IN VITRO TOXICITY AND BIOACTIVITY OF SOME PYRIMETHAMINE DERIVATIVES AS ANTI-TUBERCULOSIS AGENTS

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THESIS SUMMARY

Tuberculosis has become such a public health threat that the WHO has declared TB a global public health emergency. However, no single anti-mycobacterial agent has been introduced into widespread use since rifampicin in the 1960s.

This project is aimed at synthesising and testing of novel potential DHFR inhibitors. The testing involved the toxicity evaluation of some potential DHFR inhibitors synthesised by a previous worker and study of efficacy and toxicity of newly synthesised compounds.

The *in vitro* toxicity of two potential inhibitors was determined using human mononuclear leucocytes (MNLs) as target cells. Due to the high toxicity encountered, their structures were modified to reduce the toxicity and a series of related new compounds were synthesised.

The minimum inhibitory concentration against *Mycobacterium fortuitum* and *in vitro* toxicity for the newly synthesised compounds were also studied. Although all the compounds showed low potency, the toxicity and bioactivity data can be used as a basis in further research to yield useful information about structure-toxicity and structure-efficacy relationship.

***Keywords*:** Dihydrofolate reductase; Toxicity; Tuberculosis; Pyrimethamine derivatives; Bioactivity.

DEDICATION

I would like to dedicate this thesis to my parents, Mr. Zhongren Wei and Mrs. Lihui Li, and my wife, Mrs. Ying Li.

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ABBREVIATIONS USED

AIDS	Acquired Immure Deficiency Syndrome
ANOVA	Analysis of Variance
BCG	Bacillus Calmette-Guèrin
BSA	Bovine Serum Albunim
CDC	Centers for Disease Control and Prevention
CSM	The Committee of Safety of Medicine
DHFR	Dihydrofolate Reductase
DMF	Dimethyl Formamide
DMSO	Dimethylsulphoxide
DOTS	Directly Observed Therapy, Short-course
GSH	Glutathione
HIV	Human Immunodeficiency Virus
LAM	Lipoarabinomannan
LM	Lipomannan
MABA	Microplate Alamar Blue Assay
MCA	Medicines Control Agency
MDR	Multi-drug Resistance
MIC	Minimum Inhibitory Concentration
MNL	Mononuclear Leucocytes
MP	Melting Point
NAC	N-acetyl-L-cysteine
PBS	Phosphate Buffered Saline
PIM	Phosphatidylinositolmannosides
PYR	Pyrimethamine
PZA	Pyrazinamide
RIF	Rifampicin
SD	Standard Deviation
TAACF	Tuberculosis Antimicrobial Acquisition and Coordinating Facility, Birmingham, AL, USA
TB	Tuberculosis
THF	Tetrahydrofuran
TLC	Thin-Layer Chromatography
WHO	World Health Organisation

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1.1 Tuberculosis

1.1.1 History of the disease

Tuberculosis (TB) is known to be one of the oldest of human afflictions and few diseases have had such an extensive or continued impact on mankind. From ancient times tuberculosis has ranked amongst the most feared and dreaded of the numerous diseases that afflict the human race. It was dubbed as 'the Captain of all of these men of Death', and in India it was known as 'the King of Diseases' (Keers, 1978).

Clear evidence of lesions resulting from tuberculosis of the bone has been found in Egypt, dating back to the Early Dynastic period around 3400 B.C. 'Lung fever' and 'lung cough', which must have included tuberculosis, were noted in ancient Chinese writings as far back as 2698 B.C. with accompanying symptoms of emaciation, cough and the expectoration of blood and pus. The oldest code of laws in the world, the Code of Hammurabi, also recorded the disease. Another advanced civilization, the Indo-Aryans, have left medical records in Sanskrit, the earliest of which, the Rig-Veda which was written about 1500 B.C. and it suggests that they were acquainted with pulmonary tuberculosis.

The spread of tuberculosis throughout Europe during the 18th and 19th centuries made it one of the commonest causes of death in those days and even in the early part of this century the disease was still only too frequently met and only too seldom cured.

The discovery of the causative agent of tuberculosis by Robert Koch in 1882 led to great hopes that this disease would soon be vanquished. But, only in the 1940s and 1950s, anti-tuberculosis drugs streptomycin, β-amino salicylic acid and isoniazid (INH) were developed. These drugs gave hope to cure tuberculosis and the mortality declined annually. People started to believe that tuberculosis might disappear from the Earth eventually. But after three decades of linear decline, tuberculosis surged in the middle of 1980s all around the world due to the emergence of multi-drug resistance tuberculosis (MDR-TB) and HIV prevalence.

1.1.2 Main features

Tuberculosis, caused by the bacterium *Mycobacterium tuberculosis,* is a disease of the respiratory system, but it can affect almost any part of the body. The symptoms can include a resistant cough, feeling tired, weight loss, loss of appetite, fever, night sweats and coughing up blood.

Like the common cold, it spreads through the air. Only people who are sick with pulmonary TB are infectious. When infectious people cough, sneeze, talk or spit,

they propel TB bacilli into the air. A person needs only to inhale a small number of these to be infected.

Left untreated, each person with active TB will infect on average between 10 and 15 people every year (WHO). However, people infected with TB will not necessarily develop symptoms with the disease. The immune system isolates the TB bacilli, which are protected by a thick waxy coat, but prior to this point, the bacillus has the potential to disseminate to the kidneys, bones, meninges, and other sites where there is the potential for reactivation years later. This is what called 'latent TB infection'. The individual may not be able to eliminate the infection without taking anti-TB medications (CDC). The worldwide prevalence of latent TB is estimated to be 1.5 billion, making latent TB arguably the most prevalent bacterial infection on the planet.

Without treatment, the lifetime risk of reactivation with active disease is about 10% among persons with healthy immune systems. The risk is greater among persons whose immune system is weakened, such as HIV infection. Indeed, a person with HIV infection who has latent infection has an annual risk of 7% to 10% of developing active TB. An HIV-infected individual who becomes acutely infected with TB has a 40% risk of developing active TB within a few months of initial infection (CDC).

Tuberculosis is increasing in prevalence throughout the world and becoming more dangerous due to the newly developed multi-drug resistance tuberculosis and HIV prevalence.

Currently:

- Tuberculosis is a bigger killer than malaria and AIDS combined (2 to 3 million people per year).
- Overall, one-third of the world's population is infected with the TB bacillus.
- 7 to 8 million people around the world become sick with TB each year.
- TB is the leading cause of death among people who are HIV-positive.
- Someone in the world is newly infected with TB every second and dies of TB every 15 seconds.
- 5 - 10 percent of people who are infected with TB become sick or infectious at some time during their life.
- The death rate for untreated patients with active disease is 55%.

So serious is the global threat of tuberculosis that, in 1993, the WHO took the unprecedented step of declaring this disease a global emergency.

1.1.3 Why TB is so successful as a disease

Tuberculosis has an extraordinary impact on the economies all over the world because in general the disease attacks individuals in their working years—causing nearly 20% of all adult deaths aged 15-59 (Bloom and Murray, 1992). Prior to World War II, the disease was fatal in 50-60% of all cases (Blanchard, 1996). The discovery of the causative agent of tuberculosis, the tubercle bacillus, by Robert Koch in 1882 gave great hope that the disease would soon be eradicated. Who would, therefore, have predicted that, 111 years after Koch's discovery, the World Health Organization, far from celebrating the eventual conquest of tuberculosis, would declare it a global emergency.

Despite the fact that anti-tuberculosis drugs have been available for over half a century, tuberculosis still kills millions people every year. To explain why tuberculosis is so successful as a disease and to understand why it is so difficult to eradicate, the causative agent—*Mycobacterium tuberculosis* must be studied.

Mycobacterium is undeniably one of the most clinically important and intensively studied bacteria. The generic name *Mycobacterium* was introduced by Lehmann and Neumann in the first edition of their 'Atlas of Bacteriology' published in 1896. At that time the genus contained only two species, *Mycobacterium Leprae* and *M. tuberculosis* (Grange, 1988). Despite of many years of study, knowledge of the disease remains incomplete. Some

characteristics of this bacterium make it very difficult to cure, such as, slow growth, multiple growth stages, its waxy outer membrane and drug resistance.

1.1.3.1 Slow growth

The generation time of common fast-growing bacteria is about 20 minutes, but in the case of *M. tuberculosis,* it is about 24 hours (18 hours in the most favorable conditions devised) (Wayne, 1976).

The reason for the slow growth of *M. tuberculosis* is not clearly known. Originally, it seems that the thick waxy outer cell wall must be relatively impermeable to the uptake of nutrients as well as protective against anti-TB drugs. Studies of *M. chelonae* reveal that there are water-filled channels in its cell wall formed by a porin (Trias, 1992). These channels are crucial for the transport of bacterial growth substances, like purines and pyrimidines, which must be acquired exogenously and used for nucleic acid biosynthesis and this is directly related to the generation time (Wheeler and Ratledge, 1988).

Most antibiotics are anti-metabolites, which operate by exploiting the rapidly growing bacterial demand for nutrients for cell wall building (penicillin) and DNA assembly (DHFR inhibitors). Drugs only slow the growth of bacteria sufficiently to allow the immune system enough time to mount a response to the bacteria. As tuberculosis grows so slowly in multiple stages of growth, drugs can make little impact on bacterial numbers, and in many cases, the immune system

cannot destroy the bacteria no matter how much time it is given, partly because the waxy cell envelope protects the bacteria from being killed.

These factors, together with the different growth stages, mean that anti-tuberculosis chemotherapy must be prolonged for up to 24 months, rather than days or weeks.

1.1.3.2 Multiple growth stages

Aside from the slow generation time of *M. tuberculosis*, the metabolic activity of the bacteria make tuberculosis chemotherapy much more difficult to achieve. It was suggested that pathogenic mycobacterial populations be divided into four components: actively metabolizing and rapidly growing, semi-dormant in an acidic intracellular environment, semi-dormant in a non-acidic intracellular environment and dormant (Mitchison, 1985). Dormancy allows the bacteria to remain in the human body for years, or even decades, protected by its cell wall, which prevent drugs from permeating the cell.

Effective tuberculosis chemotherapy must include early bactericidal action against rapidly growing organisms (to both reduce the time of infectiousness and shorten the treatment period) and subsequent sterilization of the semi-dormant and dormant populations of bacilli.

Certain drugs are clearly more effective against bacteria in different phases. Isoniazid, rifampicin, streptomycin and ethambutol all individually exhibit rapid

bactericidal action against actively metabolizing organisms (Heifets, 1994). Pyrazinamide appears to be most effective against semi-dormant bacilli in acidic intracellular environments, and rifampicin can be effective against semi-dormant bacilli in non-acidic environments. Whether any single drug is effective against dormant bacilli is not clear (Blanchard, 1996).

1.1.3.3 Mycobacterial membrane structure

The *M. tuberculosis* cell is covered by a thick and waxy 'bacterial envelope', which provides not only support for the bacterial cell content but also protection from xenobiotic compounds.

The envelope consists of two distinct parts: the plasma membrane and the outer cell wall. The primary structure of the cell wall consists of a plasma membrane, which is supported by a peptidoglycan backbone. Attached to the peptidoglycan is an arabinogalactan layer, which is attached to mycolic acid (Figure 1) (Bloom, 1994).

The structure of the outer cell wall of *M. tuberculosis* is different from all other known bacterial species and is impermeable to many drugs used in the chemotherapy of tuberculosis. It is believed that the mycolic acids that extend perpendicular to the arabinogalactan/peptidoglycan cell wall as well as the associated glycolipids intercalate into a nonpermeable bilayer. This permeability barrier operates not only for drugs used in anti-TB chemotherapy, but also all

other foreign substance as well, which is one of the reason why the generation time of *M. tuberculosis* is much longer than other species.

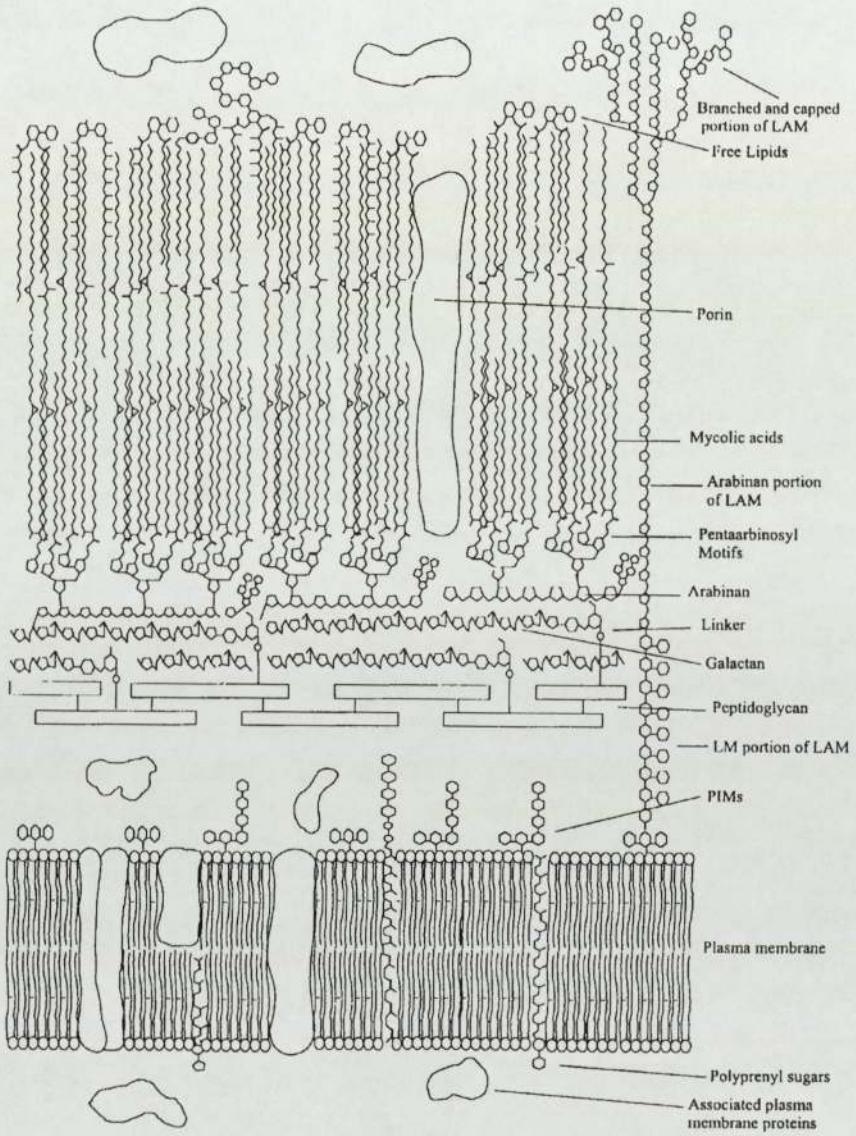


Figure 1 The *Mycobacterium tuberculosis* cell envelope (Lee et al., 1996)

1.1.3.4 Resistance

Until 50 years ago, there were no drugs to cure TB. Now, strains that are resistant to individual drugs have been documented worldwide and, what is more, strains of TB resistant to all major anti-TB drugs have emerged.

When people fail to complete standard treatment regimens, or are given the wrong treatment regimen, they may remain infectious. The bacilli in their lungs may develop resistance to anti-TB drugs due to sub-curative drug level. People they infect will have the same drug resistant strain. A particularly dangerous form of drug-resistant TB is multi-drug resistant TB (MDR-TB), which is defined as the disease due to TB bacilli resistant *in vitro* to at least isoniazid and rifampicin—the two most powerful anti-TB drugs.

MDR can be classified as primary (initial) or secondary (acquired). Primary resistance occurs with the detection of drug resistance in a patient with TB who has never been treated with anti-TB medications. This may occur as a result of random spontaneous genetic mutations, as is the case with any large population of bacteria regardless of their exposure to antibiotic agents, or through the acquisition of drug-resistant TB. Secondary MDR occurs when drug-resistant organisms develop as a result of the inappropriate use of anti-TB medications or patient noncompliance with the prescribed therapy (this presumes that the patient initially had drug-susceptible organisms) (McCray et al., 1997).

Globally, about 10% of cases of tuberculosis are resistant to one anti-TB drug (Douglas and McLeod, 1999). Drug resistance in the UK is approximately 8.9 times more common in HIV-infected patients with tuberculosis and 11.7 times more common in previously treated patients than in the general population (Kunar et al., 1997). In the United States during the past 3 decades, the prevalence of MDR-TB among individuals with pulmonary TB has steadily increased from 2% to 9% (CDC).

The duration of MDR-TB treatment is usually extended from 6 months to 18, or even 24 months (usually at least 2 anti-TB medications not previously taken). Despite a prolonged period of treatment, cure rates decrease from more than 95% to 60% or less (CDC). Of those with MDR-TB who fail to convert or relapse, 40% to 50% will likely succumb to progressive disease within 10 to 15 years (Cooper and Pappas, 1996).

While drug-resistant TB is treatable, it is often prohibitively expensive (often more than 100 times more expensive than treatment of drug-susceptible TB), and is also more toxic to patients.

1.1.3.5 HIV

HIV, human immunodeficiency virus, is accelerating the spread of TB. HIV and TB form a lethal combination, each speeding the other's progress: The prevalence of tuberculosis in HIV-infected individuals is high; correspondingly,

tuberculosis is one of the most common infections in AIDS patients. Someone who is HIV-positive is many times more likely to become seriously ill with TB than someone infected with TB who is HIV-negative. Any recently infected person is at high risk for clinical tuberculosis since half of the 10% lifetime risk of progressing from infection to active disease occurs in the first 2 years after the initial infection. For persons infected with both HIV and *M. tuberculosis*, it appears that the risk of clinical tuberculosis doubles to 20% or more. Furthermore, diagnosis of tuberculosis in persons with AIDS is often delayed (William and Shipley, 1996).

