Synthesis, Characterization
and Biological Activities of
2-Decylsulfonylacacetamide Derivatives

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Statement of Originality

This work, conducted at the School of Pharmacy, Griffith University, has not previously been submitted for the award of degree or diploma at any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the thesis itself.

Mr. Hsien-Kuo Sun (PhD Candidate)

........................................

Signature
Abstract

The major challenges of *Mycobacterium tuberculosis* treatment are the emergence of multidrug resistant or extensively drug resistant strains and the hepatotoxic adverse drug reactions of current antitubercular drugs. With an incidence of approximately 9.4 million people, tuberculosis is one of the leading causes of death by an infectious agent. 2-Decylsulfonylacetamide represents a novel class of compounds shown to have comparable antitubercular activity to the first-line antitubercular drugs isoniazid and rifampicin.

The aim of this research was to synthesize a library of 2-decylsulfonylacetamide derivatives, substituting the acetamide moiety with a variety of functional groups (methyl ester, carboxylic acid, thioamide and an alkyne); elucidate and characterise synthesised compounds using Nuclear Magnetic Resonance, Mass Spectrometry, Attenuated Total Reflectance Fourier Transform Infrared Red Spectroscopy, Differential Thermal Analysis; determine their *in vitro* antitubercular activity; evaluate and compare their *in vitro* antibacterial (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*) and antifungal (*Candida albicans*) activities; evaluate and compare *in vitro* cytotoxicity profiles using A549 (adenocarcinomic human alveolar basal epithelial cells), HeLa (cervical adenocarcinoma), Jurkat (acute T cell leukemia), LNCap (prostate carcinoma) and MCF-7 (human breast adenocarcinoma) cell lines; evaluate the *in vitro* hepatotoxicity potential of synthesised compounds to select current antitubercular agents; and assess the immunomodulating effects (*in A549* and Jurkat cells) of the synthesised 2-decylsulfonylacetamide and its derivatives using the A549
and Jurkat *in vitro* models in an attempt to characterise their potential effects on the non-specific and specific immune system.

A library of 2-decylsulfonylacetamide derivatives was successfully synthesized using a standard alkylation reaction to couple the decane thiol backbone with the acetamide functional group (yield of 90%). Meta-Chloroperoxybenzoic acid was used to oxidise the sulfide into a sulfone (yield 44%). The methyl ester derivative was synthesised by replacing the 2-bromoacetate with methyl bromoacetate as the starting material (yield 77%). The carboxylic acid derivative was synthesized by hydrolysis of the methyl ester and the alkyne derivative was synthesized by reacting the acid derivative with propargylamine using hydroxybenzotriazole and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide as catalysts (yield 67% and 91%, respectively). The thioamide derivative was synthesized by substitution of the oxygen molecule of 2-decylsulfonylacetamide with a sulfur using the Lawesson’s reagent (yield 62%). Compounds were characterised by nuclear magnetic resonance and mass spectrometry.

This study showed that the acetamide functional group was essential for 2-decylsulfonylacetamide antitubercular activity (MIC 0.73 to 2.92 μM). Substitution with other functional groups significantly reduced antitubercular activity. An interesting observation was that while the acetamide functional group was essential for antitubercular activity, the thioamide functional group enhanced antibacterial activity against *S. aureus* (MIC 8 mg/L). All other derivatives showed only moderate antibacterial activity against *S. aureus* (16 mg/L) and limited activity was observed against *E. coli*, *P. aeruginosa* and *C. albicans* (≥16 mg/L) suggesting that these compounds have a more specific mechanism of action against these organisms.
Only moderate toxicity toward mammalian cells was observed for 2-decylsulfonylacetamide and synthesised derivatives. The thioamide derivative was shown to be the most toxic toward the cell lines examined, demonstrating a significant reduction in cell viability in A549, HeLa, MCF-7 and HepG2 cells ($P < 0.05$; ANOVA). 2-Decylsulfonylacetamide and the carboxylic acid derivative were the least toxic towards A549, HeLa, MCF-7 and HepG2 cells. No reduction in cell viability was observed in LNCaP and Jurkat cells for any of the compounds synthesised, providing further evidence that observed activity was due to specific rather than general toxicity.

This study also showed that the toxic effects of these compounds in HepG2 cells were associated with the production of reactive oxygen species and mitochondrial permeability transition pore opening, leading to apoptosis, autophagy or cellular senescence. Glutathione is an important antioxidant within the cell and plays an important role in the detoxification of xenobiotics. In this study it was shown to have a protective effect against the toxicity of 2-decylsulfonylacetamide and its derivatives. 2-Decylsulfonylacetamide was found to be no more toxic toward HepG2 cells when compared to the current first-line antitubercular drug isoniazid. This agent was shown to be only slightly more toxic than hydrazine, the toxic metabolite of isoniazid, and the second-line antitubercular drug ethionamide.

The synthesised compounds were also evaluated for potential inflammatory and/or immunomodulating effects using A549 and Jurkat cells in an attempt to characterise their effects on non-specific immunity. The objective was to determine their therapeutic potential (stimulation of non-specific and specific immune response), and establishing possible risks for adverse drug reactions. The inflammatory response
was determined by measuring the release of prostaglandin E\textsubscript{2} and leukotriene B\textsubscript{4} by A549 cells, which are frequently used as a model for respiratory epithelial immune response. NF-κB, interleukin-2, interleukin-6 and tumour necrosis factor-α release by Jurkat cells were used as surrogate markers for immune response. The thioamide derivative of 2-decylsulfonylacetamide significantly increased the production of prostaglandin E\textsubscript{2}, while the methyl ester, thioamide and carboxylic acid derivatives increased leukotriene B\textsubscript{4} production in A549 cells. The increase in prostaglandin E\textsubscript{2} has been linked to the induction of apoptosis in infected macrophages, restricting bacterial replication. The apoptotic vesicles vesicles are then taken up by dendritic cells and the bacterial antigens are presented to naïve T cells, leading to activation. The activation of naïve T cells plays an important role in the outcome of tuberculosis infection through either granuloma formation to contain the infection or activation of macrophage killing of intracellular tuberculosis. Hence, it is possible that the induction of prostaglandin E\textsubscript{2} by the thioamide derivative may be of benefit to the host during tuberculosis infection. Furthermore, all the synthesised compounds increased NF-κB (p50 and p65 subunit) in the nucleus of Jurkat cells, indicating immunomodulating effects. Increases in interleukin-2, interleukin-6 and tumour tumour necrosis factor-α production in Jurkat cells were also observed. The induction of T cell activity by the synthesised compounds may potentially influence the disease outcome, hence treatment success. Considering the potential these compounds have in the treatment of tuberculosis and bacterial infection it is of interest to further investigate the immune modulating effect of these compounds. Compounds with moderate antitubercular action and specific immune modulating effects have already shown promise as potential therapeutic agents. This study showed that the difference in inflammatory and immunomodulating properties of 2-decylsulfonylacetamide and synthesised derivatives was dependent on the nature of the hydrophilic functional group.
The findings from this study indicate that the hydrophilic head of 2-decylsulfonylacetamide was an important determinant for biological activity, including antibacterial, antitubercular, mammalian toxicity, inflammatory and immunomodulating properties. Modification of the hydrophilic head significantly altered biological properties. These findings suggest that careful structural modification may allow for optimisation of the desired biological action and reduction of the undesired properties. Of all the synthesised compounds, 2-decylsulfonylacetamide was shown to possess the most potent antitubercular effects and immunomodulating action, whilst displaying least toxicity to mammalian cells. Based on the findings of this study, 2-decylsulfonylacetamide remains the ideal lead compound for further investigation and development as a potential antitubercular agent. Furthermore, the antibacterial properties of the thioamide derivatives also warrant further investigation as a potential novel antibacterial agent in the treatment of drug resistant *S. aureus*. This study provides a successful synthetic strategy for producing numerous derivatives of 2-decylsulfonylacetamide for structural activity relationship studies.
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<thead>
<tr>
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<tbody>
<tr>
<td>2-DSA/DSA</td>
<td>2-decylsulfonylacetamide</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>ADME</td>
<td>absorption, distribution, metabolism and elimination</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<tr>
<td>ADR</td>
<td>adverse drug reaction</td>
</tr>
<tr>
<td>ANT</td>
<td>adenine nucleotide translocator</td>
</tr>
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<td>AST</td>
<td>aminotransferase</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>ATR-FTIR</td>
<td>attenuated total reflectance fourier transformed infrared spectroscopy</td>
</tr>
<tr>
<td>AVO</td>
<td>acidic vesicular organelles</td>
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<td>BCG</td>
<td>Bacillus Camette Guerin</td>
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<td>BSO</td>
<td>buthionine sulfoximine/L-buthionine S, R-sulfoximine</td>
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<td>CMC</td>
<td>critical micellar concentration</td>
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<td>cytochrome P450</td>
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<td>cyclophilin D</td>
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<td>$N,N'$-dicyclohexylcarbodiimide</td>
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<td>death inducing signalling complex</td>
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<td>Dulbecco’s Modified Eagle Medium</td>
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<td>EtOAc</td>
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<td>FADD</td>
<td>Fas-associated death domain</td>
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<td>FAS</td>
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<td>fetal bovine serum</td>
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<td>FDA</td>
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<td>FMOs</td>
<td>flavin-containing monooxygenases</td>
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<td>GM-CSF</td>
<td>granulocyte macrophage colony-stimulating factor</td>
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<td>GSH</td>
<td>glutathione</td>
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<td>HCl</td>
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<td>human immunodeficiency virus</td>
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<td>heme oxygenase-1</td>
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<td>hydroxybenzotriazole</td>
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<td>horseradish peroxidase</td>
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<td>HYD</td>
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<td>IKK</td>
<td>IκB kinase</td>
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<td>INH</td>
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<td>iNOS</td>
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<td>KSase</td>
<td>ketoacyl-acyl-carrier-protein synthase I</td>
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<td>Luria-Bertani</td>
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<td>LDH</td>
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<td>LTA₄</td>
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<td>m-CPBA</td>
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<td>m-CBA</td>
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<td>MDR</td>
<td>multiple-drug resistant/multi-drug resistant</td>
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<td>MeOH</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>MIC</td>
<td>minimum inhibitory concentration</td>
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<td>Mn-SOD</td>
<td>Manganese-dependent superoxide dismutase</td>
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<td>MPT</td>
<td>mitochondrial permeability transition</td>
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<td>MR</td>
<td>mannose receptors</td>
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<td>MTP</td>
<td>mitochondria transmembrane potential</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>MRSA</td>
<td>methicillin-resistant <em>S. aureus</em></td>
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<td>MS</td>
<td>mass spectrometry</td>
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<td>sodium chloride</td>
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<td>NADH/NAD</td>
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<td>nicotinamide adenine dinucleotide phosphate</td>
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<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<td>OPA</td>
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<td>protein 53</td>
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<td>PE</td>
<td>phosphatidylethanolamine</td>
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<td>prostaglandin E&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>Description</td>
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<td>prostaglandin G&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>PGI&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>PMN</td>
<td>polymorphonuclear neutrophil</td>
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<td>PTP</td>
<td>permeability transition pore</td>
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<td>PTH</td>
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<td>rifampicin</td>
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<td>reactive oxygen species</td>
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<td>S.D.</td>
<td>standard deviation</td>
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<td>S.E.M</td>
<td>standard error of the mean</td>
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<td>sulfontransferases</td>
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<td>TEA</td>
<td>triethylamine</td>
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<td>TGA</td>
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<td>Th2</td>
<td>T helper cell type 2</td>
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<td>TCR/BCR</td>
<td>T cell receptor/B-cell receptor</td>
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<td>TLR</td>
<td>toll-like receptors</td>
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<td>TNF-α</td>
<td>tumour necrosis factor-alpha</td>
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<td>Abbreviation</td>
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<tr>
<td>Treg</td>
<td>T regulatory cells</td>
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<td>TXA₂</td>
<td>thromboxane A₂</td>
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<td>UCPs</td>
<td>uncoupling proteins</td>
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<td>VDAC</td>
<td>voltage-dependent anion channel</td>
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<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>XDR</td>
<td>extensively drug-resistant</td>
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Chapter 1. Introduction

1.1 Background

According to the World Health Organisation (WHO) tuberculosis (TB) is one of the leading causes of infectious death worldwide [1]. *Mycobacterium tuberculosis* is an obligate aerobic, slow-growing, non-spore forming and non-motile bacterium [2]. It has a unique cell wall structure containing a high lipid content, which confers resistance to chemical injury, dehydration and certain xenobiotics [3, 4]. Humans are the only natural reservoir for *M. tuberculosis*. Infection causes chronic and persistent systemic illness and is characterised by common symptoms of prolonged cough, chest pain, and hemoptysis, fever, chills, night sweats, appetite loss, weight loss, and easy fatigability. *M. tuberculosis* can invade any tissue or organ in the body [2].

More than ever, there is a clear need for the development of novel agents for the treatment of TB. A 2010 report by the WHO estimated that the worldwide disease incidence of TB was approximately 9.4 million in 2009. Currently, developing countries, which lack adequate healthcare and infrastructure, are the most affected (Table 1) [1]. Malnourishment, smoking, diabetes, alcohol intake and immunocompromised individuals are at greatest risk of contracting TB [5-7]. In 2008, the WHO estimated that *per capita* TB incidences were either stable or falling. However, steadily rising human populations offset any observed decline in incidence *per capita*. Therefore, the overall number of new cases of TB is steadily rising globally [6]. Furthermore, in regions where HIV and TB thrive, the incidence of TB is steadily increasing. It is estimated that 1.7 million people died from TB in 2009, with the
highest mortality rate reported in the African Region (mortality rate of 50 per 100,000 population) [1].

Table 1. Global Incidence, Prevalence and Mortality of TB (adapted from WHO) [1]

<table>
<thead>
<tr>
<th>WHO region</th>
<th>Incidence¹</th>
<th>Prevalence²</th>
<th>Mortality (excl. HIV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. in thousands</td>
<td>% of global total</td>
<td>Rate per 100 000 pop</td>
</tr>
<tr>
<td>Africa</td>
<td>2 800</td>
<td>30%</td>
<td>340</td>
</tr>
<tr>
<td>The Americas</td>
<td>270</td>
<td>2.9%</td>
<td>29</td>
</tr>
<tr>
<td>Eastern Mediterranean</td>
<td>660</td>
<td>7.1%</td>
<td>110</td>
</tr>
<tr>
<td>Europe</td>
<td>429</td>
<td>4.5%</td>
<td>47</td>
</tr>
<tr>
<td>South-East Asia</td>
<td>3 300</td>
<td>35%</td>
<td>180</td>
</tr>
<tr>
<td>Western Pacific</td>
<td>1 900</td>
<td>21%</td>
<td>110</td>
</tr>
<tr>
<td>Global total</td>
<td>9 400</td>
<td>100%</td>
<td>140</td>
</tr>
</tbody>
</table>

¹ Incidence is the number of new cases arising during a defined period.

² Prevalence is the number of cases (new and previously occurring) that exists at a given point in time.

TB infection occurs when an individual inhales droplets containing viable bacteria from an individual with active pulmonary infection. Only 10-30% of individuals exposed to TB contract infection. Of these individuals, 90% will harbor
dormant TB, which forms granulomas. The remaining 10% will develop active TB, with half of these occurring within 2 years. In 50% of cases, active TB will only develop many years after initial infection [8]. Human immunodeficiency virus (HIV) co-infection, however, dramatically increases the risk of activation of latent \textit{M. tuberculosis} from a 5-10% lifetime risk to a 10% annual risk [9]. HIV also increases the risk of direct infection following to re-exposure to active infection [9].

The current first-line antitubercular regimen, termed short-course chemotherapy, involves a two month intensive phase treatment with isoniazid (INH), rifampicin (RMP), ethambutol (EMB) and pyrazinamide (PZA) (Figure 1). This intensive phase is followed by a further four months of INH and RMP [10]. With this regimen, patients become non-infectious after the first few weeks, but treatment is continued in order to eradicate the slow-growing fraction of the bacilli and allow the host immune system to achieve clinical cure. One of the main issues facing this extended treatment regimen is the prevalence of adverse drug reactions (ADR), particularly hepatotoxicity, which may ultimately lead to dose-adjustment, alteration of regimen or termination of treatment. These actions may result in re-establishment of infection and contribute to the development of drug resistance [10]. Although short-course chemotherapy has been highly effective at controlling TB, it is not effective in patients infected with RMP-resistant TB or multi-drug resistant (MDR)-TB [11].
Figure 1. First-line antitubercular drugs (adapted from [11])

INH (1) is a prodrug with a simple structure consisting of a pyridine ring and a hydrazide group. 1 is a highly active agent against of *M. tuberculosis* with a MIC of 0.01-0.25 μg/mL [12]. 1 is active against both active and latent acid-fast bacilli. In addition, 1 retains activity against *M. tuberculosis* in anaerobic conditions [12]. Its exact mechanism of action is still unknown, but it has been shown to produce a number of radicals such as reactive oxygen species (ROS) and reactive organic species [13, 14]. 1 enters the cell via passive diffusion [15] and is activated by the *M. tuberculosis* enzyme KatG, which converts it to isonicotinic acid, reactive organic species (isonicotinic acyl radical or anion, some electrophillic species and acylperoxyl and pyridyl radical adducts), and a range of ROS molecules (superoxide, hydroxyl radicals) [13-18]. These metabolites interfere with numerous metabolic pathways in *M. tuberculosis* [17, 18], leading to accumulation of soluble carbohydrates and phosphate esters, inhibition of mycolic acid synthesis and phospholipid synthesis, and inhibition of
DNA and protein synthesis. Numerous studies have also shown that 1 is able to form adducts with NADH and NAD\(^+\), which in turn disrupts the respiratory chain as NAD is no longer able to function as an electron acceptor, hence interfering with various metabolic functions within \textit{M. tuberculosis} [16]. The isonicotinic acyl-NADH adduct binds tightly to the enoyl-acyl carrier protein reductase, known as InhA, which is involved in the elongation of fatty acids in mycolic acid synthesis [19]. Ketoacyl ACP synthase (KSase) is an enzyme involved in the meromycolate extension of mycolic acid has been shown to bind to 1 through an unknown mechanism [20]. The multi-mechanistic effect of 1 on \textit{M. tuberculosis} renders it a potent antitubercular drug.

2 is a synthetic compound that is similar in structure to D-arabinose and is only active against \textit{M. tuberculosis} (MIC 1.17 \(\mu\)g/mL) and other slow-growing non-tuberculosis mycobacteria [21-23]. This agent’s mechanism of action involves inhibition of arabinosyl transferase, which is involved in the polymerization of arabinose into arabinogalactan, a key component of mycobacterium cell wall [24].

3 is a prodrug that must be converted to the active pyrazinoic acid by bacterial pyrazinamidase (PZase) enzyme. The mechanism of antitubercular action involves the inhibition of fatty acid synthase I (FASI), which catalyzes the sequential condensation of acetyl-coenzyme A and malonyl-CoA to form long chain fatty acids [25]. It is capable of killing persisting tubercule bacilli, effectively shortening treatment time. 3 is only active against \textit{M. tuberculosis}, \textit{M. africanum} and \textit{M. microti} and not against \textit{M. bovis} [23]. Its activity is greatly affected by pH with a MIC of 50 \(\mu\)g/mL at pH 5.5, MIC 100 \(\mu\)g/mL at pH 5.8, and MIC of 200 \(\mu\)g/mL at pH 6.1 [26].
4 is a very potent antitubercular drug with a reported MIC from 0.025 – 2 μg/mL [27, 28]. It has a unique structure with an aromatic nucleus linked on both sides by an aliphatic bridge. Studies have shown that structural change to C-21, C-23, C-8 or C-1 results in decreased microbiological activity, while modification at C-3 does not affect activity. Currently there are three rifamycins (RMP, rifabutin, and rifapentine) in clinical use and all are based on modification at the C-3 position. 4 is active against both active and latent bacilli [29]. Unlike 1, 4 exerts its effect rapidly by binding to and inhibiting DNA-dependent RNA polymerase of mycobacteria [30]. The mycobacteria DNA-dependent RNA polymerase consists of four subunits and rifamycins bind to the β subunit, inhibiting its action and preventing RNA elongation [31]. This action also imparts 4 with activity against Staphylococcus aureus, including methicillin-resistant S. aureus (MRSA) (MIC ≤ 2mg/L) [32-34].

The emergence of MDR-TB renders current first-line drugs ineffective and increases the requirement for second-line drugs, which in general have decreased antitubercular activity and increased toxicities [35, 36].
1.2 Mycobacterium Drug Resistance

TB strains resistant to at least two of the most potent antitubercular drugs (i.e, 1 and 4) are classified as MDR. Drug-resistant TB develops largely due to inconsistent or partial treatment. Factors associated with the development of TB drug resistance include for the following: improper use of medication; failure to implement or complete proper treatment regimens; lack of consistent drug supply; and inadequate or sporadic access to treatments. In addition, poor patient concordance due to complicated regimens and extended treatment duration, contributes to its development [30, 37-39].

Further complicating the issue is the emergence of extensively drug-resistant (XDR) TB strains [37, 40]. Strains resistant to either 1 or 4, any fluoroquinolone and at least one of the three second-line injectable antitubercular drugs (i.e, capreomycin, kanamycin and amikacin) are classified as XDR [35]. Furthermore, TB/HIV co-infected patients are more likely to experience XDR, which is virtually untreatable with currently available TB drugs [37]. This emphasises the clear need for novel antitubercular agent development.

Numerous mechanisms of resistance to first-line antitubercular agents have been identified. Common genes associated with resistance to the main antitubercular agents (1, 2, 3, 4, ethionamide (ETA), prothionamide (PTH) and thiacetazone (TAC) are presented in Table 2.
Table 2. Mechanism antitubercular drug action and resistance in mycobacteria [11, 25, 41-43]

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of action</th>
<th>Common gene markers for resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH (1)</td>
<td>Inhibition of mycolic acid biosynthesis NAD metabolism</td>
<td>katG, inhA, aphC, Ndh</td>
</tr>
<tr>
<td>EMB (2)</td>
<td>Inhibition of arabinogalactan biosynthesis</td>
<td>embB</td>
</tr>
<tr>
<td>PZA (3)</td>
<td>Inhibition of fatty acid biosynthesis</td>
<td>pncA</td>
</tr>
<tr>
<td>RMP (4)</td>
<td>Inhibition of fatty acid biosynthesis</td>
<td>rpoB</td>
</tr>
<tr>
<td>ETA (5)</td>
<td>Inhibition of mycolic acid biosynthesis</td>
<td>ethA, inhA</td>
</tr>
<tr>
<td>PTH (6)</td>
<td>Inhibition of mycolic acid biosynthesis</td>
<td>ethA, inhA</td>
</tr>
<tr>
<td>TAC (7)</td>
<td>Inhibition of cyclopropanation in mycolic acid</td>
<td>ethA</td>
</tr>
</tbody>
</table>

*M. tuberculosis* resistance to 1 is linked to mutations in katG, inhA, kasA and ndh. The mutation of the katG gene is involved in the activation of 1 and is the most common cause of resistance, responsible for between 40 and 95% of 1 resistant clinical isolates [44, 45]. Mutation in katG315 is potentially favoured by the organism as it decreases the activation of 1 without the loss of catalase-peroxidase activity [46, 47]. *M. tuberculosis* has been shown to overexpress the ahpC gene to compensate for katG mutation, but the exact role ahpC plays in *M. tuberculosis* is currently unknown [48, 49]. *M. tuberculosis* is able to overcome the inhibition of InhA by overexpressing it [50]. The ndh gene was reported to encode a NADH dehydrogenase in *M. tuberculosis* and mutation in this gene alters the NADH/NAD ratio sufficiently to protect the organism from 1-mediated toxicity [51]. There are at least 16 other reported genes whose mutations have been associated with the resistance to 1. The mechanism by which these genes confer resistance still remains unclear [52].
The *M. tuberculosis* arabinosyl transferase targeted by 2 is encoded by three genes, namely: embC; embA; and embB. Mutation in the embABC gene has been shown to confer *M. tuberculosis* resistance to 2 [53]. Telenti *et al* [53] have shown that mutation of the embB codon 306 is present in approximately 50% of 2-resistant clinical isolates.

Resistance to 3 occurs in *M. tuberculosis* isolates that are defective in PZase activity. As PZase is not an essential enzyme to *M. tuberculosis*, it inherently displays a diverse range of mutations across the gene. This is suggested to be due to the lack of selective pressure on the gene product [54]. Various studies have identified that mutation in pncA plays a key role in the resistance to 3 [55-57]. Common mutations have been found in pncA 3-17, 61-85 and 132-142 region of the gene codon [58].

*M. tuberculosis* resistance to 4 occurs through a single mutation of the RNA polymerase β subunit gene (rpoB), preventing 4 binding to and inhibiting it. It is estimated that 95% of RMP-resistant *M. tuberculosis* carry this mutation [59, 60].

Due to the increased prevalence of MDR-TB, there is greater reliance on second-line drugs, which in general, are less effective and/or exhibit greater toxicities [36]. Currently, available second-line drugs include: thioamides, D-cycloserine, fluoroquinolones, riminphenazines, capreomycin, kanamycin and aminosalicylic acid [11, 35]. Currently there is a rise in drug resistance to second-line agents. There is the emergence of XDR-TB and even the more difficult to treat totally drug-resistant strain (TDR-TB) [61]. TDR-TB is defined as MDR-TB that is resistant to all first- and
second-line antitubercular drugs [61]. Cases of TDR-TB were first reported in Iran in patients infected with MDR-TB strains. Complicating the issue is that the Iranian report showed that none of the TDR-TB patients had been treated with a second-line antitubercular agent. It is currently unknown what gave rise to the TDR-TB strains in Iran [61]. With the reported emergence of TDR-TB, there is an increasing urgency to develop new drugs for the treatment of TB infection.

At this stage, both novel drug classes and novel drugs in existing antitubercular classes would be welcomed by the medical profession. Of particular relevance to this study are the highly active thioamide derivatives. The amide functional group of the compound of interest in this study could be easily modified to synthesize novel thioamide-based antitubercular compounds.

