ca, and PLA_2 activity by the drug-free control systems at time zero were 1235 ± 46, 3016 ± 83 and 278 ± 13 cpm respectively.

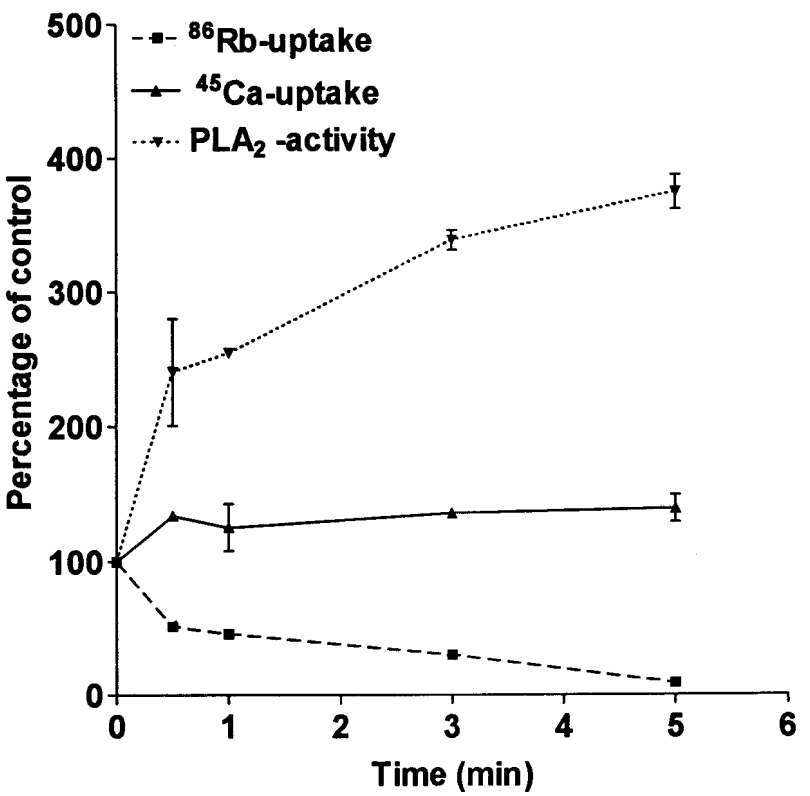


Figure 26: The time-course of altered uptake of ^{86}Rb and ^{45}Ca by, and PLA_2 activity in B4121 (2.5 µg/ml)-treated *M.aurum* A^+. The results of a single experiment with quintuplicate determinations for ^{86}Rb uptake assays and of 3-4 experiments for ^{45}Ca uptake and PLA_2 assays are expressed as the mean percentages of untreated control systems ± SEMs. The absolute values for uptake of ^{86}Rb and ^{45}Ca, and PLA_2 activity by the drug-free control systems at time zero were 1412 ± 21, 3016 ± 83 and 278 ± 13 cpm respectively.

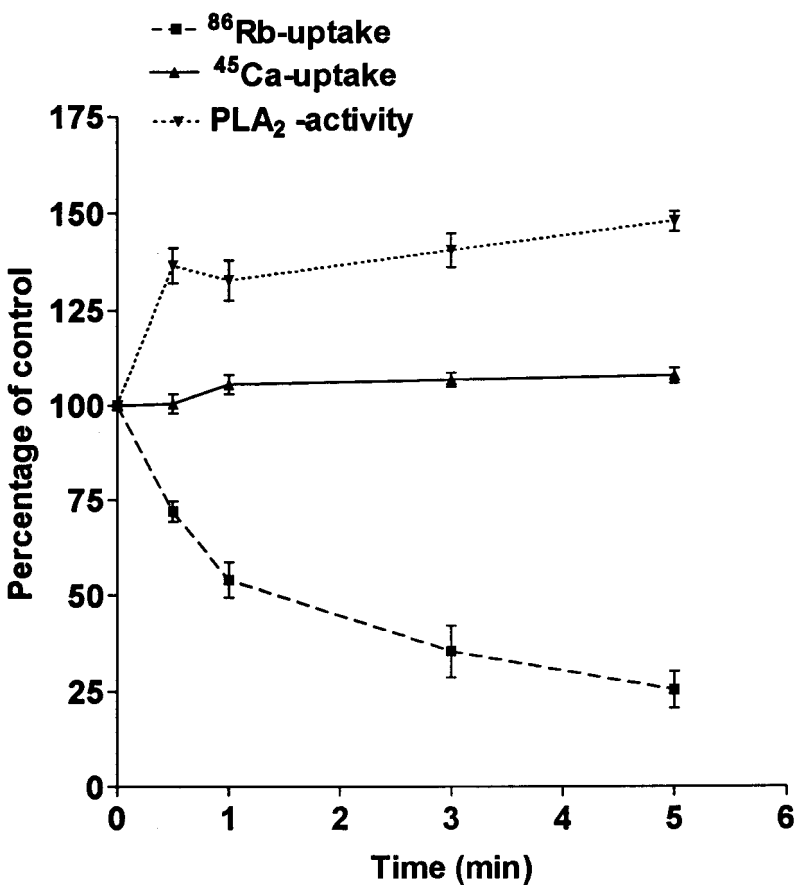


Figure 27: The time-course of altered uptake of ^{86}Rb and ^{45}Ca by, and PLA_2 activity in B4128 (0.6 µg/ml) -treated *M. aurum* A^+. The results of 3-4 experiments are expressed as the mean percentages of the untreated control systems ± SEMs. The absolute values for uptake of ^{86}Rb and ^{45}Ca, and PLA_2 activity by the drug-free control systems at time zero were 1378 ± 89, 1964 ± 84 and 278 ± 13 cpm respectively.

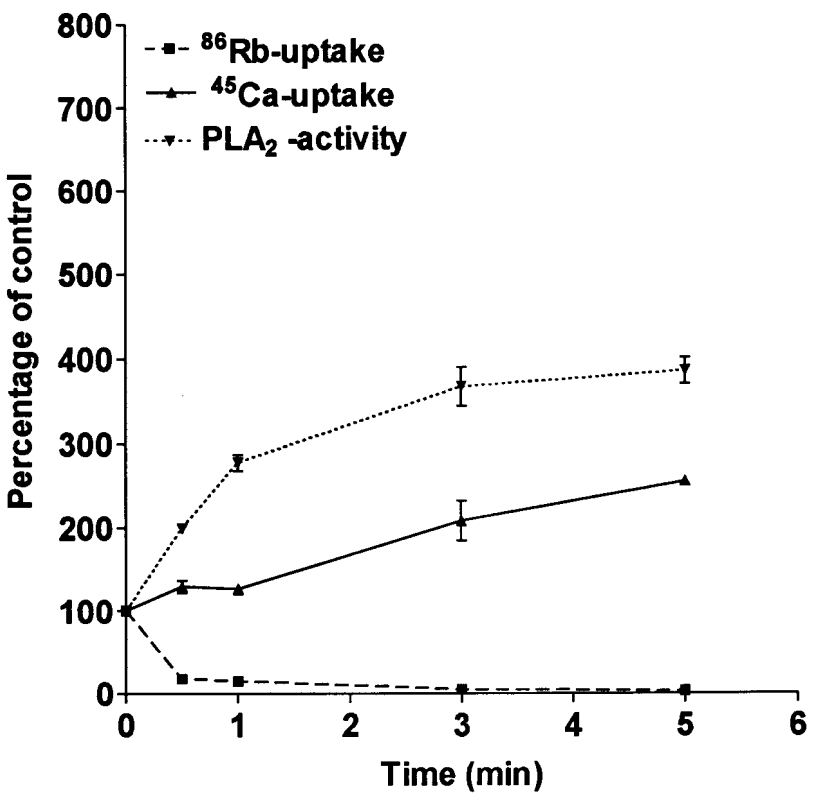


Figure 28: The time-course of altered uptake of ^{86}Rb and ^{45}Ca by, and PLA_2 activity in B4128 (2.5µg/ml)-treated *M.aurum* A^+. The results of a single experiment with quintuplicate determinations for ^{86}Rb uptake assays and of 3-4 experiments for ^{45}Ca uptake and PLA_2 activity assays are expressed as the mean percentages of the untreated control systems ± SEMs. The absolute values for uptake of ^{86}Rb and ^{45}Ca, and PLA_2 activity by the drug-free control systems at time zero were 1159 ± 32, 1964 ± 83 and 278 ± 13 cpm respectively.

Table 4: Effects of B663 and its two TMP-substituted analogues (B4121 and B4128) on the uptake of ^{86}Rb by *M.aurum* A+.

µg/ml	B663 (% of control)	B4121(% of control)	B4128 (% of control)
0.15	85±5	52±9	56±13
0.3	80±6	27±11	23±11
0.6	60±4	22±8	2±2
1.25	32±3	0	0

Results are expressed as the mean values ± standard errors of the means (SEMs) of 3-6 experiments. All values shown are significantly different ($p<0.025$-$p<0.001$) from the riminophenazine-free control system.

Table 5: Effects of B663 and its TMP-substituted analogues (B4121 and B4128) on PLA_2 activity in the virulent strain of *M.tuberculosis* H37R.

µg/ml	B663 (% of control)	B4121 (%of control)	B4128 (% of control)
0.15	103 ± 0.3	ND	120 ± 26
0.3	279 ± 26	ND	152 ± 18
0.6	645 ± 19	ND	440 ± 40
1.25	889 ± 26	ND	769 ± 30
2.5	977 ± 28	213 ± 47	2197 ± 38
5	ND	313 ± 32	ND
10	ND	569 ± 29	ND

The results of a single experiment with quituplicate determinations, are expressed as the mean percentages of the untreated control systems ± SEMs. The absolute values for PLA_2 activity by the drug-free control systems were 150 ± 23, 191 ± 36 and 150 ± 23 for B663, B4121 and B4128 respectively.

ND = not done

Table 6:The effects of EGTA on clofazimine- and B4121-mediated inhibition of uptake of ^{86}Rb by *M. aurum* A^+

Treatment	^{86}Rb-uptake (% of control)
EGTA (5mM)	102.4 ± 7
EGTA (10mM)	97.6 ± 2
EGTA (20mM)	100.0 ± 4
B663 (0.6 µg/ml)	43.0 ± 2
EGTA (5mM) and B663	44.4 ± 3
EGTA (10mM) and B663	46.4 ± 3
EGTA (20mM) and B663	48.6 ± 4
B4121 (0.6 µg/ml)	51.0 ± 6
EGTA (5mM) and B4121	61.2 ± 4
EGTA (10mM) and B4121	65.2 ± 3
EGTA (20mM) and B4121	67.0 ± 5

The results of single experiment with quintuplicate determinations are presented as the mean values ± SEMs. The absolute value for uptake of ^{86}Rb by the drug-free control system was 19823 ± 213.

Table 7: The effects of BAPTA on clofazimine- and B4121-mediated inhibition of uptake of ^{86}Rb by *M. aurum* A^+

Treatment	^{86}Rb-uptake (% of control)
BAPTA (5μM)	95.6 ± 4.5
BAPTA (10μM)	94.3 ± 3.4
BAPTA (20 μM)	89.0 ± 0.9
B663 (0.6 μg/ml)	59.2 ± 1.9
BAPTA (5 μM) and B663	54.0 ± 1.9
BAPTA (10 μM) and B663	55.2 ± 3.2
BAPTA (20 μM) and B663	55.8 ± 7.5
B4121 (0.6 μg/ml)	59.4 ± 4
BAPTA (5 μM) and B4121	63.2 ± 5
BAPTA (10 μM) and B4121	64.4 ± 4
BAPTA (20μM) and B4121	60.2 ± 4

The results of a single experiment with quintuplicate determinations are presented as the mean values ± SEMs. The absolute value for uptake of ^{86}Rb by the drug-free control system was 31136 ± 428.

Table 8: The effects of BAPTA or EGTA on clofazimine-, B4121- and B4128-mediated enhancement of PLA_2 activity in *M. aurum* A^+

Treatment	PLA_2 activity (% of control)
BAPTA (10 µM)	99.5 ± 5
EGTA (10 mM)	101 ± 11
B663 (1.25 µg/ml)	220 ± 12
B663 (1.25 µg/ml) and BAPTA (10 µM)	283 ± 20
B663 (1.25 µg/ml) and EGTA (10 mM)	372 ± 24
B4121 (2.5 µg/ml)	285 ± 34
B4121 (2.5 µg/ml) and BAPTA (10 µM)	235 ± 27
B4121 (2.5 µg/ml) and EGTA (10 mM)	219 ± 22

The results of a single experiment with quintuplicate determinations are presented as the mean values ± SEMs. The absolute value for PLA_2 activity by the drug-free control system was 1212 ± 34.