In Africa, HIV is the single most important factor determining the increased incidence of TB in the last ten years. In almost all sub-Saharan African counties, over 60% of children and 70% of adult who infected with TB were co-infected with HIV (Elliott et al., 1992). At the same time, about 8-10% of all cases of tuberculosis worldwide are related to HIV infection (Zumla and Grange, 1998).

TB is also a leading cause of death among people who are HIV-positive. It accounts for about 15% of AIDS deaths worldwide. In 1994 the Global Programme on AIDS of the World Health Organization estimated the prevalence of HIV infection among adults worldwide as around 14 million (Douglas and McLeod, 1999). At the same time, worldwide, about 1.75 billion people (i.e. one-third of the total human population) are infected with *M tuberculosis* (Sudre, et

al., 1992), and approximately 4 to 5.6 million are also infected with HIV (Raviglione, et al., 1992).

Although the bacteriological response to modern chemotherapy is the same in HIV-positive as in HIV-negative tuberculosis patients (Ackah et al., 1995), HIV-positive patients in Africa are almost four times as likely to die of tuberculosis than HIV-negative patients (Nunn, 1994). Therefore, prevention rather than cure of tuberculosis is of great importance in the HIV-positive patients.

1.1.4 Recent advances

1.1.4.1 Mapped genome

The complete genome sequence of *M. tuberculosis* has been determined and analysed, which contains around 4,000 genes (Cole et al., 1998). This achievement will give a better understanding of the biology of *mycobacteria* and the pathogenesis of tuberculosis.

In the development of anti-tuberculosis chemotherapy, old drugs could be reviewed and retested to uncover their targets and mechanism. The research into the newly discovered genes may lead to new potential drug targets, which can lead to new anti-TB drugs and chemotherapies. However, until now, four years after mapping the genome of *M. tuberculosis,* no new drug targets have been reported, let alone new drugs.

1.1.4.2 Experimental *Mycobacteria* studies

M. tuberculosis, in particular, is not an organism whose large scale culture is achievable in any but a handful of laboratories. The recent development of strains of the non-pathogenic fast-growing *M. smegmatis* that can be genetically manipulated (Blanchard, 1996) has allowed researchers to begin classical genetic studies in this organism. These approaches have clarified the mechanism of action of, and resistance to, both rifampicin and isoniazid (Levin and Hatfull, 1993). The biochemical transformations occurring in *mycobacteria* during the acquisition of drug resistance can be studied, so tremendous progress should be made in this area in the next decade.

1.1.4.3 The lack of novel anti-TB agents

Although it is over a century since the discovery of *Mycobacteria,* the sobering reality is that no new drugs have been introduced to fight tuberculosis since rifampicin in the 1960s, apart from the rifabutins, which are close structural analogues of rifampicin. What is needed is new drugs with new therapeutic targets. The need for novel anti-TB agents is now greater than ever.

1.1.5 Chemotherapy

1.1.5.1 Early work

Ever since the dawn of human medicine, ceaseless attempts have been made to cure tuberculosis. As far back as 3000 BC, bizarre remedies included the dung of animals and man, the urine of women and infants, the lungs of the hog and the ashes of hair. Unsurprisingly, these 'cures' were unsuccessful. In Europe, Roman physicians recommended bathing in human urine, eating wolf livers, and drinking elephant blood. Depending on the time and country in which they lived, patients were advised to rest or to exercise, to eat or to fast, to live in the mountains or underground.

It took over fifty years after the discovery of bacteria before any successful therapeutic agent was introduced. Prior to this time, patients had to rely on sanatorium treatment regimens, which including resting, being exposed to constant fresh air and consuming enormous amounts of food.

The sanatorium era was brought to a close by the introduction of some of the most important anti-TB drugs including streptomycin, isoniazid, rifampin and ethambutol. It was believed that TB, like infectious diseases of previous centuries, could be completely eliminated by modern chemotherapy. Indeed, the United Nations predicted the worldwide elimination of TB by the year 2025.

The hope of tuberculosis would be eliminated soon vanished after resurgence of the disease in the mid-80s. Existing chemotherapy is struggling to control tuberculosis.

1.1.5.2 Existing chemotherapy

It was not until the late 1940s that the first anti-tuberculosis drugs, streptomycin and β-amino-salicylic acid became available, accompanied by the discovery of isoniazid and pyrazinamide in 1952, rifampin in 1957 and ethambutol in 1961.

At present, the five first line anti-TB drugs are isoniazid (INH), rifampicin (RIF), streptomycin, ethambutol, and pyrazinamide (PZA). The standard regimen is INH, RIF and PZA for a 2-month intensive phase followed by INH and RIF for 4 months (Bloom et al., 1992; Snider et al., 1992; Bass et al., 1994).

Initially, tuberculosis was treated with a single drug, but not long after the emergence of drug-resistant *M. tuberculosis* strains in 1950s necessitated combination therapy. Since then, modern chemotherapies were developed to decrease tuberculosis mortality rates worldwide.

This trend continued until the mid-1980s, when the number of new reported cases began to increase. One of the reasons was that the appearance of AIDS virus in the early 1980s. Because of the weakened immune system, tuberculosis is one the most common infections in HIV-infected individuals. Another reason for the increase is MDR-TB. The MDR strains which are resistant to all the five

first line drugs have emerged and resist once to at least 2 anti-TB drugs is relatively common (10% in all cases) (Blanchard, 1996). Accompanied with the high toxicity of current anti-TB drugs, which will be discussed in detail later this chapter, existing chemotherapy certainly could not meet the need to eliminate tuberculosis. The need for new anti-TB drugs and vaccines has never been greater.

According to WHO predictions, without radical changes in our approach to tuberculosis, nearly one billion people will be newly infected, 200 million people will develop the disease, and 35 million will die from TB between 2000 and 2020.

HISTORICAL PERSPECTIVE

- In 1882, *Mycobacterium tuberculosis* bacillus was identified.
- In the 1940s, anti-TB drugs streptomycin and p-amino salicylic acid were developed.
- In the 1950s, resistant TB required combination therapy and public health policy changes.
- In 1952, isoniazid and pyrazinamide was developed.
- In 1957, rifampicin was discovered.
- In 1961, ethambutol was introduced.

- Between 1985 and 1992, large increases in TB cases occurred.

1.1.5.3 New targets

Drugs must act on a specific and unique target that is essential for bacterial survival in order to kill the bacteria.

Isoniazid inhibits the synthesis of mycolic acids. (Winder and Collins, 1970); Rifampin acts by interfering with the synthesis of mRNA by binding to the RNA polymerase. (Telenti, 1998); Ethambutol acts on the biosynthesis of arabinogalactan , the major polysaccharide of the mycobacterial cell wall (Takayama and Kilburn, 1989); Streptomycin inhibits protein synthesis of *M. tuberculosis*, which acts on ribosomes and causes misreading of the genetic code, inhibition of translation of mRNA, and aberrant proofreading (Moazed and Noller, 1987).

Knowledge of the complete genome sequence of *M. tuberculosis* gives us more scope to investigate the biology of the mycobacterium. By studying the genes, the routes of protein synthesis will be better understood and this may lead to more potential drug targets that are crucial for mycobacterial survival.

Dihydrofolate reductase (DHFR, Figure 2) is essential for folate metabolism in both eukaryotic and prokaryotic cells. The role of DHFR is to catalyse the NADPH dependent reduction of dihydrofolate to give tetrahydrofolate, a central component in the single carbon metabolic pathway (Figure 3). The

tetrahydrofolate is methylated to methylene tetrahydrofolate, which is directly involved in thymidine synthesis (assisting the methylation of deoxyuridine monophosphate to give thymidine monophosphate) and indirectly implicated in the metabolism of amino acids and purine nucleotide. Inhibition of DHFR thus inhibits the growth of rapidly dividing cells by arresting of DNA synthesis leading to cell death.

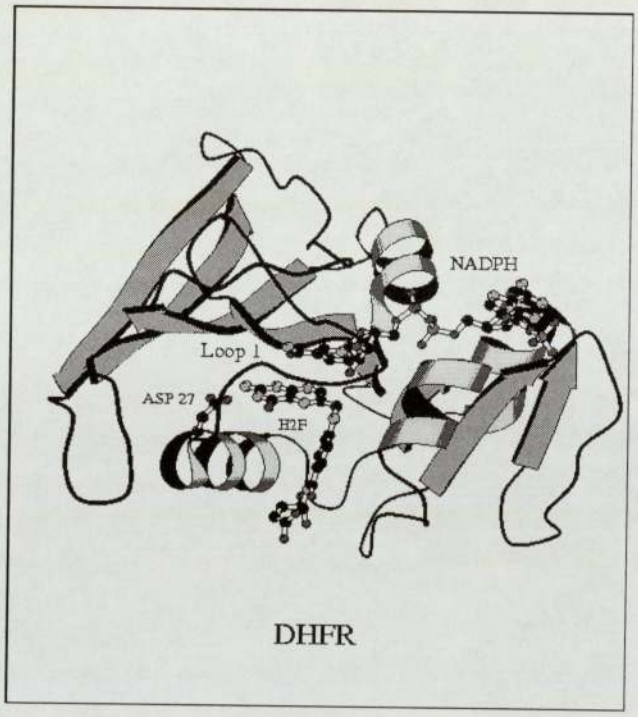


Figure 2 3D structure of dihydrofolate reductase

(http://research.chem.psu.edu/sjbgroup/dhfr.html)

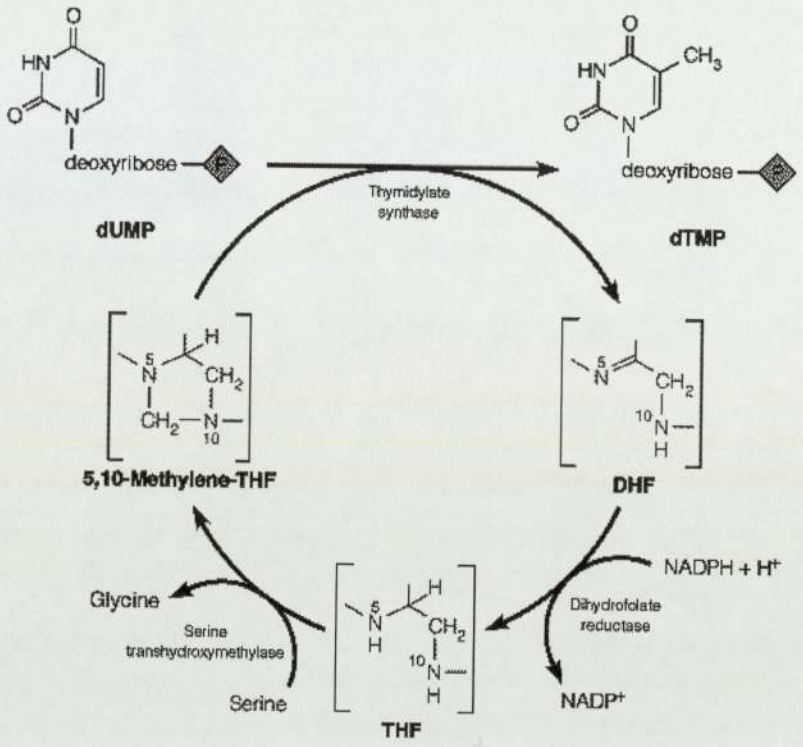


Figure 3 One-carbon transfer indicating the essential role of DHFR in the synthesis of dTTP (Mathews et al., 2000)

DHFR has already been used as a drug target for the development of anti-microbial and anti-cancer agents. Methotrexate is used in cancer chemotherapy and compounds like trimethoprim and pyrimethamine are successfully used to treat bacterial infections and malaria, respectively. Recently, there has been a growing interest in the possibility of targeting DHFR in other disease areas (Zuccottoa, et al., 2001).

DHFR is a successful drug target as it is an essential enzyme and because the structure of the enzyme varies subtly from species to species allowing selective

drug design. A large amount of structural information is available concerning *M. tuberculosis* DHFR and human DHFR (Li, et al., 2000), which suggests considerable opportunity to rationally design new selective drugs.

Pyrimethamine is a potent inhibitor of bacterial DHFR, but is only a weak inhibitor of mammalian DHFR. This agent affords an opportunity to modify its structure to selectively increase its inhibition of *M. tuberculosis* DHFR.

A series of pyrimethamine derivatives were synthesised by a former worker at Aston University, and two of them showed high activity during Minimum Inhibitory Concentration (MIC) test (Wols, 2000).

1.1.5.4 Vaccine

The existing vaccine, *Mycobacterium bovis* BCG (Bacillus Calmette-Guérin, after its discoverers), has been used extensively for many decades, but it has been found of lack effectiveness in adults (Zumla and Grange, 1998; Colditz et al., 1994). Its protective efficacy varies from 0%-80% in different regions (Fine, 1995).

Over the last several years, there has been extensive research to develop new vaccines against tuberculosis. Some experimental vaccines have been reported, but it is a huge leap from animal models to humans. Even then, there is no guarantee that the experimental vaccines will work. The good example is BCG,

which has been thoroughly tested in humans and exhibits limited protection, but is much more effective in animal models (Orme et al., 2001). It will be a long time before any new vaccine appears which might be superior to BCG.

1.1.5.5 Directly Observed Therapy, Short-course—DOTS

The WHO has advocated the DOTS strategy. Despite some notable successes (China Tuberculosis Control Collaboration, 1996), DOTS implementation has been slower than anticipated reaching only 12% of tuberculosis cases worldwide by the end of 1996 (WHO Global Tuberculosis Programme, 1998). Recent projections suggest that even if this rate of implementation were substantially increased, tuberculosis mortality will only be halved before 2030 (Murray and Saloman, 1998). Evidence is also emerging that DOTS is unable to control tuberculosis in countries with high levels of HIV infection (Kenyon et al., 1999), and in other countries it is failing to achieve adequate cure rates (Zwarenstein et al., 1998). Some strains of the disease are now thought to be virtually incurable (Pym and Cole, 1999). New anti-tuberculosis agents are needed to solve the problems of DOTS and current chemotherapy. Unfortunately, without many new drugs, the disease will not even be controlled, never mind eradicated.

1.1.5.6 Political will

Diseases like tuberculosis, malaria, HIV/AIDS and leprosy cause the greatest damage to poor and developing countries, where TB is most common. Drug

supplies may be inadequate and interrupted and there is a lack of proper supervision by qualified doctors. Unfortunately, partial or incorrect treatment for TB is worse than no treatment at all. TB bacteria that linger following incomplete therapy are likely to resist anti-tuberculosis drugs in future. Worse still, people with active cases of MDR-TB can pass those resistant organisms on to new victims.

Government commitment is essential to sustain tuberculosis control, including significant funding to support tuberculosis control programme; reporting system to monitor the programme progresses; as well as comprehensive educational programmes to train enough medical staff.

Pharmaceutical companies should be given financial incentives to conduct research in new anti-TB drug development and lower the cost of existing drugs. International organizations and industrialized countries should also provide more funds to support anti-TB programmes, as poor countries are vulnerable to tuberculosis and AIDS. Therefore, poverty is the strength of these diseases. Moreover, funds are vital to ensure accessibility of regular and uninterrupted supply of high quality tuberculosis drugs in a programme like DOTS.

1.2 Toxicity of anti-TB drugs

Toxicity is always an important issue in drug design, especially in anti-TB chemotherapy, as the duration of therapy, which contains 5 drugs, may exceed 6

months. The drugs often cause unpleasant side effects and because patients start feeling better after a month or so, not everyone completes the full course of treatment mostly due to toxicity. Lack of compliance is also a major source of the emergence of drug resistance. If the toxicity of drugs could be lowered, the compliance would increase and this will lead to less drug resistance, which would increase the cure rate.

In the case of MDR-TB therapy, the treatment generally lasts 18-24 months. High drug toxicity would be a crucial problem and it may outweigh the benefits of chemotherapy.

Existing chemotherapy including rifampicin and isoniazid in combination has proved to be highly effective in the treatment of tuberculosis, but one of their adverse effects is hepatotoxicity (Pande et al., 1996). During the studies in the 1970s, isoniazid has caused considerable alarm in Washington, D.C. where 19 of 2,231 government employees given isoniazid preventive therapy developed clinical signs of liver disease within 6 months of starting the drug; 13 were severely jaundiced and 2 died (Garibaldi et al., 1972). There have been 45 reported deaths from liver reactions to currently recommended first-line anti-TB agents since 1964, with isoniazid implicated in 25 of these deaths (Ormerod and Skinner, 1996). The risk of drug-induced hepatitis needs to be weighed against the advantages of preventing tuberculosis morbidity (Girling, 1978). The

resurgence of tuberculosis since mid-1980s also presents a challenge for old drugs.

The tragedy of this situation is that treating tuberculosis is one of the most cost effective of all heath interventions. The World Health Organisation has calculated that, unless urgent action is taken, the annual number of deaths could rise from 3 million to 4 million by the year of 2004 (WHO). We urgently need improvements in the implementation of existing strategies for tuberculosis control, with particular emphasis on delivery of effective and low-toxic treatments.