### 1.3 Thioamides

Thioamide drugs are an important class of second-line antitubercular agents that have been successfully used to treat MDR-TB [35]. This class includes ETA (5), PTH (6), and TAC (7) [62] (see figure 2).
5 and 6 are prodrugs that are structurally similar to 1, and need to be activated in order to be metabolically active. While 1 is activated by KatG, 5 and 6 are activated by the mycobacterial mono-oxygenase enzyme EthA [42, 43]. EthA mutation confers resistance to all thioamides as they are all activated by the mycobacterium enzyme EthA [42]. Similar to 1, both 5 and 6 exert their antitubercular effect on InhA thereby decreasing synthesis of the mycolic acid components of tuberculosis bacterial cell wall [42]. It exerts this effect by forming adducts with NAD, which subsequently bind and inhibit the action of InhA [43].

Like 5 and 6, 7 is also a prodrug that is activated by EthA [63]. Although its precise mechanism remains unknown, it appears to decrease cyclopropanation in mycolic acid [63]. Mycolic acid cyclopropanation is essential for viability, drug resistance, and cell wall integrity of M. tuberculosis [64]. Due to its low cost, 7 is
widely used in combination with 1 or 4 in Africa and South America, despite its known ADRs [63, 65, 66], such as gastrointestinal disturbances, hepatotoxicity and dermal hypersensitivity that may result in life-threatening skin reactions [36].

The available second-line drugs remain undesirable due to their poor activity or inherent toxicity, such as nausea, loss of appetite, abdominal pain, gastrointestinal disturbances, hepatotoxicity and life-threatening skin reactions [36]. Furthermore, the emergence of MDR- and XDR-TB has rendered second-line drugs such as 5 ineffective in some patients [42]. This once again highlights the significant need for the development of novel antitubercular compounds with unique mechanisms of action. Ideally these agents should retain activity against MDR-TB and latent disease, and have reduced host toxicity compared to existing first- and second-line antitubercular drugs [67].

1.4 β-sulfonylacetamide derivatives

β-sulfonylacetamides, also termed β-sulfonylcarboxamides, represent a novel class of antitubercular compounds, which prevent growth of mycobacterial cells, involved in the synthesis of α-substituted, β-hydroxyl fatty acids (i.e. corynemycolic acid, nocardic acid and mycolic acid). A study by Jones et al. [27] examined the effect of varying the functional groups around the sulfonyl core of these compounds and identified 2-decylsulfonylacetamide (2-DSA; C_{12}H_{25}NO_{3}S) as the most active agent. 2-DSA is also referred to as 2-decylsulfonylethanamide. Various thioamide-based compounds have been shown to have interesting antitubercular, antimicrobial and cytotoxic properties. Despite preliminary reports, little is known about the
pharmacological properties of these compounds. In this study we synthesised novel thioamide derivatives of 2-DSA and investigated their broader actions, including antimicrobial action, cytotoxicity profile, and immune-related effects. In addition, the hepatotoxic potential of these compounds was investigated as this is a severe limiting factor for existing first- and second-line agents.

The lead compound 2-DSA was previously shown to have \textit{in vitro} activity comparable to the first-line antitubercular agents, 1, 2, 3 and 4. [27] The antitubercular activity of 2-DSA was also significantly higher than the second-line agents streptomycin and thiolactomycin [27]. Importantly, 2-DSA was shown to retain activity against MDR-TB [27, 67-69]. Given the low success rate of new compounds reaching clinical use (approximately 5%) there remains a significant need for the development of new antitubercular drugs [67]. Based on preliminary evidence on the potent \textit{in vitro} antitubercular activity, derivatives of 2-DSA may fulfill these requirements.

\subsection{1.4.1 2-DSA}

The structure of 2-DSA (8a) consists of a sulfonyl core with a 10-carbon lipid chain (acyclic aliphatic tail) on one end and an acetamide group on the other (Figure 3).

\begin{center}
\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure3.png}
\caption{Structure of 2-DSA (8a)}
\end{figure}
\end{center}
Structurally, 8a can be classified as an amphiphilic compound. Amphiphiles are compounds comprised of a hydrophilic (water-loving) head and a hydrophobic (water-hating) tail (Figure 4). For 8a, the β-sulfonylacetamide functional group represents the hydrophilic head, while the fatty acid moiety corresponds to the hydrophobic tail of the compound.

![Diagram](https://via.placeholder.com/150)

**Figure 4. General structure of (A) An amphiphile, and (B) 2-DSA (8a)**

Jones *et al.* [27] investigated the influence of modifying the fatty acid chain length on the antitubercular activity of β-decylsulfonylacetamide. Antitubercular activity increased with increasing chain length to a threshold value. The optimal antitubercular activity was between 8 and 10 carbons (Figure 4). Increasing the carbon length beyond 10 yielded poorer antitubercular activity, possibly due to a reduction in aqueous solubility of the compound [27]. This observed trend is similar to the amphiphilic phenomena known as the “cut-off effect”. In this phenomena, the biological activity of amphiphilic compounds increase with increasing carbon chain lengths up to a critical point, after which potency decreases with longer chain lengths [70]. It appears that the 10-carbon aliphatic chain is optimal for antitubercular activity.
2-DSA was shown to have the highest \textit{in vitro} antitubercular activity of all the $\beta$-sulfonylacetamide derivatives tested. An initial study of $8a$ reported a MIC of 0.75 – 1.5 mg/L against the H37Rv clinical-strain of \textit{M. tuberculosis}. This activity was shown to be comparable to the MIC of $1$ (0.1 – 0.4 mg/L), $2$ (2 mg/L), $3$ (100 mg/L), and $4$ (2 mg/mL). In this study, 2-DSA was shown to possess 4 – 8 fold higher activity than $N$-octansulfonylacetamide (OSA), a related compound, against H37Rv [27]. In addition, $8a$ was shown to retain activity against anaerobically adapted latent BCG \textit{in vitro} (MIC 1.5 mg/L to 50 mg/L) [68].

![Figure 5](image-url)  

**Figure 5.** The effect of $\beta$-sulfonylacetamide chain length on \textit{in vitro} antitubercular activity. Adapted from Jones \textit{et al} [27]

Keeping the fatty acid chain length at 10 carbons and modifying the sulfonyl component of $8$ led to a more than 4-fold decrease in the \textit{in vitro} antitubercular activity against H37Rv [27]. While, this would suggest that the sulfonyl functional group is essential for antitubercular action, the influence of structural modification of the
acetamide moiety on 2-DSA antitubercular, biological activity and potential toxicity has not yet been examined and remains unknown.

It is hypothesised that the action of 2-DSA may be related to amphiphilic properties, which would confer broad-ranging toxicity rather than specific effect. Given that these compounds exhibit a “cut-off effect”, similar to amphiphilic compounds, the contribution of the amphiphilic structure to biological activity cannot be excluded. If this is the case, it is possible that these compounds will exhibit broader biological action and potential toxicity. This study therefore investigated the effect of modifying the acetamide moiety, while maintaining the ideal carbon chain length, and made comparisons in relation to their antitubercular effect, biological activity and potential for cytotoxicity.

1.5 Proposed mechanisms of β-sulfonylacetamide antitubercular action

Both 8a and N-octanesulfonylacetamide (OSA) were originally designed as inhibitors of the β-ketoacyl synthase step in fatty acid synthesis (FAS) [27, 67]. β-ketoacyl synthase catalyses FAS reactions in the biosynthesis of fatty acids in mycobacterium [27]. Mycolic acid, which is critical for cell wall formation, is an important fatty acid synthesized by the FAS reaction in the mycobacterium. OSA was originally shown to decrease the presence of mycolic acids in numerous mycobacteria, including *M. bovis, M. bovis* BCG, *M. kansasii*, *M. avium* complex and *M. paratuberculosis*. Interestingly, OSA exhibited no activity against rapid growing mycobacterial species, including *M. smegmatis, M. fortuitum, M. chelonei*, and *M. abscessus*. However, only *M. smegmatis* displayed unaffected mycolic acid production
These results, therefore suggests possible alternate mechanisms of action. A later study by Parrish et al. [67] identified that OSA may directly or indirectly interfere with ATP synthase and possibly other components of the mycobacteria respiratory chain [67]. The proposed inhibition of ATP synthase and other components of the respiratory chain would lead to reduced energy production, interruption of protein and mycolic acid synthesis. It would appear that β-sulfonylacetamide derivatives possess novel mechanisms of antitubercular action, which justifies further investigation into their biological activities.

1.6 Relevance of the amphiphilic structure

Amphiphilic compounds have been investigated for numerous clinical and scientific applications. Compounds with amphiphilic structure have been shown to exert vast biological effects, including: antibacterial; antifungal; antitubercular; antiviral; antiparasitic; ion channel modulation; and cytotoxicity [27, 67-69, 71-75]. In addition, amphiphilic compounds have been used as excipients in pharmaceutical formulations, and cosmetics and toiletries due to their surfactant or detergent properties [76, 77].

As previously outlined, amphiphiles contain a hydrophilic head and hydrophobic tail. Amphiphilic compounds can be divided into four main sub-types depending on the ionic characteristics of the hydrophilic head moiety. Amphiphile sub-types include: anionic, cationic, nonionic and amphoteric (zwitterionic) (Table 3) [77]. Anionic amphiphiles contain a hydrophilic group bearing a negative charge, while cationic amphiphiles contain a hydrophilic group bearing a positive charge. Nonionic amphiphiles have an uncharged hydrophilic group. Amphoteric amphiphiles are those
that contain a hydrophilic head moiety comprised of functional groups carrying both positive and negative charges. In the majority of cases, the presence of charge is dependent on the pH of the environment and the pKa of the amphiphile. An extensive review of published literature on amphiphilic compounds revealed that they possess distinctly different biological activities, toxicities, and physicochemical properties depending on the nature of their hydrophilic head [77-84].

The hydrophobic groups of reported amphiphilic compounds with biological or physicochemical properties of interest vary greatly. Many of these compounds contain long chain hydrocarbons (8-20 carbons), hydrophobic groups of alkybenzenes, alkynaphtalenes, fluoroalkyl groups or polydimethylsiloxanes, amongst others [79].

Table 3. Anionic, cationic, nonionic and amphoteric amphiphiles [77, 79].

<table>
<thead>
<tr>
<th>Head groups</th>
<th>Examples</th>
<th>General Structure (R = hydrophobic tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anionic</strong></td>
<td>Sulfonate</td>
<td>RSO(_3)M(^+)</td>
</tr>
<tr>
<td></td>
<td>Sulfate</td>
<td>ROSO(_3)M(^+)</td>
</tr>
<tr>
<td></td>
<td>Carboxyl</td>
<td>RCOO(^-)M(^+)</td>
</tr>
<tr>
<td><strong>Cationic</strong></td>
<td>Quaternary ammonium halides</td>
<td>R(_2)N(^-)X(^-)</td>
</tr>
<tr>
<td><strong>Nonionic</strong></td>
<td>Polyoxyethylene</td>
<td>-OCH(_2)CH(_2)O-</td>
</tr>
<tr>
<td></td>
<td>Sugars</td>
<td></td>
</tr>
<tr>
<td><strong>Amphoteric</strong></td>
<td>Sulfobetanines</td>
<td>RN(^+)(CH(_3))(_2)CH(_2)SO(_3))</td>
</tr>
</tbody>
</table>

18
Nonionic amphiphiles have been widely investigated as excipients for the delivery of drugs with poor water solubility [85]. The hydrophobic tails of amphiphiles are attracted to the hydrophobic functional groups of drugs, while their hydrophilic heads interact with water, thus encapsulating the compound for delivery [83, 84, 86-91]. When amphiphiles are surrounded by water, their hydrophobic tails face away from water molecules and their hydrophilic heads project toward them. This allows amphiphiles to form aggregates called micelles or larger layered structures, such as lipid bilayers when surrounded by water. Nonionic amphiphiles have been successfully used in formulatory research to enclose a delivery package, such as DNA or drugs.

As a result of their structure, amphiphilic molecules have the ability to dwell in the aqueous medium of living organisms and also interact with the lipid layers of their cell membranes [92]. Although, this may impart biological activity it may also lead to potential membrane toxicity. Cellular toxicity is often reported for cationic amphiphiles [81, 93-95]. The hydrophobic tail of cationic amphiphiles enhances its ability to pass through cell membranes when unionized. However, once the drug molecule is ionized, it remains embedded within the membrane and causes changes to membrane structure that may lead to cellular toxicity [93, 94]. As a consequence, cationic amphiphiles have been widely reported to induce phospholipidosis, the accumulation of phospholipids within cells [81, 93-95]. Amphiphiles with aromatic hydrophobic groups appear to be more toxic [94, 96]. With respect to 8a, it is possible that its amphophilic structure may contribute to antitubercular activity, however, it may also lead to poor selectivity and potential for cellular toxicity. Consequently, the relative selectivity of action and potential for cytotoxicity of 8a and related derivatives must be assessed.
Amphiphilic compounds, depending on their critical micellar concentration (CMC), may produce detergent effects [73, 97]. Detergent effects are associated with cytotoxicity, immunological response and irritant effects. It is possible that 8a and related compounds, based on their amphiphilic structure, may produce similar effects.

1.7 Relevance of the thioamide moiety

The thioamide functional group was identified as a rational substitute for the acetamide moiety of 2-DSA. The thioamide functional group is an important pharmacophore for the antitubercular activity of 5, 6 and 7. In addition, compounds containing this functional group have been shown to possess varying degrees of antibacterial action (spectrum and potency) [62, 98-102]. For example, sulfinemycin (9) is a thioamide with interesting antibacterial properties (Figure 6). 9, isolated from Streptomyces spp., contains a thioamide group coupled with a fatty tail. This agent was shown to possess selective activity against Gram-positive bacteria, including S. aureus [100]. Given the structural similarity of 8a to 9, it is possible that β-sulfonylacetamide compounds may possess similar unreported activity. In addition, substitution of the acetamide functional group of 8a with a thioamide moiety may impart improved activity against S. aureus.

Figure 6. Structure of sulfinemycin (9), a novel thioamide antibacterial [100]
1.8 Hypothesis, Aim and Objectives

8, a β-sulfonylacetamide, has been identified as a novel antitubercular drug with activity comparable to existing first- and second-line agents. Research thus far has focussed on the *in vitro* antitubercular activities of 8a and related β-sulfonylacetamide derivatives. The effect of modifying the sulfonyl functional group and fatty aliphatic chain on *in vitro* antitubercular activity has been reported [27]. Both the sulfonyl moiety and 10-carbon aliphatic chain were proven optimal for antitubercular activity [27, 67-69]. The effect of modifying the acetamide functional group, however, has not previously been reported [27].

Based on 8a’s amphiphilic structure, it is possible that it and its related structural derivatives may have other biological activities of interest. To date, there are no published studies on the biological selectivity of 8a and related compounds. In addition, there are no comparative investigations on the *in vitro* toxicity of 8a and derivatives to existing first- or second-line antitubercular drugs.

In this study, a library of 8a homologs was synthesized by modifying the acetamide moiety and keeping the 10-carbon aliphatic chain and sulfonyl functional group constant. This study hypothesised that the modification of 8a’s acetamide moiety would influence its selectivity of biological action and potential for host cellular toxicity and immunological response in the treatment of TB infection.
The aims and objectives of this study were as follows:

- Explore and optimise methods for the chemical synthesis of 8a and novel derivatives. In particular, substitution of the acetamide functional group of 8a with a thioamide moiety was considered rational. Additional substitutes for the acetamide moiety included the methyl ester, carboxylic acid, and alkyne functional groups. This aim was achieved through the sequential and rational modification of the reported chemical synthetic pathway for 8a. Reaction progress was monitored through thin-layered chromatography and synthesized compounds were fractioned and purified by column chromatography;

- Elucidate and characterise the structures of synthesised 8a and novel derivatives. This aim was achieved by Nuclear Magnetic Resonance (NMR), Mass Spectrometry (MS), Attenuated Total Reflectance Fourier Transform Infrared Red Spectroscopy (ATR-FTIR), and Differential Thermal Analysis (DTA);

- Determine the in vitro antitubercular activity of synthesised 8a and novel derivatives. This aim was achieved by evaluating the activity of synthesised compounds against the M. tuberculosis H37Ra clinical strain in a modified in vitro microbroth dilution assay;

- Evaluate and compare the in vitro antibacterial and antifungal activities of synthesised 8a and novel derivatives. This aim was achieved by evaluating the activities of synthesised compounds against S. aureus, E. coli, P. aeruginosa, and C. albicans using a modified microbroth dilution in vitro assay;

- Evaluate and compare the in vitro cytotoxicity profile of synthesised 8a and novel derivatives. Cytotoxicity against A549 (adenocarcinomic human alveolar basal epithelial cells), HeLa (cervical adenocarcinoma), Jurkat (acute T cell leukemia), LNCaP (prostate carcinoma) and MCF-7 (human breast adenocarcinoma) cell lines
was assessed using the resazurin viability assay and sulforhodamine B toxicity assays;

- Evaluate the *in vitro* hepatotoxicity of synthesised $8a$ and novel derivatives. This aim was achieved by evaluating the physiological and biochemical response of the HepG2 (hepatoma) cell line to $8a$ and novel derivatives. Comparisons were made to $1$, hydrazine, and $5$;

- Assess the immunomodulating effects of synthesised $8a$ and novel derivatives. Cellular responses to $8a$ and novel derivatives were assessed *in vitro* using A549 (model for epithelial response) and Jurkat (model for T-cell response) cell lines. Prostaglandin E$_2$ (PGE$_2$), leukotriene B$_4$ (LTB$_4$), tumour necrosis factor alpha (TNF-$\alpha$), NF-$\kappa$B, interleukin-2 (IL-2) and interleukin-6 (IL-6) responses were quantified by enzyme-linked immunosorbent assays (ELISAs).
Chapter 2. Synthesis and structural elucidation of 2-DSA and derivatives

2.1 Background

2-DSA (8a) represents a novel class of potential antitubercular compounds. Structurally it can also be classified as an amphiphile, with a hydrophobic fatty 10-carbon aliphatic tail and a hydrophilic head comprised of a sulfonyl acetamide moiety. Alteration of the aliphatic tail and sulfonyl component was previously shown to decrease antitubercular activity [27]. No study has reported on the biological effects associated with modifying the acetamide component of this class of drug.

This study was undertaken to synthesise 8a and a library of 2-DSA (8a) homologs by modifying the acetamide functional group (a component of the hydrophilic head). For this investigation, the chain length and sulfonyl components of 8a were kept constant. This study involved the development and optimisation of methods for the synthesis of the analogues of 8a and elucidation of their chemical structures. Two novel derivatives were synthesised in this study, a thioamide and an alkyne derivative. Attempts were made to optimise the synthesis of a methyl ester and a carboxylic acid derivative that was previously synthesised by Jones et al [27] (Figure 7). The methyl ester (8b), carboxylic acid (10), alkyne (11) and amine hydrophilic head of 2-DSA (8a) derivatives represent the different types of hydrophilic heads (anionic, ionic, and nonionic). Depending on the pH of the local environment, these derivatives could potentially exist in either the charged or uncharged form. The methyl ester (8b) and
alkyne (11) substitutes represents potential non-ionic heads, while the carboxylic acid represents an anionic head.

![Structures of 2-DSA (8a) and proposed derivatives](image)

**Figure 7. Structures of 2-DSA (8a) and proposed derivatives**

It was proposed that substitution of the acetamide moiety would significantly influence the biological activities of 8a. 8a was identified as having an amphiphilic structure, which may be contributing to its biological activity. The hydrophilic head of amphiphiles can be divided into four main sub-types based on its ionic characteristics, namely: anionic, cationic, non-ionic, and zwitterionic. These different hydrophilic heads play an important role in determining the biological activity and cytotoxicity of amphiphiles [70, 85, 103]. Cationic and anionic amphiphiles have been shown to have promising antimicrobial properties, while non-ionic amphiphiles have been used as excipients for gene and drug delivery [80, 82-85, 103-106].

The substitution of the acetamide moiety with a thioamide functional group was considered to be of particular interest in this study. Compounds with a thioamide moiety have been shown to possess varying degrees of antibacterial activity. Biologically active thioamides include the second-line antitubercular drugs ETA (5),
PTH (6), and TAC (7) and the antibacterial sulfinemycin (9) [62, 98-102]. The substitution with the thioamide moiety was considered relevant to both antibacterial and antitubercular activity of 2-DSA (8a) derivatives.

The highly lipophilic nature and associated poor aqueous solubility was considered to be the major limitation of 8a. It is likely that the aqueous solubility may limit its therapeutic potential [107-109]. The addition of an alkyne group to the acetamide moiety was considered important for the development of derivatives with greatly improved aqueous solubility. The addition of an alkyne moiety would allow for the use of copper catalyzed azide alkyne coupling chemistry, enabling the addition of water-soluble conjugates to 8a to improve its physiochemical properties [108-111]. The successful synthesis of the alkyne derivative of 8a was considered to be important for improvement of its physiochemical properties.

2.1.1 Synthetic scheme

The synthesis of 8a was reported by Jones et al [27]. Straightforward alkylation reaction of 2-bromoacetate derivatives 14a was performed with 1-decanethiol 13. The oxidation of the resulting thioester 15a was performed using meta-chloroperoxybenzoic acid (m-CPBA) as the catalyst to form the final product 8a. m-CPBA is a strong oxidising agent which is commonly used to convert sulfides to sulfoxides and sulfones [112]. The reaction was stirred for 12 h at ambient temperature to ensure the complete conversion of product to sulfone and not sulfoxide (Synthesis scheme 1).
The synthesis of methyl 2-decylsulfonylacetate (8b) was also reported by Jones et al [27]. Methyl 2-decylsulfonylacetate was synthesised by replacing 2-bromoacetate and n-decanthiol with methyl thioglycolate and 1-bromodecane in the synthesis for 8a. Jones et al [27] reported a yield of 90%. In this study, methyl thioglycolate and 1-bromodecane were replaced by methyl bromoacetate and n-decanthiol. The synthesis of the acid derivative 10 was performed using standard hydrolysis reagent sodium hydroxide (NaOH), which attacks the carbon on the carbonyl group of the 8b, forming a salt. The addition of concentrated hydrochloric acid (HCl) removes the salt, forming the acid derivative 10.

Hydroxybenzotriazole (HOBt) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) are standard peptide synthesis reagents used to activate carboxyl groups to react with amines [13-15]. By reacting 10 with propargyl amine 16 using HOBt and EDC as a catalyst the product 11 was formed (Synthesis scheme 2).

Scheme 1. Representative synthesis steps of 2-DSA (8a) and methyl 2-decylsulfonylacetate (8b). (a) K₂CO₃, CH₃CN, (b) m-CPBA, CH₂Cl₂
Scheme 2. Representative synthesis of 2-decylsulfonylacetic acid (10) and 2-decylsulfonyl-N-prop-2-ynyl-acetamide (11). (a) MeOH, 0.5 M NaOH; (b) EDC, HOBT, DCM

The synthesis of the thioamide derivative 12 required the substitution of the oxygen molecule on product 8a’s acetyl group with sulfur using a thionation reaction. Lawesson’s reagent (LR) was used as a thionating agent (Synthesis scheme 3). Lawesson reagent’s is a common and wildy used thionating agent for ketones, esters and amides to prepare thioketones, thioesters and thioamides [113].

Scheme 3. Representative synthesis of 2-decylsulfonylthioamide (12). (a) Lawesson’s reagent, toluene, reflux for 2 h

Two chemical reagents were commonly used for this reaction, P₄S₁₀ (17) and Lawesson’s reagent (LR) (20). 20 was chosen for this reaction because reactions using 17 normally require a higher temperature, a large excess of reagent and longer reaction
time [113, 114]. Thionation reaction using these 2 reagents is usually performed in refluxing benzene, toluene or xylene. When these reagents are in dissociation equilibrium, it yields 19 and 21 (Figure 8).

![Dissociation mechanism of P₄S₁₀ (18) and LR (21)](image)

**Figure 8. Dissociation mechanism of P₄S₁₀ (18) and LR (21)**

The dissociated product 19 and 21 then reacts with carbonyl functional groups to form a four-member ring intermediate, which decomposes to the corresponding thio product 23 (Figure 9 and for more information regarding LR catalysed reaction see the review [113]).
Using the outlined method four homologs of 8a were successfully synthesised and structures were confirmed using ATR-FTIR, MS and NMR.

### 2.2 Materials and Methods

The following reagents were purchased from Merck (Darmstadt, Germany): acetonitrile (CH$_3$CN); dichloromethane (DCM); diethyl ether; ethyl acetate (EtOAc) hexane; methanol (MeOH); toluene; sodium carbonate; sodium chloride (NaCl); and sodium hydroxide. 2-bromoacetamide, 2-bromomethylacetate, EDC, n-decanthiol, m-CPBA, LR and propargyl amine were purchased from Sigma Aldrich (St Louis, MO, USA). Potassium carbonate and hydrochloric acid were purchased from Ajax Finechem (Sydney, Australia). HOBt was sourced from GL Biochem (Shanghai, China). Magnesium sulphate was purchased from Chem Supply (Adelaide, Australia).

DCM, EtOAc, hexane, MeOH were purified via distillation before use. Saturated solutions of carbonate and brine were prepared by adding either excess potassium or sodium carbonate to reverse osmosis water. The saturated solution was left on the bench for 24 h before use.
2.2.1 Representative Alkylation Procedure: 3-Thiotridecanamide (16a)

Potassium carbonate (4 g, 29 mmol) was suspended in acetonitrile (40 mL). To this mixture 2-Bromoacetamide (1.2 g, 8.69 mmol) was added, followed by n-decanthiol (1.5 mL, 7.24 mmol). The mixture was stirred at room temperature for 24 h.

Potassium carbonate was then filtered off and the solvent removed under vacuum using a rotatory evaporator (35°C) leaving a white powder, which was used without purification (1.2 g, 5.19 mmol, 59.72%). The reaction process was monitored using thin layer chromatography (TLC). As the reaction progressed the decanthiol spot (Rf 3.4) disappeared and a new spot appeared showing product formation (Rf 0.06) (1:3 EtOAc/hexane, permanganate stain).