Table 9: The effects of BAPTA and EGTA in combination on B663 - and B4121-mediated enhancement of PLA_2 activity of *M. aurum* A^+

Treatment	PLA_2 activity (% of control)
B663 (1.25 µg/ml)	295 ± 62
B663 and BAPTA and EGTA	605 ± 54
B4121 (2.5 µg/ml)	790 ± 97
B4121 and BAPTA and EGTA	720 ± 108
BAPTA (10 µM) and EGTA (10 mM)	97 ± 11

The results of a single experiment with quintuplicate determinations are presented as the mean values ± SEMs. The absolute value for PLA_2 activity by the drug-free control system was 280 ± 18.

Table 10: The effects of exposure of *M. aurum* A^+ to B663 on cellular ATP levels

Concentration of B663	Cellular ATP (nanomoles/10^6 cells
Control	9.4 ± 1.25
0.15	9.3 ± 1.0
0.3	10.2 ± 1.0
0.6	9.4 ± 0.6
1.25	9.9 ± 0.8
2.5	10.1 ± 0.3
5	6.7 ± 0.4

Results of a single experiment with quintuplicate determinations for each concentration are presented as the mean values ± SEMs.

Table 11: The effects of exposure of *M.aurum* A^+ to B4121 on cellular ATP levels

Concentration of B4121	Cellular ATP (nanomoles/ 10^6 cells)
Control	7.7 ± 1.5
0.15	8.9 ± 1.2
0.3	7.7 ± 1.1
0.6	6.0 ± 0.9
1.25	4.9 ± 0.8
2.5	4.0 ± 0.5
5	3.4 ± 0.3

The results of three different experiments with quintuplicate determinations for each system are presented as the mean values ± SEM.

Table 12: Effects of exposure of *M. aurum* A^+ to B4128 on cellular ATP levels

Concentration of B4128	Cellular ATP (nanomoles/ 10^6 cells)
Control	9.0 ± 0.9
0.15	10.1 ± 0.7
0.3	9.1 ± 0.5
0.6	8.0 ± 1.0
1.25	3.5 ± 0.3
5	4.4 ± 0.1

The results of 3 different experiments, each with quintuplicate determinations for each system are presented as the mean values ± SEMs.

3.4. DISCUSSION

In the studies presented in this chapter, I have investigated the biochemical mechanisms by which B663 (clofazimine) and two novel TMP-substituted phenazines B4121 and B4128 modulate potassium (K^+) transport in mycobacteria with emphasis on microbial PLA_2 activity, Ca^{2+} fluxes and energy metabolism. Clofazimine as well as the TMP-substituted phenazines B4121 and B4128, caused a dose-related inhibition of uptake of ^{86}Rb by *M.aurum* A^+ as well as by the virulent and avirulent strains of *M.tuberculosis* H37R with the novel TMP-substituted phenazines being more active than clofazimine. However, the dose-response curves for inhibition of transport of ^{86}Rb by the avirulent strain of *M.tuberculosis* H37R were fairly similar for all three riminophenazines with clofazimine at concentrations of 0.3, 0.6 and 1.25 µg/ml being only slightly less potent than B4121 and B4128. The inability to distinguish differences in sensitivity in the uptake of ^{86}Rb by *M.tuberculosis* H37Ra treated with clofazimine, B4121 and B4128 is suprising, since this was clearly not the case with *M.tuberculosis* H37Rv and *M.aurum* A^+. The similarities in sensitivity of the avirulent mutant strain of *M.tuberculosis* H37R to B663, B4121 and B4128 in these short-duration assays of cation transport may reflect differences in cellular metabolic activity and/or membrane architecture, which may affect the activity of these TMP-riminophenazines, at least in the short term. Alternatively, clofazimine may exert its inhibitory effects on cation transport more rapidly in H37Ra than in H37Rv and *M.aurum* A^+. The results for clofazimine are in agreement with the report by De Bruyn *et al* (1996), who found that this antimicrobial agent inhibited potassium transport in Gram-positive microorganisms.

When the novel TMP-substituted phenazines and clofazimine at concentrations of 0.018-5 µg/ml were tested for their effects on the growth of *M.aurum* A^+, B4121 was found to be much more potent than clofazimine , while B4128 was of intermediate potency (Matlola, 1996). Although the concentrations of the test agents which caused inhibition of uptake of ^{86}Rb by *M.aurum* A^+ were higher than those required to inhibit bacterial growth, this probably reflects differences in exposure time of the microorganisms to the antimicrobial agents in the different assay systems. In assays of K^+ transport, the total incubation time was only 75 minutes, while in assays of

bacterial growth the incubation time was at least 48 hours. The effects of B4121 in the cation uptake assays as well as in PLA_2 assays were of a lesser magnitude than those of B663 and B4128. B4121 showed improved performance in the assays of microbial growth and this may indicate that this agent is somewhat slower-acting than the other TMP-riminophenazines, or that it has a lower level of binding to components of the culture medium.

Decreased uptake of K^+ may be a consequence of inhibition of influx, or accelerated efflux of the cation, or both. To identify which of these mechanisms are operative during exposure of mycobacteria to the riminophenazines, I investigated the effects of low, MIC concentrations of clofazimine (0.3 µg/ml), B4121 and B4128 (both at 0.15µg/ml) on K^+-influx and -efflux using *M.aurum* A^+. All three riminophenazines inhibited influx of K^+ without significantly affecting the rate of efflux of the cation. Microbial ATP levels were unaffected by the relatively brief 30 minute exposure to the riminophenazines. However, exposure of mycobacteria to higher concentrations of B663, B4121 (both at 5 µg/ml) and B4128 (at 1.25 µg/ml and 5 µg/ml) did result in a significant decrease in microbial ATP levels. This suggests that B663, B4121 and B4128 at higher concentrations have abrupt cytotoxic effects or, that alternatively at high concentrations of the riminophenazines there is increased turnover of ATP by microbial Ca^{2+}-ATPases which pump out Ca^{2+}. Taken together, these findings suggest that riminophenazine-mediated inhibition of K^+-influx appears to be a primary event that precedes microbial death, and is not a consequence of microbial damage or interference with microbial ATP metabolism resulting in leakage of the cation.

It has previously been reported that clofazimine increases the activity of PLA_2 in human neutrophils (Krajewska & Anderson, 1993), squamous carcinoma cell lines (Van Rensburg *et al*, 1993), and in Gram-positive bacteria (Van Rensburg *et al*, 1992) *in vitro*. In the present study I have investigated the mechanistic relationship which may exist between altered PLA_2 activity, K^+-uptake and Ca^{2+} uptake (a divalent cation which is normally excluded from both prokaryotic and eukaryotic cells, Norris *et al*, 1996) in mycobacteria exposed to clofazimine and the TMP-substituted phenazines, B4121 and

B4128.

Exposure of *M. aurum* A^+ to clofazimine and B4128 was accompanied by enhancement of PLA_2 activity and an increase in the uptake of Ca^{2+}, both of which occurred at concentrations of ≥ 1.25 µg/ml. Short-term exposure (5 minutes) of *M. aurum* A^+ to clofazimine or B4128 (0.6 µg/ml and 2.5 µg/ml) resulted in an immediate dose-related enhancement of microbial PLA_2, which was associated with inhibition of K^+-influx . Uptake of Ca^{2+} was delayed, only occurring after 3 minutes exposure of the mycobacteria to the riminophenazines. B4121 (2.5 µg/ml) also caused an immediate enhancement of PLA_2 and inhibition of K^+-transport. However, with this agent, at the concentrations and reaction times used, Ca^{2+} uptake was minimally affected throughout the entire time-course of the experiment. The absence of detectable Ca^{2+} uptake, or K^+-efflux suggests that the effects of the riminophenazines on microbial PLA_2 are not dependent on calcium influx, but are nevertheless related to inhibition of K^+-uptake in the setting of unaffected ATP levels.

In human lymphocytes and cancer cell lines, coincubation with riminophenazines is associated with increased activity of PLA_2 leading to generation of anti-proliferative lysophospholipids *eg* lysophosphatidylcholine (LPC) (Anderson & Smit, 1993; Van Rensburg *et al,* 1993). LPC and arachidonic acid, the primary hydrolysis products generated during cleavage of phosphatidylcholine by PLA_2, are inhibitors of Na^+K^+ - ATPase (Oishi *et al*, 1990, Okafor *et al*, 1997), an enzyme which is critically involved in the maintenance of cellular K^+ homeostasis and growth (Sweadner & Goldin, 1980). Although the exact molecular mechanism by which these effects are achieved has not been established, it has been proposed that the intra-membrane accumulation of lysophospholipids *i.e.* LPC during exposure of lymphocytes to riminophenazines results in decreased activity of the essential membrane enzyme, Na^+K^+-ATPase (Anderson & Smit, 1993).

The proposed mechanistic link between riminophenazine-mediated enhancement of microbial PLA_2 activity and inhibition of uptake of K^+ and growth was strengthened by

data from experiments using α-tocopherol. This agent, at a fixed concentration of 25 μg/ml does not appear to inhibit the activity of PLA_2 (Pentland *et al*, 1992), but rather interacts with lysophospholipids and unsaturated fatty acids to neutralize their membrane-destabilizing activity, a property not shared by α-tocopherol acetate or other lipid-soluble anti-oxidants (Kagan, 1989).

Pretreatment of *M. aurum* A^+ with α-tocopherol neutralized the inhibitory effects of clofazimine, B4121 and B4128 on both microbial K^+-transport and growth (Matlola thesis, 1996). In the present study, I have found that pretreatment of mycobacteria with α-tocopherol also antagonized the inhibitory effects of the riminophenazines on influx of Ca^{2+} into mycobacteria treated with clofazimine, while delayed addition (15 minutes after exposure to the antimicrobial agents) of α-tocopherol to riminophenazine-treated mycobacteria restored Ca^{2+} homeostasis. This observation suggests that intra-membrane accumulation of lysophospholipids eventually causes an α-tocopherol-reversible increase in permeability of the outer membrane to Ca^{2+}.This may occur indirectly as a consequence of inactivation of microbial K^+-transporters, and/or directly as a result of lysophospholipid-mediated damage to the cell membrane.

Since activation of most, but not all classes of PLA_2 is Ca^{2+}-dependent, extracellular and intracellular Ca^{2+}-chelating agents were used to investigate the involvement of this cation in riminophenazine-mediated inhibition of uptake of K^+ and enhancement of mycobacterial PLA_2 activity. Treatment of the bacteria with EGTA and BAPTA individually or in combination was found to have no antagonistic effects on B663-,or B4121-mediated inhibition of uptake of ^{86}Rb. Likewise, treatment of the bacteria with these chelating agents, either individually or in combination, did not prevent, but rather potentiated, B663- or B4121-mediated augmentation of PLA_2 activity. These results suggest that the riminophenazine-mediated enhancement of PLA_2 is a Ca^{2+}-independent event (Dennis, 1983).

Van Rensburg *et al* (1992) have demonstrated that the activity of purified PLA_2 was unaffected by either clofazimine or B669 (5 μg/ml), an observation which suggests that

the riminophenazine-mediated alterations in the hydrolysis of mycobacterial membrane phospholipids are not achieved by direct effects of these agents on PLA_2. Taken together with the insensitivity of enhancement of PLA_2 activity to EGTA or BAPTA, it is possible that riminophenazines may disrupt membrane structure, making the integral phospholipids more susceptible to attack by PLA_2. Interestingly, it has previously been reported that bilayer packing stresses of the cell membrane during phase changes increase the sensitivity of the integral phospholipids to PLA_2 (Rao, 1992; Rao & Nagaraj, 1993). It is possible therefore that riminophenzines, which are extremely lipophilic, may cause alterations in lipid packing in the outer membrane, resulting in increased susceptibility of phospholipids to PLA_2. Such effects have previously been reported for membrane-interactive antimicrobial peptides, including gramicidins (Rao, 1992). These peptides were found to induce non-bilayer phases in membranes, apparently increasing and decreasing the accessibility of the acyl chains and head groups respectively, with the net effect of enhancing PLA_2 activity (Rao, 1992).

Although relatively little is known about the K^+-transporting systems of mycobacteria, the susceptibility of these to riminophenazine-mediated inactivation suggests structural similarities to those operative in Gram-positive bacteria (De Bruyn *et al*, 1996) and eukaryotic cells (Anderson & Smit, 1993; Van Rensburg *et al*, 1993). In all three cases inhibition of K^+-transport is achieved by indirect, PLA_2-dependent mechanisms. The indiscriminate effects of the riminophenazines on K^+-transport do not, however, eliminate microbial K^+-transporters as possible novel and selective targets for antimicrobial chemotherapy. This is based on the observed absence of effects of ouabain, a selective and potent inhibitor of Na^+K^+-ATPase in eukaryotic cells, on mycobacteral growth and uptake of K^+, indicating the existence of structural differences between microbial and eukaryotic K^+-transporting systems. An ideal inhibitor of microbial K^+ transport should, however, interact directly and selectively with the cation transporter, rather than by the non-selective, indirect, PLA_2-dependent mechanism described here for the riminophenazines. The observation that ouabain increases the proliferation of mycobacteria was unexpected and requires more intensive investigation which is outwith the scope of the current study.