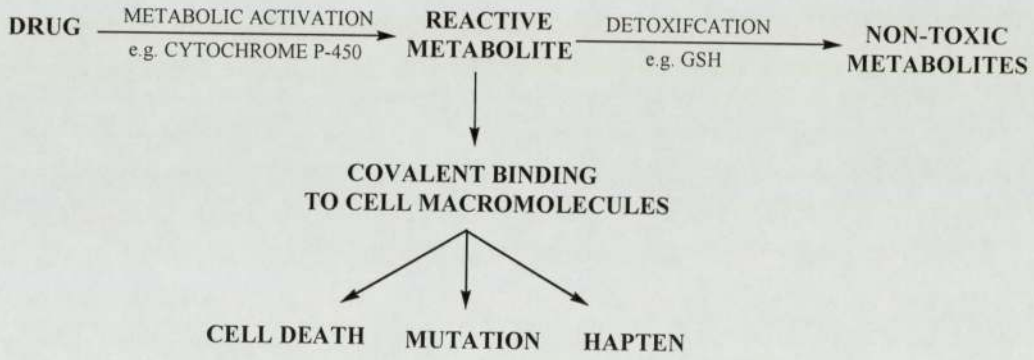
1.2.1 Toxicity assay

When evaluating drug toxicity, a predictable and reliable means of assessing drug toxicity is needed by using human cells, which could be easily obtained.

To test the toxicity of drugs, animals are often used as models. However, the problem with animal models is that animal studies do not always reflect how human tissues behave. The Committee of Safety of Medicine (CSM) of Medicines Control Agency (MCA) has withdrawn several drugs including troglitazone most recently, which is an orally active anti-diabetic agent. Serious hepatic reactions occurring during treatment, including severe hepatocellular damage, hepatic necrosis and hepatic failure, had been reported worldwide. One patient had died and another required liver transplantation (CSM/MCA, 1997).

This drug certainly showed no toxic during the toxicity test in animals. Unfortunately, drugs do not always react the same way in human body as in animals.

Therefore, we have employed the white cell toxicity assay designed by Stephen P Spielberg (1980, 1984). Scheme 1 presents the role of drug metabolism in drug toxicities. Many compounds themselves are not toxic, but can be metabolized, e.g. by a cytochrome P450 oxidase, to a reactive inter-mediate. Normally the metabolite is detoxified, e.g. by conjugation with glutathione. If a sufficient dose is given to deplete glutathione reserves, metabolites may then covalently bind to cell macromolecules with resultant toxicity and cell death. This may be manifested *in vivo* by hepatotoxicity in animals and man (Spielberg, 1984).



Scheme 1 Pathways for production and detoxification of reactive, toxic drug metabolites (Spielberg, 1984)

The mononuclear leucocytes (MNLs) were selected as easily isolated human tissue for toxicity screening. In other studies, the MNL assay successfully predicted subsequent *in vivo* toxicity (Coleman et al., 1991) and had the ability of distinguishing structurally between similar compounds (Coleman and Kohl, 1995; Coleman et al., 2000), which showed the same in this project.

However, the white cell toxicity assay is only a preliminary test for toxicity screening. We can use this *in vitro* means of toxicity assessment to screen our compounds prior to animal test. If the compound synthesised is toxic against human cells, there will be no point testing on animals. This will certainly shorten the duration of new drug discovery and reduce the cost of new drug development.

1.3 Aims of this project

The structure study of this report is based on the derivatives of pyrimethamine.

The aim of this project is to:

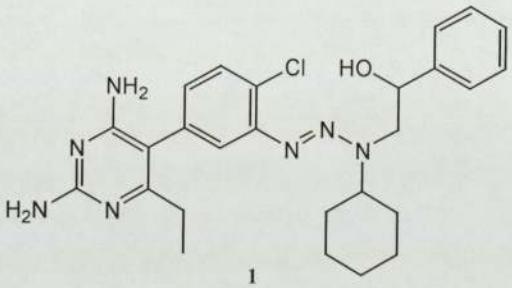
- Evaluate the toxicity of two high activity compounds synthesised by a previous worker (Wols, 2000).
- Design new low-toxicity anti-mycobacterial agents according to structure-toxicity relationship information revealed form the toxicity test.
- Evaluate the toxicity and efficacy of newly synthesised compounds.
- Discover structure-toxicity and structure-efficacy relationships.

Chapter 2. Methods

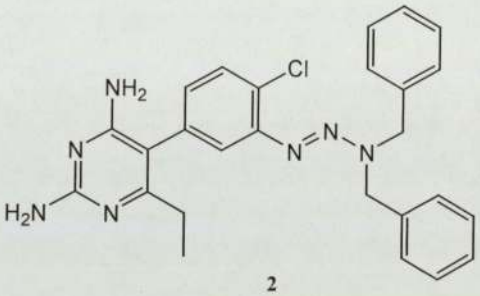
2.1 Chemistry

A series of triazenyl-substituted pyrimethamine derivatives were synthesised by a former worker (Wols, 2000) and two of them (compound **1** and **2** in Table 1) exhibited high activity during the minimum inhibitory concentration (MIC) test against *M. fortuitum*.

Table 1 Compounds synthesised by a former worker (Wols, 2000)



1

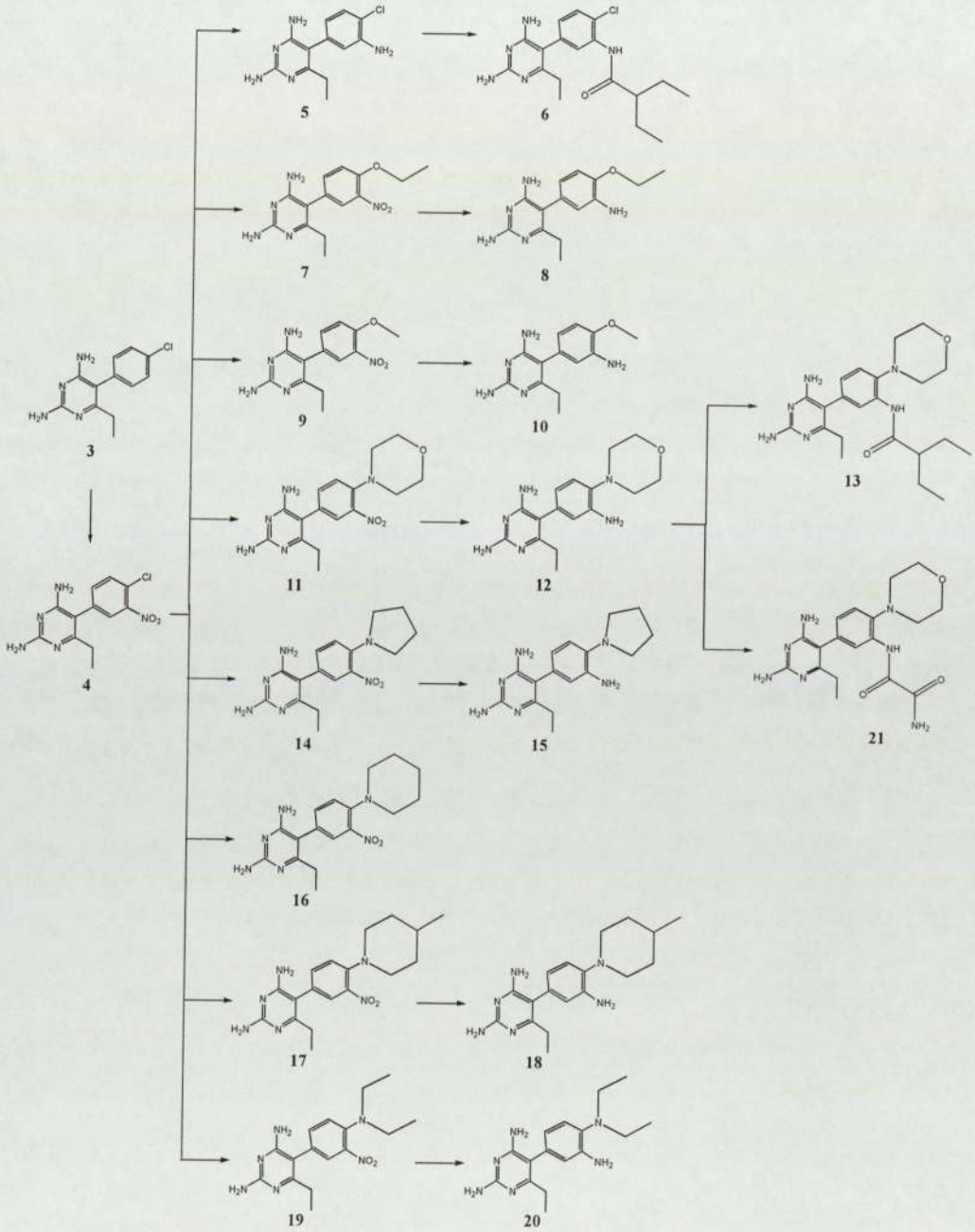


2

However, both compounds showed high toxicity against human mononuclear leucocytes (MNLs) during the toxicity test. It was believed that the toxicity comes from the triazenyl part of the molecule.

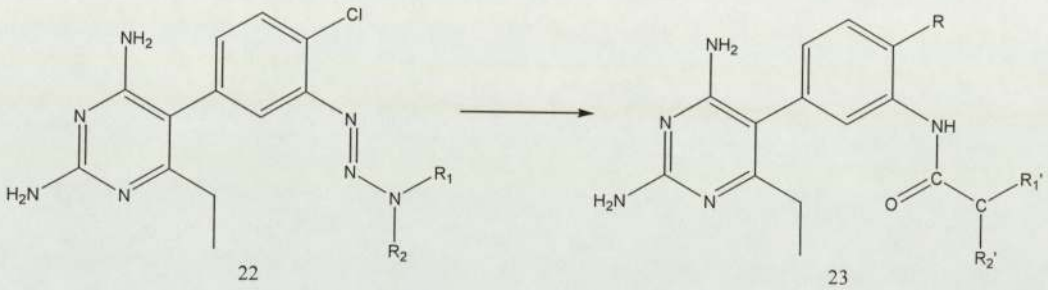
Therefore, in this project, pyrimethamine (**3**) was reacted with nitric acid and sulphuric acid to synthesise nitro-pyrimethamine (**4**). Amines and alcohols were used to replace the chlorine to give the corresponding substitutes, which were reduced to amino-pyrimethamine derivatives with tin(II) chloride. The amino-

pyrimethamine derivatives were reacted with acid chlorides to give the corresponding amides (Scheme 2), which were identified by using proton-NMR, MS and IR. Detailed chemistry can be found in Chapter 3 Experimental.



Scheme 2 Synthesis route

As can be seen in this project, nitrogen-nitrogen double bond in compounds **1** and **2** was replaced with nitrogen-carbon single bond (Scheme 3) with the hope of reducing the toxicity while retaining the efficacy.



Scheme 3 Replacement of nitrogen-nitrogen double bond with nitrogen-carbon single bond

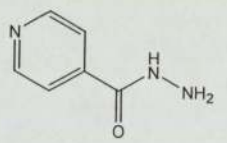
2.2 *Toxicity assay*

Newly synthesised compounds, compound **4** to **21** (Scheme 2), and compounds **1** & **2** (Table 1) were evaluated toxicologically using human mononuclear leucocytes (MNLs) as target cells.

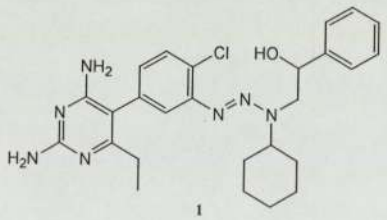
2.2.1 Chemicals

The following compounds were tested: isoniazid (**24**); compound **1**, {2,4-diamino-5-{4-chloro-3-[3-(2-hydroxy-2-phenylethyl)-3-cyclohexyltriazen-1-yl]phenyl}-6-ethylpyrimidine}; compound **2**, {2,4-diamino-5-[4-chloro-3-(3,3-dibenzyltriazen-1-yl)phenyl]-6-ethylpyrimidine} in Table 2 and all the compounds listed in Scheme 2 (compound **4** to compound **21**).

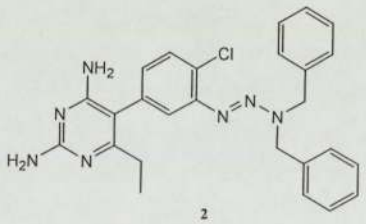
Table 2 Structures of agents used in the toxicity assay



ISONIAZID (**24**)



1



2

2.2.2 Biological materials

Blood was drawn from healthy volunteers and mononuclear leucocytes (MNLs) were isolated at room temperature using the method of Boyum (1976). The

MNLs were resuspended and incubated using HEPES buffer (pH 7.4). The cells were used within 1 hour of isolation.

Preparation of HEPES buffer:

HEPES (0.894 g), NaCl (1.828 g), KCl (0.112 g), $MgSO_4$ (0.074 g), NaH_2PO_4 (0.039 g), $CaCl_2$ (0.037 g) and Glucose (0.45 g), dissolved in 250 ml of distilled water and the pH was adjusted to 7.4.

2.2.3 Methods

Blood was collected into a tube containing heparin. 10 ml of blood was carefully layered over 5 ml Lymphoprep (Nycomed, Norway) in a 20 ml centrifugal tube. Centrifuge at 2100 rpm for 20 minutes at room temperature. After centrifugation, the mononuclear cells formed a distinct band at the sample/medium interface. The cells were removed from the interface using a pipette without removing the upper layer. The cells were diluted with PBS buffer and pellet the cells by centrifugation for 10 minutes at 1100 rpm. The supernatant was removed with pipette and the cell pellet diluted with HEPES buffer to the desired concentration.

Each compound was incubated with MNL in quadruplicate with 1 million cells per ml in HEPES buffer using DMSO as a positive control. The compounds were studied at 0.5 mM. After 1 hour of incubation, the MNL were centrifuged at 1100 rpm for 4 minutes and the HEPES buffer was replaced with fresh drug-free

buffer, which contained 5 mg/ml bovine serum albumin (BSA). Cell death was determined by trypan blue exclusion over 18 hours.

2.3 Bioactivity

Two bioactivity tests were carried out: (1) The Minimum inhibitory concentrations (MICs) of all the synthesised compounds, compounds **4** to **21**, were evaluated against *Mycobacterium fortuitum*; (2) The MICs and inhibition percentages of some compounds (listed in Table 9) were evaluated against *Mycobacterium tuberculosis* by Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF), Birmingham, AL, USA.

Mycobacterium fortuitum was initially used during the bioactivity test because *Mycobacterium tuberculosis* is a dangerous pathogen to work with. Furthermore, it grows very slowly, only doubling once every 20-24 hours, which means it will be four weeks before recognizable colonies appear (compared with 1-3 days for *M. fortuitum*). Therefore, the synthesised compounds were initially tested against *M. fortuitum* as a model for *M. tuberculosis*.

2.3.1 MIC test against *Mycobacterium fortuitum*

The Minimum inhibitory concentrations (MICs) against *M. fortuitum* were carried out by using Middlebrook 7H9 broth. Stock solution was prepared at 2.56 mg/ml in DMSO to give solution of 128, 64, 32, 16 μg/ml. A 10-fold solution

(0.26 mg/ml) was performed to give solution of 8, 4, 2, 1 μg/ml. Each tube was inoculated with *M. fortuitum* (10 μl of 10^7 CFU/ml), and incubated at 37°C for three days. Control tubes containing broth and inoculum, and broth with DMSO were also set up.

The MIC values were recorded as the minimum concentration that resulted in a 99% reduction of mycobacterial growth in the number of visible colonies.

2.3.2 MIC test against *Mycobacterium tuberculosis*

This test was conducted by TAACF (Birmingham, AL, USA).

Primary screening is conducted at 6.25 μg/ml (or molar equivalent of highest molecular weight compound in a series of congeners) against *Mycobacterium tuberculosis* H37Rv (ATCC 27294) in BACTEC 12B medium using the Microplate Alamar Blue Assay (MABA). Compounds effecting <90% inhibition in the primary screen (MIC >6.25 μg/ml) are not generally evaluated further.

Compounds demonstrating at least 90% inhibition in the primary screen are re-tested at lower concentrations against *M. tuberculosis* H37Rv to determine the actual minimum inhibitory concentration (MIC) in the MABA. The MIC is defined as the lowest concentration effecting a reduction in fluorescence of 90% relative to controls.

Chapter 3. Experimental

3.1 Toxicity screening of existing lead compounds

3.1.1 Introduction

A total of 27 triazenyl-substituted 2,4-diamino-5-(4-chlorophenyl)-6-ethylpyrimidines were synthesised by the former worker—Raoul V. Wols, 2000. During the bioactivity screening, eight compounds were active against *M. fortuitum*, including two most active compounds **1** & **2** (Figure 4). The toxicity of compounds **1** & **2** was evaluated against human MNLs.

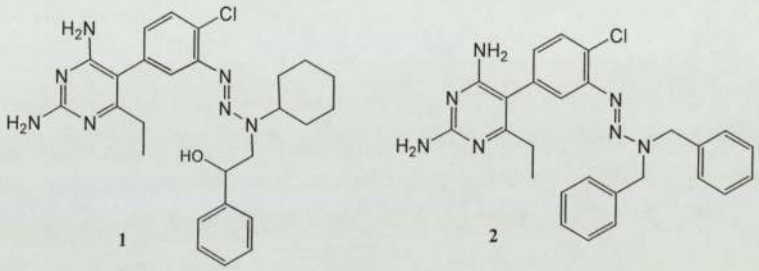


Figure 4 The two most active compounds synthesised by a former worker

One-way ANOVA with Dunnett's post test was performed using GraphPad InStat® version 3.01 for Windows 98, GraphPad Software, San Diego California USA, www.graphpad.com.