2.2.2 2-decylsulfanylacetate (16b)

2-Bromomethylacetate (1.4 mL, 12 mmol); acetonitrile (50 mL); n-decanthiol (2.1 mL, 10 mmol). The product obtained was a clear oily liquid (2.7 g, 10.97 mmol, 91.4%, Rf 0.63, 1:3 EtOAc/Hexane, permanganate stain).

2.2.3 Representation Oxidation Procedure: 2-decylsulfonylacetamide (8a)

3-Thiodecanamide (1.2 mg, 5.19 mmol) was dissolved in 150 mL dichloromethane. m-CPBA (70%, 5 g, 20.28 mmol) was slowly added to this solution at room temperature and the solution was left to stir for 12 h. The reaction mixture was then concentrated under vacuum to give a white powdery residue, which was dissolved in EtOAc (150 mL). The solution was washed with sodium carbonate (5 x 100 mL), water (1 x 100 mL) and brine (1 x 100 mL). The organic layer was dried over
anhydrous sodium sulphate and concentrated under vacuum to give a white solid, which was recrystallised from hot hexane to give white flakes (604 mg, 2.29 mmol, 44.12%). The purity of the product was determined using NMR, as any reaction by-products can be observed in the NMR spectrum.

2.2.4 Methyl 2-decylsulfonylacetate (8b)

Methyl 2-decylsulfanylacetate (1.5 g, 6 mmol); dichloromethane (200 mL); m-CPBA (70%, 5.44 g, 22.07 mmol). The resulting product was a white waxy solid. The white solid was purified through a silica column using a gradient elution (9:1; 7:1; 5:1; 3:1; 1:1 Hexane/EtOAc). The eluted product was combined and dried under vacuum yielding a white powder. (1.3 g, 4.67 mmol, 77.83 %, Rf 0.56, 1:1 Hexane/EtOAc).

2.2.5 Hydrolysis Procedure: 2-decylsulfonylacetic acid (10)

Methyl 2-decylsulfanylacetate (8b) (312 mg, 1.12 mmol) was dissolved in 42 mL MeOH. 0.5 N NaOH (60 mL) was then added to the mixture. The mixture was stirred at room temperature for 2 h and was then extracted with ether. The product was acidified with concentrated aqueous hydrochloric acid (HCl) and extracted with EtOAc (3 x 100 mL). The combined EtOAc extracts were washed with water (2 x 100 mL) and brine (1 x 100 mL). The organic layer was then dried with MgSO₄ and concentrated under vacuum to give compound 10 as a white solid. (yield 200 mg, 67.5%, Rf 0, 1:1 Hexane/EtOAc).

2.2.6 2-decylsulfonyl-N-prop-2-ynyl-acetamide (11)

2-decylsulfonylacetic acid (10) (76 mg, 0.287 mmol) was dissolved in DCM (25 mL). Then EDC (71 mg, 0.373 mmol), HOBT (58.1 mg, 0.43 mmol) and propargyl
amine (20 μL, 0.363 mmol) were added giving a cloudy solution. The mixture was stirred overnight at room temperature. DCM was removed under vacuum and the solid formed was redissolved in EtOAc (70 mL). This was then washed with 0.5 M HCl (30 mL), sodium carbonate (30 mL), and brine (40 mL). The organic layer was dried under vacuum yielding a white solid 11, was purified by column chromatography on silica gel (1% acetic acid, 1:1 Hexane/EtOAc) to give the final product (yield 79.9 mg, 91.3%, Rф 0.75, 1:1 Hexane/EtOAc).

2.2.7 Thionation Procedure: 2-decylsulfonylthioamide (12)

2-decylsulfonylacetamide (8a) (121.2 mg, 0.46 mmol) was dissolved in 20 mL toluene and 254 mg (0.628 mmol) of LR was then added to the mixture. The reaction mixture was stirred at reflux for 2 h. The reaction mixture was then allowed to cool to room temperature and the organic solvent was removed under vacuum. The product 12 was purified twice through a silica gel column (3:1 Hexane/EtOAc) (yield 80 mg, 62%, Rф 0.16, 1:1 Hexane/EtOAc).

2.3 Structural elucidation and chemical characterization

MS, NMR spectroscopy, ATR-FTIR spectroscopy, and DTA were used to identify and confirm the identity of synthesized products.
2.3.1 MS

Electron spray ionization (ESI) was performed utilizing a Bruker Daltonics esquire series 3000 mass spectrometer (Bruker Daltonik GMBH, Bremen, Germany) with esquire control software version 5 with the cone voltage set at 4000V. The ESI process produces single charged species, making it easy to identify the mass to charge ratio (m/z) peak of the parent compound or adducts of these compounds [115]. Adducts formed through ESI usually involve protons or simple solution phase anions, such as Na⁺ or Cl⁻, therefore making peak assignments simple and straightforward. Depending on the property of the compound, the detection was switched from positive to negative ion mode for optimal detection [115]. The compounds synthesised were dissolved in HPLC grade CH₃CN and filtered through 0.45 μM Teflon (PTFE) syringe filters (Phenex, USA) before direct continuous injection into the MS. The relative molecular mass of the compounds synthesised were calculated based on its molecular structure using the software Symyx Draw 4.0 (refer to table 4).

2.3.2 NMR spectroscopy

NMR spectroscopy was performed using Bruker Avance 300 MHz spectrometer (Bruker Daltonik GMBH, Bremen, Germany) with Topspin 2.1 acquisition software. The reaction products were confirmed via ¹H NMR where the proton peak assignments were used for the following: determine the structure of the synthesised compound; determine whether the reaction was completed; and identify the absence of reactants after purification. In addition, ¹³C NMR was also performed for the novel thioamide derivative 12. NMR samples were dissolved in either CDCl₃-d₆ or DMSO-d₆ (Cambridge Isotope laboratories, Andover, USA).
2.3.3 ATR-FTIR spectroscopy

ATR-FTIR spectroscopy was performed using Bruker alpha-P (Bruker Daltonik GMbH, Bremen, Germany) with Opus 6.5 acquisition and analysis software. The wavelength measured ranged from 375 to 4000 cm\(^{-1}\) at 4 cm\(^{-1}\) resolution. Each sample was measured with 24 scans. FTIR is widely used in the pharmaceutical industry as a method for molecular fingerprinting of drugs, verifying purity, and structural investigation [116, 117]. The advantage of ATR-FTIR over conventional FTIR is that it requires very little sample preparation. With FTIR the sample needs to be mixed with an alkali halide (typical KBR or KCl) using a high pressure apparatus to compress the mixture forming a glass pellet [118], while the sample preparation for ATR-FTIR simply involved placing the powdered sample in contact with the ATR crystal [116]. The resulting spectra from ATR-FTIR were used to identify the different functional groups in the synthesised and purified products.

2.3.4 Differential thermal analysis (DTA)

The DTA was performed using a Lineis STA PT 1000 calorimeter (Germany) and each sample was heated to a maximum of 350°C with a run-time of 60 min. The rate of rise in temperature was set to 5°K/min and the sampling interval was set to 1 sec. Samples were placed in porcelain crucibles and an empty porcelain crucible was used as a reference. Approximately 25 mg of sample was analysed by DTA, associated weight loss was measured by thermogravimetric analysis (TGA). TA-Win data acquisition software was used to record the data and thermograms were plotted using the Linseis Data Evaluator (version 3).
2.4 Results

2-DSA (8a) homologs were successfully synthesized using the outlined methods (Table 4). The structure of each compound was confirmed using MS, FTIR and NMR and melting point was determined using DTA.

Table 4. Molecular formula and mass of 2-decylsulfonylacetamide and its derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>Name</th>
<th>Chemical structure</th>
<th>Formula weight</th>
<th>Chemical formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>8a</td>
<td>2-DSA</td>
<td></td>
<td>263.4</td>
<td>C₁₂H₂₃NO₃S</td>
</tr>
<tr>
<td>8b</td>
<td>methyl 2-decylsulfonylacetate</td>
<td></td>
<td>278.41</td>
<td>C₁₃H₂₆O₄S</td>
</tr>
<tr>
<td>10</td>
<td>2-decylsulfonylthioamide</td>
<td></td>
<td>279.46</td>
<td>C₁₂H₂₃NO₂S₂</td>
</tr>
<tr>
<td>11</td>
<td>2-decylsulfonylacetic acid</td>
<td></td>
<td>264.38</td>
<td>C₁₂H₂₃O₄S</td>
</tr>
<tr>
<td>12</td>
<td>2-decylsulfonyl-N-prop-2-ynyl-acetamide</td>
<td></td>
<td>301.44</td>
<td>C₁₅H₂₇NO₃S</td>
</tr>
</tbody>
</table>

³H NMR proton peaks 0.86 (t, 3H), 1.22-1.4 ppm (m, 14H), 1.8 (m, 2H), 3.1 (t, 2H) and 3.8 (s, 2H) were observed across all compounds synthesised, corresponding to the lipid chain and sulfonyl moiety (Figure 12-16).
Figure 10. $^1$H NMR spectrum of 2-decylsulfonylacetamide (8a)
Figure 11. $^1$H NMR spectrum of Methyl 2-decylsulfonylacetate (8b)
Figure 12. $^1$H NMR spectrum of 2-decylsulfonylacetic acid (10)
Figure 13. $^1$H NMR spectrum of 2-decylsulfonyl-N-prop-2-ynyl-acetamide (11)
Figure 14. $^1$H NMR spectrum of 2-decylsulfonylthioamide (12)
2.4.1 2-decylsulfonylacetamide (8a)

2-DSA (8a) $^1$H NMR was performed using either CDCl$_3$-$d_6$ or DMSO-$d_6$. $^1$H NMR spectra of the CDCl$_3$-$d_6$ sample was used to compare 8a to the different synthesised derivatives and to monitor the reaction process. DMSO-$d_6$ $^1$H NMR sample was compared to $^1$H NMR peaks published by Jones et al [27], confirming the synthesised compound (Figure 15).
Figure 15. $^1$H NMR spectrum of 2-decylsulfonylacetamide (8a) in DMSO-d$_6$.

The ATR-FTIR provided the molecular fingerprint of 8a and the assignment of functional groups was used to confirm the compound synthesised (Figure 16).
Figure 16. ATR-FTIR spectrum of 2-decylsulfonylacetamide (8a)
In addition to data from $^1$H NMR and ATR-FTIR, a m/z peak of 286.9 [M+Na$^+$] (Figure 17) from the mass spectra confirmed the structure of the synthesised compound to be 8a.

![Figure 17. Positive-ion mass spectrum of 2-decylsulfonylacetamide (8a)](image)

2.4.2 Methyl 2-decylsulfonylacetate (8b)

The reaction of methyl bromoacetate with decanethiol was monitored using $^1$H NMR. As the alkylation reaction proceeded there was a shift in the BrCH$_2$ peak as it formed a SCH$_2$ bond with the decanethiol (from 3.19 to 3.92). Furthermore, the OCH$_3$ peak also shifted from 3.71 to 3.77 as the reaction proceeded to completion (Figure 11 and 18). Upon completion of the reaction the ATR-FTIR spectra also showed the disappearance of the NH$_2$ functional group and presence of peaks representing the new OCH$_3$ functional group (see Figure 19). The mass spectrum of the final product also revealed a single major peak at m/z of 301.2 [M+Na]$^+$, confirming the product synthesised as 8b (Figure 20).
Figure 18. $^1$H NMR spectrum of n-decanthiol and 2-bromo-methyl acetate
Figure 19. ATR-FTIR spectrum of methyl 2-decylsulfonylacetate (8b)
2.4.3 2-decylsulfonylacetic acid (10)

The hydrolysis of methyl-2-decylsulfonylacetic acid (8b) into 2-decylsulfonylacetic acid (10) was monitored using $^1$H NMR. The reaction was monitored by observing the loss of the OCH$_3$ at 3.77 ppm moiety as it proceeded to completion (Figure 21). The loss of the OCH$_3$ and formation of carboxyl group was also observed in the ATR-FTIR spectrum (1702.89 cm$^{-1}$ and 1408.25 cm$^{-1}$) upon the formation of 10 from 8b (Figure 22).
Figure 21. $^1$H NMR comparison of methyl-2-decylsulfonylacetate (8b) and 2-decylsulfonylacetic acid (10) showing the loss of the OCH$_3$ in the methyl acetate moiety.
Figure 22.  ATR-FTIR spectrum of 2-decylsulfonylacetic acid (10)
The mass spectrum of compound 10 revealed a peak at $m/z$ of 287.3 [M+Na]$^+$, which corresponds to the molecular mass plus sodium for the compound (Figure 23) and confirmed the synthesised target product.

![Figure 23. Positive-ion mass spectrum of 2-decylsulfonylacetic acid (10)](image)

### 2.4.4 2-decylsulfonyl-N-prop-2-ynyl-acetamide (11)

2-Decylsulfonyl-N-prop-2-ynyl-acetamide (11) was synthesised by reacting propargyl amine with 2-decylsulfonylacetic acid (10) catalysed by EDC and HOBt. The reaction was monitored by tracking shifts in proton peaks. The reaction between propargyl amine and 10 produced a number of peaks shifts in $^1$H NMR, which were used to track the reaction process. The propargyl amine $^1$H NMR spectrum shows 3 peaks; 1.294 ppm (s, 2H); 2.2 ppm (t, 1H); and 3.393 ppm (d, 2H). Upon the formation of 11 the NH$_2$ peak at 1.29 ppm disappeared and a new NH peak appeared at 6.9 ppm. In addition, the CH$_2$ attached to the amine shifted from 3.39 ppm to 4.04 ppm as the protons became shielded by the carbonyl motif. There was also a slight shift of CH$_2$ between the sulfanyl and carbonyl motif from 3.97 to 3.85 (Figure 24). The ATR-FTIR
spectrum showed the reappearance of the NH peak at 3268.95 cm\(^{-1}\) as the amine is coupled to 10 (Figure 25).

Figure 24. Comparison of the \(^1\)H NMR spectra of propargyl amine and 2-decylsulfonyl-N-prop-2-ynyl-acetamide (11) showing various peak shifts.
Figure 25. ATR-FTIR spectrum of 2-decylsulfonyl-N-prop-2-ynyl-acetamide (11)
The mass spectrum of 11 revealed a single peak at $m/z$ of 324.4 [M+Na]$^+$, thus confirming the synthesised target product (Figure 26).

**Figure 26. Positive-ion mass spectrum of 2-decylsulfonyl-n-prop-2-ynyl-acetamide (11)**

### 2.4.5 2-decylsulfonylthioacetamide (12)

The $^1$H NMR spectra revealed a number of shifts in the proton peaks as 2-decylsulfonylacetamide (8a) were converted to 2-decylsulfonylthioacetamide (12) by LR. The NH peaks shifts from 5.56 ppm and 6.47 ppm to 7.66 and 7.99, respectively due to the increased shielding by the thio motif on the amide protons. The CH$_2$ proton between the sulfonyl and carbonyl motif also become more shielded as the carbonyl group was converted to a thio group, shifting the peaks from 3.83 ppm to 4.30 ppm (Figure 27). $^{13}$C NMR spectrum also confirmed the synthesised compound to be 12, showing a C=S peak at 193.17 ppm, CH$_2$-S at 65.52 ppm, S-CH$_2$ at 52.42 and CH$_3$ at 14.07 ppm. The CH$_2$ of the alkyne chain was between 22.1 to 31.82 ppms with some of the carbon peaks overlapping each other (Figure 28). The ATR-FTIR spectra between 8a and 12 were very similar as the thioamide and carbonyl groups have similar absorbance profiles (see Figure 16 and 29).
Figure 27. $^1$H NMR spectra comparison of 2-DSA (8a) and 2-decylsulfonylthioamide (12) showing the shift in NH$_2$ and CH$_2$ peaks
Figure 28. $^{13}$C NMR spectrum of 2-decylsulfonylethioamide (12)
Figure 29. ATR-FTIR spectrum of 2-decylsulfonylthioamide (12)
Compound 12 mass spectrum revealed multiple peaks with a peak at m/z of 302 [M+Na]+ corresponding to the compound (see Figure 30).

Figure 30. Positive-ion mass spectrum of 2-decylsulfonylthioamide (12)

2.4.6 DTA results

DTA was used to determine the melting point of the synthesised compounds. Apart from 2-DSA (8a), all the compounds synthesised exhibited sharp endothermic peaks consistent with the target melting point. No additional exotherms or endotherms were observed. The DTA data recorded a melting point of 147.1°C for 8a and 53.7°C for methyl 2-sulfonyldecananoate (8b), which was the same as that reported by Jones et al [27]. 2-decylsulfonylthioamide (12) recorded a melting point of 107.2°C. The measured melting point for 2-decylsulfonylacetic acid (10) and 2-decylsulfonyl-N-prop-2-ynyl-acetamide (11) were 106.3°C and 107.6°, respectively (Figures 31 – 35). Weight loss, measured by TGA, was minimal for all compounds between 90 and 100°C (less than 5%). Melting points were considerably different between the synthesised derivatives of 2-DSA.
Figure 31. DTA of 2-decylsulfonylacetamide (8a)
Figure 32. DTA of methyl 2-sulfonylacetate (8b)
Figure 33. DTA of 3-sulfonylacetic acid (10)
Figure 34. DTA of 2-decylsulfonyl-N-prop-2-ynyl-acetamide (11)
Figure 35. DTA of 2-decylsulfonylthioamide (12)
2.5 Discussion

The sulfonyl fatty amide 2-DSA (8a) and its derivatives, methyl 2-decylsulfonyleacetate (8b) and 2-decylsulfonyleacetic acid (10), were synthesised using a method adapted and modified from Jones et al [27]. 2-Decylsulfonyl-N-prop-2-ynylacetamide (11) and 2-decylsulfonylethioamide (12) were synthesised using novel methods explored in this study. For compounds 8a, 8b and 10, various reaction steps were modified using alternative reagents and solvents in order to improve yield and explore the chemistry of sulfonyl fatty amide synthesis.

The initial alkylation step used potassium carbonate as a base to catalyse the reaction. The reaction was modified by replacing acetone/DMSO (3:1) with acetonitrile to simplify the reaction work-up, as potassium carbonate is insoluble in acetonitrile. Therefore, it can be removed via filtration through a sintered glass funnel. Synthesis of 2-decylsulfonyleacetamide (15a) using the alternative solvent produced a slightly lower product yield compared to that reported by Jones et al (16% vs. 91). This was resolved by washing the potassium carbonate multiple times with acetonitrile during the alkylation reaction.

For the synthesis of methyl 2-decylsulfonyleacetaetate (15b) Jones et al used methyl thioglycolate and 1-bromodecane as the starting material with a 99% product yield [27]. Using 2-bromomethylacetate and n-decanthiol as alternative reagents produced a slightly lower yield of 91.4%. In the method outlined by Jones et al the resulting product was purified using a silica gel column [27], while the product was purified simply through filtering through a sintered funnel when the alternative method was used.
The product 8b was synthesised through the oxidation of methyl 2-decylsulfanyacetate (15b) using m-CPBA. The reaction product was washed with sodium carbonate and water to remove excess m-CPBA and m-CBA by products. However, due to the excess amount of m-CPBA used in the reaction, even after numerous washes, the $^1$H NMR spectrum still showed the presence of m-CPBA and m-CBA. The product was then purified through a silica column using a gradient elution using hexane/EtOAc (9:1; 7:1; 5:1; 3:1; 1:1). This process was repeated 3 to 4 times to yield a pure product as confirmed via $^1$H NMR. The final yield of 77.8% was comparable to that synthesis methodology used by Jones et al with a product yield of 78% [27].

The hydrolysis of 8b to produce 10 was a straightforward reaction adapted from Jones et al [27]. When the pure compound was dried it was discovered that excess salt was present. The product was then dissolved in EtOAc and the salts were filtered off to produce a pure product 10, which was obtained after EtOAc was removed.

The synthesis of 11 requires the coupling of propargyl amine to the carboxyl moiety of 10. There are many different amine and carboxyl coupling reagents reported in the literature [119-122]. EDC, N,N'-dicyclohexylcarbodiimide (DCC), HOBut and triethylamine (TEA) are often used in peptide coupling reactions to activate the carboxyl groups to react with amines. These reagents are also to catalyse the reaction of other carboxylic acid compounds with propargyl amines [110, 111, 119, 122-129]. Based on the literature, a number of different reaction conditions using these catalysts were investigated for the synthesis of 11 [110, 111, 119, 122-129]. Of these only the combination of HOBut and EDC produced the desired product. Literature reports the use
of various combinations of HOBt and EDC to react carboxyl groups with amines [110, 111, 125] and these combinations were investigated for the synthesis of 11. 11 was purified using a silica column resulting in a relatively good yield of 91%.

The synthesis of 12 was relatively straightforward using LR, a widely used thionating reagent. The optimal reaction time at reflux was determined by allowing the reaction to continue for 8 h. Samples were taken hourly and the reaction was monitored via $^1$H NMR. It was discovered that the reaction was completed after 2 h. The product was purified by filtration through a silica gel column.

The modification of the method adapted from Jones et al enabled us to synthesize products of comparable yield to that reported in the paper [27]. The alkylation procedure using acetonitrile as the solvent simplified the workup procedure as both 15a and 15b was simply filtered through a sintered glass funnel to remove the potassium carbonate. The usage of methyl bromoacetate and 1-decanthiol as alternative reagents for the synthesis of 15b also produced a similar yield compared to the reagents used by Jones et al [27]. Although the synthesis of 11 using EDC and HOBt produced quite a good yield, exploration of its synthesis using other coupling reagents could further enhance yields.

Most substances will undergo a physical and chemical change when subjected to high temperature [130]. These changes range from simple change in state to complete decomposition and are specific for each compound. The change in enthalpy measured utilizing DTA and the unique IR pattern obtained from the FTIR provides a unique
molecular fingerprint of the synthesized compounds. The combination of MS and NMR data provides confirmation of structure of the synthesized compounds.

The long fatty aliphatic chain of 8b produces high lipophilicity, which could limit its suitability for clinical application. The addition of the alkyne functional group to the acetamide moiety (compound 11) enables the usage of copper catalysed azide alkyne coupling chemistry (click chemistry) [108, 109, 111]. This chemistry can be used to chemically conjugate 2-DSA (8b) to numerous water-soluble compounds, such as cyclodextrins [107, 109, 131] to improve its solubility. This would improve drug absorption and ease of delivery for clinical applications.

The reported chemistry provides a framework for further work into the synthesis and modification of 2-DSA homologs. The chemistry used to synthesise 11, provides an important avenue for research into the synthesis of various prodrugs for pharmaceutical application [111].
Chapter 3. *In vitro* antitubercular screening

3.1 Background

2-DSA was previously reported to possess high *in vitro* activity against *M. tuberculosis* H37Rv (MIC 0.75 – 1.5 μg/mL). The observed activity of 2-DSA (8a) was comparable to INH (MIC 0.1 – 0.4 μg/mL), RMP (MIC 2 μg/ml), EMB (2 μg/mL) and PZA (100 μg/mL). Altering the aliphatic chain length, amide and sulfonyl moiety was shown to significantly decrease *in vitro* antitubercular activity [27]. The thioamide functional group has also previously been shown to be an important pharmacophore for the antitubercular activity of ETA, PTH and TAC [62]. This current study investigated if modification of the acetamide functional group altered antitubercular activity.

3.2 *M. tuberculosis* H37

The *M. tuberculosis* H37 variants are widely used as reference strains in mycobacteriology [132-134]. H37 was originally isolated in 1905 from a 19 year-old male patient with chronic pulmonary TB. In 1934, H37 was dissociated into “virulent” (Rv) and “avirulent” (Ra) strains [133, 135]. Both strains of H37 have been used as reference strains for studying virulence and pathogenesis of TB [134].

Traditionally, the primary screening of potential antitubercular compounds has been performed against the more virulent H37Rv strain [136]. More recently, researchers have provided sound evidence that *M. tuberculosis* H37Ra is a suitable surrogate for the more virulent H37Rv strain in primary screening of compounds for
antitubercular activity. MICs for the majority of clinical antitubercular drugs were shown by researchers to be comparable between H37Ra and H37Rv [28]. H37Ra was subsequently used for antitubercular screening in this study.

### 3.3 Materials and Methods

The antitubercular activity testing was conducted by Dr Richard J. Payne from the School of Chemistry at The University of Sydney (NSW, Australia) and Dr Nicholas West from Medicine, Central Clinical School, Centenary Institute of Cancer Medicine And Cell Biology (NSW, Australia) using methods adapted from Collins et al [28]. The H37Ra (ATCC 25177) clinical strain of *M. tuberculosis* was used to screen for antitubercular activity.

Stock solutions of the test compounds were prepared in DMSO and diluted twofold with 7H9GC broth (no Tween 80) to prepare final concentrations ranging from 2 μg/mL to 1000 μg/mL. Suitable vehicle controls were included. Antitubercular testing was performed in 96 well black plates (back view plates; Packard Instruments Company, Meriden, Conn) in order to minimize background fluorescence. The outer well of the 96 well black plates were filled with sterile water to prevent dehydration of the experimental wells.

Frozen H37Ra was initially diluted 1:20 in BACTEC 12B medium, followed by a 1:50 dilution in 7H9GC. This culture was added to the wells at a final bacteria titre of 50,000 CFU/mL. Wells containing drugs alone were included to detect and account for autofluorescence of test compounds. Additional controls included bacteria and medium
only containing wells. Plates were incubated at 37°C and at day 14, 20 μL of 10 × AlamarBlue® solution (Alamar biosciences/Accumed, Westlake, Ohio) and 12.5μL of 20% Tween 80 were added to all wells. The plates were then incubated for a further 24 h at 37°C. Following which fluorescence was measured at excitation of 530 nm and emission at 590 nm on a fluorescence microplate reader. Experiments were conducted in triplicate.

3.3.1 Data analysis

Results were calculated as the percentage growth relative to vehicle controls and represented as the mean ± SD. Statistical analysis was performed with one-way ANOVA followed by Dunnett’s post-test. Statistical significance was accepted at a probability level of $P < 0.05$. An MIC$_{50}$ of 25 μM was set as the threshold for activity of interest.