In conclusion, the results presented in this chapter have demonstrated that exposure of *M. aurum* A^+ as well as the *M.tuberculosis* H37R strains to the riminophenazines results in dose-related enhancement of microbial PLA_2 activity, which was associated with inhibition of K^+-influx. Uptake of Ca^{2+} by mycobacteria was unaffected, or minimally affected, by the riminophenazines at concentrations of <1.25 µg/ml, while higher concentrations resulted in increased uptake of the cation in the setting of decreased microbial ATP levels in bacteria exposed to the TMP-substituted phenazines, B4121 and B4128 as well as clofazimine. These results suggest that the anti-mycobacterial activities of clofazimine, B4121 and B4128 are related to a Ca^{2+}-independent increase in the activity of mycobacterial PLA_2, leading to interference with microbial K^+-transport.

CHAPTER 4

EFFECTS OF LYSOPHOSPHATIDYLCHOLINE ON THE GROWTH OF AND POTASSIUM UPTAKE BY *M.aurum* A^+, COMPARED WITH THAT OF *S.aureus*

4.1. INTRODUCTION

Lysophospholipids are generated during the cleavage of membrane phospholipids by PLA_2, and have potent membrane-destabilizing effects on eukaryotic cells, as well as inhibitory effects on Na^+,K^+-ATPase, the primary K^+ transporter in eukaryotic cells (Oishi *et al*, 1990; Okafor *et al*, 1997). Although the exact molecular mechanism by which these effects are achieved has not been established, it has been proposed that lysophospholipids may compromise the activity of Na^+,K^+-ATPase by interfering with essential interactions of this cation transporter with boundary phospholipids in the inner membrane (Oishi *et al*, 1988).

In the previous chapter I reported that clofazimine, as well as the two TMP-substituted phenazines, inhibited the uptake of K^+ by mycobacteria which was associated with increased activity of microbial PLA_2. On the basis of the following observations a mechanistic relationship between these events was proposed: I) the time-courses of increased activity of PLA_2 and inhibition of K^+ transport were superimposable ii) both effects were observed over the same concentration ranges of all three test riminophenazines and iii) α-tocopherol, which neutralises lysophospholipids generated during cleavage of membrane phospholipids by PLA_2, protected mycobacterial K^+-uptake against the inhibitory effects of the riminophenazines. This latter observation also implicates lysophospholipids as being potential mediators of the antimicrobial activity of the test riminophenazines.

The experiments presented in the current chapter were designed to investigate the effects of reagent lysophosphatidylcholine (LPC) on K^+-uptake by and growth of the non-pathogenic mycobacterium, *M.aurum* A^+. *Staphyloccocus aureus*, which has previously been shown to be sensitive to LPC (De Bruyn *et al*, 1996) was included for comparison. I have also measured and compared lysophospholipase (lysophospholipid-degrading enzyme) activity in *M.aurum* A^+ and *S.aureus*.

4.2. MATERIALS AND METHODS

4.2.1. LYSOPHOSPHATIDYLCHOLINE

Reagent LPC was dissolved in distilled water to a stock concentration of 1mg/ml. Subsequent dilutions were made in distilled water and LPC was used at final concentrations ranging from 0.25-2.5 µg/ml and 1.25-10 µg/ml for the potassium transport assays for *S.aureus* and *M.aurum* A^+ respectively and 0.5-5 µg/ml for growth studies for both microorganisms.

4.2.2. CHEMICALS AND REAGENTS

Unless otherwise indicated, all chemicals used were obtained from the Sigma Chemical Co., St Louis, MO, USA.

4.2.3. MICROORGANISMS

M.aurum A^+ was obtained from Institut Pasteur, Paris, France, while *S.aureus* ATCC 25923 was kindly supplied by the South African Institute for Medical Research.

4.2.4. BACTERIAL GROWTH STUDIES

The effects of LPC on the growth of *S.aureus* and *M.aurum* A^+ were investigated using a rapid and sensitive radioassay (Van Rensburg *et al*, 1992). Following overnight culture of the bacteria in nutrient broth (Becton Dickinson & Company, Cockeysville, MD, USA), the bacterial cells were resuspended in 0.15 M phosphate-buffered saline (PBS), pH 7.4, to obtain a final concentration of 10^7 cfu/ml. The bacterial suspensions were then exposed to 0.5-5 µg/ml of LPC in 2 ml PBS for 30 min at 37°C. An equal volume of double-strength nutrient broth containing 0.5 µCi of radiolabelled amino acids (L-amino acid mixture, L-^{14}C[U], specific activity (55.2 mCi/mmol), Du Pont-NEN Research Products, Boston, MA, USA) was then added to each tube. The tubes of *S.aureus* and *M.aurum* A^+ were incubated for 3 hours and 48 hours respectively at 37°C, after which the bacteria were centrifuged and washed twice in PBS. The extent of incorporation of radiolabelled amino acids into the bacterial pellets was measured in a liquid scintillation spectrometer.

4.2.5. ^{86}Rb-UPTAKE STUDIES

^{86}Rb (Rubidium-86 chloride, specific activity (3.56 mCi/mg), Du Pont-NEN Research Products, Boston, MA, USA) was used as a tracer for measuring K^+-uptake by the bacteria. *S.aureus* and *M.aurum* A^+ were cultured for overnight and for 4 days respectively before being harvested, washed and resuspended in glucose- and K^+-free buffer (KONO buffer) to a concentration of 3.5 X 10^9cfu/ml for *S.aureus* and 0.5 X 10^7cfu/ml for *M.aurum* A^+, which in both case equates to a protein concentration of 0.01mg/ml. After 15 minutes, the bacteria were exposed to LPC (0.5-2.5 and 1.25-10 µg/ml for *S.aureus* and *M.aurum* A^+ respectively) at 37°C for 30min. The bacteria were then concentrated by centrifugation and resuspended in 2 ml KONO buffer supplemented with 22 mM glucose and ^{86}Rb (2 µCi/ml). Uptake of the cation was measured using a fixed incubation time of 45 min at 37°C. After incubation, the bacteria were washed twice with cold PBS and the pellets finally disrupted by adding 0.4 ml pre-heated 5% trichloroacetic acid (TCA). Radioactivity was assayed for in a liquid scintillation spectrometer and the net uptake was taken as the difference in uptake of ^{86}Rb in the tubes incubated at 37°C and the controls kept on ice. The effects of pretreatment with a fixed concentration (25 µg/ml) of α-tocopherol (vitamin E) on the uptake of ^{86}Rb by the control and LPC-treated bacteria were also investigated. In these experiments α-tocopherol was added 1 min before the LPC (1µg/ml and 5µg/ml for *S.aureus* and *M.aurum* A^+ respectively).

4.2.6. PHOSPHOLIPID BREAKDOWN

LPC breakdown by *S.aureus* and *M.aurum* A^+ was measured as a reduction in the concentration of added radiolabelled substrate, using a high-performance thin-layer chromatography (HPTLC) method (Bradova *et al*, 1990; Van Rensburg *et al*, 1992). The bacteria were resuspended in Hanks balanced salt solution, with 1.25 mM calcium chloride to concentrations of 10^9 cfu/ml for *S.aureus* and 10^7 cfu/ml for *M.aurum* A^+, after which 0.25 ml aliquots of the bacterial suspensions were added to tubes containing 0.75 ml HBSS, 2.5 µg/ml cold carrier LPC and 0.25µCi/ml lysophosphatidylcholine (lysopalmitoylphosphatydyl-choline, L-α-[palmitoyl-1-^{14}C], specific activity, 40-60 mCi/mmol). After 15, 30, 45, 60, and 90 minutes incubation at

37°C, the reactions were terminated by the addition of 2ml chloroform:methanol (2:1 [vol/vol]) and the fatty acid-containing lower phase was removed and evaporated to dryness under nitrogen. The samples were stored at -20°C until assayed.

The evaporates were reconstituted with 40µl of chloroform:methanol (2:1 [vol/vol]) containing 0.01mM LPC to facilitate detection and spotted onto silica gel 60F 254-precoated HPTLC plates (Merck, Darmstadt, Germany). The plates were developed twice in chloroform: methanol:isopropanol:0.25% KCl:ethyl acetate (30:9:25:6:18 [vol/vol/vol/vol/vol]). After exposure to iodine vapours the phospholipids were localized and the silica was removed and assayed for radioactivity in a liquid scintillation spectrometer.

4.2.7. EXPRESSION AND STATISTICAL ANALYSIS OF RESULTS

The results are expressed as the mean value ± standard error of the mean (SEM) for each series of experiments, usually as percentage of the corresponding drug-free control system. Levels of statistical significance were calculated using the Student paired t-test (paired t statistic).

4.3. RESULTS

Effects of lysophosphatidylcholine on the growth of *M.aurum* A^+ and *S.aureus*

The effects of LPC on the growth of *M.aurum* A^+ and *S.aureus* are shown in Table 13, Page 114. The growth of *M.aurum* A^+ was minimally affected by LPC at all the concentrations tested. LPC caused significant inhibition of the growth of *S.aureus* at concentrations of 2.5µg/ml and 5µg/ml ($p \leq 0.0001$).

Effects of lysophosphatidylcholine on ^{86}Rb-uptake by *M.aurum* A^+ and *S.aureus*

Exposure of *M.aurum* A^+ and *S.aureus* to concentrations of 0.25-10 µg/ml LPC resulted in dose-related inhibition of uptake of ^{86}Rb with *S.aureus* being considerably more sensitive to the lysophospholipid than *M.aurum* A^+ (Table 14, Page 115). Uptake of ^{86}Rb by *M.aurum* A^+ was significantly inhibited by 5 µg/ml of LPC and higher ($p \leq 0.0006$-

$p \leq 0.0003$) while significant inhibition of ^{86}Rb-uptake in the case of *S.aureus* occurred at a concentrations of 0.5µg/ml of LPC and higher ($p \leq 0.003$-$p \leq 0.0001$).

Alpha-tocopherol was found to protect *M.aurum* A^+ and *S.aureus* against the inhibitory effects of LPC. The mean uptakes (percentage of control) of ^{86}Rb by *M.aurum* A^+ exposed to LPC (10µg/ml) in the absence and presence of α-tocopherol were 18.3 ± 2.6 and 69.7 ± 4.6 respectively. The mean percentages of ^{86}Rb-uptake in *S.aureus* exposed to LPC (0.5µg/ml) in the absence and presence of α-tocopherol were 35.9 ± 1.5 and 82.6 ± 0.7 respectively.

Effects of lysophosphatidylcholine breakdown by *M.aurum* A^+ and *S.aureus*

The kinetics of breakdown of LPC by lysophospholipase in *M.aurum* A^+ and *S.aureus* are presented in Table 15, Page 116. *M.aurum* A^+ was highly efficient in degrading LPC with approximately 86% of the labelled LPC being degraded within the first 15 minutes. No further breakdown by *M.aurum* A^+ was noted. In the case of *S.aureus*, 21.9% of labelled LPC was degraded in 15 min and 54.3% in 90 min.

Table 13: Effects of lysophosphatidylcholine on the growth of *M..aurum* A^+ and *S.aureus*

LPC (µg/ml)	*M.aurum* A^+ (% of control)	*S.aureus* (% of control)
0.5	98.9 ± 3.1	110.6 ± 6.9
1	88.8 ± 2.1	99.3 ± 7.7
2.5	79.9 ± 3.1	54.8 ± 2.9
5	80.1 ± 1.9	0.3 ± 0.2

The results of 3 experiments with quintuplicate determination are presented as the mean percentages of the untreated control systems ± SEMs. The absolute values for the LPC-free control systems were 438 ± 18 and 1763 ± 58 cpm for *M.aurum* A^+ and *S.aureus* respectively.

Table 14: Effects of lysophosphatidylcholine on ^{86}Rb-uptake by *M.aurum* A^+ and *S.aureus*

LPC (µg/ml)	*M.aurum* A^+ (% of control)	*S.aureus* (% of control)
0.25	ND	83.1 ± 1.0
0.5	ND	35.9 ± 1.5
1	ND	21.9 ± 1.5
1.25	82.5 ± 4.3	ND
2.5	77.6 ± 3.8	4.7 ± 0.5
5	42.6 ± 3.7	ND
10	18.3 ± 2.6	ND

The results of 3-6 experiments with triplicate determinations for each concentration are presented as the mean percentages of the untreated control systems ± SEMs. The absolute values for the LPC-free control systems were 10290 ± 92 and 67168 ± 328 cpm for *M.aurum* A^+ and *S.aureus* respectively.