3.1.2 Results

The incubation of the human MNLs with the compounds showed that compared with control (4.85±0.64% as Mean±SD, n=15), compound **1** ($P<0.01$) and compound **2** ($P<0.01$) caused significant cell death (Table 3 and Figure 5).

Table 3 Toxicity data of compounds synthesised by a former worker

Compound	Cell death (%)				
	Sample 1	Sample 2	Sample 3	MEAN	SD
Pyrimethamine (PYR)	10.49	12.98	11.85	11.77	1.25
Isoniazid (INH)	9.83	8.98	14.1	10.97	2.74
1	71.9	79.5	73	74.8	4.11
1+Glutathione (GSH)	77.7	81.59	-	79.65	-
2	77.27	79.55	81.6	79.47	2.17
2+Glutathione	80.42	79.55	-	79.99	-
2+N-acetyl-L-cysteine (NAC)	65.18	78.05	70.37	71.2	6.48

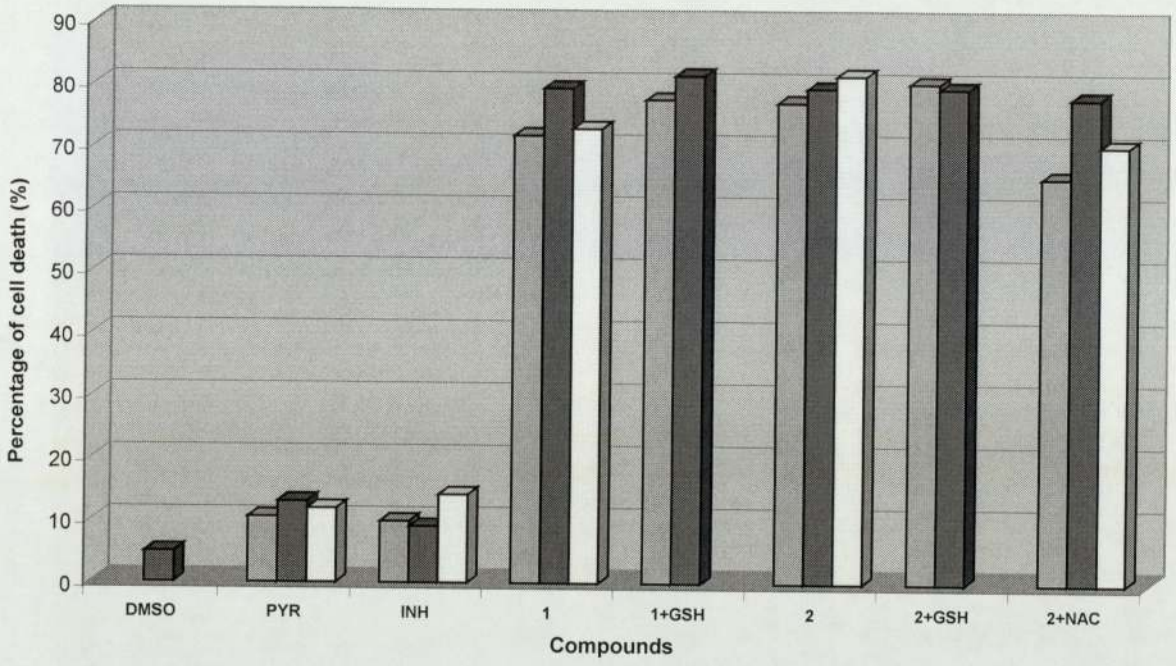


Figure 5 Toxicity chart

To investigate the mechanism of toxicity, glutathione (GSH) and N-acetyl-L-cysteine (NAC) were incubated together with MNLs and compounds.

Glutathione is one of the most important factors in the cellular defence against toxic chemicals. It is present in high concentrations in most mammalian cells. A wide variety of reactive metabolites can be conjugated with GSH. Depletion of intracellular GSH is know to be one of the most critical effects of toxic injury, since loss of glutathione protection against reactive intermediates rapidly leads to cell death.

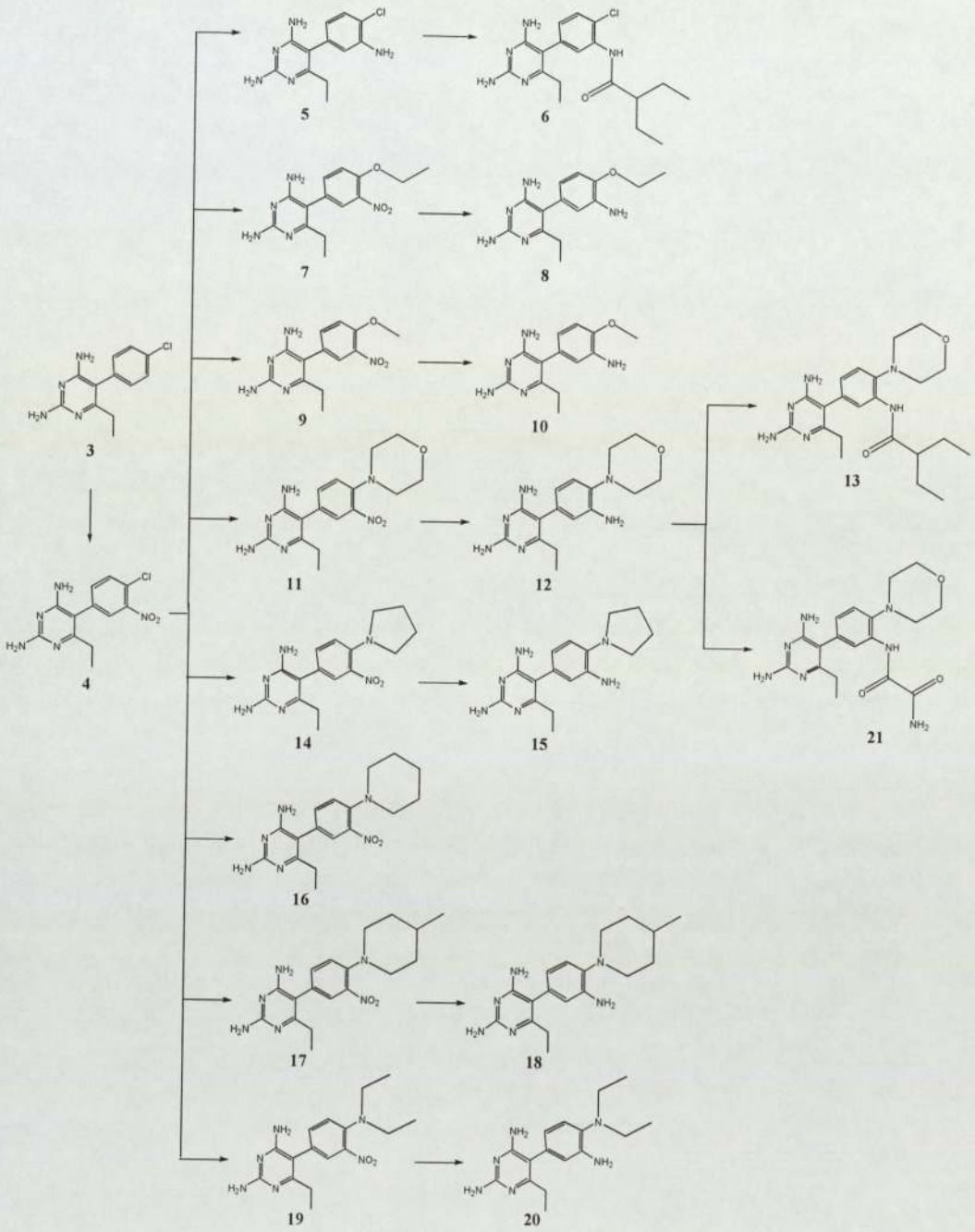
However, added glutathione cannot enter the cells due to the size of the molecule. N-acetyl-L-cysteine (NAC) is a precursor of GSH. It can penetrate into cells and be biosynthesised to GSH to offer protection against toxic chemicals.

The results (Table 3) showed no difference as to whether the MNLs were incubated with or without GSH or NAC, which means that neither GSH nor NAC could offer any protection to the MNLs. Therefore, compounds **1** & **2** were unlikely to be chemically reactive. The toxicity may come from the compounds themselves or due to other metabolic reasons.

3.2 Chemistry

3.2.1 Synthesis of new compounds

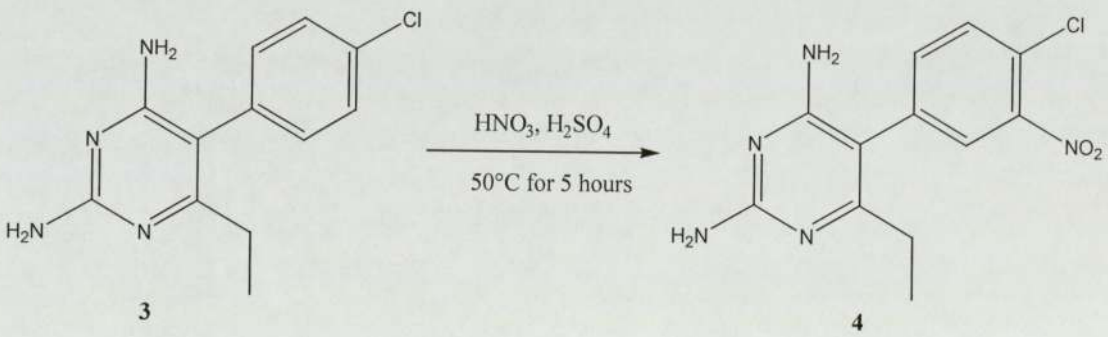
As described in section 3.1, the two most potent compounds (**1** & **2**) were proved very toxic. The synthetic route (Scheme 4) employed in this project was described below.



Scheme 4 Synthesis route

3.2.1.1 Synthesis of Nitro-pyrimethamine (4) (Scheme 5)

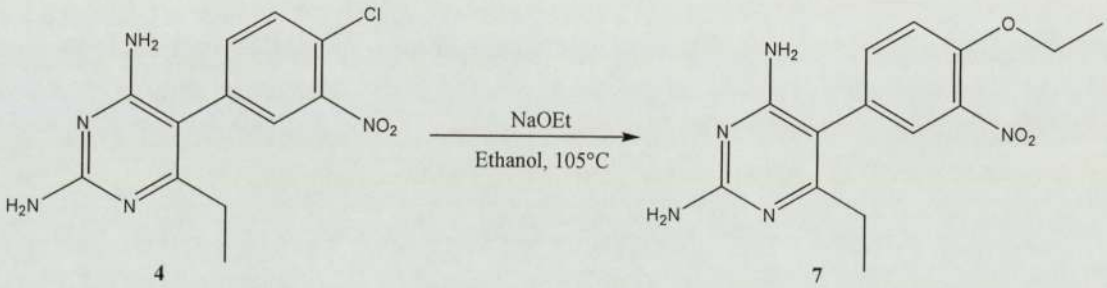
5-(4-Chloro-3-nitrophenyl)-6-ethylpyrimidine-2,4-diamine: 'nitro-pyrimethamine' (4). Nitric acid (d 1.42; 90ml) was added slowly to stirred sulphuric acid (d 1.84; 90ml). Pyrimethamine (**3**) (30.0g) was added slowly (15 min) to the mixture of acids. The mixture was heated at 50 °C for 5h and kept at room temperature overnight. The syrupy golden solution was poured into a mixture of concentrated aqueous ammonia and ice in an ice bath. The resulting solid was collected by filtration and rinsed with water (200 ml). The product was dissolved in stirred ethanol (200 ml) heated and refluxed for half an hour, then filtered through filter paper. The ethanol was volatilised and a yellow solid was collected, which was dried under vacuum. Yield: 97.7%.



Scheme 5 Synthesis of Nitro-pyrimethamine (4)

3.2.1.2 Synthesis of Ethoxy-nitropyrimethamine (7) (Scheme 6)

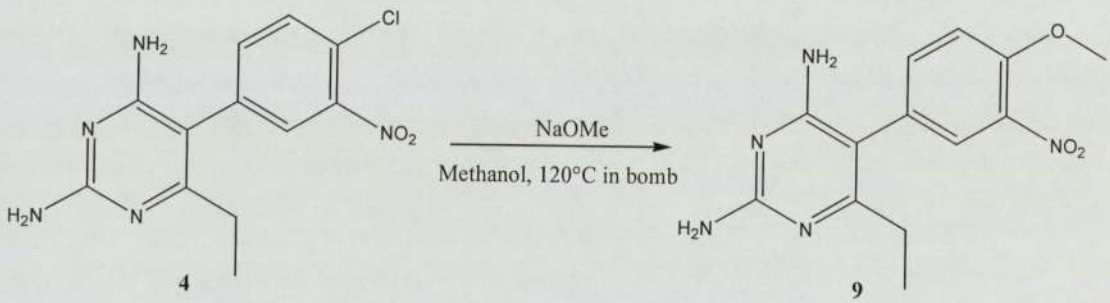
2,4-Diamino-5-(4-ethoxy-3-nitrophenyl)-6-ethylpyrimidine **(7).** Nitro-pyrimethamine (**4**) (10 g) and ethanol (400 ml) were added in a round-bottom flask and heated, stirred until it dissolved. Sodium ethoxide (23 g) was added into the flask and heated at reflux at 105°C (18 h). Ethanol was distilled and brown complex was collected. Distilled water was added into the flask to dissolve the inorganic material. The brown-cream solid was collected, which was dried under vacuum. Yield: 71%.



Scheme 6 Synthesis of Ethoxy-nitropyrimethamine (7)

3.2.1.3 Synthesis of Methoxy-nitro-pyrimethamine (9) (Scheme 7)

2,4-diamino-6-ethyl-5-(4-methoxy-3-nitrophenyl)pyrimidine (**9**). Nitro-pyrimethamine (**4**) (2.8 g), sodium methoxide (6.1 g) and methanol (100 ml) were added into a bomb and heated at 120°C over 2.5 days. The mixture in the bomb was cooled down by ice bath. Methanol was removed by evaporation under reduced pressure. Distilled water was introduced to dissolve the sodium methoxide and dark yellow solid was collected by filtration. Yield: 87.3%.

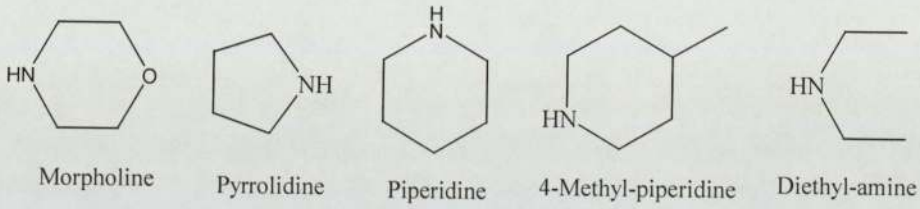


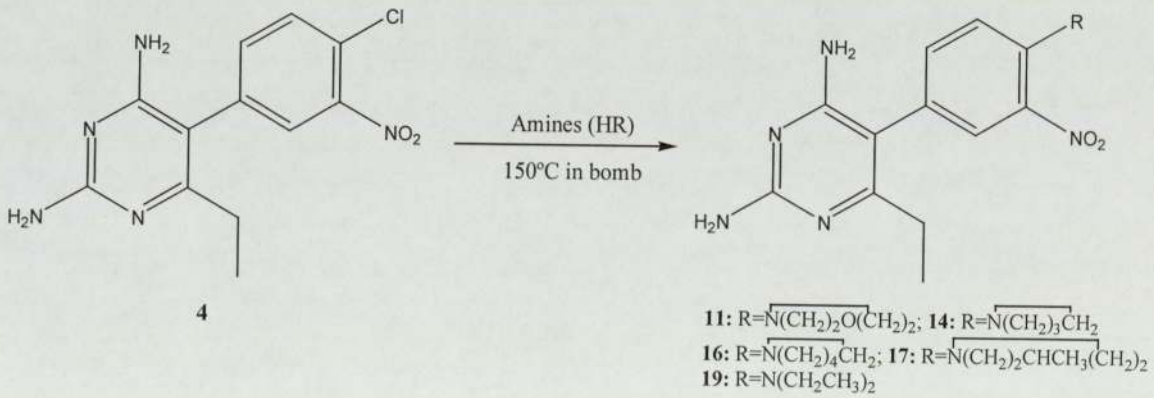
Scheme 7 Synthesis of Methoxy-nitro-pyrimethamine (9)

3.2.1.4 Synthesis of Amino-substituted nitro-pyrimethamines (Scheme 8)

Amino-substituted Diamino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidine. Nitro-pyrimethamine (**4**) was dissolved in different amines (Table 4) and added into a 100-ml stainless steel bomb, which was heated in sand bath at 150°C (24 h). The reaction mixture was cooled with ice bath. Precipitation occurred after kept at room temperature for 24 hours and was filtrated and rinsed with the solvent in which the compound is not soluble. Yield: 62-76%.