3.4 Results

The antitubercular activity of compounds was examined in vitro against the H37Ra clinical strain of *M. tuberculosis* using AlamarBlue® for detection. Compounds 8b, 10, and 11 were shown to have no activity against H37Ra when compared to vehicle controls. Results for the comparative antitubercular activity of compounds 8a and 12 are presented in Figure 36. Compound 8a was shown to possess high activity against H37Ra in vitro (MIC$_{50}$, 6 μM). Maximum growth inhibition was observed between 10 and 125 μM (IC$_{50}$ = 1.99 μM; $\alpha = 1$). The MIC$_{90}$ against H37Ra for compound 8a was approximately 15 μM (4 μg/mL). 25% inhibition of H37Ra growth was observed even at a concentration as low as 3.75 μM.
Figure 36. Percentage growth inhibition (relative to vehicle control) of the H37Ra strain of *M. tuberculosis*. Results represent the mean of replicate experiments (n=3) and SD were less than 5%.

In comparison, compound 12 produced significantly less growth inhibition ($P < 0.01$). A maximum growth inhibition of 25% was reported at a concentration of 125 μM ($IC_{50} = 15.16 \mu M; \alpha = 0.26$). Increasing the concentration of compound 12 produced no additional increase in observed inhibition of H37Ra growth. No inhibition of H37Ra growth was observed for compounds 8b, 10 and 11.
3.5 Discussion

The *M. tuberculosis* H37Ra strain has been widely used in the primary screening of compounds for antitubercular activity [28]. The *in vitro* antitubercular activity of compound 8a and 12 in this study was therefore assessed against H37Ra.

The findings of this study complement those reported by Jones *et al* [27]. Compound 8a was shown to have high activity against the attenuated *M. tuberculosis* H37Ra strain in this study. Jones *et al* previously reported that 2-DSA inhibited the growth of H37Rv. The reported MIC for 8a against H37Rv was 0.73 to 2.92 μM (0.75 to 1.5 μg/mL). Our findings confirm the *in vitro* antitubercular activity of 2-DSA (8a) against H37Ra. The activity of compound 8a against H37Ra has not previously been reported. The observed activity against H37Ra was however significantly lower than the reported findings for H37Rv. The observed difference in activity could be, in part, due to the reported phenotypic and genetic differences between H37Rv and H37Ra [134]. Differences in antitubercular activity against H37Ra and H37Rv have already been reported [28]. In addition, this current study employed AlamarBlue® for assessment of growth inhibition, whereas the previous report utilised the BACTEC system. AlamarBlue® has been previously shown to underestimate antibacterial activity, often yielding higher MIC values [28]. Nevertheless, the findings from this and previous studies suggest that 2-DSA (8a) appears to retain good activity against both H37 strains. The observed activity of compound 8a against H37Ra appears to be lower than the first-line antitubercular agents. The literature reported MIC values for INH, RMP and EBM against H37Ra were 0.4, 0.6 and 14.9 μM, respectively. However, the observed 2-DSA (8a) activity against H37Ra was greater than the second-line agent ETA (MIC 17.9 μM).
The antitubercular activity of the thioamide derivative (12) of 2-DSA (8a) was shown to be significantly lower. Although the thioamide compound retained some activity against H37Ra, its potency was well below that of 2-DSA. Differences in lipophilicity between 2-DSA (8a) and compound 12 were theorised to contribute to the altered antitubercular activity. Comparisons of the calculated Log P values (Molinspiration Cheminformatics) between these compounds suggested otherwise. The calculated Log P values for 2-DSA (8a) and compound 12 were 2.9 and 3.1, respectively. This minimal difference between calculated Log P values was therefore unlikely to explain the large difference in observed activity. Based on the findings of this study, it is therefore proposed that the acetamide functional group of 2-DSA (8a) is crucial for retaining activity against M. tuberculosis H37Ra. The lack of antitubercular activity of the methyl ester and carboxylic acid derivatives provides further evidence to substantiate this claim. If this is the case, the action of compound 8a is unlikely to be due to an amphiphile-like effect, which is expected to non-specifically disrupt cell membrane and outer wall integrity.

2-DSA (8a) has again been shown to be an important lead compound for further development of antitubercular drugs. The acetamide, sulfonyl and C-10 aliphatic functional groups appear to be crucial for antitubercular activity. The major limitation of this compound remains its relatively high lipophilicity (Log P of 2.9) and aqueous solubility problems.

Compound 11, synthesised in this study, contains an alkyne addition to the acetamide moiety of 2-DSA (8a). This alkyne moiety provides a platform for the addition of various water-soluble conjugates to 8a in an attempt to improve its
physicochemical properties. Given the observed importance of the acetamide moiety for antitubercular activity, the influence of added conjugates on the activity of 8a will need to be assessed. It is likely that activity will be sacrificed for the improvement of physicochemical properties by the addition of water-soluble conjugates.
Chapter 4. *In vitro* antibacterial and antifungal screening

4.1 Background

Compounds synthesised in this study were screened for antibacterial and antifungal activities (minimal inhibitory concentrations, MIC\textsubscript{90}) against a broad spectrum of microorganisms. The objective of this investigation was to assess the selectivity of the antimicrobial activity of 2-DSA and derivatives. Currently there are no published studies on the antibacterial or antifungal properties of the compounds synthesised in this study.

2-DSA has an amphiphilic structure, which could possibly lead to interaction with and disruption of the cell membrane structure. This effect could potentially lead to non-specific cellular toxicities and broad antimicrobial activities. Numerous reports, detailing the antibacterial activities of amphiphilic compounds [75, 80, 104], support this hypothesis. A structural analysis of these compounds reveals that, in general, they possess a variety of hydrophilic heads (nonionic, cationic, anionic and zwitterionic) linked to hydrophobic tails with chain lengths between C\textsubscript{10} and C\textsubscript{14}. 2-DSA and derivatives share good structural homology to these antibacterial amphiphilic compounds [77].

The antibacterial activity of the thioamide derivative of 2-DSA was of particular interest due to its structural similarity to sulfinemycin [100]. Sulfinemycin was reported to have activity (MICs of 128 μg/mL) against a limited number of Gram-positive bacteria, including *S. aureus* (SSC 82-24), *S. aureus* (SSC 82-57) and *S. epidermis* (ATCC 12228). Sulfinemycin was shown to have little or no activity against Gram-
negative bacteria [100]. Despite the amphiphilic structure of sulfinemycin, it appears to possess selective antimicrobial properties. Based on this finding, it was proposed that 2-DSA and derivatives may possess some degree of antimicrobial specificity [100, 137].

Based on the antimicrobial activities of amphiphilic compounds, the antibacterial and antifungal screening of 2-DSA and derivatives was considered feasible. In order to evaluate selective action, Gram-positive, Gram-negative and yeast organisms were included in the screening of 2-DSA and derivatives. Gram-positive bacterium included S. aureus (ATCC 25923) and Gram-negative bacteria included E. coli (ATCC 8739) and P. aeruginosa (ATCC 27853). Compounds were also screened against the yeast, C. albicans (ATCC 10231).

4.2 Materials and Methods

2-DSA (8a), methyl 2-decylsulfonylacetate (8b), 2-decylsulfonylacetie acid (10), 2-decylsulfon-y-N-prop-2-ynyl-acetamide (11) and 2-decylsulfonylthioamide (12) were screened for antibacterial and antifungal activity using a fluorescence-based in vitro microbroth dilution method. The antibacterial and antifungal assays were adapted from the published methods of Andrews [138] and Sarker et al [139]. This methodology has been extensively validated and routinely used in our laboratory. Resazurin (blue) is a non-fluorescent dye that is reduced to resorufin (pink) by metabolic activity within the cell. The metabolic product resorufin is a highly fluorescent compound, which allows for sensitive detection. Activity can be effectively measured by either colorimetric (λ = 574 nm) or fluorometric (excitation 570 nm; emission 590 nm) detection. The integration of resazurin into microbroth dilution antibacterial and antifungal assays has been shown to produce reliable results, with good correlation to other methodologies.
Numerous studies have confirmed the direct relationship between the reduction of resazurin and the proliferation of living organisms [139-145].

4.2.1 Reagents

*S. aureus* (ATCC 25923), *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC 27853) and *C. albicans* (ATCC 10231) were donated by Mr Zoran Klipic (Institute for Glycomics, Griffith University). Luria-Bertani (LB) broth was purchased from Oxoid (Hampshire, England) and the antibiotics (dicloxacillin sodium, vancomycin hydrochloride, gentamicin sulfate, ciprofloxacin hydrochloride monohydrate, ketoconazole) used for validating the assay were sourced from Sigma Aldrich (St Louis, MO, USA). All other chemicals and reagents were of analytical grade and sourced from Sigma Aldrich (St Louis, MO, USA). MilliQ water was sterilized by autoclaving at 121°C for 20 min. 96 well plates were purchased from Nunc (New York, USA).

4.2.2 Preparation of antibiotic and compound stock solutions

Antibiotic stock solutions were prepared at 1000 mg/L in solvents recommended by Andrews [138]. Dicloxacillin, vancomycin, gentamicin and ciprofloxacin were dissolved in water and ketoconazole was dissolved in methanol. The synthesized compounds 8a, 8b, 10, 11 and 12 were dissolved in DMSO at a concentration of 1000 mg/L. Stock solutions were aliquoted and stored at 4°C and used within 1 month of preparation.
4.2.3 Preparation of bacterial and yeast culture

A single colony of streaked bacteria or fungi was transferred to 1 mL of sterile single strength broth and incubated overnight for 18 h. After which, the culture was adjusted to 0.5 McFarland with sterile single strength LB broth (~0.1Abs @ 600 nm). The culture was subsequently diluted 1:100 with sterile single strength LB broth for seeding of assay wells.

4.2.4 Preparation of Resazurin stock

16.13 mg of resazurin powder was dissolved in 100 mL of sterile water to prepare a 704 μM resazurin stock solution. This resazurin stock solution was stored at 2-4°C and was used within 1 month of preparation.

4.2.5 Antibacterial and antifungal assay

Antibacterial and antifungal assays were performed in 96 well microtitre plates. On the day of the experiment, antibiotic and test compounds stock solutions were diluted to 64 mg/L with sterile water to prepare working stock solutions. 37.5 μL of these antibiotic and test compound working solutions were transferred to the wells and sequentially diluted with water to prepare the following final concentrations: 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 mg/L. Appropriate vehicle/solvent controls were included in each assay. 37.5 μL of sterile double strength broth was subsequently transferred to all wells. 75 μL of prepared bacterial or fungal culture was then transferred to the test wells. 75 μL of sterile single strength LB broth was transferred to the negative control wells. The final volume for each well was 150 μL.
The plates containing bacteria were covered and incubated at 37°C shaking at 100 rpm for 18 h. The plates containing yeast were covered and incubated at 30°C shaking at 100 rpm for 24 h. After incubation, 10 μL of resazurin stock solution was added to all wells. The plates were then incubated for a further 1 h at 37°C shaking at 100 rpm. Fluorescence was measured (excitation 485 nm, emission 535 nm) using a Fluoroskan Ascent microplate fluorometer (Thermo Scientific, Victoria, Australia). Each concentration of antibiotics used was repeated in triplicate and assays were performed on three separate occasions.

4.2.6 Data analysis

Percentage viability (relative to vehicle controls) was calculated from corrected fluorescent values. The MIC value of antibiotic controls and test compounds were determined as the first dilution at which at least 90% of growth was inhibited (MIC₉₀). Results are represented as the median value of replicate experiments.

Statistical analysis was performed using GraphPad Instat 3 (GraphPad Software, San Diego, CA, USA) on median value of the replicated experiments (triplicates). The Kruskal-Wallis non-parametric analysis of variance on ranks was used to determine whether there were significant differences among susceptibilities. Mann-Whitney tests were performed to determine specific differences between antibiotics and test compounds (Mann-Whitney test). Differences were considered significant when $P < 0.05$. 
4.3 Results

Median MIC\(_{90}\) results for control antibiotics and test compounds (8a, 8b, 10, 11, and 12) against *S. aureus* (ATCC 25923), *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC 27853) and *C. albicans* (ATCC 10231) are presented in Table 5. Antibiotics and test compounds were screened between the concentrations of 0.125 and 16 mg/L.

Table 5. Median MIC\(_{90}\) (mg/L) of control antibiotics and test compounds against representative Gram-positive and Gram-negative bacteria and yeast species

<table>
<thead>
<tr>
<th>Antibiotic/Test compound</th>
<th><em>S. aureus</em> ATCC 25923</th>
<th><em>E. coli</em> ATCC 8739</th>
<th><em>P. aeruginosa</em> ATCC 27853</th>
<th><em>C. albicans</em> ATCC 10231</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dicloxacillin</td>
<td>0.5 mg/L</td>
<td>&gt;16 mg/L</td>
<td>&gt;16 mg/L</td>
<td>&gt;16 mg/L</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>4 mg/L</td>
<td>&gt;16 mg/L</td>
<td>&gt;16 mg/L</td>
<td>&gt;16 mg/L</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>8 mg/L</td>
<td>4 mg/L</td>
<td>2 mg/L</td>
<td>&gt;16 mg/L</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>2 mg/L</td>
<td>0.125 mg/L</td>
<td>0.5 mg/L</td>
<td>&gt;16 mg/L</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>&gt;16 mg/L</td>
<td>&gt;16 mg/L</td>
<td>&gt;16 mg/L</td>
<td>0.5 mg/L</td>
</tr>
<tr>
<td><strong>8a</strong></td>
<td>16 mg/L</td>
<td>&gt;16 mg/L</td>
<td>&gt;16 mg/L</td>
<td>&gt;16 mg/L</td>
</tr>
<tr>
<td><strong>8b</strong></td>
<td>16 mg/L</td>
<td>&gt;16 mg/L</td>
<td>&gt;16 mg/L</td>
<td>&gt;16 mg/L</td>
</tr>
<tr>
<td><strong>10</strong></td>
<td>16 mg/L</td>
<td>&gt;16 mg/L</td>
<td>&gt;16 mg/L</td>
<td>&gt;16 mg/L</td>
</tr>
<tr>
<td><strong>11</strong></td>
<td>&gt;16 mg/L</td>
<td>&gt;16 mg/L</td>
<td>&gt;16 mg/L</td>
<td>&gt;16 mg/L</td>
</tr>
<tr>
<td><strong>12</strong></td>
<td>8 mg/L</td>
<td>&gt;16 mg/L</td>
<td>&gt;16 mg/L</td>
<td>&gt;16 mg/L</td>
</tr>
</tbody>
</table>

*The same value was obtained for all the replicates at each concentration.

The resazurin-based fluorescent microbroth dilution method was shown to be effective in determining the MIC\(_{90}\) and spectrum of activity of control antibiotics, thereby validating the screening assays [138]. The antibiotic controls used for this assay produced slightly higher MIC\(_{90}\) values against the representative organisms tested when
compared to reported literature [138]. However, determined MIC\textsubscript{90} values for tested antibiotics fall within the limits set by the Clinical and Laboratory Standards Institute (CLSI) guidelines [146].

With exception of compound 11, all test compounds inhibited the growth of the Gram-positive bacteria \textit{S. aureus} (ATCC 25923). Test compounds 8a, 8b, and 10 produced significant growth inhibition at a concentration of 16 mg/L ($P < 0.05$). Compound 12 displayed the highest activity against \textit{S. aureus} (ATCC 25923) with an observed MIC\textsubscript{90} of 8 mg/L ($P < 0.01$). Test compounds (8a, 8b, 10, 11, and 12) failed to produce any significant inhibitory effects against the representative Gram-negative bacteria within the concentration range tested. Even at a concentration of 16 mg/L, none of the test compounds (8a, 8b, 10, 11, and 12) displayed any significant activity against the representative yeast, \textit{C. albicans} (ATCC 10231) when compared with the controls ($P > 0.05$).

### 4.4 Discussion

Based on the structural similarity of 2-DSA and its derivatives to amphiphilic antibiotics, it was proposed that these agents may possess broad antibacterial and antifungal activity. To date, there are no published studies that describe the antibacterial or antifungal activities of 2-DSA and its derivatives synthesised in this study.

The \textit{in vitro} antibacterial and antifungal screening of the test compounds provided evidence that their activity appears to be relatively selective. The synthesised compounds displayed no activity against the representative Gram-negative bacteria
tested. In addition, these compounds lacked any significant effect on *C. albicans* (ATCC 10231). In contrast, 2-DSA (8a), methyl 2-decylsulfonylacetate (8b), 2-decylsulfonylacetic acid (10) and 2-decylsulfonylthioamide (12) displayed reasonable activity against the Gram-positive organism, *S. aureus* (ATCC 25923). The results of this study appear to be similar to the reported findings of sulfinemycin, which also had limited activity against Gram-positive bacteria [138]. With exception of compound 11, the activity of the synthesised compounds against *S. aureus* (ATCC 25923) were greater than that reported for sulfinemycin (MIC 128 mg/L) [100].

The addition of the alkyl group to the acetamide moiety of 2-DSA, surprisingly led to a significant loss of activity against *S. aureus* (ATCC 25923). This finding raises particular concerns about the feasibility of this compound retaining antimicrobial activity, especially after the addition of water-soluble conjugates. Structural modifications may need to adopt a prodrug principle in order to retain biological activity. These must be assessed by further explorative studies.

Substitution of the acetamide group of 2-DSA with the thioamide moiety led to a 2-fold reduction of MIC\textsubscript{90} against *S. aureus* (ATCC 25923). The observed activity for compound 12 against *S. aureus* (ATCC 25923) was comparable to gentamicin, however, well below the antistaphylococcal penicillin, dicloxacillin (0.5 mg/L).

The findings of this *in vitro* antibacterial and antifungal study suggest that the actions of compound 8a, 8b, 10, and 12 may not merely be associated with mechanisms that disrupt the integrity of cell membranes or cell walls. The selectivity indicates a
more specific mechanism of action. 2-DSA was designed as a inhibitor for the KAS enzyme in mycobacterium fatty acid synthesis II (FASII) pathway involved in mycolic acid synthesis [27]. The enzymes involved in the FASII system contain a high degree of conservation and is regarded as a valid target for development of broad spectrum antibiotics [147]. Hence, it is possible the observed antibacterial activity against *S. aureus* was through interference with bacterial FAS. Species differences between the *M. tuberculosis* and *S. aureus* FAS enzymes may explain the difference in activity due to different functional groups in 2-DSA derivatives [147, 148]. This is exemplified by the compound platensimycin. Platensimycin is a secondary metabolite from *Streptomyces platensis* and its mechanism of action was through the inhibition of FabF, FabH and KAS enzymes of the FASII pathway [148]. It has been shown to exhibit strong antibacterial activity against *S. aureus* (1mg/L), *Enterococcus faecalis* and *Streptoccus pneumonia* [148]. When tested against *M. tuberculosis* it exhibited an MIC of 12 mg/L [148]. Further research, however, is required to confirm this for our test compounds and to determine how effective these agents are against other Gram-positive bacteria and clinical strains. Based on this preliminary study, further investigation into the antibacterial activity of compound 12 is warranted. The methods of synthesis outlined in this study provide the capability to produce numerous analogs of compound 12 for the investigation of structural activity relationship.
Chapter 5. *In vitro* cytotoxicity testing of 2-DSA and synthesized derivatives

5.1 Background

*In vitro* cytotoxicity screening was undertaken to evaluate the selective activity of 2-DSA and the derivatives synthesised in this study. Due to their amphiphilic structure, it was proposed that these agents may possess non-selective toxicity towards mammalian cells. Amphiphilic compounds, depending on their structural composition, have been shown to possess varying degrees of toxicity towards mammalian cells [73, 81, 93, 94, 97]. Based on 2-DSA’s proposed action against *M. tuberculosis* [27], it was also hypothesised that these compounds may possess activity against mammalian FAS. Although normal tissues have low levels of fatty acid synthesis, a number of recent studies have demonstrated surprisingly high levels of FAS expression in a wide variety of human malignancies and their precursor lesions, including carcinoma of the colon, prostate, ovary, endometrium, and breast. The differential expression of FAS between normal tissues and cancer has led to the notion that FAS is a target for anticancer drug development [149, 150]. 2-DSA and the derivatives synthesised in this study bear resemblance to many inhibitors of FAS [151-154], known to induce apoptosis in cancer cells [149].

To date there are no published reports on the cytotoxic activities of 2-DSA and the derivatives synthesised in this study. Human alveolar adenocarcinoma (A549), human cervical carcinoma (HeLa), human breast adenocarcinoma (MCF-7), human
prostate adenocarcinoma (LNCaP) and human T cell lymphoblast-like cells (Jurkat) were selected for cytotoxicity screening.

5.2 Materials and methods

2-DSA (8a), methyl 2-decylsulfonylacacetate (8b), 2-decylsulfonylacetic acid (11) and 2-decylsulfonylthioamide (12) were screened for cytotoxic effects against adherent A549, MCF-7, HeLa and LNCaP cell lines using sulforhodamine B (SRB) colorimetric assay. The non-adherent Jurkat cell line viability was assessed by a fluorescence-based resazurin proliferation/viability assay.

5.2.1 Materials

A549, MCF-7, HeLa, LNCaP and Jurkat cell lines were obtained from ATCC (Manassas VA, USA). Complete Dulbecco’s Modified Eagle Medium (DMEM)/F12, DMEM, high glucose RPMI 1640 medium, L-glutamine, sodium pyruvate, HEPES, and Penicillin-Streptomycin solution were purchased from Invitrogen (Victoria, Australia). All other chemicals and reagents were of analytical grade and sourced from Sigma Aldrich (St Louis, MO, USA). MilliQ water was sterilized by autoclaving at 121°C for 20 min. 96 well plates used in this study were purchased from Nunc (New York, USA).

5.2.2 Compound stock solutions

The synthesised compounds 8a, 8b, 10 and 12 were dissolved in DMSO at a concentration of 1 mM. Stock solutions were aliquotted and stored at 2-4°C and used within 1 month of preparation.
5.2.3 Cell cultures

Adherent A549 cells were grown and maintained at 37°C with 5% CO₂ in complete DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 500 U/mL Penicillin-Streptomycin. Adherent HeLa and MCF-7 cells were grown and maintained at 37°C with 5% CO₂ in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine and 500U/mL Penicillin-Streptomycin. Non-adherent Jurkat and adherent LNCaP cells were grown and maintained at 37°C with 5% CO₂ in high glucose RPMI 1640 medium (Invitrogen Australia) supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 500 U/mL Penicillin-Streptomycin.

5.2.4 Cell viability assay

For screening of the test compounds, cells were seeded at 1 × 10⁴ trypan blueexcluding cells/mL in 96 well microtiter plates. 180 μL of cells, at appropriate cell density, were transferred to all experimental wells. 20 μL of the test compounds (at final concentrations of 5, 50 and 100 μM) or vehicle controls were added to each well, making up a final volume of 200 μL. Concentrated stock solutions of the test compounds were prepared in DMSO ensuring that the final solvent concentration remained below 1%. DMSO concentrations below 1% were shown to not affect cell viability of any of the tested cell lines during assay validation. Percentage cell viability was then assessed by either the colorimetric sulforhodamine B (SRB) or fluorescentbased resazurin assays.

The SRB colorimetric assay was used as the primary measure of cell viability for adherent A549, MCF-7, HeLa and LNCaP cells. SRB is a protein staining assay,
which measures the cellular protein content of adherent or suspension cultures. SRB is a bright pink aminoxanthene dye with two sulfonic groups. Under acidic conditions, the two sulfonic groups bind to basic amino acids of cellular proteins, providing a colorimetric evaluation of the estimated total protein mass in relation to the cell number. The SRB assay has been shown to detect cell densities as low as 1,000 to 2,000 cells per well, with sensitivity comparable with several routinely used fluorescent assays. In addition, the SRB assay exhibits linearity over a dynamic range of cell densities [51, 52].

The SRB assay kits were purchased from Sigma Aldrich (St Louis, MO, USA). The cells were treated with test compounds for 24 h for screening. After which, 50 μL of the 50% trichloroacetic acid (TCA) solution was added to each well to growth medium and incubated for 1 h at 4°C to fix the cells. For non-adherent Jurkat cells, plates were centrifuged at 200 g for 5 min at room temperature prior to the addition of TCA. Too much variability was observed for control plates of Jurkat cells during the validation of the SRB assay. As a result, Jurkat cell viability was assessed using the fluorescent-based resazurin assay. The supernatant was removed and cells washed several times with water to remove TCA and serum proteins. Plates were subsequently air dried overnight. 50 μL of SRB staining solution was added to cells for 20 min and then removed by washing with 1% acetic acid solution until all unincorporated dye was removed. Plates were again air dried overnight. 200 μL of Tris base solution was added to each well and incubated for 5 min at room temperature on a IKA gyrator shaker (Crown Scientific, Minto, NSW) to enhance dye mixing. Absorbance was measured using a Titertek Multiskan MKII absorbance plate reader (Huntsville, USA) at an absorbance of 492 nm.
Reduction of the redox dye resazurin to resorufin was used as the primary measuring tool for the measuring of cell viability of non-adherent Jurkat cells. Resazurin has been widely used in the measurement of cell viability in toxicity assays [139-143, 155]. Resazurin is a non-florescent dye, which is reduced to a highly fluorescent pink dye, called resorufin, by mitochondria, microsomal and cytosolic oxidoreductases [156]. Therefore, any toxic compound that impairs cell viability and proliferation will affect the capacity of cells to metabolize resazurin to resorufin. The rate at which resazurin is reduced has been shown to be proportional to number of viable cells present [142]. Resazurin has been reported to be non-toxic to cells and stable in culture medium, thereby allowing extended monitoring of cell proliferation [141].

Following a 24 h treatment, 20 μL of 484μM resazurin stock solution was added to each well. After a 3 h incubation at 37°C in 5% CO₂, reduction of resazurin to resorufin was determined by fluorescence (excitation 530 nm; emission 590 nm) using a Fluoroskan Ascent microplate fluorometer (Thermo Scientific, Victoria, Australia). Appropriate cell free controls were included. Under all conditions tested, the extent of resazurin reduction was directly proportional to the viable cell counts.