ND = not done

Table 15: Kinetics of breakdown of lysophosphatidylcholine by *M.aurum* A^+ and *S.aureus*

Time (minutes)	*M.aurum* A^+ (% of $time_o$)	*S.aureus* (% of $time_o$)
15	14.5 ± 2.6	78.1 ± 1.5
30	12.6 ± 1.7	71.4 ± 2.1
45	12.3 ± 1.4	62.6 ± 0.9
60	9.7 ± 1.1	68.8 ± 9.9
90	12.0 ± 1.7	45.7 ± 2.2

The results of 2 different experiments with quintuplicate determinations for each time point are presented as the mean percentages of the time zero value ± SEMs. The absolute value for time zero was 183747 ± 801 cpm.

4.4. DISCUSSION

Lysophospholipids, are, together with unsaturated fatty acids the primary phospholipid degradation products, generated by PLA_2 and have been shown to have antimicrobial properties, particularly against Gram-positive bacteria (Van Rensburg *et al*, 1992; De Bruyn *et al*, 1996). In this chapter, the effects of LPC on the growth of and uptake of K^+ by *M.aurum* A^+ have been described and compared with the corresponding effects of this agent on *S.aureus*. With respect to K^+ transport, *M.aurum* A^+ was considerably less sensitive to the inhibitory effects of LPC than was *S.aureus*. LPC at concentrations of 0.25 µg/ml and upwards caused dose-related inhibition of uptake of K^+ by *S.aureus*, which was almost total (95%) at 2.5 µg/ml. In the case of *M.aurum* A^+ this level of inhibition of K^+ transport was not achieved even at 10 µg/ml (approximately 82% inhibition).

LPC also caused dose-related inhibition of the growth of *S.aureus*, although growth was somewhat less sensitive than K^+-uptake with inhibition being detected at 2.5 µg/ml LPC and being almost total at 5 µg/ml of lysophospholipid. This difference suggests that inhibition of bacterial growth may only occur at a minimum threshold value for inhibition of K^+ transport (*ie.* > 80% inhibition according to the data in Table 13 and 14). Alternatively, unidentified constituents in nutrient broth may antagonise or reverse the inhibitory effects of LPC on K^+ transport in *S.aureus*. In keeping with this, the correlation between LPC-mediated inhibition of K^+-uptake and growth in *M.aurum* A^+ was also not absolute, possibly for the same reasons. Pretreatment of the bacteria with α-tocopherol, which complexes with and neutralises lysophospholipids (Kagan, 1989), prevented LPC-mediated inhibition of uptake of K^+.

Differences in microbial lysophospholipase activity were identified as the possible biochemical mechanism of the differential sensitivity of *M.aurum* A^+ and *S.aureus* to the inhibitory actions of exogenous LPC on K^+ transport and growth. While degradation of LPC (presumably to saturated fatty acid and glycerophosphocholine) was relatively slow in *S.aureus*, inactivation of the lysophospholipid occurred quickly in *M.aurum* A^+ being maximal (85% degradation) within 15 min. This observation is in agreement with

previous reports that mycobacteria possess surface lysophospholipase activity which enables these microorganisms to degrade extracellular phospholipids which may enable mycobacteria to utilise host phospholipids (Wheeler and Ratledge, 1992). While these observations support the proposed involvement of lysophospholipids in the indirect, PLA_2- associated antimicrobial activity of the riminophenazines, the differential effects of B663 and the TMP-substituted phenazines on bacterial growth and K^+ transport relative to those of added, exogenous LPC require explanation. The most likely explanation for the greater sensitivity of the mycobacteria to the riminophenazines is that these agents probably cause sustained generation of endogenous lysophospholipids which may be less accessible to enzymatic inactivation, as opposed to a one-off addition of exogenous LPC which may be rapidly degraded.

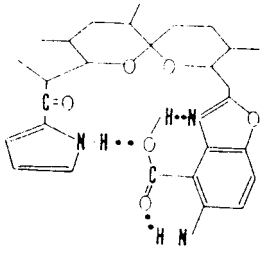
In conclusion, these data lend support to the contention that lysophospholipids are possible mediators of the anti-mycobacterial action of the riminophenazines. The unravelling of the *M.tuberculosis* genome may enable identification of the gene(s) encoding lysophospholipase activity in this microorganism. The acquisition of deletion mutants (if this is not a lethal mutation) would be of enormous assistance in validating these conclusions, since, theoretically, they should be more susceptible to the anti-mycobacterial action of exogenous lysophospholipids.

CHAPTER 5

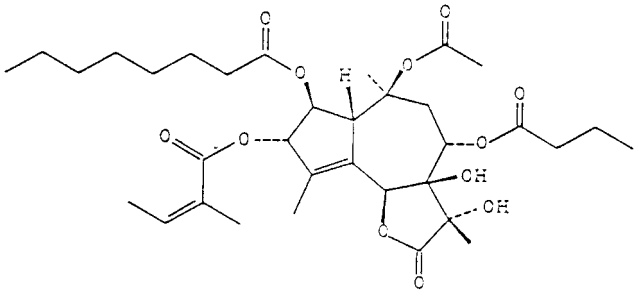
INVESTIGATION OF THE ANTIMYCOBACTERIAL POTENTIAL OF CONVENTIONAL CALCIUM MOBILIZING STIMULI (THE CALCIUM IONOPHORE A23187 AND THAPSIGARGIN)

5.1. INTRODUCTION

In this chapter I have extended my research into the anti-mycobacterial potential of activation of endogenous PLA_2 by using two different types of calcium-mobilizing stimuli *viz.* the calcium ionophore A23187, a classic activator of PLA_2 (Swendsen *et al*, 1983) and thapsigargin, a specific inhibitor of the endomembrane Ca^{2+}-ATPase (Thatsrup, 1990), which mobilize extracellular and intracellular Ca^{2+} respectively. Calcium ionophore A23187 is a carboxylic acid antibiotic which is crystallized from broths of *Streptomyces chartreusensis* as the magnesium plus calcium salt and can be converted to and crystallized as the free acid (Reed & Lardy, 1972). The function of the ionophore is to bind the divalent cation and transport it through the outer membrane thus facilitating the entry of ions (Youatt, 1993). Thapsigargin is a naturally occurring sesquiterpene lactone, which is isolated from the umbrelliferous plant *Thapsia garganica* (Rasmussen *et al*, 1978). Thapsigargin is known to be a very useful pharmacological tool for the study of intracellular Ca^{2+} storage and release. It causes leakage of Ca^{2+} from intracellular stores in eukaryotic cells by inhibiting the endomembrane Ca^{2+}-ATPase, resulting in increased concentrations of cytosolic Ca^{2+} (Thastrup, 1990). The molecular structures of the calcium ionophore A23187 and thapsigargin are shown in figure 29.



a) Calcium ionophore A23187



b) Thapsigargin

Figure 29: The structures of calcium ionophore A23187 and thapsigargin

5.2. MATERIALS AND METHODS

5.2.1. ANTIMICROBIAL AGENTS

The calcium ionophore A23187 and thapsigargin were dissolved in dimethyl sulphoxide (DMSO) to give stock solutions of 0.5 mM and 1mM respectively. Subsequent dilutions were made in DMSO and calcium ionophore was used at final concentrations of 0.1-1μM for bacterial growth studies, potassium transport and PLA_2 activity assays and at 0.5-10μM for calcium transport assays. Thapsigargin was used at final concentrations of 0.1-10 μM for growth studies assays and at 1-20 μM for cation (Rb^+ and Ca^{2+}) transport and PLA_2 activity assays.

5.2.2. CHEMICALS AND REAGENTS

Unless otherwise indicated, all chemicals used were obtained from Sigma Chemical Co., St Louis, MO, USA.

5.2.3. MYCOBACTERIAL STRAINS

The rapidly proliferating, non-pathogenic mycobacterium *M. aurum* A^+ was obtained from the Institut Pasteur, Paris, France. Two strains of *M. tuberculosis* (H37Rv and its mutant H37Ra) were obtained from the Medical Research Council, Tuberculosis Research Institute, Pretoria, South Africa.

5.2.4. BACTERIAL GROWTH STUDIES

Following the culture of the *M.aurum* A^+ for 4 days and the *M.tuberculosis* H37R strains for 14 days in Middlebrook 7H9 broth, the bacterial cells were resuspended in 0.15 M PBS, pH 7.4, to obtain a McFarland Number 1 opacity density (3×10^8 cfu/ml). These suspensions were diluted 10-fold and 5-fold in the case of *M.aurum* A^+ and the *M. tuberculosis* H37R strains, respectively. One hundred microlitres of the diluted suspensions were inoculated into the vials containing 12B TB medium with or without calcium ionophore A23187 (0.1 - 1 μM) or thapsigargin (0.1-10 μM).

The effects of a fixed concentration (25 μg/ml) of α-tocopherol on the growth of control

and A23187-treated bacteria were also investigated. α-Tocopherol was added to the bacteria 5 minutes before A23187 (0.5 μM) and the vials incubated for 72 hrs prior to being assayed using the BACTEC 460 TB system.

5.2.5. Rb^+-UPTAKE STUDIES

In all the experiments described here, ^{86}Rb was used as a tracer for measuring K^+-uptake. *M.aurum* A^+ and the *M.tuberculosis* H37R strains were harvested, washed and resuspended in glucose-free KONO buffer to the equivalent of 0.5×10^7 and 1×10^7 cfu/ml respectively. One millilitre of the cell suspension was then added to 9 ml of KONO buffer and the cultures were treated with calcium ionophore A23187 (0.1-1μM) or thapsigargin (1-20 μM) at 37°C for 30 min. Appropriate solvent controls were included. The mycobacteria were then concentrated by centrifugation and resuspended in 2 ml KONO buffer supplemented with 22 mM glucose and ^{86}Rb (2 μCi/ml). Specific activity of ^{86}Rb is 3.56 mCi/mg. The effects of the test agents on K^+-transport were then assessed using a fixed incubation time of 45 min for *M.aurum* A^+ and 90 min for *M.tuberculosis* at 37°C. After incubation, the mycobacteria were washed twice with ice cold PBS and the pellets finally disrupted by adding 0.4 ml of pre-heated 5% TCA. Radioactivity was assayed for in a liquid scintillation spectrometer and uptake of K^+ was taken as the difference in uptake of ^{86}Rb in the bacteria incubated at 37°C and the controls kept on ice.

The effects of pre-treatment with a fixed concentration (25 μg/ml) of α-tocopherol on the uptake of ^{86}Rb by control and A23187- or thapsigargin-treated mycobacteria were also investigated. Alpha-tocopherol was added to the bacteria 1 minute before the calcium ionophore A23187 (0.5 μM) or thapsigargin (5 μM).

5.2.6. Ca^{2+}-UPTAKE STUDIES

The *M.aurum* A^+ and *M. tuberculosis* H37R strains were cultured for 4 days and 14 days respectively, before being harvested, washed and resuspended in Ca^{2+}-free Hanks balanced salt solution (HBSS) to a concentration of 1×10^7 cfu/ml for *M. aurum* A^+ and 2×10^7 cfu/ml for the *M. tuberculosis* H37R strains. The bacteria were exposed

to 4 µCi/ml ^{45}Ca (calcium-45-chloride, specific activity, 28.84 mCi/mg) containing 20 µM cold, carrier calcium chloride ($CaCl_2$) for 15 minutes at 37°C. Following addition of the calcium ionophore A23187 (0.5 - 10 µM) or thapsigargin (1 - 20 µM), the mycobactera were incubated for a further 15 minutes at 37°C, after which the reactions were terminated by the addition of ice-cold PBS. The cells were then washed with PBS and assayed for incorporated ^{45}Ca following disruption of the pellets by the addition of 0.4 ml of pre-heated 5% TCA. The incorporated ^{45}Ca was assayed for in a liquid scintillation spectrometer. To eliminate the complicating effects of non-specific binding of the radiolabelled cation to the bacteria, net uptake of Ca^{2+} was taken as the difference in uptake of ^{45}Ca in the tubes at 37°C and the controls kept on ice. In kinetics studies, the time-course of Ca^{2+}-uptake by control or A23187 (5 µM)-treated bacteria was measured at 10 seconds, 30 seconds, 1, 5, and 15 minutes after addition of A23187.

The effects of α-tocopherol (25 µg/ml) on the uptake of ^{45}Ca by control and A23187- or thapsigargin-treated *M.aurum* A^+ were also investigated. In these experiments, α-tocopherol was added 5 minutes before or 15 minutes after the calcium ionophore A23187 (5 µM) or 5 minutes before thapsigargin (10 µM).