Table 4 Amines reacted with nitro-pyrimethamine



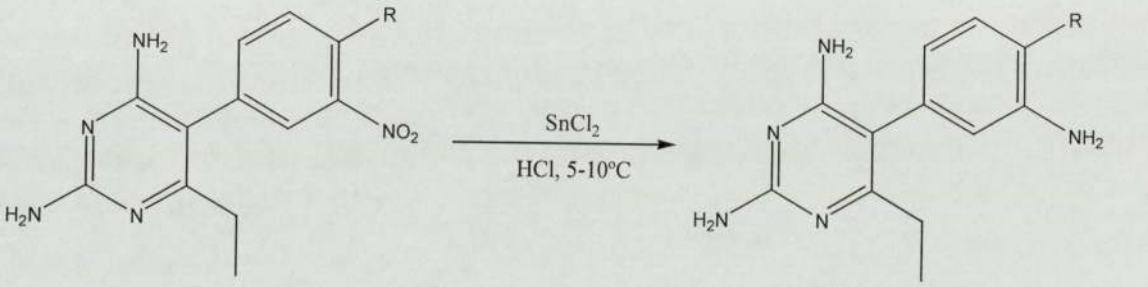


Scheme 8 Synthesis of Amino-substituted nitro-pyrimethamines

3.2.1.5 Synthesis of Amino-pyrimethamines (Scheme 9)

Diamino-5-(3-amino-4-chlorophenyl)-6-ethylpyrimidine substitutes.

Substituted pyrimethamines from the above procedures (3.2.1.1- 3.2.1.4) were added in small portions to a stirred solution of tin(II) chloride dihydrate in hydrochloric acid (10 M) at 5-10°C. The mixture was stirred overnight at room temperature and the white stannic complex collected. A solution of the complex in hot water was basified to pH 12 with sodium hydroxide-ice (10 M). The white stannic complex was collected by filtration and rinsed with water. Yield: 70-85%.



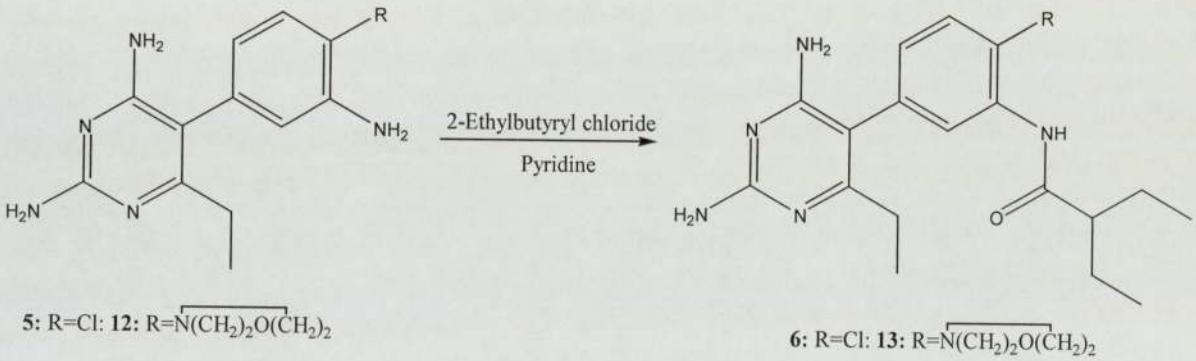
4: R=Cl; **7:** R=OCH_2CH_3; **9:** R=OCH_3
11: R=$\overline{N(CH_2)_2O(CH_2)_2}$; **14**: R=$\overline{N(CH_2)_3CH_2}$
17: R=$\overline{N(CH_2)_2CHCH_3(CH_2)_2}$; **19**: R=$N(CH_2CH_3)_2$

5: R=Cl; **8**: R=OCH_2CH_3; **10**: R=OCH_3
12: R=$\overline{N(CH_2)_2O(CH_2)_2}$; **15**: R=$\overline{N(CH_2)_3CH_2}$
18: R=$\overline{N(CH_2)_2CHCH_3(CH_2)_2}$; **20**: R=$N(CH_2CH_3)_2$

Scheme 9 Syntheses of Amino-pyrimethamines

3.2.1.6 Synthesis of Pyrimethamine amides (Scheme 10)

Pyrimethamine amides. The mixture of pyridine and substituted diamino-5-(3-amino-4-chlorophenyl)-6-ethylpyrimidine were stirred at room temperature in a round-bottom flask and 2-ethyl-butyryl chloride (2 equivalence) was added into the mixture. After 3 hours, concentrated aqueous ammonia (10 ml) was added into the mixture and kept at room temperature without stirring. The mixture was left overnight. White solid was collected by filtration and dried under vacuum. Yield: <20%.



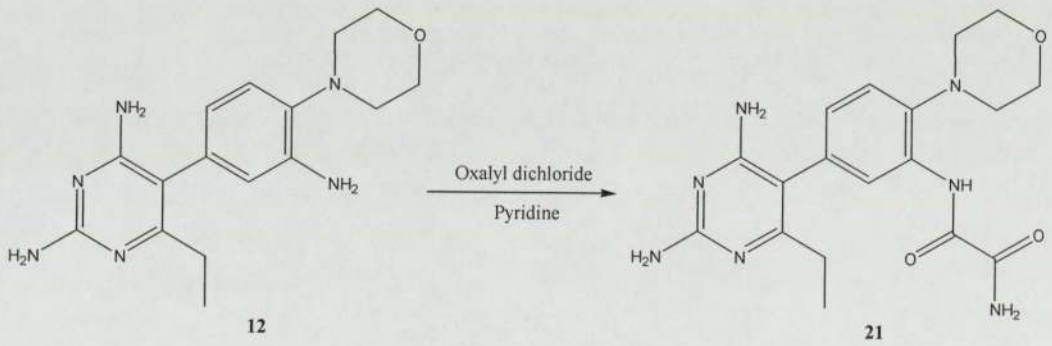
5: R=Cl: **12:** R=$\overline{N(CH_2)_2O(CH_2)_2}$

6: R=Cl: **13:** R=$\overline{N(CH_2)_2O(CH_2)_2}$

Scheme 10 Synthesis of Pyrimethamine amides

3.2.1.7 Synthesis of N-[5-(2,4-Diamino-6-ethyl-pyrimidin-5-yl)-2-morpholin-4-yl-phenyl]-oxalamide (21) (Scheme 11)

N-[5-(2,4-Diamino-6-ethyl-pyrimidin-5-yl)-2-morpholin-4-yl-phenyl]-oxalamide (21). The mixture of pyridine and 5-(3-amino-4-morpholin-4-yl-phenyl)-6-ethyl-pyrimidine-2,4-diamine (**12**) were stirred at room temperature in a round-bottom flask and oxalyl dichloride (2 equivalence) was added into the mixture. After 3 hours, concentrated aqueous ammonia (10 ml) was added into the mixture and kept at room temperature without stirring. The mixture was left overnight. White solid was collected by filtration and dried under vacuum. Yield: <20%.



12 21

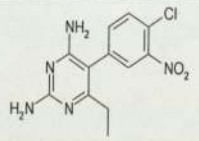
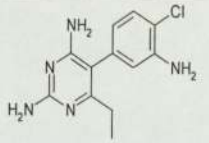
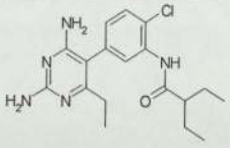
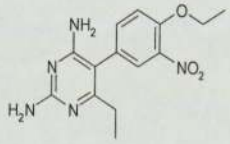
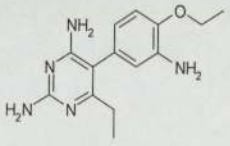
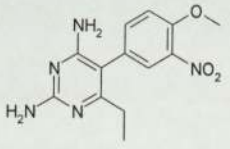
Scheme 11 Synthesis of compound 21

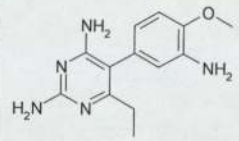
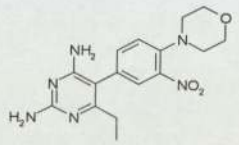
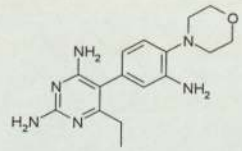
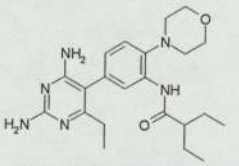
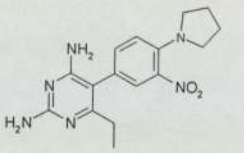
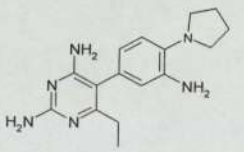
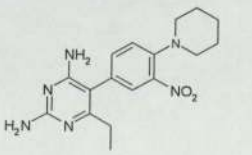
3.2.2 General methods

Proton NMR spectra were obtained on a Bruker AC 250 instrument operating at 250 MHz as solution in d^6-DMSO and referenced from δDMSO=2.50 ppm. Infrared spectra were recorded as KBr discs on a Mattson 3000 FT-IR spectrophotometer. Atmospheric pressure chemical ionization mass spectrometry (APCI-MS) was carried out on a Hewlett-Packard 5989B quadrupole instrument connected to an electrospray 59987A unit with an QPCI accessory and automatic injection using a Hewlett-Packard 1100 series autosampler. Melting points were obtained using a Reichert-Jung Thermo Galen hot stage microscope and corrected. TLC systems for routine monitoring of reaction mixtures and confirming the homogeneity of analytical samples employed Kieselgel 60F254 (0.25 mm) with 20% methanol in ethyl acetate as developing solvent.

3.2.3 Results

Table 5 Compounds data: Structure, Chemical Name & Melting Point (MP)

Compound	Structure	Chemical name	MP (°C)
4	 Molecular Weight =293.71 Molecular Formula =C12H12ClN5O2	2,4-diamino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidine	234-235
5	 Molecular Weight =263.73 Molecular Formula =C12H14ClN5	2,4-diamino-5-(3-amino-4-chlorophenyl)-6-ethylpyrimidine	219-220
6	 Molecular Weight =361.88 Molecular Formula =C18H24ClN5O	N-[2-Chloro-5-(2,4-diamino-6-ethyl-pyrimidin-5-yl)-phenyl]-2-ethyl-butyramide	224-226
7	 Molecular Weight =303.32 Molecular Formula =C14H17N5O3	2,4-diamino-5-(4-ethoxy-3-nitrophenyl)-6-ethylpyrimidine	263-265
8	 Molecular Weight =273.34 Molecular Formula =C14H19N5O	2,4-diamino-5-(3-amino-4-ethoxyphenyl)-6-ethylpyrimidine	176-177
9	 Molecular Weight =289.30 Molecular Formula =C13H15N5O3	2,4-diamino-6-ethyl-5-(4-methoxy-3-nitrophenyl)pyrimidine	277-279

10	 Molecular Weight =259.33 Molecular Formula =C14H19N4O	2,4-diamino-5-(3-amino-4-methoxyphenyl)-6-ethylpyrimidine	264-265
11	 Molecular Weight =344.38 Molecular Formula =C16H20N6O3	6-ethyl-5-(4-morpholin-4-yl-3-nitro-phenyl)-pyrimidine-2,4-diamine	246-248
12	 Molecular Weight =314.39 Molecular Formula =C16H22N6O	5-(3-amino-4-morpholin-4-yl-phenyl)-6-ethyl-pyrimidine-2,4-diamine	235-237
13	 Molecular Weight =412.54 Molecular Formula =C22H32N6O2	N-[5-(2,4-Diamino-6-ethyl-pyrimidin-5-yl)-2-morpholin-4-yl-phenyl]-2-ethyl-butyramide	257-259
14	 Molecular Weight =328.38 Molecular Formula =C16H20N6O2	6-ethyl-5-(3-nitro-4-pyrrolidin-1-yl-phenyl)-pyrimidine-2,4-diamine	233-234
15	 Molecular Weight =298.41 Molecular Formula =C17H24N5	5-(3-amino-4-pyrrolidin-1-yl-phenyl)-6-ethyl-pyrimidine-2,4-diamine	208-210
16	 Molecular Weight =342.40 Molecular Formula =C17H22N6O2	2,4-diamino-6-ethyl-5-(3-nitro-4-piperidinophenyl)pyrimidine	214-216

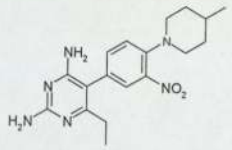
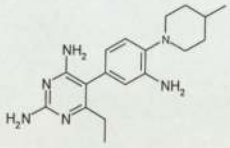
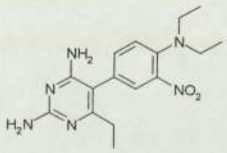
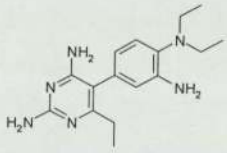
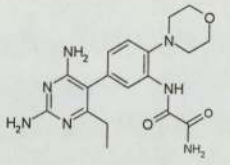
17	 Molecular Weight =356.43 Molecular Formula =C18H24N6O2	6-Ethyl-5-[4-(4-methyl-piperidin-1-yl)-3-nitro-phenyl]-pyrimidine-2,4-diamine	241-242
18	 Molecular Weight =326.45 Molecular Formula =C18H26N6	5-[3-Amino-4-(4-methyl-piperidin-1-yl)-phenyl]-6-ethyl-pyrimidine-2,4-diamine	178-180
19	 Molecular Weight =330.39 Molecular Formula =C16H22N6O2	5-(4-Diethylamino-3-nitro-phenyl)-6-ethyl-pyrimidine-2,4-diamine	170-172
20	 Molecular Weight =300.43 Molecular Formula =C17H26N5	5-(3-Amino-4-diethylamino-phenyl)-6-ethyl-pyrimidine-2,4-diamine	174-176
21	 Molecular Weight =385.43 Molecular Formula =C18H23N7O3	N-[5-(2,4-Diamino-6-ethyl-pyrimidin-5-yl)-2-morpholin-4-yl-phenyl]-oxalamide	275-277

Table 6 Compounds data: NMR & MS

Compound	NMR (ppm)	MS APCI$^+$, m/z=$(M+H)^+$
4	0.96 (t, 3H, J=7.5 Hz, CH_3), 2.11 (q, 2H, J=7.5 Hz, CH_2), 5.85 (s, 2H, NH_2), 5.98 (s, 2H, NH_2), 7.49 (dd, 1H, J=8.2 Hz, Ar-H), 7.77 (d, 1H, J=8.3, Ar-H), 7.85 (d, 1H, Ar-H)	294
5	0.97 (t, 3H, J=7.5 Hz, CH_3), 2.13 (q, 2H, J=7.5 Hz, CH_2), 5.35 (s, 2H, NH_2), 5.56 (br, 2H, NH_2), 5.86 (s, 2H, NH_2), 6.33 (dd, 1H, J=8.0 Hz, Ar-H), 6.60 (d, 1H, Ar-H), 7.21 (d, 1H, J=8.2 Hz, Ar-H)	264
6	0.76 - 1.00 (overlapping m, 9H, CH_3), 1.38 - 1.61 (overlapping m, 6H, CH_2), 2.14 (q, 2H, J=7.5, CH_2), 5.61 (br, 2H, NH_2), 5.92 (br, 2H, NH_2), 6.99 (dd, 1H, J=8.8 Hz, Ar-H), 7.43 (d, 1H, Ar-H), 7.51 (d, 1H, J=8.2 Hz, Ar-H), 9.51 (s, 1H, NH)	362
7	0.96 (t, 3H, J=7.5 Hz, CH_3), 1.35 (t, 3H, J=6.7 Hz, CH_3), 2.10 (q, 2H, J=7.5 Hz, CH_2), 4.23 (q, 2H, J=6.9, CH_2), 5.71 (br, 2H, NH_2), 5.89 (br, 2H, NH_2), 7.30 - 7.45 (overlapping q, 2H, Ar-H), 7.61 (s, 1H, Ar-H)	304
8	1.00 (t, 3H, J=7.4 Hz, CH_3), 1.35 (t, 3H, J=6.9 Hz, CH_3), 2.17 (q, 2H, J=7.5 Hz, CH_2), 4.01 (q, 2H, J=5.1 Hz, CH_2), 4.75 (br, 2H, NH_2), 5.91 (br, 2H, NH_2), 6.2 - 6.34 (overlapping m, 3H, NH_2 and Ar-H), 7.30 - 7.45 (overlapping q, 2H, Ar-H), 7.61 (s, 1H, Ar-H)	274
9	0.96 (t, 3H, J=7.5 Hz, CH_3), 2.15 (q, 2H, J=7.5 Hz, CH_2), 3.95 (s, 3H, CH_3), 5.69 (br, 2H, NH_2), 5.89 (br, 2H, NH_2), 7.37 - 7.47 (overlapping m, 2H, Ar-H), 7.61 (d, 1H, Ar-H)	290