5.2.5 Data analysis

Results were calculated and represented as the percentage viable cells, relative to vehicle controls. Reported values represent the mean ± S.E.M of replicate experiments performed on different days. Statistical analysis was performed using GraphPad Instat 3 (GraphPad Software, San Diego, CA, USA). One-way ANOVA followed by Dunnett’s
test were used to assess statistical differences between treatments. Statistical significance was accepted at a probability level of $P < 0.05$.

5.3 Results

The cytotoxicity of the test compounds was assessed *in vitro* against A549, HeLa, MCF-7, LNCaP and Jurkat mammalian cell lines. The test compounds were screened at concentrations of 5, 50 and 100 μM. Cell viability was determined by comparing fluorescence of treatment wells to their respective vehicle controls.

The effect of the test compounds (8a, 8b, 10 and 12) on percentage viability of A549 human alveolar adenocarcinoma cells is presented in Figure 37. All test compounds appeared to affect A549 cell viability in a concentration-dependent manner, however observations were only statistically significant for compounds 8a and 12. Statistically significant reductions in cell viability were observed at both 50 (28.8 ± 7.3%) and 100 μM (31.6 ± 7.4%) concentrations for compound 8a. Compound 12 produced maximal observed inhibitory effect, reducing A549 cell viability by 50 ± 12.5% at the concentration of 100 μM ($P < 0.05$). Although 50 μM of compound 12 reduced viability by 23.3 ± 13.31%, this observation was not statistically significant. Compounds 8b and 10 tended to reduce viability at 50 and 100 μM concentrations, however, these observed effects were not statistically significant when compared to vehicle controls ($P > 0.05$). Reduction in A549 cell viability at the 100 μM concentration were 30.7 ± 11.2% and 24.1 ± 12.5% for compounds 8b and 10, respectively.
Figure 37. Percentage viability of A549 cells treated with test compounds 8a (A), 8b (B), 10 (C) and 12 (D). Each bar represents mean ± S.E.M. (n=3). Symbols (*) indicate statistical significance of $P < 0.05$ as compared to vehicle control.

The effect of test compounds (8a, 8b, 10 and 12) on percentage viability of HeLa human cervical carcinoma cells is presented in Figure 38. Compounds 8b, 10 and 12 produced a concentration-dependent reduction in viability of HeLa cells. At a concentration of 100 $\mu$M, compound 8a reduced cell viability by $25.4 \pm 13\%$ ($p > 0.05$). Compound 8b produced statistically significant ($P < 0.001$) reduction in cell viability at 5 ($13.5 \pm 2.2\%$), 50 ($18 \pm 2.5\%$) and 100 $\mu$M ($31.9 \pm 4.4\%$) when compared to the vehicle controls. Less marked reduction in viability was observed for compound 10 at 5 ($8.4 \pm 2.21%; P < 0.05$), 10 ($15.1 \pm 3.9%; P < 0.001$) and 100 $\mu$M ($16.9 \pm 3.4%; P <$
Compound 12 had maximal effect on HeLa cell viability, with a $61 \pm 14.2\%$ reduction observed at $100 \mu M \ (P < 0.05)$. The average reduction of cell viability at 5 and $50 \mu M$ concentrations of compound 12 were greater than other test compounds, but not statistically significant due to high deviation (S.E.M. > 20%).

![Graphs A, B, C, D](image URLs)

**Figure 38.** Percentage cell viability of HeLa treated with test compounds 8a (A), 8b (B), 10 (C) and 12 (D). Each bar represents mean ± S.E.M. (n = 3). Symbols (*) and (**) indicate statistical significance of $P < 0.05$ and $P < 0.001$, respectively, as compared to vehicle control.

The effect of test compounds (8a, 8b, 10, and 12) on percentage viability of MCF-7 human breast adenocarcinoma cells is presented in Figure 39. Compounds 8a and 10 tended to produce a subtle, but not statistically significant, reduction in viability of MCF-7 cells. At a concentration of $100 \mu M$, compounds 8b and 12 reduced viability
by 27.9 ± 10.2% ($P < 0.05$) and 41.0 ± 9.5% ($P < 0.01$), respectively. No significant alterations in cell viability were observed for lower concentrations of compounds 8b and 12.

**Figure 39.** Percentage viability of MCF-7 cells treated with test compounds 8a (A), 8b (B), 10 (C) and 12 (D). Each bar represents mean ± S.E.M. ($n = 3$). Symbols (*) and (**) indicate statistical significance of $P < 0.05$ and $P < 0.01$, respectively, as compared to vehicle controls.

The effect of test compounds (8a, 8b, 10, and 12) on percentage viability of LNCaP human prostate adenocarcinoma cells is presented in Figure 40. No statistical differences in LNCaP cell viability were observed for any of the compounds tested. The effects of test compounds on LNCaP viability displayed a high degree of variability, particularly at the 5 μM concentration (S.E.M. > 15%). This observation was not
corrected by increasing the number of replicate experiments. At a concentration of 5 μM, compounds 8a, 10 and 12 tended to increase the viability of LNCaP cells, however, this observation was not statistically significant due to the relatively high deviation ($P > 0.05$). At the 50 (18.4 ± 11.5%) and 100 μM (20.4 ± 11.6%) concentrations, compound 12 produced a subtle, but not statistically significant ($P > 0.05$), decrease in cell viability when compared to the vehicle control.

![Figure 40](image1.png)

**Figure 40.** Percentage viability of LNCaP cells treated with test compounds 8a (A), 8b (B), 10 (C) and 12 (D). Each bar represents mean ± S.E.M. (n = 4).

The effect of the test compounds (8a, 8b, 10, and 12) on percentage viability of Jurkat human T cell lymphoblast-like cells is presented in Figure 41. None of the test compounds produced statistically alterations in viability of Jurkat cells at any of the concentrations tested when compared to controls. At 5 μM concentrations, compounds
8a (28.9 ± 26%) and 10 (31.9 ± 18.7%) tended to cause subtle, but variable, increases in cell viability when compared to the vehicle controls.

![Graphs showing viability assays for compounds 8a, 8b, 10, and 12.]

Figure 41. Percentage viability of Jurkat treated with test compounds 8a (A), 8b (B), 10 (C) and 12 (D). Each bar represents mean ± S.E.M. (n=3).

5.4 Discussion

The effects of 2-DSA (8a), methyl 2-decylsulfonylacetate (8b), 2-decylsulfonylacetic acid (10) and 2-decylsulfonylethioamide (12) on mammalian cell viability were studied using in vitro viability assays. The activities of these compounds were assessed in a series of mammalian immortalised cancer cell lines, including: human alveolar adenocarcinoma (A549), human cervical carcinoma (HeLa), human
breast adenocarcinoma (MCF-7), human prostate adenocarcinoma (LNCaP) and human T cell lymphoblast-like (Jurkat) cells.

2-DSA (8a) and its derivatives displayed variable cytotoxicity to the cell lines selected for screening. This study found that A549, HeLa and MCF-7 cells were more sensitive to the effects of compound 8a and its derivatives. In comparison, LNCaP and Jurkat cells appeared to be more resistant. From the results obtained, it is clear that alteration of the acetamide head influences the cytotoxic selectivity. As a group, the synthesised compounds do not appear to possess broad unselective toxicity.

A549 human alveolar adenocarcinoma cells showed a moderate reduction in viability when treated with compound 8a. The relevance of this effect, in terms of potential respiratory toxicity or anticancer potential, needs to be established by further studies. A549 cell are widely used as a model for airway response to drug and other stimuli [157-159].

With the exception of MCF-7, compound 8b and 10 produced similar effects on the viability of HeLa and A549 cells. The cytotoxic effects of compound 8b appeared to be slightly less selective than compound 10. This may be explained by the higher lipophilicity of the methyl ester compared to the free carboxylic acid. In addition, the methyl ester remains as a nonionic amphiphile, irrespective of solution pH. It is possible that the free carboxylic acid of compound 10 could ionise and form an anionic species, which could influence toxicity. Nevertheless, the observed activity is low and unlikely to lead to novel cancer therapeutics.
2-decylsulfonylthioamide (12), the thioamide derivative of 2-DSA (8a), had significant concentration-dependent effects on the viability of A549, MCF-7 and HeLa cells. The substitution of the acetamide functional group of 2-DSA (8a) with a thioamide moiety appears to have extended cytotoxicity. The effect of compound 12 on the viability of A549, HeLa and MCF-7 suggests that further investigations should be undertaken to determine the mechanism and establish its therapeutic potential.

2-DSA (8a), compound 8b, and 10 produced a subtle observed increase in Jurkat human T cell lymphoblast-like cell viability. Although the effect was subtle and not significant, it raised concerns regarding the potential immunological effects these drugs could induce. Therefore, it is important to examine the extent and the potential mechanisms behind these observed effects.
Chapter 6. Hepatotoxicity

6.1 Background

Current TB drug treatment faces two major challenges, namely: increasing drug resistance of *M. tuberculosis* and high incidence of hepatotoxicity [160]. Due to the lack of alternative treatments, first-line drugs continue to remain in the market despite well documented episodes of hepatotoxicity [161]. INH-induced hepatotoxicity manifests as hepatocellular steatosis, necrosis, hepatocyte vaculation and glutathione (GSH) depletion [162-167]. RMP has been shown to interfere with bilirubin excretion, leading to hyperbilirubinemia [168]. This is characterised by hepatocellular changes, ranging from spotty to diffuse necrosis with more or less complete cholestasis [169]. RMP- and PZA-combination treatment-induced hepatotoxicity includes focal cholestasis, fibrosis, micronodular cirrhosis, lymphocytic infiltration and bridging necrosis [170]. Furthermore, rise in *M. tuberculosis* resistance to first-line antitubercular drugs has led to the use of more toxic, sometimes less effective, second-line antitubercular drugs, such as ethionamide [35, 36]. As mentioned previously, it would be ideal to replace these agents with novel compounds that are equally effective against TB, but possess less toxicity potential.

Jones *et al* [27] demonstrated the antitubercular activity of 2-DSA was comparable to many first-line antitubercular drugs, suggesting that it is a potential replacement drug candidate for RMP-resistant mycobacterium infection [67]. 2-DSA was developed as an inhibitor of the tubercular mycolic acid synthesis enzyme β-ketoacyl synthase (KAS) [27]. Recent evidence suggest that 2-DSA-mediated
inhibition of mycobacterial growth may be ascribed to the disruption of mycobacterial ATP synthesis [68]. However, little is known about the toxicity profile of these compounds. The amphiphilic structure of these compounds suggests potential cytotoxic effects against mammalian cells due to its ability to insert and remain within the cell membrane, potentially leading to changes in membrane structure [81, 82, 93, 95, 104].

6.2 Antitubercular drug hepatotoxicity

The reported incidence of hepatotoxicity during multidrug TB treatment varies considerably, ranging between 2 and 28% depending on the study population [171]. INH, RMP and PZA are first-line drugs with reported hepatotoxicity. Of these, INH-induced toxicity is the most prevalent [172]. INH is metabolised in the liver by the cytosolic hepatic enzyme N-acetyltransferase-2 (NAT2). NAT2 phenotype can be divided into either fast or slow acetylator [173-176]. It was observed that individuals with a slow NAT2 phenotype had a significantly higher incidence of hepatotoxicity and an increase in serum AST during INH and RMP combination treatment [177]. Lin et al [178] showed that NAT2 phenotype varies greatly between different ethnic origins, correlating to the observed variability in incidence of hepatotoxicity between different populations studied [171].

NAT2 acetylates INH into acetylisoniazid, which is further hydrolyzed by amidohydrolase into acetylhydrazine and isonicotinic acid. Acetylhydrazine is then either hydrolyzed by amidohydrolase into hydrazine (HYD) or acetylated by NAT2 into diacetylhydrazine, a non-toxic metabolite that is excreted in urine (Figure 42). In addition, INH can also be directly hydrolysed into isonicotinic acid and HYD by amidohydrolase [171, 179]. In individuals with the slow type NAT2 phenotype, more
INH is available for direct hydrolysis to HYD. In addition, acetylhydrazine accumulates in the liver and can be converted into HYD. Hence, individuals with slow NAT2 phenotype have a higher risk of developing hepatotoxicity as they are predisposed to produce more HYD.

Figure 42. Metabolism of isoniazid (adapted from [171, 179])

An animal study revealed that HYD was the major cause of INH-associated hepatotoxicity [162]. In humans with the slow acetylator NAT2 phenotype, both INH
and acetylhydrazine could be detected in urine between 24-32 h, after a single 300 mg dose of INH. In contrast, individuals with the fast acetylator NAT2 phenotype, an increase in acetylhydrazine and diacetylhydrazine were detected in the urine [176]. Thus confirming the importance of the NAT2 phenotype in the risk of developing INH induced hepatotoxicity.

CYP2E1 has also been associated with INH-induced hepatotoxicity [180-182]. Both animal and human studies showed that INH and its metabolite HYD induce CYP2E1 activity [179, 182]. The generation of free radicals from hydrazine has been suggested to be a possible mechanism [179, 180].

Although RMP on its own rarely causes hepatotoxicity, its use in combination with INH has been associated with an increased risk of hepatotoxicity. RMP is not only a potent inducer of CYP450, but it also has been shown to induce the hydrolysis of isoniazid to HYD [183]. This correlates with the observation that rifampicin combined with INH in individuals with slow acetylator NAT2 phenotype results in increased toxicity [184].

Regarding PZA-induced hepatotoxicity, there have been suggestions that combination drug treatment regimen of PZA with INH and RMP is associated with higher frequency of hepatotoxicity. It has also been suggested that higher doses of PZA in combination treatments leads to an increased risk of hepatotoxicity [185, 186]. However, the mechanism by which PZA induces hepatotoxicity is currently unknown, but has been suggested to involve either PZA directly or one of its metabolites [171].
ETA is a second-line antitubercular drug that has been used to replace INH in INH-resistant TB infections. As mentioned previously, ETA (a thioamide) bears structural similarity to 2-DSA and the derivatives synthesised in this study. Like all second-line drugs, toxicity limits its widespread use [10] and the hepatotoxicity of ETA remains a topical subject [187]. A recent study showed that ETA is metabolised by flavin-containing monooxygenases (FMOs) in both human and mice into an S-oxide (ETASO) form, which results in altered redox cycling and depletion of GSH and NADPH thereby producing oxidative stress and associated toxicity [188].

6.3 Heptacellular toxicity study

Drug-induced hepatotoxicity is difficult to predict and remains a major cause for failures during drug development. Of the new drugs approved by the U.S. Food and Drug Administration (FDA), between 1994 and 2006, 36 (2-3% of total) were withdrawn from the market due to safety concerns, with the majority of adverse drug reactions (ADR) being hepatotoxicity and cardiotoxicity [189]. Predictive toxicology screening assays for identifying biomarkers and providing mechanistic assessments allow earlier identification of potential liabilities and can result in recommendations to minimize these risks during lead optimization. Toxicity testing using in vitro monolayer cell models has made significant progress over the past 30 years. HepG2 cell lines are now routinely used in drug hepatotoxicity testing, providing a system for testing compound effects on cell proliferation, apoptosis, steatosis, cytokine secretion and other inflammatory markers. HepG2 cells are human hepatic carcinoma cell line derived from the liver tissue of a human [190, 191]. It has been one of the most widely used cell lines to study the metabolism and toxicity of drugs as it retains many of the specialized characteristics of normal human hepatocytes. These include the synthesis and secretion
of plasma proteins [192], lipoproteins [193], and metabolism of cholesterol [194]. More importantly HepG2 expresses several drug metabolising enzymes including cytochrome (CYP) P450-dependent monooxygenases [195-197], although at a lower level compared to primary hepatocytes from organ cell culture [191]. Nevertheless, it also expresses mixed function oxidase [196] and possesses a complete set of phase II enzymes [198]. With the expectation of UDP-glucuronosyltransferases Westerink et al [198] demonstrated that the level of various phase II enzymes such as sulfotransferases (SULT) 1A1, 1A2, 1E1, mGST-1, glutathione S-transferase GST, NAT1 and epoxide hydrolase (EPHX1) were only modestly different between HepG2 and cryopreserved primary human hepatocytes. Interestingly, a previous report by Grant et al [196] found that HepG2 cells express high levels of UDP-glucuronyltransferases activity and low levels of SULT activity when compared with that of human hepatocytes. These differences in enzyme activity may in part be due to mRNA levels and activities of phase I and II enzymes being highly dependent on the source and culture conditions [191].

In order to validate the use of HepG2 for in vitro metabolism of drug studies Brandon et al [199] examined the genetic polymorphism of phase I and phase II enzymes. The author concluded that in spite of the presence of genetic polymorphisms, there is no real effects influencing the activity of metabolising enzymes and that interpretation of drug metabolism studies should be interpreted keeping in mind of the presence of genetic polymorphisms [199]. Furthermore, the author also found that similar other reports, HepG2 cells only expresses low level of SULT and NAT2 activity [199].
Despite having low levels of CYP P450 enzymes, HepG2 cells are still widely used for toxicology studies due to its ability to carry biotransformation of xenobiotic compounds. It is also metabolises mutagens and carcinogens and carries no p53 mutation, which enables the cells to activate DNA damage responses, induce growth arrest and initiate apoptosis [200]. Furthermore, various studies demonstrated good correlation between in vitro toxicity screening using HepG2 cells and in vivo hepatotoxicity [201, 202].

6.4 Mitochondrial dysfunction and cellular toxicity studies

Mitochondrial dysfunction plays an important role in the toxicity of many drugs [189]. Mitochondria produce approximately 95% of the cells energy in the form of ATP (adenosine triphosphate). It does this through a complicated electron chain transport system, where energy is stored through the conversion of ADP to ATP [203, 204]. The mitochondria consists of an outer and inner membrane [205]. The outer membrane surrounds the organelle, while the inner membrane contains numerous folds called crestae, which increases its surface area exposed to the fluid contents or the matrix of the mitochondria. The metabolic enzymes present in the matrix provide ATP energy generation for cellular function [205].

The outer membrane of the mitochondria is freely permeable to a variety of substrates and metabolites, while the inner membrane is highly impermeable, requiring specific protein carriers to translocate molecules. This allows the mitochondria to maintain an H⁺ electrochemical gradient used for the generation of ATP [189, 204]. The loss of membrane impermeability has been associated with cell death and mitochondrial permeability transition (MPT) may be a key event in this process [161,
MPT has been shown to play a role in both the apoptotic and necrotic cell death. However, while the molecular mechanism by which MPT occur remains largely unknown. The most commonly accepted model involves the opening of a channel complex known as the permeability transition pore (PTP). The PTP consists of the voltage-dependent anion channel (VDAC: outer membrane channel), the adenine nucleotide translocator (ANT: inner membrane channel), cyclophilin D, (Cyp D), and other molecules, which are critical for MTP to occur [207-209]. Some reports suggest the number of mitochondria undergoing MPT determines whether the cell undergoes apoptotic or necrotic cell death [207, 208].

An increasing number of studies have revealed that drug impairment of mitochondria function is involved in drug-induced cellular toxicity [210]. For many years it has been recognized that INH-induced hepatotoxicity was due to the metabolism of INH to acetylhydrazine and HYD [171, 179]. Furthermore, the co-administration of RMP has also been shown to enhance HYD production through enzyme induction which increases the risk of liver toxicity [183, 184]. The exact mechanism by which HYD induced hepatotoxicity remained unknown. In 2006 a study by Chowdhury et al [161] shed some light on this mystery. The study found that apoptosis of hepatocytes in INH and RMP treated mice correlates to an increase in hepatocyte oxidative stress and MPT. In the same study it was also observed that the level of antioxidants plays an important role in determining the degree of oxidative stress. This shows that antioxidant levels can play a role in determining the level of cell death [161].

The impairment of fatty acid β-oxidation will also result in mitochondrial dysfunction and associated hepatotoxicity. Within the mitochondria, fatty acid β-
oxidation plays an important role in the metabolism of fatty acids and ATP generation. The disruption of fatty acid β-oxidation leads to the accumulation of tiny lipid vesicles within the cytoplasm of hepatocytes. Over time this can lead to microvesicular steatosis [211] and disruption of cellular respiration [212]. Several drugs have been shown to inhibit the fatty acid β-oxidation process. These include several cationic amphiphiles drugs such as amiodarone, perhexiline and diethylaminoethoxhexesterol [213]. The inhibition of β-oxidation and cellular respiration leads to a decrease in ATP production and an increase in ROS production in the mitochondria, which oxidise fat deposits causing lipid peroxidation [212].

These and other studies in published literature demonstrate that mitochondrial dysfunction has been increasingly implicated as a cause of drug-induced cellular toxicity [189]. It is therefore important that potential drug compounds be screened for mitochondrial toxicity during development.

6.5 Markers of cell death

Cell death occurs as a natural part of the cell lifecycle or due to the exposure to toxic chemicals, withdrawal of growth factors, hypoxia and ischemia. Depending on the cause and severity of the toxicity effect of these adverse stimuli cells may undergo apoptosis, necrosis, autophagy or cellular senescence [213-217].

Apoptosis is an orderly process of cell death involving margination and condensation of nuclear chromatin, cytoplasmic shrinkage and the formation of apoptotic bodies [217]. There are two pathways to apoptotic cell death, the intrinsic
(intracellular) and the extrinsic (extracellular) pathway. The intrinsic pathway is triggered in response to cellular stress, such as DNA damage and involves the B cell leukemia/lymphoma 2 (Bcl-2) protein families and downstream mitochondrial signal. The extrinsic pathway is triggered by an extracellular signal which interacts with specialized cell surface death receptors [218, 219].

In the intrinsic pathway, the p53 protein is activated in response to cellular stress, such as DNA damage, which leads to the activation of pro-apoptotic proteins BAX and BAK and a decrease in the level of the anti-apoptotic protein family Bcl-2. This leads to the opening of PTP, MTP and release of pro-apoptotic proteins from the mitochondrial intermembrane space into the cytosol, which includes cytochrome c, Smac/DIABLO and serine protease HtrA2/Omi. These pro-apoptotic proteins then activate various caspases such as caspase 9, which activates other caspases (including caspase 3) resulting in the disassembly of the cell [218-220].

The extrinsic pathway is activated when pro-apoptotic ligands such as apoptosis ligand 2/ tumour necrosis factor related apoptosis-inducing ligand (Apo2/TLRAIL) binds to specialized pro-apoptotic membrane receptors such as DR4 and DR5. There are also other apoptotic receptor and ligand combinations such as FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5. Upon activation, each receptor ligand combination forms the death-inducing signalling complex (DISC) by recruiting the adaptor Fas-associated death domain (FADD) and procaspase 8 and 10. The formation of the DISC results in the autocatalytic activation of pro-caspase 8 and 10, which releases activated caspase molecules into the cytoplasm. The active caspase then activates effector caspase 3, 6 and 7, which execute the apoptosis process [218-220].
Unlike apoptosis, which is a tightly regulated ATP-dependent process, necrosis is recognised as a passive externally driven (infection, toxins or physical trauma) process that lacks any metabolic self-involvement. Typical features of cells undergoing necrosis include irreversible changes in the nuclei, loss of cytoplasmic structure, dysfunction in various organelles and cytolysis as a result of cellular swelling. The release of intracellular content of the necrotic cell into the extracellular space can cause further injury and death to adjacent cells, which may result in inflammation and the infiltration of pro-inflammatory cells into the region leading to further tissue damage [217, 220]. Although, apoptosis and necrosis differ with regard to mechanism and morphology, the two processes may overlap in some instances. For example, during the apoptotic process, a decrease in the availability of caspase or intracellular ATP may trigger a switch to necrosis [220].

Autophagy involvement in cell death or survival has been recognised over the past several years. In cellular autophagy, the cells switch to a catabolic metabolic program in which it trades cellular constituents for energy production during periods of nutrient stress [217]. Autophagy may also be a survival mechanism for cells during nutrient stress and share some regulatory processes with apoptosis. For example, hematopoietic cells that are dependent on extracellular signal interleukin 3 (IL-3) for survival. In the absence of IL-3, the cell undergoes macroautophagy allowing it to survive for a number of weeks [221].

The formation of autophagosomes is the hallmark of autophagy and is regulated by a series of protein complexes composed of atg gene products, such as the Atg1/ULK1 complex (Atg1 in yeast and ULK1 in mammals). There are over 30 atg genes.
genes identified in yeast and at least 11 orthologs in mammals. Binding of ULK1 to rapamycin (mTOR) complex 1 (MTORC1) inhibits autophagy when nutrients are abundant. During starvation MTORC1 disassociates from the ULK1 complex freeing it to trigger the formation of autophagosomes. The formation of autophagosomes is divided into the nucleation and expansion stage. Autophagosome nucleation is mediated by a complex containing Atg6 (Beclin 1 in mammals) which recruits class II phosphatidylinositol 4-kinase VPS34 to generate phosphatidylinositol 3-phosphate. The expansion of autophagosomes involves two protein conjugation systems and many Atg proteins. The ubiquitin-like molecule Atg8 (LC3 in mammals) and Atg12 are activated by Atg7 leading to their conjugation to the lipid phosphatidylethanolamine (PE) and Atg5 respectively in a multi-step process which also involves Atg3, Atg4, and Atg10. This process gives rise to nascent autophagosome membranes which form around organelles to be degraded when it subsequently fuses with a lysosome, forming an autolysosome. The breakdown products including amino acids, lipids, nucleosides and carbohydrates are released into the cytosol where they are available for synthetic and metabolic pathways [216, 222].

Several processes that are involved in apoptosis have also been shown to regulate autophagy. For example, p53, a potent inducer of apoptosis was shown to be involved in the activation of cellular senescence through the activation of p21, can also induce autophagy through increased expression of the gene DRAM. Another example is Bel-2, which inhibits apoptosis by blocking Bax activation and the release of cytochrome c, can inhibit autophagy through interaction with Atg 6/Beclin 1. It is not known whether these two mechanisms occur at the same time or if one is more important than the other. Nevertheless, despite being separate mechanism of cell death,
there are some cross regulatory process between autophagy and apoptosis. These cross regulatory process is essential in determining which mechanism of cell death is activated under certain circumstances [216].