5.2.7. FURA-2 SPECTROFLUORIMETRY

This procedure, which is widely used to monitor fluctuations in cytosolic Ca^{2+} concentrations in eukaryotic cells (Grynkiewicz *et al*, 1985), and which has limited application in prokaryotic cells (Norris, 1996) was used to measure Ca^{2+} -fluxes in *M. aurum* A^+ exposed to the non-fluorescent 4-bromo derivative of A23187 and to thapsigargin (both at 20 µM and 40µM). Using procedures which were established in preliminary experiments, the bacteria (1 x 10^7 cfu/ml) were loaded with FURA-2/AM (Calbiochem Corp. La Jolla, CA, USA) at a final concentration of 4 µM in Ca^{2+}-free HBSS for 30 minutes at 37°C. The FURA-2-loaded bacteria were then transferred to cuvettes which were placed in the thermoregulated cuvette-holder of a Hitachi-10S spectrofluorimeter with excitation and emission wavelengths set at 340 nm and 500nm respectively. After a stable baseline was obtained, A23187 or thapsigargin was added

to the bacteria and the subsequent alterations in FURA-2 fluorescence monitored over a 5 - 10 minute period. To identify the origins of the intracellular Ca^{2+}, these experiments were repeated using FURA-2 loaded bacteria to which 5mM of the extracellular Ca^{2+}-chelating agent, EGTA was added immediately before treatment with A23187 or thapsigargin. In some experiments the bacteria were pretreated with α-tocopherol (25 µg/ml), while in others this agent was added 3 minutes after thapsigargin.

5.2.8. PHOSPHOLIPASE A_2 ACTIVITY

PLA_2 (EC 3.1.1.4; phosphatidylcholine 2-acyl-hydrolase) activity in the mycobacteria was measured according to release of [^{14}C] arachidonate from the C-2 position of added phosphatidylcholine. Release of [^{14}C]-arachidonate was measured by a high-performance thin-layer chromatography (HPTLC) method (Bradova *et al*, 1990; Van Rensburg *et al*, 1992). Mycobacteria were suspended in HBSS containing 1.25 mM $CaCl_2$ to concentrations of 1 x 10^7 and 2 x 10^7 cfu/ml for the *M. aurum* A$^+$ and the *M. tuberculosis* H37R strains respectively. Phosphatidylcholine (L-α-1-palmitoyl-2-arachidonyl, [arachidonyl-1-^{14}C], specific activity (180-240 Ci/mmol), 0.5 µCi/ml) was added to the bacterial suspensions which were then incubated for 30 minutes at 37°C after which 0.25 ml aliquots were added to tubes containing 0.75 ml HBSS, followed by addition of the calcium ionophore A23187 (0.1-1 µM) or thapsigargin (1 - 20 µM). After incubation for a further 15 minutes, the reactions were terminated by the addition of 2 ml chloroform:methanol (2:1 [vol/vol]) and the fatty acid-containing lower phase was removed and evaporated to dryness under nitrogen. The samples were stored at -20°C until assayed.

The evaporates were reconstituted with 40 µl of chloroform:methanol (2:1 [vol/vol]) containing 0.01 mM arachidonate standard and spotted onto silica gel 60-precoated HPTLC plates. The plates were developed in chloroform:acetone (96:4 [vol/vol]). After exposure to iodine vapours, the arachidonate spots were localised and the silica was removed and assayed for radioactivity in a liquid scintillation spectrometer.

5.2.9. EXPRESSION AND STATISTICAL ANALYSIS OF RESULTS

The results of each series of experiment are expressed as the mean values ± the standard error of the mean (SEM). Levels of statistical significance were calculated by the paired Student's *t*-test.

5.3. RESULTS

Effects of the calcium ionophore A23187 and thapsigargin on the growth of *M. aurum* A^+ and the *M. tuberculosis* H37R strains

The susceptibility of *M. aurum* A^+ and the *M. tuberculosis* H37R strains to the calcium ionophore A23187 is shown in Figures 30, 31 and 32 (pages 129, 130 and 131) respectively. All the strains tested were susceptible to A23187, with *M. aurum* A^+ being the most susceptible. The growth of all the strains was inhibited in a dose-related manner by A23187 which achieved statistical significance at concentrations of 0.1 - 1 µM. The p-values for A23187-treated *M. aurum* A^+ were $p \leq 0.02$ - $p \leq 0.0001$ while those for the A23187-treated *M. tuberculosis* H37Ra strain were $P \leq 0.03$ - $P \leq 0.0001$.

The growth of *M.aurum* A^+ was inhibited, albeit incompletely, in a dose-related manner by thapsigargin (Figure 34; page 133).

α-Tocopherol (25 µg/ml) was found to neutralise the inhibitory effects of A23187 (0.5 µM) on the growth of *M.aurum* A^+. The mean percentage of growth relative to the control, were 2.0 ± 0.07 and 101.8 ± 8.3 for *M.aurum* A^+exposed to A23187 alone or to A23187 following pretreatment with α-tocopherol respectively.

Effects of the calcium ionophore A23187 and thapsigargin on K^+ -uptake by mycobacteria

The results are shown in Figures 30, 31, 32 and 34 (pages 129, 130, 131 and 133). Exposure of *M.aurum* A^+ and the avirulent strain of *M.tuberculosis* to A23187 (0.1 - 1 µM) resulted in a significant dose-related inhibition of the uptake of ^{86}Rb ($P \leq 0.0001$ and $p \leq 0.004$ - $p \leq 0.0007$ respectively). Uptake of ^{86}Rb by *M.tuberculosis* H37Rv was

also inhibited by A23187 at concentrations of 0.1 to 1 µM. Exposure of *M.aurum* A^+ to thapsigargin (1 - 20 µM) resulted in a significant dose-related inhibition of the uptake of ^{86}Rb ($p \leq 0.04$- $p \leq 0.03$).

Alpha-tocopherol (25 µg/ml) was found to protect the mycobacteria against the inhibitory effects of both A23187 (0.5 µM) and thapsigargin (5 µM) on the uptake of ^{86}Rb. The mean uptakes (percentage of control) of ^{86}Rb by the avirulent strain of *M.tuberculosis* H37R exposed to A23187 in the absence and presence of α-tocopherol were 8.6 ± 0.8 and 78.1 ± 0.5 respectively. The mean percentage of ^{86}Rb-uptake in the mycobacteria exposed to thapsigargin in the absence of α-tocopherol was 88.0 ± 4.0. When *M.aurum* A^+ was pretreated with α-tocopherol, the mean percentage uptake was 135.3 ± 6.2 for thapsigargin.

Effects of the calcium ionophore A23187 and thapsigargin on Ca^{2+} -uptake by *M. aurum* A^+ and *M. tuberculosis* H37Ra

The results of experiments designed to investigate the effects of A23187 and thapsigargin on Ca^{2+}-uptake by the mycobacteria are shown in Figures 30, 31, 32 and 34 (pages 129, 130, 131 and 133). Treatment of the mycobacteria with A23187 at concentrations of 0.5 -10 µM caused a dose-dependent increase in the uptake of ^{45}Ca. The uptake of ^{45}Ca by A23187-treated *M.aurum* A^+ was higher than that of the A23187-treated *M. tuberculosis* H37R strains. Exposure of *M.aurum* A^+ to thapsigargin (1-20 µM) also resulted in an increase in the uptake of ^{45}Ca. The effects of A23187 (5µM) on the kinetics of uptake of ^{45}Ca by *M.aurum* A^+ are shown in Figure 33 (page 132). Exposure to A23187 caused an immediate and sustained increase in uptake of ^{45}Ca.

Exposure of *M.aurum* A^+ to α-tocopherol, prior to treatment with A23187 (5 µM) or thapsigargin (10µM) respectively, inhibited influx of ^{45}Ca into the mycobacteria. The mean percentage of ^{45}Ca-uptake in the bacteria exposed to A23187 in the absence of α-tocopherol was 437 ± 49 relative to the untreated control. When *M.aurum* A^+ was pretreated with α-tocopherol , the mean percentages uptake of ^{45}Ca was 112 ± 4 for A23187. Delayed addition of α-tocopherol, 15 minutes after exposure to A23187 and

I minute prior to termination of the experiment, resulted in efflux of ^{45}Ca from the mycobacteria. The mean percentages of uptake of ^{45}Ca by *M.aurum* A^+ treated with A23187 alone or in combination with α-tocopherol were 437 ± 49 and 192 respectively. The mean pecentage of ^{45}Ca-uptake in the bacteria exposed to thapsigargin in the absence of α-tocopherol was 252 ± 32 relative to the untreated control. When *M.aurum* A^+ was pretreated with α-tocopherol, the mean percentage uptake of ^{45}Ca was 122 ± 16 for thapsigargin.

Effects of the calcium ionophore A23187 and thapsigargin on FURA-2 fluoresence of *M. aurum* A^+

The effects of A23187 and thapsigargin on the FURA-2 fluorescence responses of *M.aurum* A^+ in the presence and absence of α-tocopherol are shown in Figures 35 and 36 (pages 134 and 135) respectively. Exposure of the FURA-2-loaded bacteria to A23187 resulted in an abrupt increase in fluoresence intensity, which then subsided to about $^2/_3$ of the peak value followed by a more gradual decline (20μM) or a plateau (40 μM) indicative of a secondary influx of the cation (Figure 35). Pretreatment of the bacteria with α-tocopherol did not affect the abruptly occurring increase in fluoresence intensity, but completely inhibited the apparent secondary influx (Figure 35).

Exposure of *M. aurum* A^+ to thapsigargin resulted in an abrupt increase in fluoresence intensity which reached a sustained plateau after 3 minutes (Figure 36). Pretreatment of the bacteria with α-tocopherol caused a substantial reduction in fluoresence intensity, while addition of this agent 5 minutes after the thapsigargin resulted in an immediate decline in fluoresence and a return to base-line values (Figure 36).

When these experiments were repeated using bacteria suspended in HBSS containing 5mM EGTA, no increase in fluorescence intensity was observed following the addition of A23187, while the thapsigargin-activated responses were unchanged (Figure 37; page 136).

Effects of the calcium ionophore A23187 and thapsigargin on the PLA_2 activity of *M. aurum* A^+ and the *M. tuberculosis* H37R strains

The effects of A23187 and thapsigargin on PLA_2 activity are shown in Figures 30, 31,32 and 34 (pages 129, 130, 131 and 133). Exposure of *M.aurum* A^+ , and the avirulent and virulent *M.tuberculosis* H37R strains to A23187 (0.1-1 µM) resulted in a dose-related increase in PLA_2 activity according to increased release of [^{14}C]-arachidonate from A23187-treated bacteria. Enhanced release of radiolabelled-arachidonate was also observed on exposure of the *M.aurum* A^+ to thapsigargin (1-20 µM).

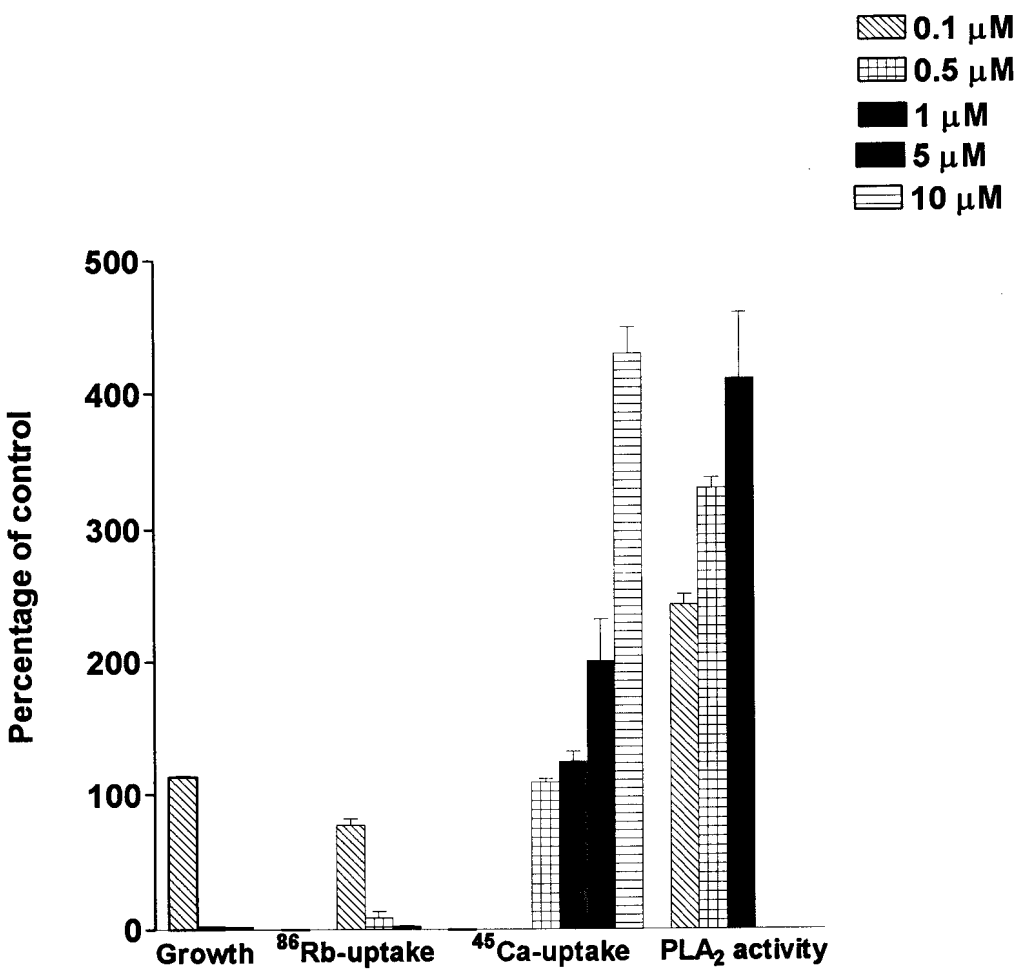


Figure 30: Effects of the calcium ionophore A23187 on the growth of, cation uptake (^{86}Rb and ^{45}Ca) by and PLA_2 activity of *M.aurum* A^+. The results of three experiments each with triplicate determinations for growth assays and ^{86}Rb -uptake assays and two experiments with duplicate and triplicate determinations for PLA_2 activity and ^{45}Ca -uptake respectively, are expressed as the mean percentages of the untreated control systems ± SEMs. The absolute values for uptake of ^{86}Rb and ^{45}Ca, growth and PLA_2 activity by the drug-free control systems were 279419 ± 4871, 4995 ± 326 , 514 ± 21 and 370 ± 21 cpm respectively.