10	0.97 (t, 3H, J=7.5 Hz, CH_3), 2.11 (q, 2H, J=7.5 Hz, CH_2), 3.78 (s, 3H, CH_3), 6.32 (dd, 1H, J=8.0 Hz, Ar-H), 6.45 (d, 1H, Ar-H), 6.84 (d, 1H, J=8.2 Hz, Ar-H)	260
11	0.99 (t, 3H, J=7.5 Hz, CH_3), 2.12 (q, 2H, J=7.5 Hz, CH_2), 3.03 (t, 4H, J=4.4 Hz, CH_2), 3.72 (t, 4H, J=4.3 Hz, CH_2), 5.70 (br, 2H, NH_2), 5.90 (br, 2H, NH_2), 7.32 - 7.42 (overlapping m, 2H, Ar-H), 7.59 (d, 1H, Ar-H)	345
12	0.98 (t, 3H, J=7.5 Hz, CH_3), 2.15 (q, 2H, J=7.5 Hz, CH_2), 2.83 (t, 4H, J=4.4, CH_2), 3.76 (t, 4H, J=4.3, CH_2), 4.83 (br, 2H, NH_2), 5.33 (br, 2H, NH_2), 5.77 (br, 2H, NH_2), 6.36 (d, 1H, J=7.9, Ar-H), 6.51 (d, 1H, Ar-H), 6.93 (d, 1H, J=8.0, Ar-H)	315
13	0.83 - 1.01 (overlapping m, 9H, CH_3), 1.41 - 1.59 (overlapping m, 6H, CH_2), 2.11 (q, 2H, CH_2), 2.86 (t, 4H, J=4.4, CH_2), 3.81 (t, 4H, J=4.3, CH_2), 5.46 (br, 2H, NH_2), 5.86 (br, 2H, NH_2), 6.93 (dd, 1H, Ar-H), 7.21 (d, 1H, J=8.2 Hz, Ar-H), 7.71 (s, 1H, Ar-H), 8.90 (s, 1H, NH)	413
14	0.98 (t, 3H, J=7.5 Hz, CH_3), 1.82 (t, 4H, J=6.9 Hz, CH_2), 2.13 (q, 2H, J=7.4 Hz, CH_2), 3.07 (t, 4H, J=7 Hz, CH_2), 5.63 (br, 2H, NH_2), 5.85 (br, 2H, NH_2), 7.10 (d, 1H, Ar-H), 7.25 (dd, 1H, Ar-H), 7.48 (d, 1H, Ar-H)	329
15	0.98 (t, 3H, J=7.5 Hz, CH_3), 1.86 (s, 4H, CH_2), 2.15 (q, 2H, J=7.4 Hz, CH_2), 3.00 (t, 4H, CH_2), 4.66 (br, 2H, NH_2), 5.29 (br, 2H, NH_2), 5.75 (br, 2H, NH_2), 6.33 (dd, 2H, Ar-H), 6.47 (d, 1H, Ar-H), 6.90 (d, 1H, Ar-H)	299
16	0.96 (t, 3H, J=7.5 Hz, CH_3), 1.61 (overlapping m, 6H, CH_2), 2.10 (q, 2H, J=7.5 Hz, CH_2), 2.95 (overlapping m, 4H, CH_2), 5.67 (br, 2H, NH_2), 5.88 (br, 2H, NH_2), 7.20 - 7.31 (overlapping m, 2H, Ar-H), 7.53 (d, 1H, Ar-H)	343

17	0.95 - 1.72 (overlapping m, 12H, CH, CH_2 and CH_3), 2.12 (q, 2H, J=7.5 Hz, CH_2), 2.82 (t, 4H, CH_2), 5.69 (br, 2H, NH_2), 5.89 (br, 2H, NH_2), 7.27 - 7.36 (overlapping m, 2H, Ar-H), 7.54 (d, 1H, Ar-H)	357
18	0.95 - 1.72 (overlapping m, 12H, CH, CH_2 and CH_3), 2.13 (q, 2H, J=7.5 Hz, CH_2), 3.06 (d, 4H, CH_2), 4.70 (br, 2H, NH_2), 5.29 (br, 2H, NH_2), 5.75 (br, 2H, NH_2), 6.33 (dd, 1H, J=7.9 Hz, Ar-H), 6.47 (d, 1H, Ar-H), 6.90 (d, 1H, Ar-H)	327
19	0.95 - 1.18 (overlapping m, 9H, CH_3), 2.12 (q, 2H, J=7.5 Hz, CH_2), 3.12 (q, 4H, J=7.1 Hz, CH_2), 5.70 (br, 2H, NH_2), 5.89 (br, 2H, NH_2), 7.27 - 7.36 (overlapping m, 2H, Ar-H), 7.48 (d, 1H, Ar-H)	331
20	0.92 - 1.02 (overlapping m, 9H, CH_3), 2.17 (q, 2H, J=7.5 Hz, CH_2), 2.91 (q, 4H, CH_2), 4.79 (br, 2H, NH_2), 5.40 (br, 2H, NH_2), 5.79 (br, 2H, NH_2), 6.35 (d, 1H, J=7.8 Hz, Ar-H), 6.49 (s, 1H, Ar-H), 6.98 (d, 1H, Ar-H)	301
21	0.96 (br, 3H, CH_3), 2.84 (br, 4H, CH_2), 3.76 (br, 4H, CH_2), 5.41 (br, 2H, NH_2), 5.85 (br, 2H, NH_2), 6.9 (br, H, Ar-H), 7.3 (br, 1H, Ar-H), 8.4 (br, 1H, COOH), 10.28 (Br, 1H, NH)	386

Table 7 Compounds data: Yield & IR

Compound	Yield	IR (v/cm-1)
4	34.7 g, 118 mmol, 98 %	3604, 3479, 3318, 3133, 2969, 1632, 1560, 1447, 1343, 1054, 808
5	3.4 g, 12.9 mmol, 85%	3465, 3318, 3166, 1629, 1554, 1437, 812
6	<20%	3492, 3465, 3302, 3145, 3083, 2964, 2933, 2871, 1660, 1625, 1562, 1504, 1438, 1406
7	7.32 g, 24.1 mmol, 71%	3467, 3396, 3320, 3140, 2979, 2939, 1641, 1581, 1140, 1346, 1259, 1162, 1013, 810
8	0.76 g, 2.78 mmol, 67%	3441, 3328, 3174, 2974, 2934, 2874, 1616, 1560, 1437, 1245, 1201
9	0.43 g, 1.49 mmol, 87%	3473, 3448, 3317, 3157, 2970, 2944, 2873, 2844, 2752, 1654, 1635, 1579, 1570, 1545, 1456, 1442, 1344, 1263
10	0.25 g, 0.96 mmol, 59%	3454, 3363, 3313, 3158, 2978, 2933, 1633, 1571, 1434, 1276, 1241, 1016, 810
11	4.3 g, 12.49 mmol, 73%	3779, 3321, 3157, 3108, 2971, 2885, 2854, 2765, 1633, 1576, 1523, 1440, 1343, 1238, 1112, 808

12	3.22 g, 10.23 mmol, 88%	3531, 3424, 3379, 3351, 3319, 3146, 2966, 2861, 2829, 1652, 1588, 1571, 1552, 1458, 1441, 1281, 1227, 1197, 1111
13	<20%	3467, 3412, 3350, 2955, 2838, 1680, 1649, 1605, 1577, 1433, 1114
14	0.35 g, 1.06 mmol, 62%	3471, 3369, 3323, 3153, 2964, 2750, 2457, 1631, 1563, 1576, 1448, 1257, 1182, 877, 812
15	0.3 g, 1.0 mmol, 43%	3579, 3448, 3400, 3315, 3182, 2968, 2935, 2869, 2831, 1620, 1562, 1442, 1321, 1272, 1122, 814
16	0.36 g, 1.06 mmol, 62%	3473, 3317, 3161, 2951, 2840, 2809, 2740, 2632, 2524, 2430, 2154, 1888, 1816, 1631, 1571, 1558, 1450, 1332, 1226, 806
17	1.96 g, 5.51 mmol, 62%	3467, 3319, 3161, 2951, 2921, 2863, 2806, 1631, 1562, 1444, 1344, 1220, 808
18	0.58 g, 1.78 mmol, 56%	3483, 3448, 3384, 3307, 3157, 2946, 2921, 2865, 2802, 2738, 1604, 1560, 1438, 1282, 1203, 1143, 883, 810
19	1.63 g, 4.9 mmol, 76%	3452, 3319, 3108, 2978, 2931, 2875, 1635, 1563, 1558, 1490, 1483, 1442, 1346, 1286, 1230,
20	0.91 g, 3.02 mmol, 63%	3480, 3383, 3320, 3170, 2970, 2931, 2868, 2822, 1607, 1559, 1437, 1280, 1243, 813
21	<20%	3596, 3440, 3346, 3302, 3230, 3176, 2968, 2922, 2860, 2831, 1674, 1633, 1600, 1573, 1438, 1226, 1109

3.3 Toxicity

During the toxicity test, three different donors were used for each compound. This compensates for natural inter-individual variations, which have been seen in previous studies in humans (Coleman et al, 1991). Direct incubation of the human MNLs with the compounds showed that compared with the DMSO control (1.03±1.3%, as Mean±SD, N=16), compound **20** ($P<0.05$), compound **18** ($P<0.01$) and compound **19** ($P<0.01$) caused significant cell death (Table 8 & Figure 6). Other synthesized compounds (**4** to **17**, and **21**) cause no significant direct toxicity.

Table 8 Toxicity data

Compound	Cell death (%)			
	Sample 1	Sample 2	Sample 3	Mean±SD
Pyrimethamine	1.2	3.2	1.6	2±1.06
4	0.7	0.6	4.7	2±2.34
5	1.4	1.5	0.9	1.27±0.32
6	0.8	0.6	0.8	0.73±0.12
7	4	0.9	1	1.97±1.76
8	0.3	0.5	1	0.6±0.36
9	1.4	0.6	1.1	1.03±0.4
10	2.1	1	1.3	1.47±0.57
11	0.5	0.4	1.2	0.7±0.44
12	1	1.4	9.1	3.83±4.57
13	1.9	0.9	7.5	3.43±3.56
14	0.8	0.7	0.8	0.77±0.06
15	0.7	0.7	5.3	2.23±2.66
16	0.4	0.2	0.4	0.33±0.12
17	1.3	5.5	1.2	2.67±2.45
18	100	100	79.3	93.1±11.95
19	100	100	89	96.33±6.35
20	8	4.2	10.5	7.57±3.17
21	0.4	0.4	0.6	0.47±0.12

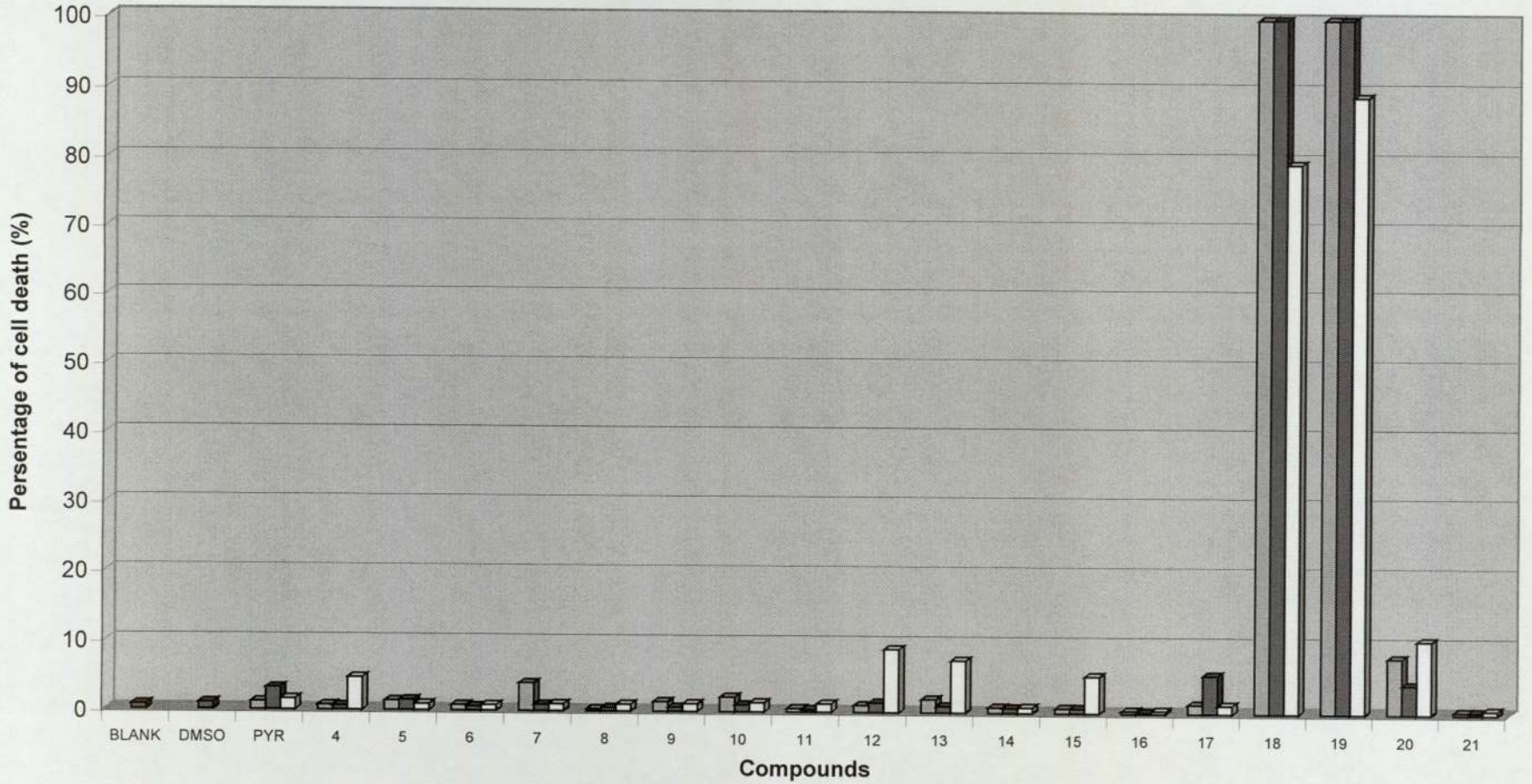


Figure 6 Toxicity chart

3.4 Efficacy

3.4.1 MIC test against *Mycobacterium fortuitum*

The initial MIC test against *Mycobacterium fortuitum* showed that only six compounds gave some bioactivity—compound **18**: 32-64 μg/ml; compounds **6**, **16**, **17**, **19** and **20**: 64-128 μg/ml. Other 14 compounds gave no bioactivity at the concentration of 128 μg/ml (Table 9).

Table 9 MICs against *Mycobacterium fortuitum*

Compound	MIC (μg/ml)	Compound	MIC (μg/ml)
Isoniazid	16-32	**13**	>128
4	>128	**14**	>128
5	>128	**15**	>128
6	64-128	**16**	64-128
7	>128	**17**	64-128
8	>128	**18**	32-64
9	>128	**19**	64-128
10	>128	**20**	64-128
11	>128	**21**	>128
12	>128		

3.4.2 MIC and percentage of inhibition test against *Mycobacterium tuberculosis* (by TAACF)

Primary screening of the *in vitro* evaluation of anti-*Mycobacterium tuberculosis* activity was conducted at 6.25 μg/ml and no synthesised compound (**4** to **21**) showed >90% inhibition in the primary screen. This means all the synthesised compounds had MICs >6.25 μg/ml. However, some compounds showed part inhibition against *M. tuberculosis*—compound **9**: 6%; compound **15**: 7%; compound **8** and **16**: 18%; compound **17**: 27% (Table 10).

Table 10 Percentages of inhibition (% Inh) against *Mycobacterium tuberculosis*

Compound	% Inh.	Compound	% Inh.
4	n/a*	**13**	0
5	n/a	**14**	0
6	0	**15**	7
7	0	**16**	18
8	18	**17**	27
9	6	**18**	n/a
10	0	**19**	n/a
11	0	**20**	n/a
12	0	**21**	n/a

*n/a: not available

Chapter 4. Discussion

4.1 Chemistry

4.1.1 Design of synthetic route

This project is aimed at synthesising and testing of novel low-toxicity anti-mycobacterial compounds, which are likely to act by inhibition of mycobacterial DHFR.

Some compounds were synthesised by a previous worker (Wols, 2000), which were found to be active against *M. fortuitum.* Two of them (compounds **1** & **2**, Figure 7) showed high bioactivity (4-8, 8-16 μg/ml). However, they also were shown to exhibit very high toxicity (74.8±4.71%; 79.47±2.17%) when exposed to human MNLs compared with control values (4.85±0.64%) (Table 3). Glutathione and N-acetyl-L-cysteine could not offer any protection for cells. Although they are very active against *M. fortuitum*, research on those compounds was curtailed due to their high toxicity.

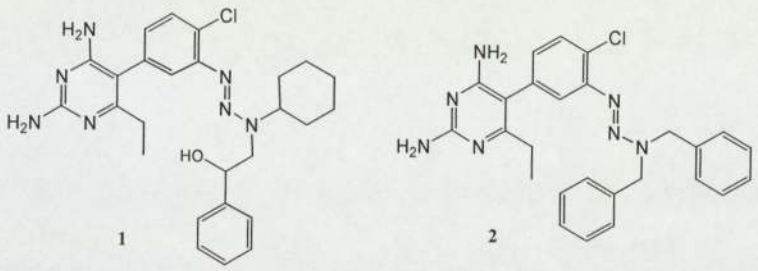
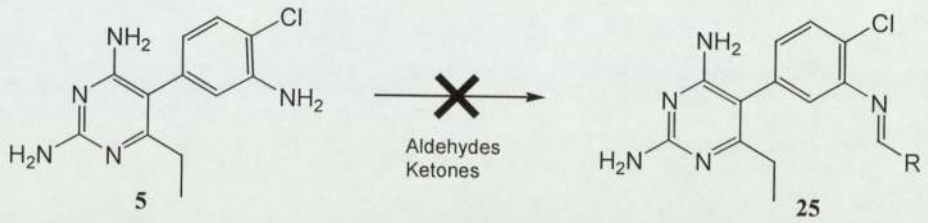


Figure 7 Triazenes with antimycobacterial activity

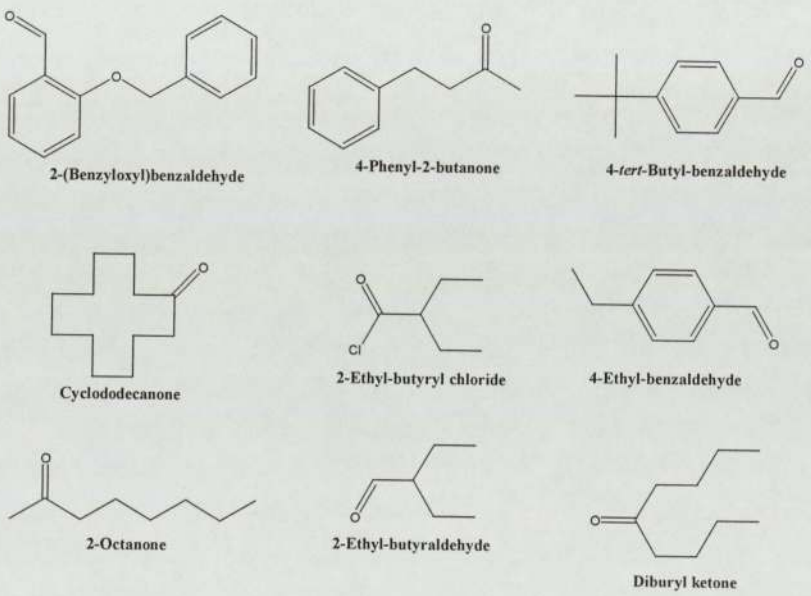
The triazenyl part of the molecule may play a very important role in the mechanism of toxicity. It was reported that they are potential substrates for metabolic dealkylation to mutagenic monoalkyltriazenes (Chan et al., 2002). Therefore, the triazenyl part was replaced by a peptide bond to give a series of analogous compounds with the hope of reducing the toxicity whilst retaining the antimycobacterial activity.