Alternatively, cells could also respond to damage from toxic compounds by cell cycle arrest during the G1 phase in a process known as cellular senescence [223]. Cellular senescence can be detected by examining specific molecular senescence associated β-galactosidase, senescence-associated heterochromatin foci, accumulation of lipofuscin granules and the lack of DNA replication [224-228].

A wide range of cellular damage could lead to cellular senescence. These include telomere shortening, DNA damage and oxidative stress, which then activate two major effector pathways, the p53 and pRB pathway. Activation of p53 in cellular senescence leads to the increase of cyclin-dependent kinase inhibitor p21 inhibiting cell cycle progression [223, 229]. The pRB pathways involve activation of p16, which binds to the E2F family of transcription factors, which regulates cell cycle progression [223, 224]. The involvement of p53 in apoptosis, autophagy and cellular senescence indicate some level of cross-talk between these three mechanisms at the p53 level, although the exact mechanism by which p53 activity select apoptosis, autophagy or cellular senescence is not clear [216, 223].

Apoptosis, necrosis, autophagy and cellular senescence all play an important role in the lifecycle of a cell. Recent studies have shown that these processes are all linked by a cellular regulatory process [216, 217].
It is clear from the preceding discussion that INH and other first-line antitubercular drugs have been widely reported to induce hepatotoxicity in patients. Therefore it is imperative that a novel agent with comparable antitubercular activity and reduced host toxicity be developed soon. Based on previous and current antitubercular studies, 2-DSA showed similar antitubercular activity compared to current first-line drugs. The aim of this study was to assess and compare the potential hepatotoxicity of 2-DSA to current antitubercular drugs by screening for in vitro biochemical and molecular markers of cell death using HepG2 cells.

6.6 Materials and Methods

2-DSA (8a), methyl 2-decylsulfonylacetate (8b), 2-decylsulfonylacetic acid (10) and 2-decylsulfonylthioamide (12) were screened for hepatotoxic effects against adherent HepG2 cell lines using a fluorescent-based resazurin proliferation viability assay. The results were confirmed by a SRB colorimetric assay. INH, ETA and hydrazine sulfate were included for comparison. Hydrazine sulfate, the salt of INH toxic metabolite HYD will henceforth be referred as HYD.

Various markers for cell death (LDH, JC-1, DCF, caspase 3, β-galactosidase activity, acidic autophagic vacuoles staining) were used to investigate the type of cellular damage caused by the test compounds.

6.6.1 Materials

HepG2 cells were donated by Professor Greg Anderson from Queensland Institute of Medical Research (QIMR), Iron Metabolism Laboratory. RPMI 1640
medium, L-glutamine, sodium pyruvate, HEPES, and Penicillin-Streptomycin solution were purchased from Invitrogen (Victoria, Australia). All other chemicals and reagents were of analytical grade and sourced from Sigma Aldrich (St Louis, MO, USA). MilliQ water was sterilized by autoclaving at 121°C for 20 min. 96 well plates used in this study were purchased from Nunc (New York, USA).

6.6.2 Compound stock solutions

Synthesised compounds 8a, 8b, 10, 11, 12 and ETA were dissolved in DMSO at a concentration of 100 mM. Stock solutions were aliquoted and stored at 2-4°C and used within one month of preparation. INH and HYD were dissolved in sterile PBS at a concentration of 750 mM and 100 mM, respectively. Fresh solutions were prepared before each experiment.

6.6.3 Cell culture

Adherent HepG2 cells were grown and maintained at 37°C with 5% CO₂ in high glucose RPMI 1640 medium (Invitrogen Australia) supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 500 U/mL Penicillin-Streptomycin.

6.6.4 Cell viability assay

For screening of the test compounds, cells were seeded at $1 \times 10^4$ trypan blue-excluding cells/mL in 96 well microtiter plates. 180 μL of cells, at appropriate cell density, were transferred to all experimental wells. 20 μL of the test compounds (at
final concentrations of 5, 50 and 100 μM) or vehicle controls were added to each well, making up a final volume of 200 μL. Concentrated stock solutions of the test compounds were prepared in DMSO to ensure that the final solvent concentration remained below 1%. DMSO concentrations below 1% were shown to not affect cell viability of any of the tested cell lines during the assay validation. Percentage cell viability was then assessed by fluorescent-based resazurin assays and confirmed by the colorimetric SRB assay.

The SRB assay was performed according to the manufacturer’s instruction. SRB assay kits were purchased from Sigma Aldrich (Missouri, USA). After a 24 h treatment, 50 μL of the 50% TCA solution was added to each well and incubated for 1 h at 4°C to fix the cells. After incubation, the supernatant was removed and the cells were washed 3 times with water to remove TCA and serum proteins. Plates were air dried overnight. SRB staining solution (50 μL) was then added to cells for 20 min and then washed with 1% acetic acid solution until all the unincorporated dye was removed. Plates were again air dried overnight. After which 200 μL of Tris base solution was added to each well and incubated for 5 min at room temperature on a IKA gyrator shaker (Crown Scientific, Minto, NSW) to enhance dye mixing. Absorbance (492 nm) was measured using a Titertek Multiskan MKII absorbance plate reader (Huntsville, USA).

Following a 24 h treatment, 20 μL of 440 μM resazurin stock solution was added to each well. After a 3 h incubation at 37°C in 5% CO₂, reduction of resazurin to resorufin was determined by fluorescence (excitation 530 nm; emission 590 nm) using a
Fluoroskan Ascent microplate fluorometer (Thermo Scientific, Victoria, Australia). Appropriate cell free controls were included. Under all conditions tested, the extent of resazurin reduction was directly proportional to viable cell counts.

6.6.5 GSH analysis

Glutathione is an intracellular non-protein thiol that plays an important role in the defence against reactive oxygen species (ROS) within the cell. It does this through multiple mechanisms, such as conjugation with free radicals; reduction of protein thiols, detoxification of toxic by-products and elimination from the cell [230, 231]. The enzyme Glutathione S-transferase plays an important role in the detoxification of certain xenobiotics by covalently binding glutathione to toxic compounds which allows it to be excreted from the cell [179, 231-233].

In order to reduce intracellular pool of GSH depletion HepG2 cells were pre-treated with 50 or 100 μL L-buthionine S, R-sulphoximine (BSO) before the addition of test compounds. Previous studies have shown that at 2.5 mM BSO is not toxic to HepG2 and the cells remain viable and capable of proliferating up to 5 days after treatment [230, 234].

In this study the reduction of intracellular GSH was confirmed using o-phthalaldehyde (OPA) in an assay adapted from Hissin, et al [235]. At pH 8 OPA will react with GSH yielding a highly fluorescent product, which was detected using fluorescent plate reader. HepG2 cells were seeded at a density of 5×10⁵ cells/mL in 25 cm² tissue culture flasks. To optimise GSH reduction with BSO the experiment was
setup as shown in the table 6 below. After either 24 or 48 h, cells were washed with ice cold PBS and detached from the flask using a cell scraper; and transferred into 2 mL microfuge tubes. The cells were then centrifuged at 17,000 g for 5 min. The resulting cell pellet was resuspended in 0.1 M sodium phosphate-5 mM EDTA and the cells were then lysed by placing it in liquid nitrogen for 1 min and allowed to thaw. A 100 μL aliquot of the lysate was taken for protein determination. 100 μL of 25% phosphoric acid was added to the remaining lysate and vortexed to precipitate proteins. The samples were then centrifuged at 4°C at 17,000 g for 30 min to obtain the supernatant for GSH measurement.

Table 6. Experimental set up of GSH reduction assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flask 1</td>
<td>Control – no BSO</td>
<td></td>
</tr>
<tr>
<td>Flask 2</td>
<td>50 μM BSO</td>
<td>24 h</td>
</tr>
<tr>
<td>Flask 3</td>
<td>100 μM BSO</td>
<td></td>
</tr>
<tr>
<td>Flask 4</td>
<td>Control – no BSO</td>
<td></td>
</tr>
<tr>
<td>Flask 5</td>
<td>50 μM BSO</td>
<td>48 h</td>
</tr>
<tr>
<td>Flask 6</td>
<td>100 μM BSO</td>
<td></td>
</tr>
</tbody>
</table>
The final assay mixture was composed of 10 μL of the cell supernatant, 10 μL of OPA and 180 μL of sodium phosphate-EDTA in a 96 well clear black plate. The samples were mixed thoroughly using an orbital shaker and incubated for 15 min at room temperature. A GSH standard curve was prepared by diluting a 40 mM GSH stock into standard concentrations, ranging between 0.15-2.0 nmol/ml. 10 μL of the standards and 10 μL OPA were added to 180 μL of sodium phosphate-EDTA buffer. Standards were then transferred to 96 well clear black plates and measurements were taken together with cellular samples. The level of fluorescence was measured with Fluoroskan Ascent microplate fluorometer (Thermo Scientific, Victoria, Australia) excitation of 350 nm and emission of 420 nm. The data was then analysed by MARS Data Analysis Software. The amount of GSH present was determined from the standard curve. The reduction of GSH by BSO was determined by comparing the amount of GSH present between BSO treated and untreated cells. The resulting GSH reduction level was then normalised for total protein present.

6.6.6 Analysis of lactate dehydrogenase

The activity of lactate dehydrogenase (LDH) released into culture media was used as an indicator of cell membrane integrity [236]. LDH was measured using a LDH assay kit (Cayman Chemical Company, Ann Arbor, USA). All steps were performed according to the manufacturer’s instruction.

Cells were seeded at a density of $1 \times 10^4$ cells/well in 120 μL of culture medium, with or without test compounds, in a 96 well plate. After a 24 h treatment time plates were centrifuged at 400 g for 5 min and 100 μL of cell supernatant was transferred to a
new 96 well plate along with 100 μL of the standards provided by the kit. 100 μL of the reaction solution was then added to each well, after which the plate was incubated at room temperature shaking gently on an orbital shaker for 30 min. Absorbance (450 nm) was then determined using Titertek Multiskan FC absorbance plate reader (Thermo Scientific, Victoria, Australia).

6.6.7 Monitoring of cellular oxidative stress by DCF-DA

The oxidization of non-florescent 2’,7’-dichlorodihydrofluorescein diacetate (DCF-DA) into fluorescent 2’,7’-dichlorofluorescein (DCF) is an indicator of the presence of ROS.

10 mM 2’,7’-dichlorodihydrofluorescein diacetate (DCF-DA) stock solution was prepared in DMSO and diluted to a final concentration of 10 μM in PBS. Cells were cultured and treated with test compound as mentioned in 6.6.4. After incubation, the culture media was removed and 100 μL of DCF-DA (10 μM) was added to each well. The cells were incubated for 30 min with the dye at 37°C. After which the dye was removed and the cells were washed twice with PBS. Fluorescence was then measured using a Fluoroskan Ascent microplate fluorometer plate reader (Thermo Scientific, Vic, Australia) at excitation 485 nm, emission 535 nm.

6.6.8 Measurement of intracellular lipid accumulation

Intracellular lipid accumulation in HepG2 cells was used as an index for steatosis [237]. In this study the measurement of intracellular lipid accumulation was determined using a steatosis colorimetric assay kit (Cayman chemical company, Ann
Arbor, USA). All experimental steps were performed according to the manufacturer’s instruction.

HepG2 cells were seeded and treated with the test compound as mentioned before (6.6.4), 25 μM of chloroquine was used as the positive control for intracellular lipid accumulation. After a 48 h treatment cells were fixed and washed twice with the wash solution provided. The wells were then allowed to dry completely and stained with 75 μL of Oil red O for 20 min. The Oil red O solution was then removed and the cells were washed 3 times with distilled water followed by 2 washes with wash solution. The wells were then allowed to dry completely. Quantification of lipid accumulation was performed by the addition of 100 μL dye extraction solution to each well. The wells were gently mixed for 30 min and the absorbance was measured at 550 nm using Titertek Multiskan FC absorbance plate reader (Thermo Scientific, Victoria, Australia).

6.6.9 Determination of acidic vesicular organelles formation – Acridine Orange Stain

Acidic vesicular organelles (AVO) or autophagolysosomes is one of the key characteristic of cells undergoing autophagy and were visualized by acridine orange staining [238]. Although this is not conclusive for autophagy is was deemed appropriate for this explorative investigation.

Cells were seeded in 6 well plates at the density of $5 \times 10^5$ cell/well. After a 24 h treatment with the test compounds, cells were stained with 1 μg/mL of acridine orange for 30 min. The cells were then washed twice with PBS and fluorescence visualized
using a Leica DM2000 fluorescence microscope (Leica Microsystems, Victoria, Australia).

### 6.6.10 Measurement of β-galactosidase activity as a marker for cellular senescence

β-galactosidase activity is a common indicator of cellular senescence. X-Gal staining of β-galactosidase was measured using a senescence cell histochemical staining kit (Sigma Aldrich, St Louis, USA). The assay was performed according to manufacturer instructions.

HepG2 cells were seeded and treated with the test compounds for 24 h. Cells were then washed, fixed and stained for β-galactosidase activity overnight at 37°C. Images were captured using a Leica DM2000 fluorescence microscope (Leica Microsystems, Victoria, Australia). Multiple random fields of view were captured for each treatment condition. Cells were scored using ImageJ 1.43u and SA-β-gal positive or negative and represented as a percentage relative to control conditions.

### 6.6.11 Assessment of Mitochondrial Function – JC-1 Assay

The mitochondria play an important role in the generation of energy in the form of ATP for the cell, as well as playing an important role in the induction of apoptosis. The loss of MPT has been shown to be a key event that occurs during this process, making it an important marker for the measurement of mitochondrial function and as an indicator of cell health. 5,5′6,6′-tetrachloro-1,1′3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) is a green dye that can selectively enter the mitochondria and reversible change its colour from green to red as the
membrane potential increases. In healthy cells with high mitochondrial transmembrane potential JC-1 spontaneously form complexes and aggregates within the mitochondria to produces an intense red fluorescence. In unhealthy cells with low mitochondria transmembrane potential (MTP) JC-1 remains monomeric and shows only green fluorescence allowing easy differentiation between healthy and unhealthy cells.

JC-1 working solution (2 mM) was prepared in culture medium and mixed thoroughly by vortexing vigorously followed by centrifugation at 400 g for 2-3 min. Cells were cultured and treated with the test compounds as mentioned in 6.6.4. After 24 h incubation, the culture media was removed and washed once with PBS. Cells were then stained with 1 μM JC-1 for 30 min. The cells were then washed twice with PBS and the fluorescent measured using a Fluoroskan Ascent microplate fluorometer plate reader (Thermo Scientific, Vic, Australia) at excitation 530 nm, emission 590 nm (red); excitation 485 nm, emission 535 nm (green).

6.6.12 Assessment of apoptosis using Caspase 3 activity

Caspase 3, a marker for apoptosis, was measured using a fluorescence assay kit (Cayman chemical company, Ann Arbor, USA). All steps were performed according to the manufacturer’s instructions.

Cells were seeded at 5 × 10^4 cell/well in 100 μL of culture medium in 96 well plates. Twenty microliters of test compounds (at final concentration of 0.1, 1 or 75 mM) or vehicle control were added to each well. Following a 24 h treatment the plates were centrifuged at 800 g for 5 min and the supernatant was then aspirated. Cells were lysed
and centrifuged at 800 g for 5 min and 90 μL of the supernatant from each well was transferred to a corresponding well in a new black 96 well plate and 10 μL of caspase 3 assay buffer was then added to the appropriate wells. Finally 100 μL of the caspase 3 substrate solution was added to each well and the fluorescence was measured after 30 min using a Fluoroskan Ascent microplate fluorometer plate reader (Thermo Scientific, Vic, Australia) at excitation 485 nm and emission 535 nm.

6.6.13 Protein determination

The total intracellular protein level present were determined using Coomassie Brilliant Blue G-250 dye (Bradford Reagent), which is able to bind to proteins [239]. Standards of 0 – 0.5 mg/mL of BSA were prepared for the protein concentration standard curve. The intensity of the protein bound dye was measured by absorbance at 550 nm using a Fluoroskan Ascent microplate fluorometer plate reader (Thermo Scientific, Vic, Australia) at excitation 485 nm and emission 535 nm.

6.6.14 Data analysis

The results were calculated and represented as the percentage viable cells relative to the appropriate vehicle control. The reported percentage values presented as the means ± S.E.M of replicate experiments performed on different days and mean ± S.D. for single experiment (multiple repeats in the same experiment). Statistical analysis was performed using GraphPad Instat 3 (GraphPad Software, San Diego, CA, USA). One-way ANOVA followed by Dunnett’s test were used to assess statistical differences between treatments. Statistical significance was accepted at a probability level of $P < 0.05$. 
6.7 Results

The synthesised compounds (8a, 8b, 10, 11 and 12), INH, ETA and HYD were assessed for hepatotoxicity in HepG2 cells. The protective effect of GSH on 2-DSA-induced toxicity was examined by reducing intracellular GSH levels. ROS and MPT were examined as the possible mechanisms of toxicity. Apoptosis and cellular senescence were investigated as a possible mechanism of cell death due to the observed toxicity. Potential involvement of autophagy was also investigated.

6.7.1 Cell viability measurements

Resazurin (non-fluorescent) reduction to resorufin (highly fluorescent) has been shown to directly correlate to number of metabolically active cells present in the culture [140, 142]. Compounds 8a, 8b, 10, 11 and 12 (5, 50 and 100 μM); INH (5, 50, 100, 40,000 and 75,000 μM); ETA (5, 10, 50 and 100 μM); HYD (50, 100, 500 and 1000 μM) were screened for toxicity to HepG2 cells and compared to their respective vehicle controls. The reduction of HepG2 cell viability by compounds 8a, 8b, 10, 11, 12, INH, ETA and HYD were presented in Figures 43 to 51 for GSH+ (not GSH reduced) HepG2 cells and

Figures 43 to 50 for GSH- (GSH reduced) HepG2 cells.
Figure 43. Compound 8a induced toxicity treated in GSH+ HepG2 cells. A) 24 h, B) 48 h, C) 72 h. Each bar represents mean ± S.E.M. (n = 3). Symbol (*) indicates statistical significance as compared to vehicle control, $P < 0.05$.

The effect of 8a on percentage viability of GSH+ HepG2 cell is presented in Figure 43. At 100 μM 8a reduced cell viability by 30 ± 28.32% after a 24 h treatment ($P <0.05$) (Figure 43). No reduction of cell viability was observed between 0 to 50 μM after a 24 h treatment and 0 to 100 μM after 48 and 72 h treatments.
Figure 44. Compound 8b induced toxicity in GSH+ HepG2 cells. A) 24 h, B) 48 h, C) 72 h. Each bar represents mean ± S.E.M. (n = 3)

The effect of compound 8b on the percentage viability of GSH+ HepG2 cell is presented in Figure 44. Compound 8b was non-toxic to HepG2 cells between 0 to 100 μM after 24, 48 and 72 h treatments.
Figure 45. Compound 10 toxicity in GSH+ HepG2 cells. A) 24 h, B) 48 h, C) 72 h. Each bar represents mean ± S.E.M. (n = 3)

The effect of compound 10 on the percentage viability GSH+ HepG2 cell is presented in Figure 45. Compound 10 was non-toxic to HepG2 cells between 0 to 100 μM after 24, 48 and 72 h treatments.
Figure 46. Compound 11 toxicity in GSH+ HepG2 cells. A) 24 h, B) 48 h, C) 72 h.

Each bar represents mean ± S.E.M. (n = 3)

The effect of compound 11 on the percentage viability of GSH+ HepG2 cell is presented in Figure 46. Compound 11 was non-toxic to HepG2 cells between 0 to 100 μM after 24, 48 and 72 h treatments.
The effect of compound 12 on the percentage viability of GSH+ HepG2 cell is presented in Figure 47. Compound 12 was non-toxic to HepG2 cells between 0 to 100 μM after 24 and 72 h treatments. However, after a 48 h treatment significant toxicity was observed at 100 μM (61 ± 13% viability, $P < 0.01$), but not between 0 to 50 μM.
Figure 48. INH-induced toxicity in GSH+ HepG2 cells. A) 24 h, B) 48 h, C) 72 h. Each bar represents mean ± S.E.M. (n = 3). Symbol (****) indicates statistical significance as compared to vehicle control, $P < 0.001$.

The effect of INH on the percentage viability of GSH+ HepG2 cells is presented in Figure 48. INH was non-toxic to HepG2 cells between 0 to 100 μM after 24, 48 and 72 h treatments. At 40,000 and 75,000 μM, INH was significantly toxic to HepG2 cells at 48 h and 72 h, but not at 24 h ($P < 0.001$). At 48 h INH reduced cell viability by $30 \pm 11\%$ at 40,000 μM and $38 \pm 13\%$ at 75,000 μM. At 72 h INH reduced cell viability by $30 \pm 6.3\%$ at 40,000 μM and $34 \pm 26\%$ at 75,000 μM.
Figure 49. ETA induced toxicity in GSH+ HepG2 cells. A) 24 h, B) 48 h, C) 72 h.

Each bar represents mean ± S.E.M. (n = 3)

The effect of ETA on the percentage viability of GSH+ HepG2 cells is presented in Figure 49. ETA was non-toxic to HepG2 cells between 0 to 100 μM after 24, 48 and 72 h treatments.
Figure 50. HYD induced toxicity in GSH+ HepG2 cells. A) 24 h, B) 48 h, C) 72 h.

Each bar represents mean ± S.E.M. (n = 3).

The effect of HYD on the percentage viability of GSH+ HepG2 cells is presented in Figure 50. HYD was non-toxic to HepG2 cells between 0 to 100 μM after 24, 48 and 72 h treatments.

6.7.2 Results for GSH reduction

The cellular GSH level was reduced in HepG2 cells to minimize its detoxification ability through binding to reactive metabolites. BSO was used to inhibit the synthesis of GSH in HepG2 cells. HepG2 cells were treated with either 50 μM or 100 μM of BSO for 24 or 48 h to reduce GSH levels.
Figure 51. **Effect of BSO on the GSH reduction in HepG2 cells.** % Cellular GSH level was calculated relative to vehicle control at corresponding time point. The GSH level was normalized to protein content by the Bradford assay. Each bar represents the mean ± S.D (n = 3). Symbols (*), (**), and (***), indicate statistical significance as compared to vehicle control, *P* < 0.05, **P** < 0.01 and ***P*** < 0.001. *P* values were calculated using ANOVA with the program GraphPad Prim version 3.

As shown in Figure 51, at 100 µM of BSO GSH levels were reduced by 30 ± 1% and 40 ± 7% compared to vehicle control GSH levels when treated with BSO over 24 and 48 h, respectively (*P* < 0.001 and *P* < 0.01, respectively). No toxicity or morphological changes were observed for BSO treated cells.
For the reduction of GSH levels in HepG2 cytotoxicity studies conducted in this thesis, cells were treated with 100 μM of BSO for 24 h before the addition of test compounds.

INH was screened at concentrations of 5, 50, 100, 40,000 and 75,000 μM; ETA was screened at concentrations of 5, 10, 50 and 100 μM; HYD was screened at 50, 100, 500 and 1000 μM; and 8a, 8b, 10, 11 and 12 were screened at 5, 50, 100 μM and the cell viability of treatment wells were compared to their respective vehicle controls. The reduction of HepG2 cell viability by compounds 8a, 8b, 10, 11, 12, INH, ETA and HYD in GSH reduced HepG2 cells is presented in Figure 52 to 59.

![Figure 52. Compound 8a induced toxicity in GSH- HepG2 cells. A) 24 h, B) 48 h, C) 72 h. Each bar represents mean ± S.E.M. (n = 3). Symbol (*) indicates statistical significance as compared to vehicle control P < 0.05.](image-url)
The effect of compound 8a on the percentage viability of GSH- HepG2 cell is presented in Figure 54. Compound 8a was non-toxic to GSH- HepG2 cells between 0 to 100 μM after 24 and 72 h treatments. However, at 48 h compound 8a was toxic to HepG2 cells at 100 μM demonstrating a 27 21.6% decrease in cell viability. No toxicity was observed between 0 to 50 μM after 48 h.

Figure 53.  Compound 8b induced toxicity in GSH- HepG2 cells.  A) 24 h, B) 48 h, C) 72 h. Each bar represents mean ± S.E.M. (n = 3). Symbol (*) indicates statistical significance as compared to vehicle control, \( P < 0.05 \).
The effect of compound \(8b\) on the percentage viability of GSH-HepG2 cell is presented in Figure 53. Compound \(8b\) was non-toxic to GSH-HepG2 cell between 0 to 100 mM after 24 and 72 h treatments. However, at 48 h compound \(8b\) was toxic to HepG2 cells at 100 \(\mu\)M demonstrating a 19 ± 3.21% decrease in cell viability. No toxicity was observed between 0 to 50 \(\mu\)M after 48 h.

![Graph A]

**Figure 54.** Compound 10-induced toxicity in GSH-HepG2 cells. A) 24 h, B) 48 h, C) 72 h. Each bar represents mean ± S.E.M. (n = 3).

The effect of compound 10 on the percentage viability of GSH-HepG2 cells is presented in Figure 54. Compound 10 was only slightly toxic to GSH-HepG2 between 0 to 100 \(\mu\)M after 24, 48 and 72 h treatments (not statistically significant).
The effect of compound 11 on the percentage viability of GSH- HepG2 cells is presented in Figure 55. Compound 11 effects on GSH- HepG2 cell viability was concentration- and time-dependent. After 24 h treatment compound 11 was non toxic at 0 to 50 μM but significantly toxic to GSH- HepG2 cells at 100 μM (80 ± 7.2% viability, P < 0.05). After a 48 h treatment a greater decrease in viability was observed, 104 ± 10.7% at 5μM; 65 ± 30.05% at 50 μM; and 49 ± 20.5% at 100 μM (P<0.05). At 72 h the decrease in cell viability was less than that observed at 48 h, 108 ± 1.16% at 5 μM; 76.5 ± 37.5% at 50 μM and 50 ± 29.8% at 100 μM.
Figure 56. Compound 12 induced toxicity in GSH- HepG2 cells. A) 24 h, B) 48 h, C) 72 h. Each bar represents mean ± S.E.M. (n = 3). Symbol (*) indicates statistical significance as compared to vehicle control, $P < 0.05$.