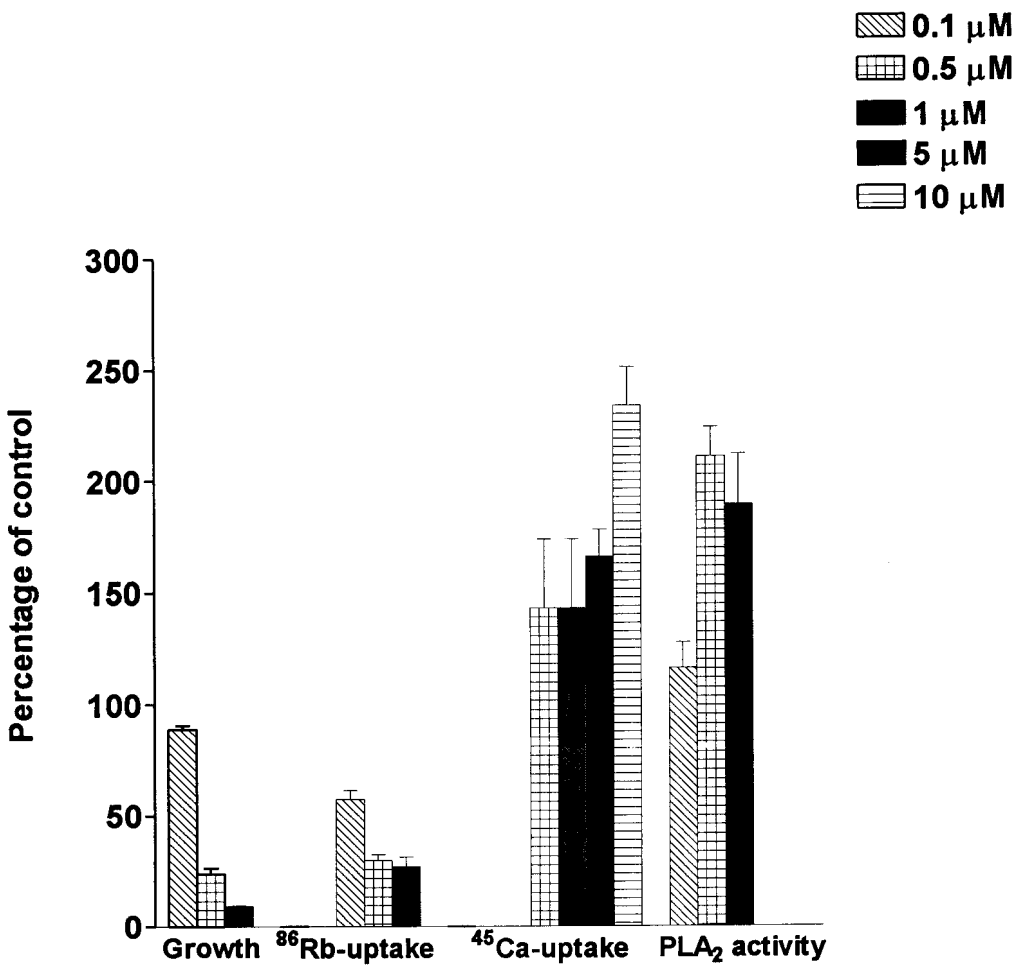


Figure 31: Effects of the calcium ionophore A23187 on the growth of, cation uptake (^{86}Rb and ^{45}Ca) by and PLA_2 activity of the avirulent strain of *M.tuberculosis* H37R. The results of three experiments with triplicate determinations for growth and cation uptake assays and of a single experiment with triplicate determinations for PLA_2 activity assays, are expressed as the mean percentages of the untreated control systems ± SEMs. The absolute values for uptake of ^{86}Rb and ^{45}Ca, growth and PLA_2 activity by the drug-free control systems were 26639 ± 1861, 4172 ± 62, 259 ± 9 and 142 ± 14 cpm respectively.

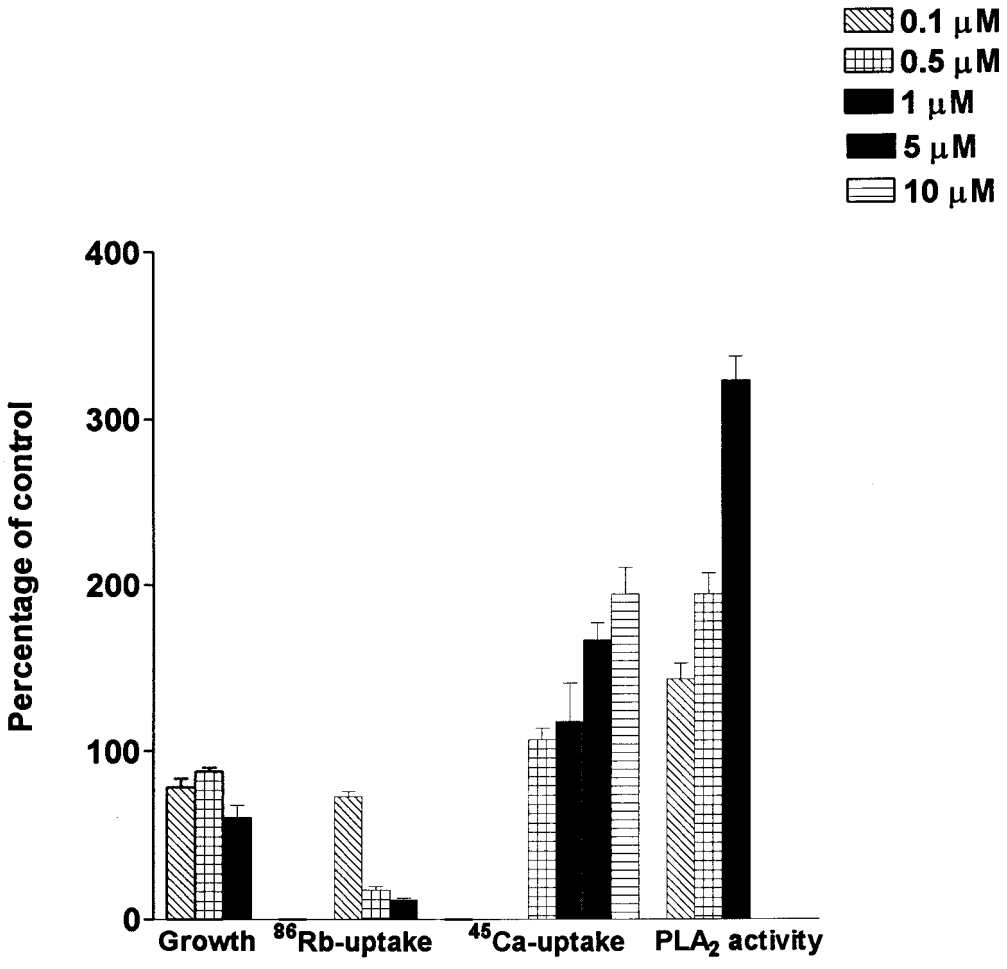


Figure 32: Effects of the calcium ionophore A23187 on the growth of , cation uptake (^{86}Rb and ^{45}Ca) by, and PLA_2 activity of the virulent strain of *M.tuberculosis* H37R. The results of 1 or 2 experiments with triplicate and quintuplicate determinations, are expressed as the mean percentages of the untreated control systems ± SEMs. The absolute values for uptake of ^{86}Rb and ^{45}Ca, growth and PLA_2 activity by the drug-free control systems were 112482 ± 7246, 4232 ± 53, 259 ± 13 and 156 ± 24 cpm respectively.

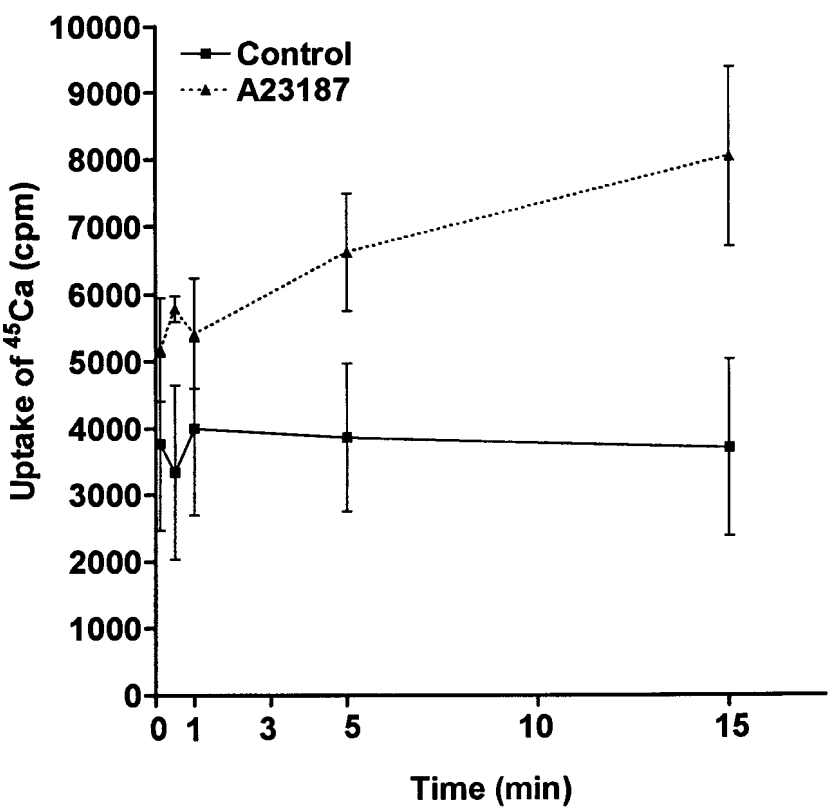


Figure 33: The kinetics of Ca^{2+}-uptake by *M.aurum* A^{+} in the presence or absence of the calcium ionophore A23187 at a fixed concentration of 5 µM. The results of a single experiment with duplicate determinations for each time point are expressed as the mean actual counts (cpm) ± SEMs.