Amino-pyrimethamine (**5**) was used to react with the following aldehydes and ketones (Table 11) in different conditions, in butanol at 129°C and in a bomb at 200°C as well, to form a nitrogen-carbon double bond (**25**) (Scheme 12). But the TLC results indicated that the presence of the starting material in all the products was unavoidable, which means the reaction rate of these reactions were very low. Therefore, this synthetic route had to be discarded and reconsidered.



Scheme 12 Attempted reaction of amino-pyrimethamine with aldehydes and ketones

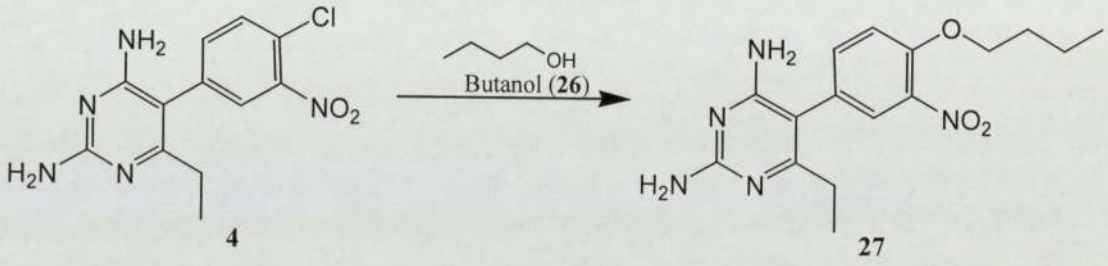
Table 11 Aldehydes, ketones and acid chloride reacted with amino-pyrimethamine



It is possible that the electronegative chlorine atom deactivated the adjacent amino group thus was unable to attach the carbonyl groups of the aldehydes and

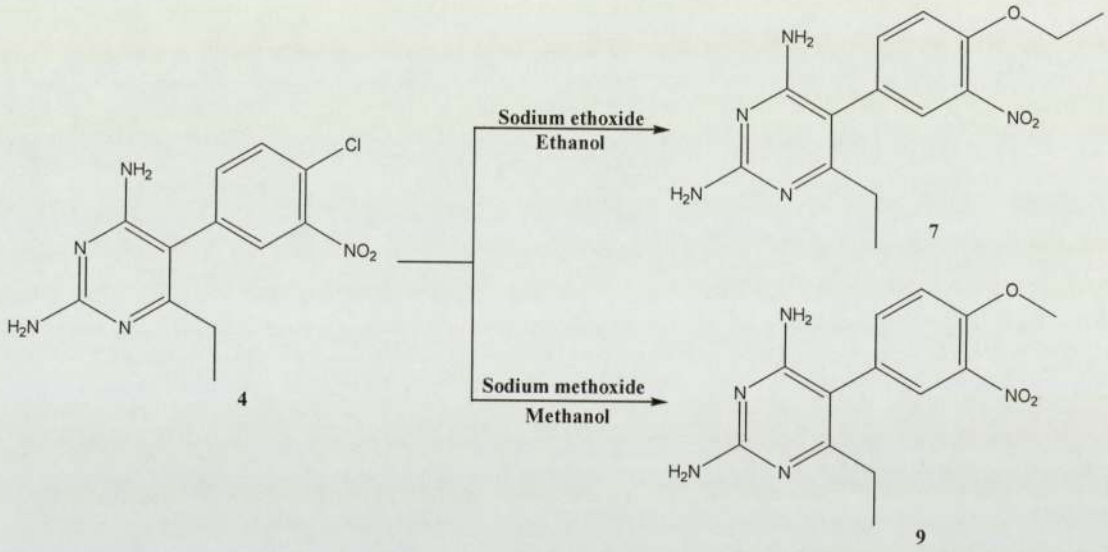
ketones presented. Electropositive groups, amines and -O-R groups, were used to replace the chlorine to make the amino group more active.

Butanol (**26**) was reacted with nitro-pyrimethamine (**4**) (Scheme 13). The reaction was carried out in a 100-ml stainless steel bomb at high temperature and pressure. After 48 hours of reaction, TLC showed that there was still a small amount of starting material not reacted. Column chromatography (15% methanol in ethyl acetate as mobile phase) was used to separate the product (**27**) from the starting material, but NMR showed that the product was still impure. The product of this reaction was not used for further reactions.



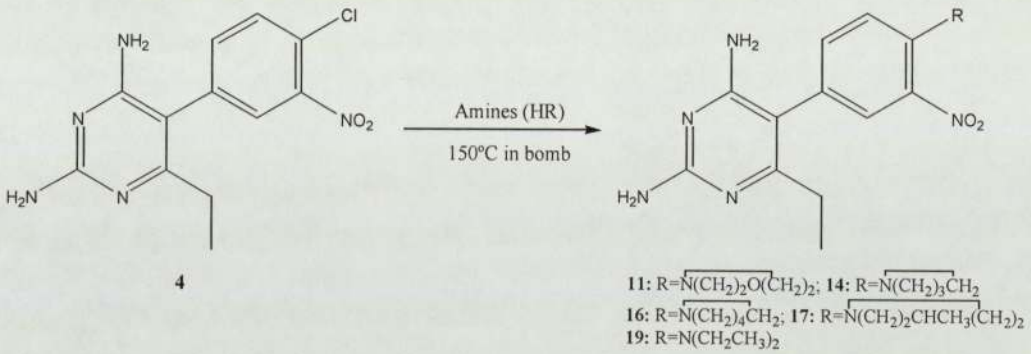
Scheme 13 Nitro-pyrimethamine (4) reacted with butanol (26)

Another two reactions with sodium ethoxide and sodium methoxide were carried out in corresponding alcohol (Scheme 14), and there was no difficulty in separating the product from reaction mixture. Pure products were isolated and verified by NMR.



Scheme 14 Nitro-pyrimethamine (4) reacted with sodium ethoxide and methoxide

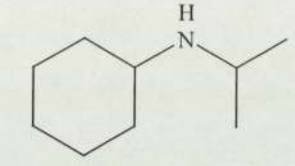
During the synthesis of amine-substituted nitro-pyrimethamines (Scheme 15), several amines (Table 12) were used as starting material. The reactions were generally carried out in a bomb at 120°C using amines as solvent and reactant at the same time. Although most of the amines successfully reacted with nitro-pyrimethamine (**4**), as the products dissolved in corresponding amines, it was very hard to separate the product from its amine solvent.



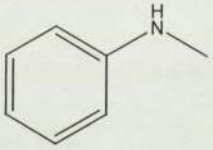
Scheme 15 Synthesis of amine-substituted nitro-pyrimethamines

Among the 11 amines used in the reaction, five amines (diethyl amine, piperidine, 4-methyl piperidine, morpholine and pyrrolidine) were successfully reacted with nitro-pyrimethamine (**4**) and the products were purified to give amine-substituted nitro-pyrimethamines for further reactions.

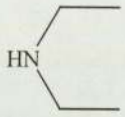
Table 12 Amines reacted with nitro-pyrimethamine



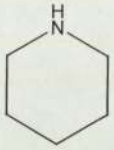
Cyclohexyl-isopropyl-amine



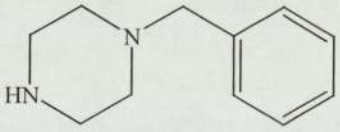
Methyl-phenyl-amine



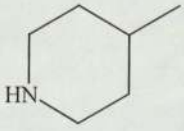
Diethyl-amine



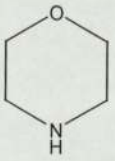
Piperidine



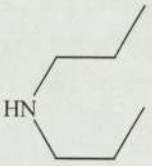
1-Benzyl-piperazine



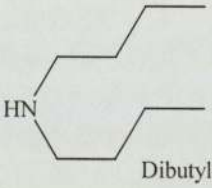
4-Methyl-piperidine



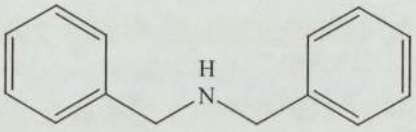
Morpholine



Dipropyl-amine



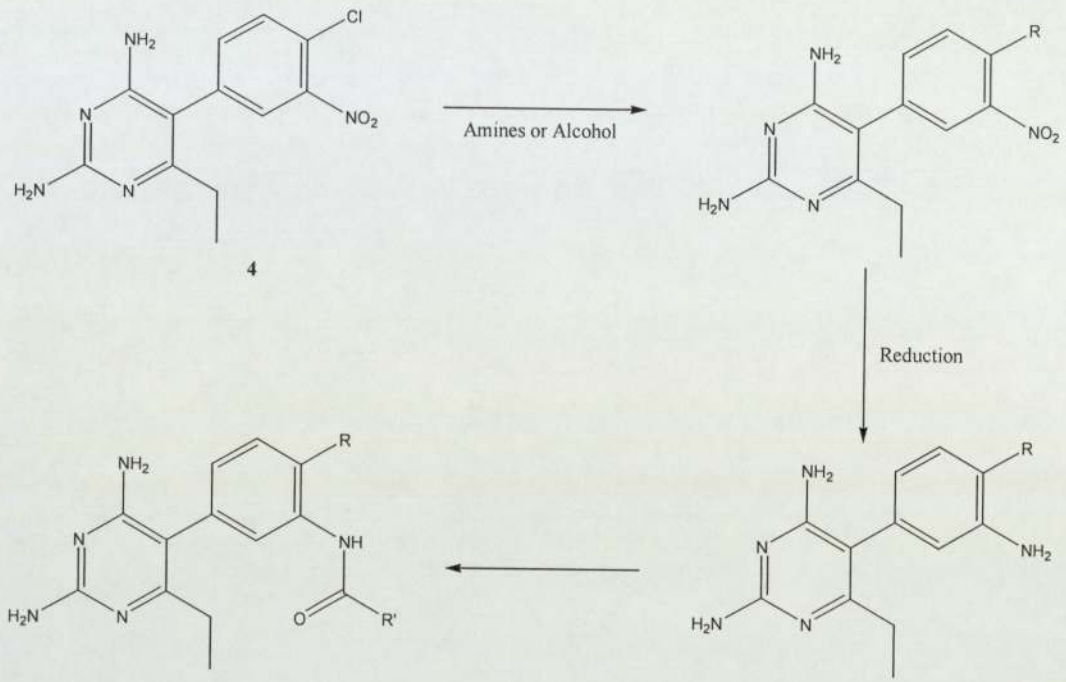
Dibutyl-amine



Dibenzyl-amine

Pyrrolidine

A series of substituted nitro-pyrimethamines (Scheme 16) was synthesised by using the above method, reacting nitro-pyrimethamine (**4**) with amines and alcohols. Then, these products were reduced to corresponding amino-pyrimethamines, which were reacted with different compounds to form amides.



Scheme 16 Synthesis of amides from substituted amino-pyrimethamines

Different conditions were employed during the reaction with amino-pyrimethamines:

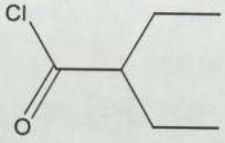
- Different solvents: tetrahydrofuran (THF), ethanol, dimethyl formamide (DMF) and pyridine. Pyridine was the only solvent that dissolved amino-pyrimethamines.

- Different temperature: The reactions in pyridine were carried out at room temperature, 50°C, 80°C and 95°C. The correlation was found to be: the higher the temperature, the more complicated the products would be.

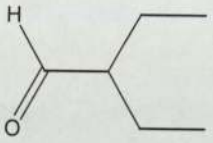
Therefore, all the amide-forming reactions were carried out at room temperature.

- Different reactants: 2-ethyl-butyryl chloride (**28**), 2-ethyl-butyraldehyde (**29**) and diethylcarbamoyl chloride (**30**) (Table 13) were reacted with amino-pyrimethamine to see which reactant is more reactive. According to the duration of reaction and the purity of product, 2-ethyl-butyryl chloride (**28**) was the most reactive one. The other two reactants could not give single product according to TLC and NMR results.

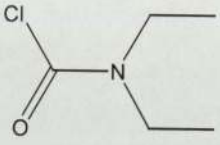
Table 13 Compounds reacted with substituted amino-pyrimethamines to attempt to give amides and imine



2-Ethyl-butyryl chloride (**28**)



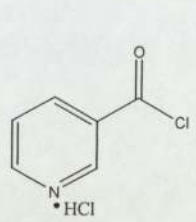
2-Ethyl-butyraldehyde (**29**)

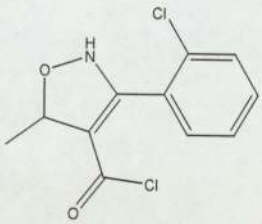


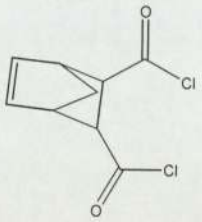
Diethylcarbamoyl chloride (**30**)

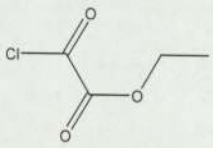
As described above, acid chlorides were chosen to react with substituted amino-pyrimethamines. Total 11 acid chlorides (Table 14) were reacted with amino-pyrimethamine (**5**) and morpholine substituted amino-pyrimethamine (**12**) in pyridine at both room temperature and 95°C, but all acid chlorides reacted with all three amino groups of the compounds due to TLC.

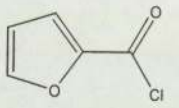
Table 14 Acid chlorides and sulphonyl chlorides reacted with amino-pyrimethamine

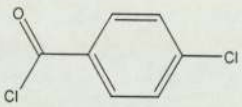

Nicotinoyl chloride

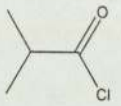

3-(2-Chloro-phenyl)-5-methyl-2,5-dihydro-isoxazole-4-carbonyl chloride

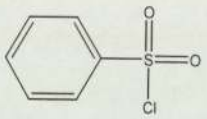

Bicyclo[2.2.1]hept-5-ene-2,3-dicarbonyl dichloride

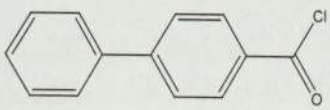

Chloro-oxo-acetic acid ethyl ester

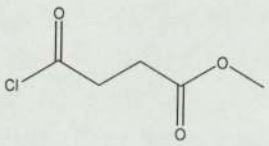

Furan-2-carbonyl chloride

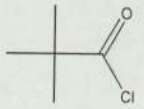

4-Chloro-benzoyl chloride


Isobutyryl chloride


Benzenesulfonyl chloride


Biphenyl-4-carbonyl chloride


3-Chlorocarbonyl-propionic acid methyl ester


2,2-Dimethyl-propionyl chloride

As it was very difficult to prevent the other two amino group from reaction with acid chloride, protective groups were used, BOC-ON and Z-protection, to react with nitro-pyrimethamine. But no matter how much protective compound added, the starting material, nitro-pyrimethamine, could not react totally and the separation of starting material and product with protective groups was difficult.

4.1.2 Summary

It was intended to synthesise a number of amides, which would have nitrogen-carbon single or double bond to reduce the toxicity to the human MNLs, but only

three amides were synthesised after using many different starting materials, such as amines, ketones, aldehydes and acid chlorides, and different conditions.

In summary, there were three main problems faced during the synthesis.

- The first was that the amino group in amino-pyrimethamine (**5**) was very inactive and the chlorine had to be replaced by amines or alcohol to push electron towards amino group to activate it. This prolonged the whole period of chemistry and made the synthesis more complicated.

- The second problem was that substituted amino-pyrimethamines had very poor solubility in general solvents, such as chloroform, ethanol, THF, DMF, toluene and etc. The only one solvent could be used was pyridine, but the solubility was also not good enough for all compounds. That was also one reason why there were substituted eight amino-pyrimethamines synthesised but only two of them reacted with acid chlorides.

- The final problem was that the reaction condition of substituted amino-pyrimethamines with acid chlorides was very difficult to control. The three amino groups in the molecule reacted with acid chloride instead of one. Due to the great similarity of the products' structures, the separation of them by using chromatographic method was impossible.

Finally, eighteen compounds were synthesised (compounds **4** to **21**) and only three of them were amides (Compounds **6**, **13** and **21**). All the compounds synthesised were evaluated against the toxicity and bioactivity.