The effect of compound 12 on the percentage cell viability of GSH- HepG2 cell is presented in Figure 56. Compound 12 was non-toxic to GSH- HepG2 cells between 0 to 50 μM after a 24 h treatment and between 0 to 100 μM after 48 and 72 h treatments. However, compound 12 was significantly toxic at 100 μM after 24 h treatment, showing a 10 ± 1.2% decrease in cell viability ($P < 0.05$).
Figure 57.  **INH induced toxicity in GSH- HepG2 cells.** A) 24 h, B) 48 h, C) 72 h. Each bar represents mean ± S.E.M. (n = 3). Symbol (**) and (***) indicate statistical significance as compared to vehicle control, $P < 0.01$ and $P < 0.001$

The effect of INH on the percentage viability of GSH- HepG2 cell is presented in Figure 57. INH was non toxic to GSH- HepG2 cell at concentrations between 0 to 1,000 $\mu$M after 24, 48 and 72 h treatments. Interestingly, an increase in cell viability was observed at 48 and 72 h between 50 and 1000 $\mu$M (50 $\mu$M, 48 h, 110 ± 12.8% vs 72h, 122 ± 23.6%; 100 $\mu$M, 48 h, 111 ± 12.8% vs 113 ± 10.1%; and 1000 $\mu$M, 48 h, 102.3 ± 14.3% vs 121 ± 27.8%). At 7,500 $\mu$M INH decreased cell viability after a 24 h treatment by 23 ± 7% ($P < 0.01$); after 48 h treatment by 15 ± 3.2% and after 72 h by 20 ± 17%. When treated with 37,500 $\mu$M and 75,000 $\mu$M of INH, cells showed a time dependent decrease in the viability of GSH reduced HepG2 cell was observed ($P < 0.001$).
At 37,500 μM of INH, cell viability was at 32 ± 7.5% after a 24 h treatment; 24 ± 7.4% after a 48 h treatment; and 17 ± 9.6% after a 72 h treatment (vs. vehicle control). At 75,000 μM of INH, cell viability was at 33 ± 7.2% after a 24 h treatment; 29 ± 6.8% after a 48 h treatment; and 15 ± 8.4% after a 72 h treatment.

![Graph A](A) 24 h, ![Graph B](B) 48 h, ![Graph C](C) 72 h. Each bar represents mean ± S.E.M. (n = 3). Symbol (*) indicates statistical significance as compared to vehicle control, $P < 0.05$

**Figure 58. ETA induced toxicity in GSH- HepG2 cells.** A) 24 h, B) 48 h, C) 72 h. The effect of ETA on the percentage viability of GSH- HepG2 cell is presented in Figure 58. ETA was non-toxic to GSH- cells between 0 to 50 μM after a 24 h treatment, but was significantly toxic at 1,000 μM (67 ± 6% viability, $P < 0.05$). After 48
and 72 h treatments statistically significant toxicity was observed between 0 to 1,000 μM of ETA.

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**Figure 59. HYD induced toxicity in GSH- HepG2 cells.** A) 24 h, B) 48 h, C) 72 h.

Each bar represents mean ± S.E.M. (n = 3). Symbol (*) indicates statistical significance as compared to vehicle control, $P < 0.05$

The effect of HYD on the percentage viability of GSH- HepG2 cell is presented in Figure 59. Between the concentrations of 0 to 500 μM HYD was non toxic to GSH-HepG2 cells after 24, 48 and 72 h treatments. At 1000 μM HYD reduced cell viability by 36 ± 25% after a 24 h treatment ($P < 0.05$); 49 ± 36.3% after 48 h; and 40 ± 27.2% after 72 h.
6.7.3 Results for analyses of lactate dehydrogenase

LDH is an intracellular enzyme located in the cytosol. The levels of LDH in the blood have been used as measurement of tissue breakdown as it is released into surrounding medium upon cellular damage or lysis. Hence, the level of LDH in culture medium is a good indicator of cell membrane integrity, as a measurement of cytotoxicity [236, 240]. In this study, LDH activity in the culture medium was measured as an indicator of cell toxicity (Figures 60 and 61).

**Figure 60. Measurement of LDH activity in the culture medium as an indication of cell damage or lysis.** A) represents GSH+ HepG2 cells; B) represents GSH- HepG2 cells. Cells were treated with 0.1 mM of the relevant test compounds. Each bar represents mean ± S.D. (n = 3). Symbols (*), (**) and (***) indicate statistical significance as compared to vehicle control, \( P < 0.05 \), \( P < 0.01 \) and \( P < 0.001 \).
Figure 61. Measurement of LDH activity in the culture medium as an indication of cell damage or lysis. A) represents GSH+ HepG2 cells; B) represents GSH- HepG2 cells. Symbols (**) and (***) indicate statistical significance as compared to vehicle control $P < 0.01$ and $P < 0.001$.

The effect of the test compounds (8a, 8b, 10, 11 and 12), INH, ETA and HYD on LDH activity of HepG2 cells are presented in Figures 60 and 61. A subtle increase in basal LDH activity of GSH reduced HepG2 cell vehicle control when compared to non-reduced HepG2 cell vehicle control (1700 $\mu$U and 1000 $\mu$U, respectively). With the exception of compound 8a and 11 all the 2-DSA derivative tested showed some increase in LDH activity in the HepG2 cell culture media with GSH- HepG2cells showing a higher level of LDH activity. In GSH+ HepG2 cells, the level of LDH
activity in compound 8b was 2252 μU (P<0.05); 11 was 2153 μU; and 12 was 204 7μU.

In GSH- HepG2 cells, the level of LDH activity in compound 8b was 1876 μU (P > 0.001); 5 was 1721 μU (P > 0.01); and 7 was 1493 μU.

INH and ETA was non-toxic to GSH+ and GSH- HepG2 at 0.1 mM. At INH’s reported in vitro IC₅₀ level of 75 mM [241, 242] both GSH+ and GSH- HepG2 cells showed a significant increase in LDH activity compared to vehicle control (5157.6 μU and 3887 μU, respectively, P < 0.001) (Figure 61). When the cells were examined under the microscope they showed gross change in morphology and a lower cell number (data not shown). GSH+ and GSH- cells treated with HYD, also showed a significant increase in LDH activity (0.1 mM, P <0.001 and 1 mM, P < 0.01). At 0.1 mM the measured level of activity was 2800 μU (GSH+) and 1990 μU (GSH-). At 1 mM the measured level of activity was 1172 μU (GSH+) and 2332 μU (GSH-).

6.7.4 Results of monitoring of cellular oxidative stress – DCF

H₂DCF-DA is a widely used reagent to evaluate cellular oxidative stress. H₂DCF-DA is a non-florescent lipophilic ester that easily crosses the plasma membrane of the cell into the cytosol where it is cleaved by intracellular esterase forming H₂DCF. In the presence of reactive oxygen species within the cell as the cell undergoes oxidative stress H₂DCF is oxidised into DCF. The brightness of the fluorescence observed is used to indicate the extent by which ROS was present.

To determine if the observed decrease in cell viability in the resazurin assay was due to ROS production. GSH-depleted and non-GSH-depleted HepG2 cells were
treated with the test compounds for 24 h. After which the cells were treated with H₂DCF and the level of fluorescence measured as an indicator of ROS production (Figure 62 and 63).

**Figure 62.** Relative fluorescence of HepG2 cells treated with test compounds versus untreated cells. Cells undergoing oxidative stress will fluoresce bright green. A) represents GSH+ HepG2 cells; B) represents GSH- HepG2 cells. Cells were treated with 0.1 mM of the relevant test compounds. Symbols (*), (**) and (***) indicate statistical significance as compared to vehicle control, \( P < 0.05, P < 0.01 \) and \( P < 0.001 \).
Figure 63. Relative fluorescence of HepG2 cells treated with test compounds versus untreated cells. Cells undergoing oxidative stress will fluoresces bright green. A) represents GSH+ HepG2 cells; B) represents GSH- HepG2 cells. Symbols (*) and (***)) indicate statistical significance as compared to vehicle control, $P < 0.05$ and $P < 0.001$

The effect of the test compounds (8a, 8b, 10, 11 and 12), INH, ETA and HYD on ROS production is presented in Figure 62 and 63 as change in fluorescence. GSH+ HepG2 cells compounds 8a (124%, $P < 0.05$), 10 (131%, $P < 0.01$) and 11 (151%, $P < 0.001$) significantly increased the level of ROS production compared to vehicle control. When intracellular GSH was reduced in HepG2 cells, compound 12 markedly increased ROS production in the cell (132%, $P > 0.05$). Compounds 8a (124%), 8b (111%), 10
(115%) and 11 (130%), also increased ROS in GSH- HepG2 cells, however only 8a ($P > 0.01$) and 11 ($P > 0.001$) were shown to be statistically significant (Figure 64).

In GSH+ HepG2 cells treated with INH, ETA and HYD at 0.1 mM showed no increase in ROS compared to vehicle control (Figure 62). While in GSH- HepG2 cell treated with 0.1 mM INH ETA and HYD a statistically significant decrease in ROS was observed (INH, 75%, $P > 0.01$; ETA, 74%, $P > 0.01$; and HYD 79%, $P > 0.05$). At a higher concentration of INH (75 mM) and ETA (1 mM) there seemed to be a marked increase in ROS in GSH-reduced cells (320% and 165%, respectively, $P < 0.001$) (Figure 63), indicating an increase in ROS production. However, HYD showed a decrease in ROS (80%, $P > 0.05$). Interestingly non-GSH-reduced cells when treated with 75 mM INH showed no increase in ROS when compared to vehicle control (86%). While at 1 mM ETA treated cells showed significant increase in ROS and 1 mM HYD showed some increase in ROS when compared to vehicle control (150%, $P < 0.01$ and 118%, respectively) (Figure 63).

6.7.5 Results for measurement of intracellular lipid accumulation

One of the most common histological markers of liver damage is steatosis or the accumulation of fatty vesicles within hepatocytes [243]. Visual examination of GSH-HepG2 cells treated with INH and 2-DSA derivates revealed clear vacuoles within the cells closely resembling fatty vesicles from fatty liver. Therefore, we investigated this using Oil red O dye to stain the cells to confirm whether the vacuoles formed were fatty vesicles. Chloroquine is an antimalarial drug, known to cause steatosis was used as the positive control. Figure 64 and 65 presents the effect of compounds 8a, 8b, 10, 11 and 12’s on HepG2 intracellular lipid level compared to INH, ETA and HYD.
Figure 64. Quantification of lipid accumulation within HepG2 cells by measuring the absorbance (550 nm) of Oil Red O dye. A) represents GSH+ HepG2 cells; B) represents GSH- HepG2 cells. Cells were treated with 0.1 mM of the relevant test compound or 0.025 mM chloroquine. Each bar represents mean ± S.D. (n = 3). Symbol (*) indicates statistical significance as compared to vehicle control, $P < 0.05$. 
Figure 65. Quantification of lipid accumulation within HepG2 cells by measuring the absorbance (550 nm) of Oil Red O dye. A) represents GSH+ HepG2 cells; B) represents GSH- HepG2 cells. Each bar represents mean ± S.D. (n = 3). Symbol (*) indicates statistical significance as compared to vehicle control, *P < 0.05.*

The effect of the test compounds (8a, 8b, 10, 11 and 12), INH, ETA and HYD on HepG2 cell lipid accumulation is presented in Figure 64 and 65. An increase in red stain stained intracellular lipid vesicles (as represented by absorbance at 550 nm relative to vehicle control) in both GSH- and GSH+ HepG2 cells after a 48 h treatment period (GSH+, vehicle control 0.072 AU; chloroquine, 0.081 AU; 8a, 0.078 AU; 8b, 0.078 AU; 10, 0.076 AU; 11, 0.064 AU; 12, 0.072 AU; INH 75 mM, 0.061 AU; INH 0.1 mM;
0.067 AU; ETA 1 mM, 0.055 AU; ETA 0.1 mM 0.066 AU; HYD 1 mM, 0.065 AU; and HYD 0.1 mM, 0.066 AU. GSH-, vehicle control, 0.052 AU; chloroquine, 0.072 AU; 8a, 0.058 AU; 8b, 0.063 AU; 10, 0.063 AU; 11, 0.060 AU; 12, 0.064 AU; INH 75 mM, 0.054 AU; INH 0.1 mM, 0.065 AU; ETA 1 mM, 0.056 AU; ETA 0.1 mM 0.063 AU; HYD 1 mM, 0.061 AU; and HYD 0.1 mM 0.067 AU). Of the compounds tested, chloroquine showed a significant increase in intracellular lipid vesicles in GSH- HepG2 cells compared to vehicle control (0.07 AU, \( P < 0.05 \)). This result suggests that GSH reduction in HepG2 cells leads to a greater increase in accumulation of lipid vesicles compared to non GSH reduced cells.

### 6.7.6 Results for determination of acidic vesicular organelles formation – Acridine Orange Stain

One of the key characteristic of cells undergoing autophagy is the formation of AVO or autophagolysosomes through the fusion of phagosomes with lysosomes. The formation of AVOs can be detected by fluorescent microscopy through the staining of AVO with the weak base acridine orange. Normal cells stained with acridine orange will display a green fluorescence. When cells are undergoing autophagy, acridine orange will protonate and accumulate in acidic compartments such as autophagolysosomes, giving off a bright red fluorescence.

HepG2 cells were stained with acridine orange in order to determine whether the loss of cell viability observed for INH, ETA, HYD and 2-DSA and its derivatives were mediated by autophagy. Cells were treated with 0.1 mM of test compounds for 24 h and were then stained with acridine orange and observed under a fluorescent microscope.
The effect of the test compounds (8a, 8b, 10, 11 and 12) on the formation of AVO in HepG2 cell stained with acridine orange is presented in Figure 66. Of the 2-DSA derivatives examined, only compound 8b and 10 showed a marked increase in red fluorescence indicating an increase in AVO formation. Compound 8a, 11 and 12 showed a similar level of red fluorescence compared to vehicle control.
Figure 67. Fluorescent microscopy of GSH+ HepG2 cells. Acidic autophagic vacuoles are stained red. A) vehicle control B) INH 1mM C) ETA 0.1 mM D) ETA 1 mM E) HYD 0.1 mM.

The effect of the INH, ETA and HYD on the formation of AVO in HepG2 cell stained with acridine orange is presented in Figure 67. When treated with vehicle control, GSH+ HepG2 cells mostly green with a sporadic red fluorescence. A similar pattern of green and red fluorescence was observed in INH, ETA 0.1 mM and HYD treated cells. When GSH+ HepG2 cells were treated with 1 mM ETA an increase in the level of red fluorescence was observed. Indicating that at 1mM, ETA may cause cell to form AVO and possibly undergoing autophagy as a mechanism of cell death or survival.
Figure 68. Fluorescent microscopy of GSH- HepG2 cells. Acidic autophagic vacuoles are stained red. A) vehicle control B) compound 8a C) compound 8b D) compound 10 E) compound 11 F) compound 12.

The effect of the test compounds (8a, 8b, 10, 11 and 12) on the formation of AVO in HepG2 cell stained with acridine orange is presented in Figure 68. From the figure it is clear that of the 2-DSA derivates only compound 8b and 10 treated GSH-HepG2 cells increased in red fluorescence when compared to vehicle control. In contrast when compared to vehicle control no increase in red fluorescence was observed in cells treated compound 8a, 11 and 12.
Figure 69. Fluorescent microscopy of GSH- HepG2 cells. Acidic autophagic vacuoles are stained red. A) vehicle control B) INH 0.1 mM C) ETA 0.1 mM D) HYD 0.1 mM

The effect of the INH, ETA and HYD on the formation of AVO in HepG2 cell stained with acridine orange is presented in Figure 69. The figure clearly shows that vehicle control treated GSH- HepG2 cells had a lower level of red fluorescence compared to vehicle control treated GSH+ HepG2 cells. In figure 69, a subtle increase in red fluorescence was observed in INH and ETA treated GSH- HepG2 cells when compared to vehicle control. In contrast a dramatic increase in red fluorescence was observed in HYD treated cells. As autophagy has been linked to other mechanism of cell death, including apoptosis and senescence, HepG2 cells were also assessed for β-galactosidase (senescence), caspase 3 and JC-1 (apoptosis) activity level.
6.7.7 Result of Measurement of β-galactosidase activity as a marker for cellular senescence

β-galactosidase activity is an indicator of cellular senescence. Although there have been some reports questioning the viability of X-Gal staining of β-galactosidase as a reliable indicator of cellular senescence. The aim of this assay was only to investigate whether GSH depletion and the synthesized 2-DSA derivatives increased β-galactosidase activity as a possible indicator of cellular senescence. The percent ratio of β-galactosidase stained cell when treated with compounds 8a, 8b, 10, 11 and 12 compared to INH, ETA and HYD are presented in the table below.

Table 7. Percent ratio of β-galactosidase activity in GSH+ and GSH- HepG2 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GSH+</th>
<th>GSH-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>29%</td>
<td>25%</td>
</tr>
<tr>
<td>Compound 4a</td>
<td>24%</td>
<td>25%</td>
</tr>
<tr>
<td>Compound 4b</td>
<td>22%</td>
<td>3%</td>
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<td>Compound 10</td>
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<td>Compound 12</td>
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<td>9%</td>
</tr>
<tr>
<td>Isoniazid 1 mM</td>
<td>37%</td>
<td>35%</td>
</tr>
<tr>
<td>Isoniazid 0.1 mM</td>
<td>35%</td>
<td>39%</td>
</tr>
<tr>
<td>Ethionamide 1 mM</td>
<td>39%</td>
<td>18%</td>
</tr>
<tr>
<td>HYD 1 mM</td>
<td>31%</td>
<td>20%</td>
</tr>
</tbody>
</table>
Of the 2-DSA derivatives investigated, in GSH+ HepG2 cells only compound 11 demonstrated an increase in β-galactosidase staining (41%) (Table 7). Compound 8a (24%), 8b (22%), 10 (21%) and 12 (22%) showed similar level of β-galactosidase staining compared to vehicle control (Table 7). In GSH- HepG2 cells treatment with compound 6 showed an increase in β-galactosidase staining compared to vehicle control (33%). Interestingly, apart from compound 8a (25%), compound 8b (3%), 11 (12%), 12 (9%) and ETA (18%) decreased in β-galactosidase staining when compared to vehicle control treated HepG- cells (Table 7).

Compared to vehicle control, ETA and HYD treated cells increased β-galactosidase staining in GSH+ HepG2 cells while GSH- cells showed a decrease in β-galactosidase staining (GSH+ ETA, 39%; HYD 31% and GSH- ETA, 18% and HYD 20%). While INH showed an increase in β-galactosidase staining in GSH- cell compared to GSH+ cells (GSH+, 1 mM 37% and 0.1 mM 35%; GSH- 1 mM, 35% and 0.1 mM, 39%) (Table 7).

6.7.8 Results of assessment of mitochondrial permeability– JC-1 assay

In healthy cells JC-1 forms aggregate within the mitochondria, which fluoresces red. However, when the cell is undergoing apoptosis or is damaged and loses mitochondria membrane potential JC-1 will leak out of the mitochondria and remain in the cytoplasm in a monomeric form, which fluoresces green. HepG2 cells were treated with the test compounds over 24 h and were then stained with JC-1 for 30 min. The percentage of ratio of red to green fluorescence was used as an indicator of mitochondria membrane integrity. Figure 70 and 71 presents the percent ratio of red to
green fluorescence in HepG2 cells treated with the compounds 8a, 8b, 10, 11 and 12 compared to INH, ETA and HYD.

**Figure 70.** Percent ratio of red to green fluorescent cells compared to vehicle control. A) represents GSH+ HepG2 cells; B) represents GSH- HepG2 cells. Cells were treated with 0.1 mM of the relevant test compounds. Each bar represents mean ± S.D. (n=3). Symbol (*) indicates statistical significance as compared to vehicle control, P<0.05.
Figure 71. Percent ratio of red to green fluorescent cell compared to vehicle control. A) represents GSH+ HepG2 cells; B) represents GSH- HepG2 cells. Each bar represents mean ± S.D. (n=3). Symbols (*) and (*** ) indicate statistical significance as compared to vehicle control, P<0.05 and P<0.001.

The effect of the test compounds (8a, 8b, 10, 11 and 12), INH, ETA and HYD on the membrane permeability of HepG2 mitochondria was assessed using JC-1 staining and presented in Figure 70 and 71. Of the 2-DSA derivatives tested, compound
8a and 11 did not decrease JC-1 aggregation within the mitochondria in GSH+ cells. However, when the GSH was reduced, a decrease in red fluorescence was observed in compound 7 (109% in non GSH depleted and 72% in GSH depleted cells). Compound 8a showed similar levels of JC-1 aggregation within the mitochondria in GSH+ and GSH- cells (112% and 117% respectively). Compound 8b showed the most dramatic decrease in red fluorescence in both GSH+ and GSH- cells (Figure 70). 8b showed a 48% red to green ratio for GSH- cells (P>0.05) compared to 71% in GSH+ depleted cells. A similar level of red green fluorescence ratio was observed in both GSH+ and GSH-cells treated with compound 12 (70% and 67% respectively). Compound 6 did not seem to have a dramatic effect on the HepG2 cells’ mitochondrial potential as indicated by 91% (GSH+) and 95% (GSH-) red to green fluorescence ratios.

Figure 70 and 71 showed that GSH- cells treated with 75 mM and 0.1 mM INH reduced the red green fluorescence ratio by 70% and 10%, while for GSH+ cells no decrease in red fluorescence was observed. In GSH- cells 1 mM ETA demonstrated an increase in mitochondria damage when compared to GSH+ cells (63% vs. 88% respectively) (Figure 73). GSH reduction also showed a remarkably increase in mitochondria damage in HYD treated HepG2 cells. A red to green fluorescence ratio of 48% vs. 69% was observed when cells were treated with 1mM HYD in GSH- and GSH+ cell, respectively (Figure 73). The same pattern was also observed at 0.1 mM HYD 73% for GSH- vs. 85% for GSH+ cells (Figure 72).

A decrease in mitochondrial potential can often lead to the release of cytochrome c and other apoptotic signal proteins from the mitochondria. Caspase 3 is
released late in the apoptotic process and is used as an indicator of whether apoptosis was the mechanism of cell death in 2-DSA, its derivatives, INH, ETA and HYD toxicity.

6.7.9 Results of assessment of apoptosis using Caspase 3 activity

Apoptotic cell death is a highly regulated event and is regulated by a variety of cellular signalling pathways. Key apoptotic events include the margination and condensation of nuclear chromatin, cytoplasmic shrinkage and the formation of apoptotic bodies [217]. Caspases play an important role in the apoptotic event. Initiator caspases (caspase 2, 8, 9, and 10) are involved in starting the apoptotic cascade and the effector caspase (caspase 3, 6, and 7) are involved in the disassembly of the cell. There are multiple pathways leading to caspase activation [216]. Caspase 3 activity was measured to determine whether INH, ETA, HYD and the 2-DSA derivatives induced cytotoxicity were due to apoptotic cell death. The results are presented in Figures 72 and 73.
Figure 72. Caspase 3 activity as a marker for apoptosis was measured using relative fluorescence of HepG2 cells treated with test compounds versus vehicle control. Values were corrected for cell viability measured using the resazurin assay. A) represents GSH+ HepG2 cells; B) represents GSH- HepG2 cells. Cells were treated with 0.1 mM of the relevant test compound. Each bar represents mean ± S.D. (n = 3). Symbols (*), (**) indicate statistical significance as compared to vehicle control, $P < 0.05$, $P < 0.01$. 
Figure 73. Caspase 3 activity as a marker for apoptosis was measured using relative fluorescence of HepG2 cells treated with test compounds versus vehicle control. Values were corrected for cell viability measured using resazurin assay. A) represents GSH+ HepG2 cells; B) represents GSH- HepG2 cells. Each bar represents mean ± S.D. (n = 3). Symbol (*** ) indicates statistical significance as compared to vehicle control P < 0.001.

The effect of the test compounds (8a, 8b, 10, 11 and 12), INH, ETA and HYD on HepG2 cell apoptotic cell death is assessed by measuring caspase 3 activity and are presented in Figures 72 and 73. GSH reduction increased HepG2 cells sensitivity to the cytotoxic effect of the test compounds. When compared to GSH+ HepG2 cells a
marked increase in caspase-3 activity was observed in GSH- HepG2 cells. Of the 2-DSA derivatives tested only the parent compound 8a (1027 RFU) didn’t demonstrated a higher level of caspase-3 activity in GSH- HepG2 cells, while the compounds 8b (1767 RFU), 10 (2077 RFU), 11 (1961 RFU) and 12 (1956 RFU) increased the caspase level compared to vehicle control (1380 RFU) (Figure 72). In GSH+ cells compounds 8a (1449 RFU), 8b (1376 RFU), 10 (1375 RFU), 11 (1204 RFU) and 12 (1956 RFU) showed similar level of caspase 3 activity compared to vehicle control (1315 RFU) (Figure 72).

Figure 73 showed that 75 mM of INH significant increased the caspase 3 activity in both GSH reduced and non reduced HepG2 cells compared to vehicle control (3300 RFU in GSH- and 2842 RFU in GSH+ cell, \( P < 0.001 \)). Comparing between GSH+ and GSH- HepG2 cells treated with 1 mM ETA and HYD. A higher level of caspase 3 activity was observed in GSH reduced HepG2 cells (ETA, GSH+, 1465 RFU and GS-, 1943 RFU; HYD, GSH+ 1736 RFU and GSH- 1939 RFU) (Figure 73). ETA at 0.1 mM caused a significant increase in caspase 3 activity within the cell compared to vehicle control (2236 RFU and 1380 RFU, respectively; \( P < 0.001 \)) (Figure 72).
### 6.7.10 Summary of Results

#### Table 8  Summary of results 1

<table>
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<th>Drugs tested (0.1mM)</th>
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<th>48 h</th>
<th>72 h</th>
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#### Table 9 Summary of results 2

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6.8 Discussion

A major limitation of current front-line antitubercular drugs is, the high incidence of hepatotoxicity [160]. Therefore, it is imperative that newly developed antitubercular agents be screened for potential hepatotoxicity. 2-DSA has been shown to have comparable antitubercular activity to current antitubercular drugs RMP and ETA [27, 28]. However, its effects on the liver are unknown. Therefore, 2-DSA and its derivatives were screened for potential hepatotoxicity in an *in vitro* model utilising HepG2 cells. Furthermore, mechanisms underlying this toxicity were also investigated.