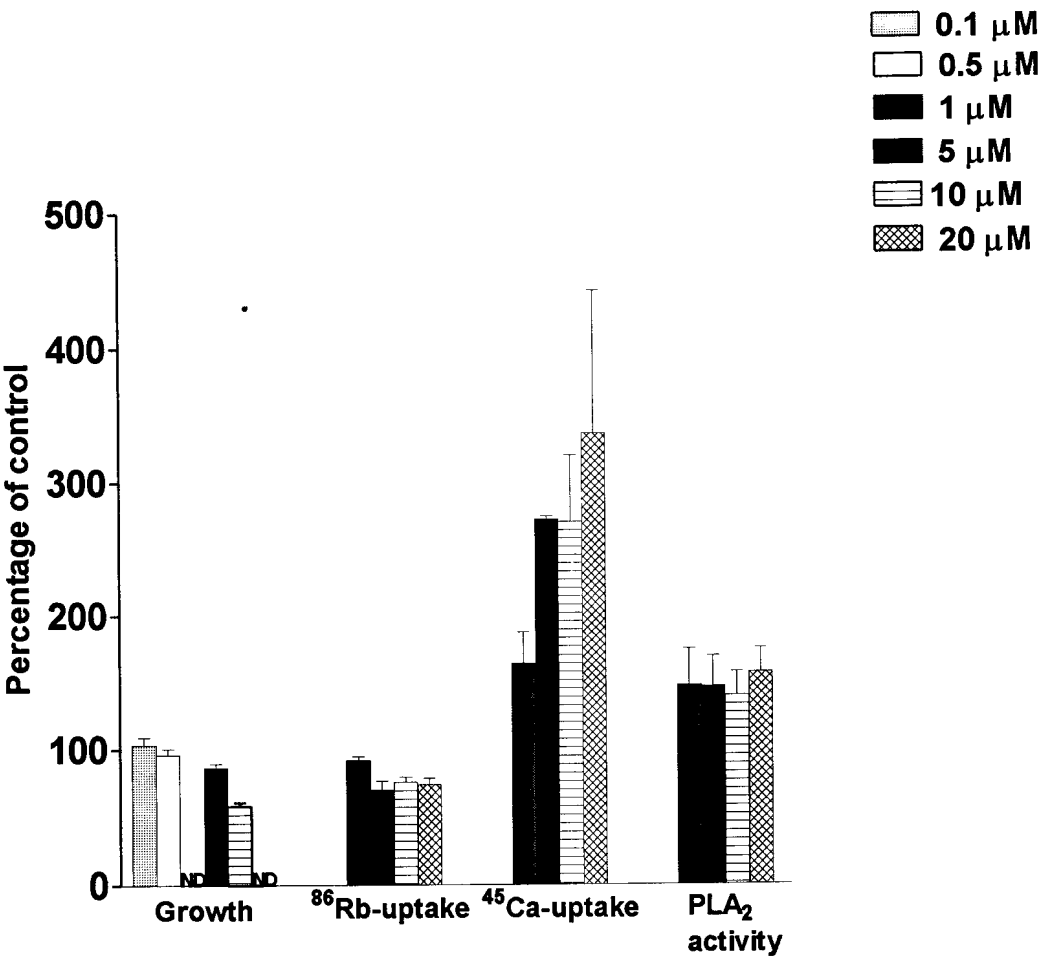


Figure 34: Effects of thapsigargin on the growth of, cation uptake (^{86}Rb and ^{45}Ca) by and PLA_2 activity of *M.aurum* A^+. The results of three experiments with triplicate determinations for cation uptake assays and of a single experiment with triplicate determinations for growth studies and PLA_2 activity assays, are expressed as the mean percentages of the untreated control systems ± SEMs. The absolute values for uptake of ^{86}Rb and ^{45}Ca, growth and PLA_2 activity by the drug-free control sytems were 61193 ± 6099, 4572 ± 183, 490 ± 12 and 943 ± 63 cpm respectively.

ND = not done

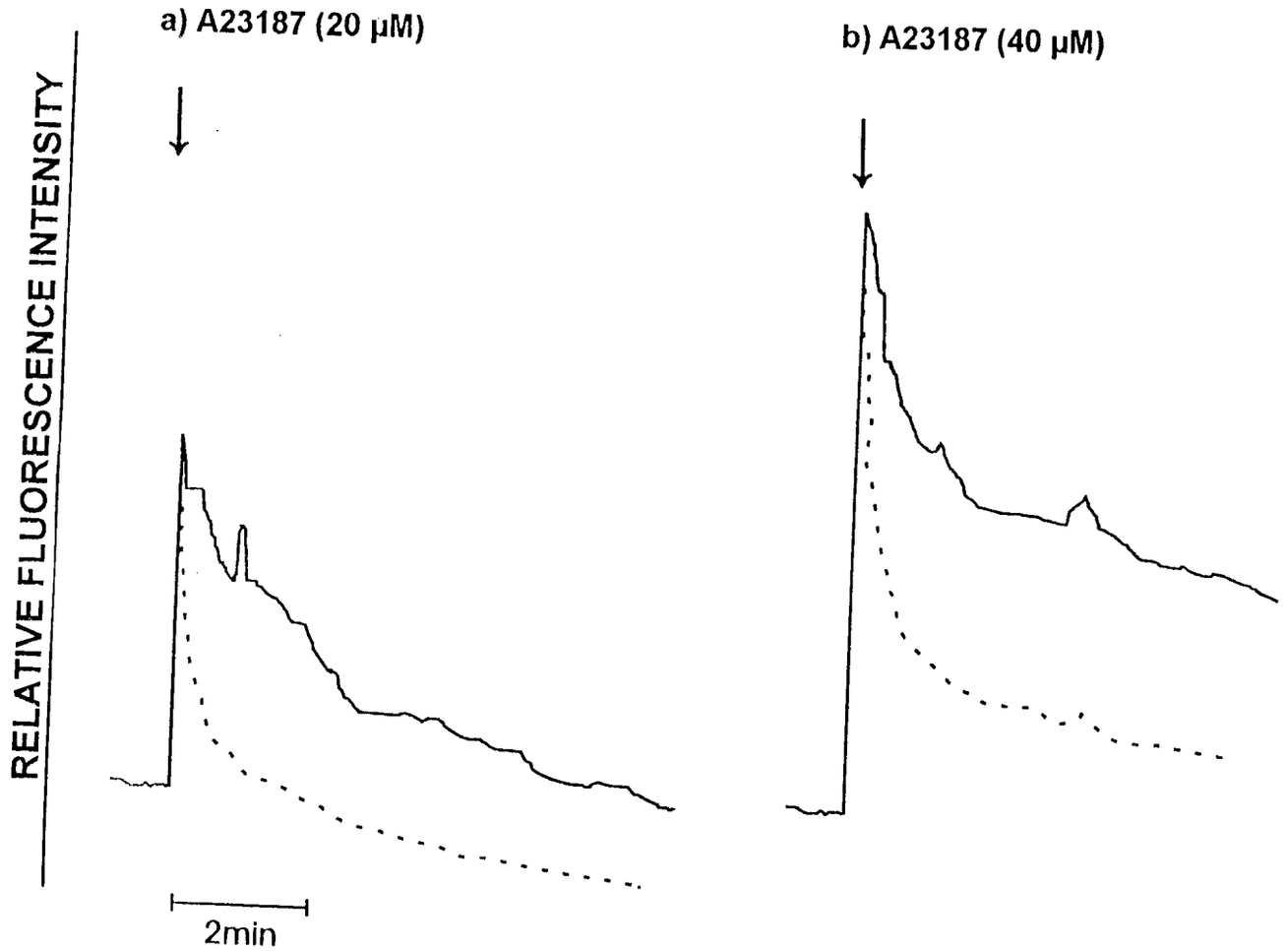


Figure 35: Calcium ionophore A23187-activated FURA-2 fluorescence responses of *M.aurum* A^+ in the presence (- - - -) and absence (___) of alpha-tocopherol.

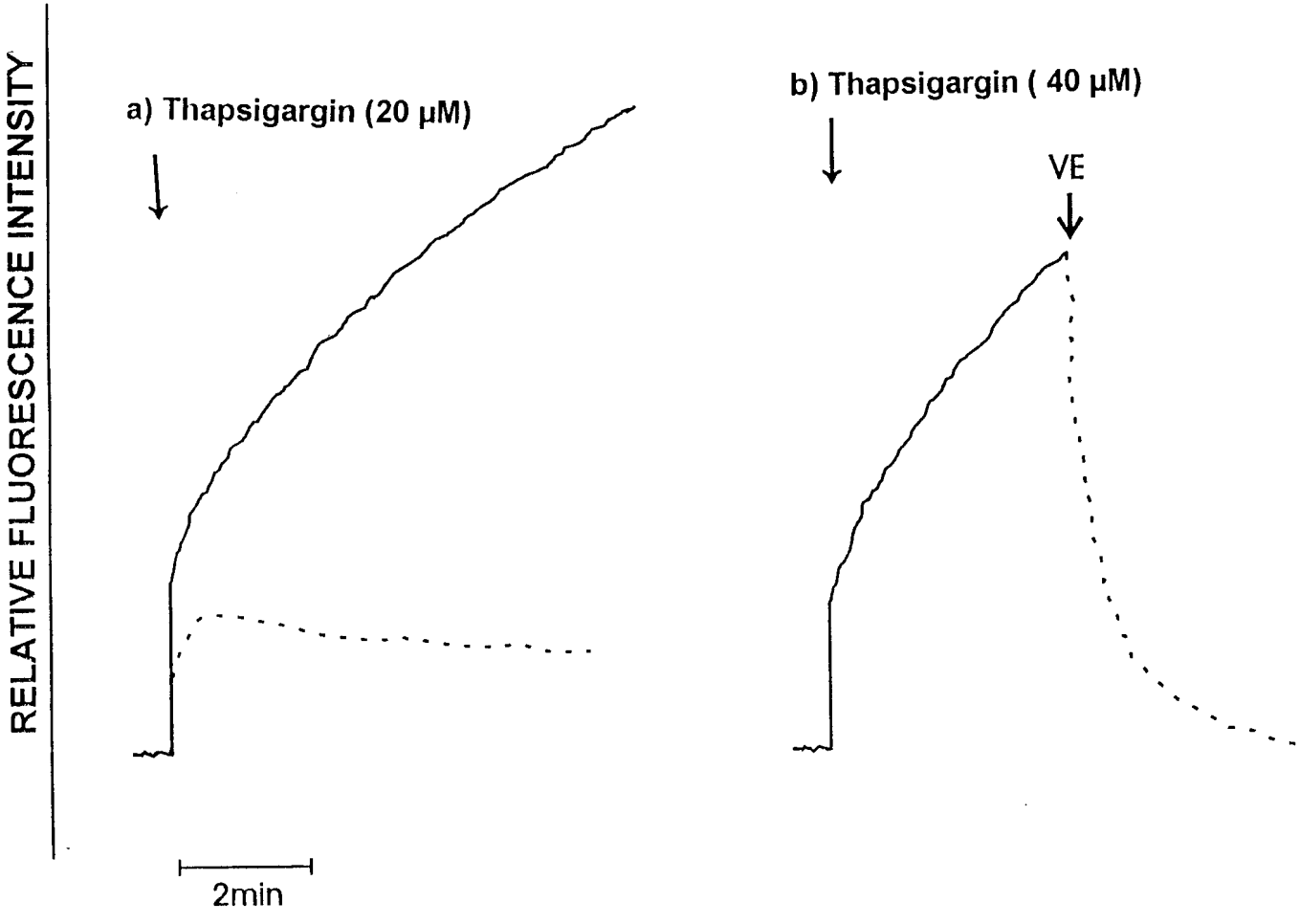


Figure 36: Thapsigargin-activated FURA-2 fluorescence responses of *M.aurum* A^+ in the presence (- - - -) and absence (___) of alpha-tocopherol.