4.2 Toxicity

Toxicity is an important issue in drug design. If the drug is toxic to humans, it cannot be developed further. Especially in anti-tuberculosis chemotherapy, as the duration of treatment can exceed 12 months, or with MDR-TB perhaps 18-24 months. During this long period, even minor drug toxicity may result in serious adverse effect and the compliance may fall dramatically. In anti-tuberculosis chemotherapy, this will certainly promote MDR-TB.

As compounds **1** & **2** showed high toxicity, a series of compounds were synthesised in the hope of reducing the toxicity whilst retaining the efficacy.

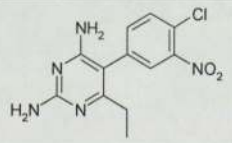
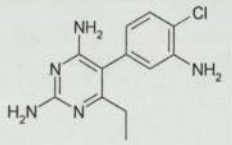
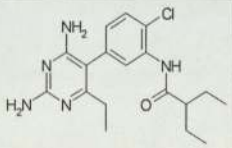
The final solvent concentration of DMSO in the incubations was 1%. Compounds were studied at the concentrations (in DMSO) at 0.5 mM. Although projected effective therapeutic concentrations are likely to be below 50 μM, compounds were studied to their solubility limits, thus maximising the opportunity for toxicity detection.

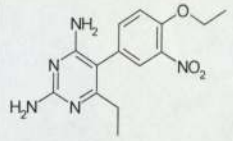
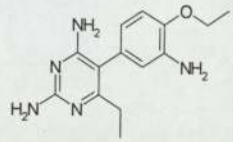
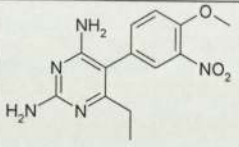
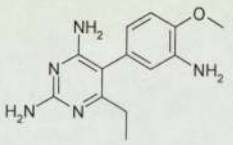
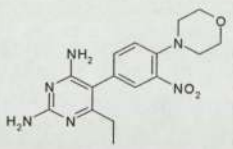
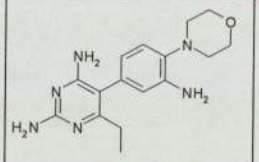
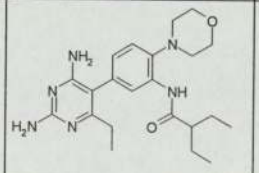
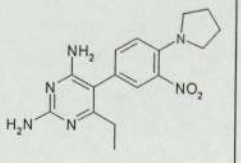
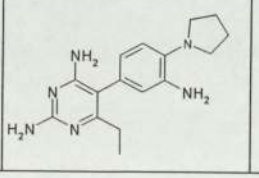
As the cells used in the toxicity assay from three different donors for each compound, we encountered considerable variation in our cytotoxicity assays in

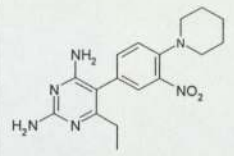
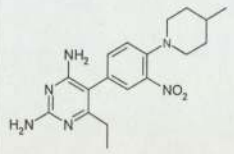
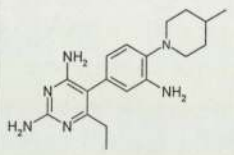
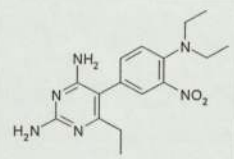
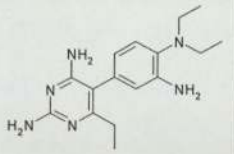
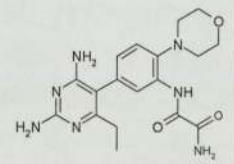
this study. However, the assays were able to distinguish consistently between two almost identical compounds. For example, compound **17** & **18**, the later compound showed high MNL toxicity (93.1±11.95%) compared with the former one (2.67±2.45%). Indeed, in other reports, human MNL toxicity has been shown to be highly sensitive to differing structural analogues (Coleman and Kohl, 1995), even with amidrazone derivatives differing only by a methylene group (Coleman et al., 1999). In addition, our previous studies with MNL have been reproducible and predictive of subsequent *in vivo* toxicity (Coleman et al., 1991; Tingle et al., 1990).

The compound data were summarised in Table 15 for easy reference.

Table 15 Compounds data summary: Compound Number, structure, Toxicity, LogP, MICs and percentage of inhibition (% Inh)

Compound	Structure	Toxicity (% as MEAN±SD)	LogP	MICs (μg/ml)	% Inh
4		2±2.34	1.46	>128	n/a*
5		1.27±0.32	2.34	>128	n/a
6		0.73±0.12	4.11	64-128	0

7		1.97±1.76	1.32	>128	0
8		0.6±0.36	1.99	>128	18
9		1.03±0.4	0.97	>128	6
10		1.47±0.57	1.66	>128	0
11		0.7±0.44	0.52	>128	0
12		3.83±4.57	1.67	>128	0
13		3.43±3.56	3.43	>128	0
14		0.77±0.06	1.51	>128	0
15		2.23±2.66	2.38	>128	7

16		0.33±0.12	1.97	64-128	18
17		2.67±2.45	2.17	64-128	27
18		93.1±11.95	3.13	32-64	n/a
19		96.33±6.35	1.86	64-128	n/a
20		7.57±3.17	2.74	64-128	n/a
21		0.47±0.12	1.16	>128	n/a

4.2.1 Structure-toxicity relationship

From the compound structures and the toxicity data (Table 15), no obvious structure-toxicity relationship was shown.

As can be seen, only compounds **18** & **19** were significantly toxic and compound **20** was toxic ($P<0.05$) compared with DMSO control. There was only one difference in the structures of compounds **17** & **18**, the nitro group in compound

17 was reduced to amino group in compound **18**, but they exhibited completely different results in toxicity test. However, in the case of compounds **19** & **20**, the situation was reversed as the compound with a nitro group (compound **19**) was much more toxic than the one with an amino group (compound **20**).

However, after carefully examining the data, we could reveal some relationships from the data:

1. There were three amides synthesised and none of them showed high toxicity during the screening. Although it was revealed that they were not active against both *M. fortuitum* and *M. tuberculosis,* this is still a partial success in reducing the toxicity of triazenes as the triazenyl part of the structure was replaced with nitrogen carbon-carbon single bond.

2. Among the non-toxic compounds, the 4-position of the benzene ring could be either an electron withdrawing group, such as chloride, or electron donating groups, such as morpholine, tertiary amines and alkoxy groups. This means the molecule could tolerate both electron withdrawing and donating group at the 4-position of benzene ring in toxicity screen.

The lipophilicity (logP) of the compounds (calculated by CS ChemDraw 5.0, Cambridge Soft Corporation) was examined with toxicity data (Table 15), but no obvious relationship could be established (Figure 8).

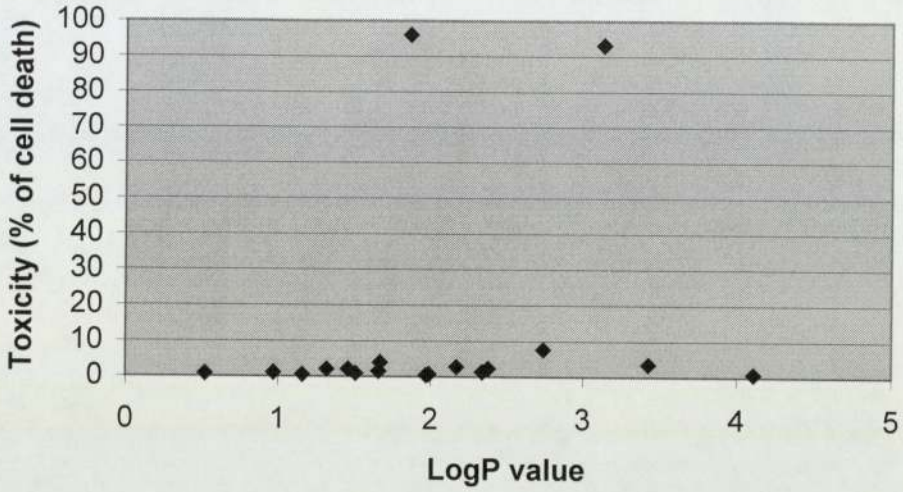


Figure 8 LogP-toxicity relationship chart

4.3 Efficacy

The efficacies of all compounds were evaluated against *M. fortuitum* while some against *M. tuberculosis* (Table 15).

Isoniazid (**24**) was also tested against *M. fortuitum* and the MIC tested was 16-32 μg/ml while the reported MIC of INH is 3 μg/ml (Billington et al., 1998). This may suggest that all the MICs might be lower than the value showed in Table 9.

Just like the toxicity data, no obvious structure-efficacy relationship could be found in both tests against *M. fortuitum* and *M. tuberculosis.*

Compounds **18** & **19** showed high activity and high toxicity, but compound **17** shows relatively high activity in both tests against *M. fortuitum* and *M. tuberculosis* and exhibited no significant toxicity against human MNLs. It seems that ring (in compounds **16**, **17** & **18**) or ring similar structure (in compounds **19** & **20**) at the 4 position of the benzene ring is important in exhibiting efficacy.

As to logP-efficacy relationship (data listed in Table 15), different trends were shown between tests against *M. fortuitum* and *M. tuberculosis.* As shown in Figure 9, the optimum logP value is between 3 and 4 during the MIC test against *M. fortuitum*. However, in percentage on inhibition test against *M. tuberculosis* (Figure 10), the most potent compounds have the logP value at around 2-2.5, which is lower than the result for *M. fortuitum*.

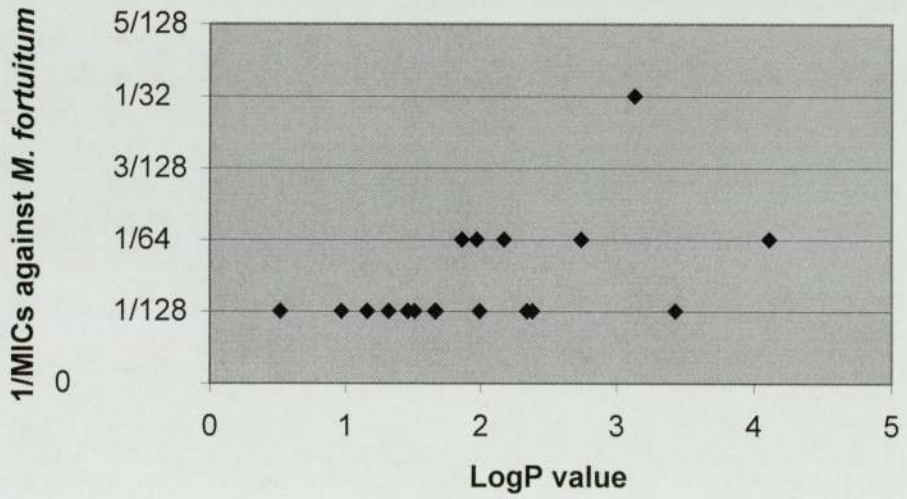


Figure 9 LogP-1/MICs relationship chart against *M. fortuitum*

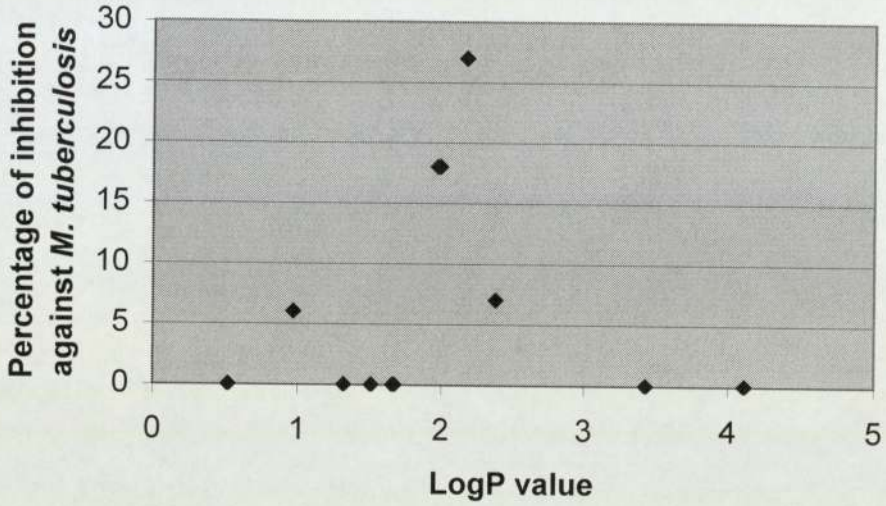
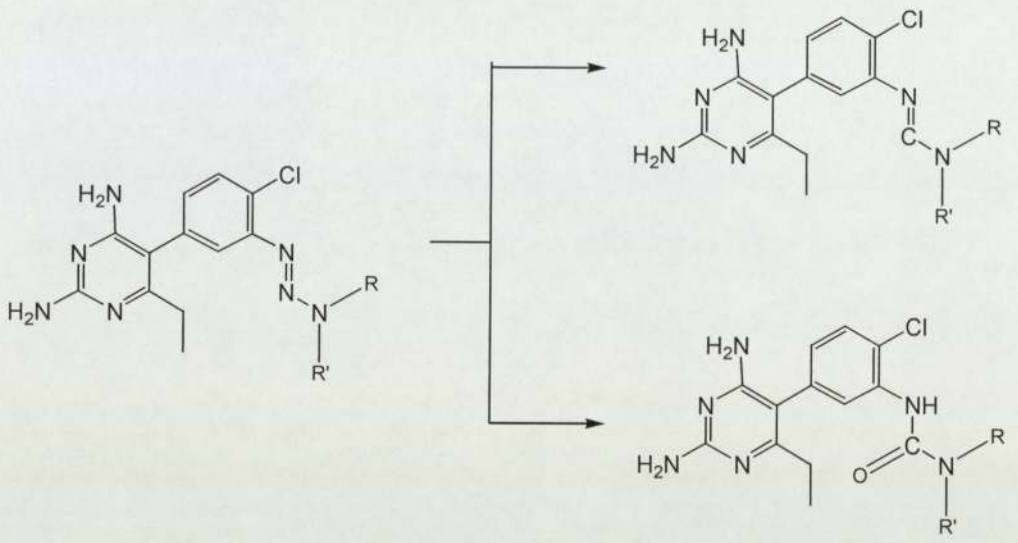


Figure 10 LogP-percentage of inhibition relationship chart against *M. tuberculosis* (by TAACF)

Although all the compounds showed low potency against *M. fortuitum* and *M. tuberculosis*, the toxicity and bioactivity data can be used in further research to give some useful information about structure-toxicity and structure-bioactivity relationship.

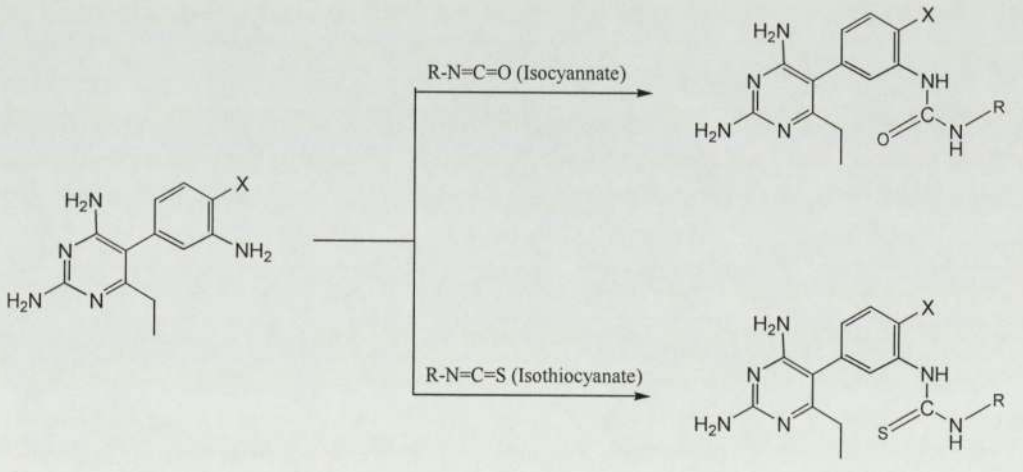
4.4 Future studies

The synthetic route of this project still needs refining to produce more compounds, especially corresponding to the triazenes (Scheme 17). By slightly altering the triazenyl group of compounds **1** & **2** might have a better chance to produce potent anti-mycobacterial agents with low toxicity



Scheme 17 Compounds corresponding to triazenes

Other routes (Scheme 18) could be used in future studies by reacting substituted amino-pyrimethamine with isocyanates or isothiocyanates, which could give more compound and of course more information on toxicity/efficacy-structure relationship.



Scheme 18 Other synthetic routes

Interestingly, the efficacy results against *M. fortuitum* and *M. tuberculosis* were slightly different (see Table 9 MICs against *Mycobacterium fortuitum* and Table 10 Percentages of inhibition (% Inh) against *Mycobacterium tuberculosis*). The logP-efficacy relationships of the screening against these two mycobacteria gave different patterns (see Figure 9 LogP-1/MICs relationship chart against *M. fortuitum* and Figure 10 LogP-percentage of inhibition relationship chart against *M. tuberculosis* (by TAACF)). Therefore, the use of *M. fortuitum* to predict efficacy against *M. tuberculosis* perhaps should be examined.

In summary, with three exceptions, this series of pyrimethamine derivatives showed low toxicity to human MNLs. Although overall efficacy was unsatisfactory, it is a contribution of an ongoing programme. The future studies should be based on the structure of the non-toxic compounds: refining their efficacies whilst retain their low toxicity.

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