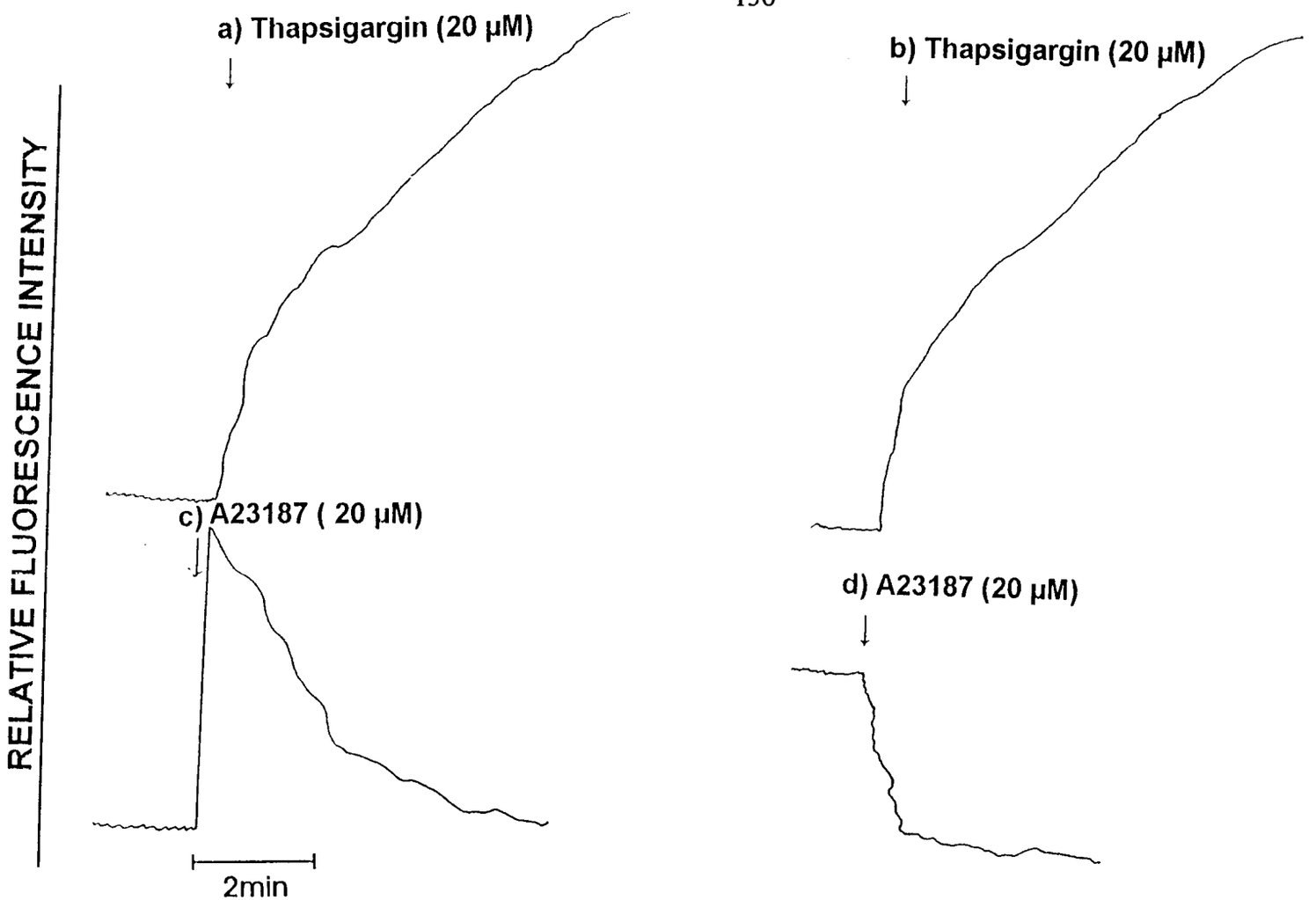


Figure 37: Thapsigargin (a and b)- and calcium ionophore (c and d) - activated FURA-2 fluorescence responses of *M.aurum* A^+. The FURA-2 loaded bacteria were suspended in HBSS (a and c) and HBSS containing EGTA (b and d).

5.4. DISCUSSION

The results presented in the previous chapter demonstrated the apparent involvement of endogenous microbial PLA_2 in the antimycobacterial action of clofazimine and the TMP-substituted phenazines. These effects were achieved by a Ca^{2+}-independent mechanism, probably resulting from interactions of the riminophenazines with outer membrane phospholipids which enhanced the susceptibility of these (phospholipids) to hydrolysis by PLA_2. The results presented in the current chapter are those of experiments which were designed to confirm and extend my initial studies on endogenous PLA_2 and K^+-transporters as potential targets for anti-mycobacterial chemotherapy. For these experiments I used the Ca^{2+} ionophore A23187, a classical activator of Ca^{2+}-dependent PLA_2 (Swendsen *et al*, 1983) to further investigate enhancement of endogenous PLA_2 activity as a mechanism of disruption of cation transport and antimicrobial activity in mycobacteria. This lipophilic agent induces non-physiologic influx of Ca^{2+} by transporting extracellular cation into cells. In this component of the study I also used thapsigargin, a highly selective inhibitor of the endo-membrane Ca^{2+}-ATPase in eukaryotic cells (Thastrup *et al*, 1990).

As was the case with the riminophenazines, the growth of *M.aurum* A^+, as well as that of the *M.tuberculosis* H37R strains, was inhibited in a dose-related manner by the calcium ionophore A23187. The growth of the virulent strain of *M.tuberculosis* H37R was, however, somewhat less sensitive to A23187 than that of the other mycobacterial strains. The relative unresponsiveness of this strain to A23187 may reflect differences in cellular metabolic activity and/or membrane structure which may affect the uptake and/or activity of A23187. In most respects (enhanced PLA_2, inhibition of uptake of K^+ and growth) the effects of A23187 and those of the riminophenazines on mycobacteria were similar. The only notable differences were related to the effects of these agents on Ca^{2+} influx. Whereas influx of Ca^{2+} levels were delayed events in mycobacteria treated with clofazimine, B4121 and B4128, they were abrupt (within 5 seconds) and prolonged following exposure to A23187. Influx of Ca^{2+} into A23187-treated mycobacteria was observed with both the radiometric and the FURA-2 procedures. Higher concentrations of A23187 were required with FURA-2 method, which may

indicate lower sensitivity relative to the radiometric procedure. As was the case with the riminophenazine-treated bacteria, the inhibitory effects of K^+ were neutralized by α-tocopherol, while uptake of Ca^{2+} was prevented and reversed by this agent. Taken together, these observations demonstrate that A23187 inhibits mycobacterial growth by causing Ca^{2+}-dependent activation of PLA_2 with resultant dysregulation of membrane-associated cation transporters.

The effects of thapsigargin on mycobacterial growth, K^+ transport, Ca^{2+} fluxes and PLA_2 activity were similar but much less striking than those of A23187. As was the case with A23187, α-tocopherol also inhibited the accumulation of Ca^{2+} in thapsigargin -treated mycobacteria. Although the efffects of thapsigargin on Ca^{2+} fluxes are suggestive of the presence of internal Ca^{2+} stores modulated by an endo-membrane Ca^{2+}-ATPase in mycobacteria, there are clearly some interpretational problems with this conclusion. Firstly, the concentrations (10-20 μM) of thapsigargin at which these effects were observed are considerably higher than those which are required to inhibit the endo-membrane Ca^{2+}-ATPase of eukaryotic cells (Lytton *et al*, 1991). This may reflect differences in uptake of the inhibitor, or decreased sensitivity of the target. Secondly, the failure of EGTA to inhibit the FURA-2 fluorescence responses of thapsigargin-treated mycobacteria is surprising. If thapsigargin was affecting a putative intracellular, Ca^{2+}-ATPase-modulated Ca^{2+} storage organelle, then fluorescence could reasonably have been expected to increase initially and then subside as the Ca^{2+} was extruded from the bacteria. The fact that this did not happen could indicate that thapsigargin, at the concentration used, may inhibit the outer membrane Ca^{2+}-ATPase in addition to, or as opposed to, inhibition of putative endo-membrane Ca^{2+}-ATPase. This proposed mechanism would not, however, be compatible with the observation that α-tocopherol reversed thapsigargin-induced Ca^{2+} overload in the mycobacteria. An alternative possibility, which is more compatible with the available data, is that thapsigargin does indeed promote the release of Ca^{2+} from poorly-defined storage organelles in mycobacteria and that extrusion of the cation is prevented by PLA_2/lysophospholipid-induced inhibition of the outer membrane Ca^{2+}-ATPase which is prevented and reversed by α-tocopherol. These very preliminary, but nevertheless provocative

findings with thapsigargin suggest the existence of Ca^{2+}-storage systems in mycobacteria and merit further, future, intensive investigation. Irrespective of the intracellular site of action of molecular target of thapsigargin, the results of this component of the study support the contention that endogenous mycobacterial PLA_2 regulates bacterial growth.

In conclusion the results presented in this chapter taken together with those in the previous chapter, clearly demonstrate that dysregulation of mycobacterial PLA_2 whether it be due to increasing the vulnerability of membrane phospholipids to hydrolysis by this enzyme, as appears to be the case with the TMP-substituted phenazines, or by promoting its activation by increasing intracellular Ca^{2+} concentrations, results in inhibition of bacterial growth, presumably by interfering with outer membrane cation transporters.

CHAPTER 6

CONCLUDING COMMENTS

6.1. CONCLUDING COMMENTS

The studies presented in this thesis have identified two novel TMP-substituted phenazines, B4121 and B4128, which possess extracellular and intracellular antimycobacterial activities which are superior to those of the prototype riminophenazine, clofazimine. The most important finding with regard to the *in vitro* activity of these riminophenazines is that none of the clinical *M.tuberculosis* isolates, including the multidrug resistant strains, showed resistance to these agents. It is interesting to note that, both B4121 and B4128 were found to possess intraphagocytic activity which was superior to that of both clofazimine and rifampicin, the latter being a potent first-line anti-tuberculosis drug with high activity in human macrophages (Crowle *et al*, 1988). The intracellular anti-mycobacterial actvity of other newly developed riminophenazines, without TMP substitution, is less impressive (Jagannath *et al*, 1995; Reddy *et al*, 1996).

The antimycobacterial activity of clofazimine and its two TMP-substituted derivatives B4121 and B4128 against mycobacteria appears to be mediated by a Ca^{2+}-independent increase in the activity of mycobacterial PLA_2, leading to interference with microbial K^+ -transport. The enhancement of endogenous PLA_2 activity as a mechanism of disruption of cation transport and antimicrobial activity in mycobacteria was further investigated using calcium ionophore A23187 and thapsigargin as an alternative strategy to augment PLA_2. Calcium ionophore A23187 caused Ca^{2+}-dependent activation of PLA_2 which also resulted in dysregulation of membrane-associated cation transporters. Even though its antimycobacterial activity was less impressive, the data obtained with thapsigargin also support the contention that endogenous mycobacterial PLA_2 regulates bacterial growth. The findings with thapsigargin also suggest the existence of poorly defined Ca^{2+}-storage systems in mycobacteria. Taken together, the antimycobacterial activity of the riminophenazines and Ca^{2+}-elevating stimuli (*i.e.* calcium ionophore A23187 and thapsigargin) both of which appear to converge on PLA_2 clearly demonstrate that dysregulation of mycobacterial PLA_2 results in inhibition of bacterial growth, presumably by interfering with outer membrane cation transporters.

There are clearly several aspects originating from my laboratory research which I will focus on in the future. These are:

i) to test the anti-mycobacterial activity of B4121 and B4128 in murine models of experimental chemotherapy. Although these agents are not direct or selective inhibitors of mycobacterial K^+ transport (and are probably more useful as probes of mycobacterial PLA_2 activity and K^+ transport, as well as relationships between these) they have two properties, which in my opinion jusitify further investigation in these systems. Firstly, they are extremely active at sub-microgram concentrations against intracellular *M.tuberculosis*, secondly they are active against multidrug resistant strains of this microbial pathogen. These studies would be dependent on the acquisition of data from preliminary toxicity/dosing/tissue distribution studies which would be undertaken to identify the most appropriate vehicles, routes of administration, maximum tolerable doses, as well as the concentrations of these agents which are attainable in serum and organs, particularly the lungs. If the results of these experiments are satisfactory, the chemotherapy experiments could be designed using intra-nasal and/or aerosol models of *M.tuberculosis* infection.

ii) a particularly exciting extension of my current research would be to identify the relative importance of the two putative K^+ transporters, Rv2691 and Rv2692 in *M.tuberculosis*. This would involve the generation of targeted K^+ transport mutants (deletion/inactivation) in which each transporter (or both) is selectively inactivated. These K^+ transport mutants would also be useful in identifying if one or both (or neither) is susceptible to endogenous PLA_2. Characterization of the gene products may also facilitate the development of selective and direct inhibitors of mycobacterial K^+ transport. Moreover, whole-genome DNA microarray technology, which is currently being used to identify genetic differences between closely related mycobacteria, may be particularly useful in characterising those deletions which affect K^+ transport (Behr *et al*, 1999).

iii) the recent identification of four genes encoding phospholipase C in *M.tuberculosis* is also a particularly exciting development (http:www.pasteur/fr/Bio/TubercuList/). If a candidate gene encoding PLA_2 is identified in *M.tuberculosis* then the generation of a PLA_2 inactivation/deletion mutants would (if not a lethal mutation) be useful to further characterize the involvement of this enzyme in riminophenazine-mediated inactivation of K^+ transport since such a mutant, if the proposed mechanism of action is correct, should be insensitive to the riminophenazines.

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GenomicTbinformation is centralised in TubercuLIst, **http://www.pasteur.fr/Bio/TubercuList/**

APPENDIX 1:

KONO BUFFER:

KONO buffer (pH) (modified from Epstein & Kim, 1971) is the minimal media employed in the K^+-uptake studies. No potassium or nitrogen is added. However, the final buffer contains approximately 20µM of K^+ due to contamination of the sodium salts.

KONO buffer consists of: Na_2HPO_4, 46mM
NaH_2PO_4, 23mM
$MgSO_4$, 0.4mM
$FeSO_4$, 0.6mM
dH_2O

APPENDIX 2:

McFarland Turbidity Standard:

The McFarland standard is a standard with which the turbidity of the bacterial suspension is compared to that of the McFarland standard to ensure a constant inoculum size.

0.1ml of 1% Barium chloride is added to 9.9ml of sulphuric acid to obtain a McFarland No. 1 turbidity standard.