Resazurin reduction to resorufin was used as an indicator of HepG2 cell viability. 2-DSA (8a) and compound 8b decreased the viability of non GSH reduced cells (GSH+) HepG2 cells at 24 h. However, between 48 h and 72 h compounds 8a and 8b were non-toxic to HepG2 cells. This suggests that over time HepG2 cells were able to metabolise these compounds into non-toxic metabolites. It is also possible that over time the HepG2 cells became less sensitive to the toxic effect of these compounds. Similar changes in sensitivity to toxic compounds were also observed in long-term toxicity studies using fibroblasts and epithelial cells [244, 245]. In contrast, when GSH levels were reduced compound 8a and 8b showed increased toxicity toward HepG2 cells. Furthermore, compounds 10 and 11 were only toxic to HepG2 cells upon GSH reduction. Compound 12 appears to be unaffected by intracellular GSH levels as it was equally toxic to both GSH+ and GSH- HepG2 cells. These results demonstrates that intracellular GSH levels is important reducing the potential toxic effect compounds 8a, 8b, 10 and 11 in HepG2 cells.
In comparison, ETA and HYD were shown to be less toxic than 2-DSA and its derivatives, while INH was only toxic to HepG2 cells at suprapharmacological doses. Similar to 2-DSA and its derivatives, INH, ETA and HYD toxicity was significantly enhanced upon reduction of intracellular GSH levels. INH is a prodrug that is only toxic after being metabolized into its toxic metabolite HYD [162]. HepG2 expression of INH metabolizing enzymes such as CYP 450 and NAT2 has been reported to be lower than that of human liver or primary hepatocytes, hence suprapharmacological doses of INH were required to induce toxicity in our in vitro model [191, 241, 242]. In addition, GSH reduction and CYP3A4 up regulation in HepG2 cell has been reported to increase sensitivity to INH toxicity as shown in this and other studies [246]. Furthermore, GSH depletion has also been reported to increase HYD-induced HepG2 toxicity [247].

GSH is an important cellular antioxidant and has been shown to protect cells from xenobiotic toxicity [246, 248, 249]. Although cells possess various enzymatic and non enzymatic systems such as, dismutase, catalase, glutathione S-transferases (GST), transferrin, lactoferrin, GSH, cysteamine and cysteine, GSH and GSH related enzymes are considered the most important in humans and play an important role in the detoxification of xenobiotics [250].

To further investigate this protective role of intracellular GSH increased lipid accumulation (steatosis) and LDH were used as surrogate markers of hepatotoxicity. Consistent with observations using resazurin increased intracellular lipid accumulation was seen in GSH reduced cells (GSH-) HepG2 exposed to 2-DSA and its derivatives. The same trend was also observed in INH, ETA and HYD treated HepG2 cells. The
accumulation of intracellular lipid formation is a well established histological marker for liver damage [243]. Drug-induced steatosis is often reversible but prolonged exposure can cause macrovesicular steatosis. Chronic macrovesicular steatosis can evolve into steatohepatitis ultimately leading to cirrhosis and liver failure [237]. The INH-induced increase in intracellular lipid accumulation in HepG2 cells consistent with previously published reports on INH-induced steatosis in rabbits [161, 251] and cirrhosis in humans [163, 252]. The observed increase in INH-induced steatosis in rabbits was also accompanied by an increase in plasma triglyceride levels [161, 251]. In humans apart from cirrhosis, severe INH-induced hepatotoxicity also includes submassive and massive necrosis in the liver [163, 252].

2-DSA and its derivatives are amphiphilic in structure. Amphiphiles with cationic hydrophilic head groups have been shown to be able to embed themselves into cellular membranes and disrupt membrane structures leading to cellular toxicity [93, 94, 96, 253]. Therefore, the release of LDH into the surrounding medium as an indicator of membrane damage was used to investigate whether 2-DSA and its derivative’s toxicity was through membrane damage due to its amphiphilic nature. LDH release is is widely considered to be a reliable marker for drug-induced toxicity, as increased serum LDH levels are often observed in patients with INH and ETA induced liver damage [254-256].

The 2-DSA derivatives had variable effects on HepG2 cell membrane integrity. This suggests that these compounds induce cell toxicity via different mechanisms. Compounds 8b, 11 and 12 were shown to increase LDH activity independent of GSH levels. In contrast the resazurin reduction assay the toxicity of compounds 8b and 11 was dependent on GSH levels. GSH reduction increased the toxic effect of 8b and 11.
Interestingly, compound 8a showed an increase in LDH release in GSH+ cells but not in GSH- cells, contrary to its observed toxicity toward GSH- cell in resazurin assay. It is possible that the toxicity of compounds 8b and 11 toward HepG2 cells was based on its amphiphilic structure causing membrane damage leading to the release of LDH into the medium [253, 257]. However, the observed toxicity of compounds 8a, 10 and 12 to HepG2 cells may be via a different mechanism. It is also possible that the 2-DSA derivatives induce cellular damage through a mechanism that is not affected by the intracellular GSH level.

Decreased intracellular GSH levels enhanced the release of LDH in HepG2 cells treated with INH, ETA and HYD. However, in cells treated with INH and ETA, increased LDH release was only observed at higher drug concentrations compared to 2-DSA and its derivatives. HYD showed a significantly increase in LDH release at comparable drug concentrations to 2-DSA and its derivatives. In comparison to INH, ETA induced higher levels of LDH release. The observation with HYD is consistent with a previous study using primary rat hepatocytes. However, the minimum dose of HYD that was required to induce toxicity in rat hepatocytes shown to be significantly higher (20 mM) compared with the dose used in this study to induce toxicity [258, 259]. This was not expected as CYP enzymes have been associated with HYD induced toxicity through the formation of free radicals and HepG2 cells only expresses low level of CYP compared to rat hepatocytes [179, 191, 260]. This suggests that other mechanism and factors of HYD metabolism was involved in HepG2 cells sensitive toward HYD toxicity.
While the hepatoxic properties of INH and ETA are well established, mechanisms underlying this toxicity remain poorly defined. The literature suggests that INH-induced toxicity to HepG2 cells was through ROS production and subsequent MPT, and apoptosis [241, 261, 262]. It is also possible that ETA-induced hepatotoxicity occur as a result of oxidative stress. ETA metabolism produces sulfenic acid and carbodiimide metabolites which potentially decrease intracellular GSH levels resulting in oxidative stress and subsequent toxicity [36]. Since oxidative stress appears to mediate toxicity to both INH and ETA we investigated its role in the toxicity of 2-DSA and its derivatives to HepG2 cells [241, 261, 262].

Various reports have shown that depletion of GSH results in increased ROS production [263-265]. Hence, it is plausible to suggest that the observed toxicity of 2-DSA and its derivatives in GSH- HepG2 cells were due to an increase in ROS production. However, this was not the case, as compound 8a, 8b, 10 and 11 increased ROS irrespective of intracellular GSH levels. In contrast compound 8b, 10 and 11 were only toxic to GSH- HepG2 cells, while the toxicity of compound 8a toxicity was independent of intracellular GSH levels. Interestingly, the increased ROS production by compound 5 was shown to be dependent on the intracellular GSH level, while the resazurin assay showed that it was toxic to HepG2 cell irrespective of intracellular GSH levels. This suggests that more than one mechanism of toxicity was involved and that the toxicity of these compounds is independent of ROS. In comparison to current antitubercular drugs, GSH reduction increased INH-induced ROS production. In contrast, HYD and ETA increased ROS production in GSH+ cells. One possible explanation is that, some studies have observed that cells preserve mitochondrial GSH when cytosolic/nuclear GSH stores are completely depleted. Although GSH is
synthesized in the cytosol, it cannot enter or leave the mitochondria except by active transport, thus preserving mitochondrial GSH levels [266-268]. This is possible as BSO does not directly deplete GSH level but rather inhibit GSH synthesis. Furthermore, various reports have shown that mitochondrial GSH only contributes 10 to 15% of the cell’s total GSH pool, and when the volume of the mitochondrial matrix is taken into account, the concentration of mitochondrial GSH is similar to that of the cytosol [264, 265, 269]. It is possible that the reduction of intracellular GSH levels in this study induced GSH sequestering leading to protection against ROS production. Furthermore, it is known that BSO does not readily enter the mitochondria. Hence, it is possible that the mitochondria GSH levels were preserved and regenerated from its oxidised form within the mitochondria [264]. Therefore, if the cells were completely depleted of mitochondrial GSH or were treated with HYD, 8b, 10 and 11 over a longer time period, an increase in ROS production may be observed as the mitochondrial GSH is depleted. Another possibility is that the increase in ROS production induces the up regulation of other endogenous antioxidants such as manganese-dependent superoxide dismutase (Mn-SOD), heme oxygenase-1 (HO-1) [270-273]. In murine fibrosarcoma cell lines the up regulation of Mn-SOD has been shown to be protective against ROS mediated cell death [274]. In human and sheep neuronal tissues, an increase in ROS was shown to enhance Mn-SOD expression [275].

These results supports our suggestion that while the reduction of GSH levels may increase ROS production for some compounds this may not always be the case as the cell may either sequester GSH within the mitochondria to reduce ROS or upregulate other antioxidants defences, such as uncoupling proteins (UCPs) as a mechanism of
survival [273]. It is also possible that the observed toxicity with some of our compounds is independent of ROS production.

Mitochondrial dysfunction is the key to many drug toxic events and the formation of MPT has been shown to trigger a cascade of intracellular signals that can lead to apoptosis, necrosis, autophagy or cellular senescence [263, 276, 277]. Therefore we investigated whether the toxicity of 2-DSA cause MPT in HepG2 cells through oxidative stress. The reduction of intracellular GSH increased MPT in cells treated with the compound 8b, 10, 11, 12, INH, HYD and ETA. HYD showed a greater induction of MPT compared to INH and the effect was comparable to compound 10, 11 and 12. Compound 8b showed the greatest induction of MPT while compound 8a did not affect mitochondrial permeability of HepG2 cells, but did increase ROS in GSH- HepG2 cells. This suggests that MTP for some of the compounds is independent of cytosolic ROS production, while the increase in MPT upon GSH reduction suggests that the level of mitochondrial GSH may be essential in preventing MPT. Mitochondrial GSH-S-transferase (GSTs) and phospholipid hydroperoxide glutathione peroxidase (Gpx4), a membrane-associated enzyme partly localized in the intermembrane space of the mitochondria have been shown to be important in protecting mitochondrial membranes from oxidative stress [273, 278, 279]. The presence of GSTs and Gpx4 enzymatically enhance the metabolic reaction between GSH and oxidant species preventing oxidative damage to mitochondrial membranes [265, 278]. In addition the inner mitochondrial protein ANT, a key component of the PTP, has three cysteine residues in the 57, 160 and 257 position. Cys\textsuperscript{160} and Cys\textsuperscript{257} of ANT are vulnerable to oxidation-induced disulfide cross-linking, which results in the opening of the mitochondrial permeability
transition pore complex. GSH has been shown to be able to prevent this cross linking from occurring [280].

The production of ROS has also been implicated in the accumulation of intracellular lipids in steatosis in xenobiotic toxicity. Accumulation of intracellular lipid occurs due to either direct disruption of enzymes involved in fatty acid β-oxidation or through the disruption of mitochondrial function. Reduction in mitochondrial function due to mitochondrial damage may be due to MPT or ROS generation, which indirectly disrupts the fatty acid β-oxidation process. [237] Hence, it is possible that the observed increase in lipid accumulation with 2-DSA and its derivatives in GSH- cells were due to an increase in ROS production and/or MPT.

These results suggest that reduction of GSH, increased ROS production and MPT may play a role in the toxicity of 2-DSA and its derivatives. Disruption of MPT is an important event in apoptosis [273], while increased ROS production can lead to cell death by either apoptosis, autophagy or cellular senescence [226, 281]. Further complicating the relationship between these cell death pathways is that new evidence suggests that these pathways are not exclusive and can occur in parallel influencing each other [223, 282]. Using HepG2 cells we investigated the involvement of apoptosis, autophagy and cellular senescence in 2-DSA and its derivatives induced toxicity.

Mitochondrial damage, ROS production and the intracellular depletion of GSH has been shown to influence the type of cell death in in vitro models [226, 273, 281, 283]. Upon GSH reduction, increased apoptosis as measured by caspase 3 activity was
observed in HepG2 cells treated with 2-DSA and its derivatives. Similar results were observed in INH, ETA and HYD treated cells. Of the current antitubercular drugs used in this study, INH has previously been shown to cause apoptosis in HepG2 cells using annexin V staining and the measurement of caspase activation [241, 261, 262]. There have been suggestions that GSH depletion predispose cells to apoptosis through an increase in susceptibility to MPT and activation of effector caspases (caspase 3, 6 and 7) [283]. The increase in ROS leading to disruption of MPT releases pro-apoptotic proteins such as cytochrome c, smac/DIABLO and the serine proteases HtrA2/Omi, which activates various caspases such as caspase 9 which in turn activates other caspases, such as caspase 3 that is involved in the disassembly of the cell [218, 220]. In addition, this also suggests that the observed increase in ROS and MPT may ultimately lead to apoptotic cell death which may be the mechanism by which 2-DSA and its derivatives are toxic to HepG2 cells.

Autophagy, senescence and apoptosis exist in a complex, interlinked and tightly regulated pathways to cell death [282]. Autophagy has been shown both as a regulator of cell death and survival. It plays an important role in the maintaince of homeostasis and cell growth through the recycling of intracellular proteins and the degradation of damaged organelles and proteins. The formation of AVOs, a hallmark of autophagy, is stimulated by various stimuli such as amino acid deprivation, ROS and infection [284]. Furthermore, it has also been shown that when intracellular GSH has been depleted, cells can induce autophagy as a mean to replenish intracellular GSH [285]. Of the compounds tested, only compound 8b, 10 and ETA treated cells showed an increase in AVOs.
While cells may undergo autophagy as a survival mechanism, in response to increased oxidative stress, prolonged autophagy could ultimately result in cell death [222, 277, 286]. However, in our model the synthesis of GSH in the cytosol was inhibited by BSO. The production of ROS and intracellular GSH levels has been linked to autophagy, apoptosis and cellular senescence. It is well established that some tumour cell lines exposed to cytotoxic drugs, avoid apoptosis and cellular senescence by undergoing autophagy [223]. However, that is unlikely in this study as increased caspase-3 activity was observed with all of the test compounds upon GSH depletion. Furthermore, a decrease in AVOs accumulation was observed in GSH- cells. This suggests that the increased cellular damage in GSH reduced cells may favour the apoptotic pathway [277]. A similar conclusion can be drawn from the lack of AVOs in INH and HYD treated cells, suggesting that these compounds do not cause autophagy. In addition previous reports have already shown that INH-induced apoptosis cell death is responsible for its toxicity [241, 261].

Interestingly, disruption of cellular GSH homeostasis has also been previously reported to lead to the development of premature cellular senescence [287]. Senescent cells undergo cell cycle arrest and are not responsive to mitogenic stimuli [288]. Of the 2-DSA derivatives, compound 10 increased β-galactosidase activity of GSH- HepG2 cells. This suggests that intracellular GSH level may be important in preventing the induction of cellular senescence by compound 10. Compound 11 was shown to increase β-galactosidase activity irrespective of GSH levels, while compound 8b, 11 and 12 decreased β-galactosidase activity upon GSH reduction. As cellular senescence and apoptosis are both influenced by ROS production and GSH level, it is possible that in these compound’s toxic effects in GSH- cells favours apoptosis instead of cellular
senescence as the mechanism of cell death. ETA and HYD showed similar trends to compound 8b, 11 and 12. While INH increased β-galactosidase activity in HepG2 cells irrespective of intracellular GSH levels.

It is possible that induction of β-galactosidase activity following treatment with 2-DSA and its derivatives was a protective mechanism [223]. It has been previously reported that during cellular senescence there is an altered response to apoptotic stimuli, such as down regulation of caspase 3. Non-senescent WI-38 fibroblasts were shown to be sensitive to serum withdrawal leading to apoptotic cell death. It was observed that senescent WI-38 fibroblasts were insensitive to serum withdrawal and showed an increase in anti-apoptotic bcl-2 protein and down-regulation of caspase 3. Duration of ROS production has been theorised to contribute toward cellular senescence by causing macromolecular damage, which if not repaired, accumulates ultimately leading to cell cycle arrest. Increased ROS production also results in mitochondrial DNA damage and telomere shortening leading to cellular senescence [288]. Hence the antioxidant activity of GSH is an important protective mechanism in preventing cellular senescence. It has also been suggested that the extent of ROS generation rather than antioxidant level may act as a determinant of whether cells undergo senescence [289]. However, the results from this study did not show an increase in ROS production by compound 6 upon GSH reduction, suggesting other mechanisms may be involved. Furthermore, an increase in caspase 3 activity was observed in 2-DSA derivatives following GSH reduction. In addition, the INH-induced hepatotoxicity is known to be mediated by apoptosis [241, 261]. Together these observations support the idea that apoptosis modulates toxicity in HepG2 cells exposed to the test compounds. Furthermore a small subset of cells may undergo cellular senescence to avoid cell death.
A variety of factors may influence whether cells undergo apoptosis, autophagy or cellular senescence. This study found that only some of the compounds tested induced cellular autophagy (either as survival or death) and/or cellular senescence. All the compounds tested increased caspase 3 activity, suggesting that apoptosis is the main mechanism of cell death. The level of intracellular GSH may be essential in preventing cellular damage from the compounds tested. MPT was shown to play an important role in the toxicity of these compounds. The results suggest that only compound 12’s toxicity toward HepG2 was independent of GSH levels. While compound 8b and 11’s amphiphilic structure may play a key role in its toxicity toward HepG2 cells due to the observed increase in LDH release from the HepG2 cells. Furthermore, while the production of ROS may play a role in cell death, in this study it was found that the test compounds toxicity was independent of ROS levels. One suggestion is the sequestering of intracellular GSH within the mitochondria by the cell when GSH levels were low. The increase in ROS and MPT in HepG2 cells combined with increased caspase 3 activity suggests that apoptosis is the main mechanism of cell death induced by the compounds tested. Further supporting this theory is that although AVO formation was induced by compounds 8b, 10 and ETA. However, upon GSH reduction there is a decrease in AVO formation, which in combination with increase caspase 3 production suggests that the cells favour apoptotic cell death pathways. These results demonstrate the importance of GSH in reducing the toxicity of the compounds tested toward HepG2 cells and that apoptosis may be the main mechanism of cell death.
Chapter 7 *In vitro* immunological and inflammatory responses to 2-DSA and synthesized derivatives

7.1 Background

The aim of this study was to evaluate selected *in vitro* inflammatory and/or immunomodulating effects of 2-DSA and the synthesised derivatives. Through the use of surrogate markers of immune/inflammatory response, the test compounds were evaluated for therapeutic potential, possible influence on TB disease progression and risk of ADR. The commercially available antitubercular drugs INH and ETA were also included in the screening process for comparison. INH has been shown to impair immune response and its use has been linked to serious immune-related hypersensitivity reactions leading to severe skin disorders [290-293]. In contrast, little is known about the effects of ETA, a second-line antitubercular drug, on inflammatory and immune responses. Prostaglandin E₂ (PGE₂) and leukotriene B₄ (LTB₄) were selected as surrogate markers of inflammatory response and NF-κB, interleukin-2 (IL-2), interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α) were selected as surrogate markers of immune response. These markers were selected on the basis of their reported role in TB disease progression [294, 295].

The lung is the primary site of TB infection and the release of inflammatory mediators PGE₂ and LTB₄ appears to play a contributing role in immunopathogenesis of pulmonary TB [294, 296]. PGE₂ and LTB₄ have also been shown to be involved in various inflammatory diseases such as arthritis, asthma, inflammatory bowel disease and allergen-induced airway hyper responsiveness/inflammation [297-299]. In the lung,
PGE$_2$ plays an important role in the regulation of inflammatory and immune responses [300]. During inflammation, PGE$_2$ in conjugation with histamine can increase vascular permeability, induce hyperalgesia in the peripheral nervous system, induce febrile response, inhibit platelet aggregation and directly influence the inflammatory immune response [299, 301, 302]. PGE$_2$ has also been shown to suppress T-cell proliferation [303-305], inhibit the production of cytokines such as TNF-$\alpha$ and IL-12 [306, 307], down-regulate dendritic cell function [308], alter antigen presentation by inhibiting the major histocompatibility complex (MHC) class II [309], promote cellular response toward T-helper$_2$ (Th$_2$) phenotype, and promote IL-4 and IL-5 production [310, 311]. Recent studies also suggest that PGE$_2$ can suppress the immune system through the promotion of T suppressor cell function [312, 313]. In the late stage of disease, high concentrations of PGE$_2$ contribute to down-regulation of cell-mediated immunity and permit TB disease progression [296]. PGE$_2$ up-regulates complement receptors (CR) and mannose receptors (MR) expression and function, which increase the ability of mycobacteria to adhere to macrophages. In contrast, LTB$_4$’s effect on the immune system appears to be stimulatory. During inflammation, the release of LTB$_4$ has numerous effects on immune function, including: neutrophil chemotaxis; B lymphocyte activation; antibody production; T cell proliferation; IL-2 production; and modulation of T helper and T suppressor cell function [298]. In addition, LTB$_4$ has been shown to increase both phagocytic and bactericidal activities of alveolar macrophages, which may be important in the pathogenesis of TB infection [300, 314].

T cells are crucial regulators of immune function and have been shown to play a central role in the management of TB infection [295]. Activated T-cells have been shown to promote macrophage killing of intracellular $M.\text{tuberculosis}$ by secreting INF-
γ. In addition, T cells play a central role in the formation of granuloma, which limits the spread of TB infection [315, 316]. Their importance is illustrated by the increased risk of developing active infection and reactivation of dormant TB in HIV infected patients [317]. NF-κB, IL-2, IL-6, and TNF-α, which are important for the regulation of T-cell function and have particular relevance to TB, were selected for screening.

NF-κB is a family of transcription factors that play a central role in the activation and regulation of immune response [318]. In T-cells, the activation of NF-κB induced gene transcription plays an important role in T-cell response. NF-κB has been shown to participate in the transcriptional activation of IL-2 and the α-chain component of IL-2 receptor [319]. The NF-κB family includes NF-κB1 (p50/105); NF-κB2 (p52/p100); p65 (RelA); RelB; and c-Rel [318]. Activation of NF-κB mediated transcription involves the nuclear translocation of cytoplasmic complexes inducing the transcription of pro-inflammatory genes [320]. The most common activated form of NF-κB is the heterodimer consisting of a p50 or p52 subunit and a p65 subunit [321]. In animal gene knockout models, different members of the NF-κB family have been shown to regulate specific immune pathways. For example, compromised B-cell function was observed in p50/p105 and p51/p100 knockout mice and IκBα lead to multifocal inflammation [321]. The p65 subunit was shown to play a role in the constitutive production of IL-6 and is essential for the development of B-cells and stimulation of T-cell proliferation [321, 322]. Thymic atrophy was observed in RelB knockout mice, while c-Rel knockout lead to reduced cytokine production in T-cell and macrophages. Finally, deficiency in humoral response was observed in Bcl-3 knockout mice [321]. In this study Jurkat cells, a T-cell line expressing p50 and p65 subunits of NF-κB, was used to investigate 2-DSA and its derivatives effect on immune response.
TNF-α is a potent stimulator of the inflammation process and is believed to play multiple roles in immune and pathological responses in TB [295]. TNF-α is released by macrophages, T-cells, neutrophils, mast cells and endothelium under different circumstances [323]. Release of TNF-α induces the production of various chemokines and adhesion molecules attracting different immune cells to the site of release [324]. During TB infection, TNF-α secreted by CD4+ T-cells and macrophages has been shown to be essential in the maintenance of the granuloma structure in TB. TNF-α is often a major factor in host-mediated destruction of lung tissue [295]. Mice deficient in TNF-α show poorly formed lesions containing many neutrophils and increased severity of pathology, which is not in proportion to the increase in bacterial load [325-327]. In humans, arthritis patient treated with TNF-α inhibitors have been shown to have an increased rate of M. tuberculosis reactivation [328]. These studies demonstrate the importance of TNF-α in controlling TB infection.

IL-2 plays a pivotal role in generating an immune response. It does this by stimulating the expansion of the pool of lymphocytes specific for an antigen. IL-2 is produced by T-cells that have been activated by an antigen or mitogen to promote proliferation. When deprived of IL-2, activated T cells have been shown to undergo apoptosis; hence it also plays an important regulator role in the maintenance of T-cell activity [329]. In the innate immune system IL-2 promotes NK cell proliferation and the production of IFN-γ, TNF-α and granulocyte macrophage colony-stimulating factor (GM-CSF). IFN-γ and IL-2 produce a synergistic effect in augmenting NK cell cytolytic activity [329]. IL-2 can influence the course of mycobacterial infections alone or in combination with other cytokines [295].
IL-6 plays multiple roles in the immune response and has been implicated in the host response to *M. tuberculosis*. Associated responses include inflammation, haematopoiesis and differentiation of T-cells [295].

Human alveolar adenocarcinoma (A549) cells were chosen as the *in vitro* model to study the effects of drugs on PGE$_2$ and LTB$_4$. A549 cells are widely used to study the inflammatory responses of drugs and have previously been shown to express a variety of inflammatory mediators, including PGE$_2$ and LTB$_4$ [157, 330, 331]. The human T cell lymphoblast-like cell (Jurkat) was selected for *in vitro* assays, based on its proven ability to express NF-κB, TNF-α, IL-2, and IL-6 when activated and has been widely used by various investigators to examine the effects of drugs on T-cell function and response [332-337]. The findings of this *in vitro* screening study provide preliminary data on the immunomodulating effects of 2-DSA and its derivatives in the context of TB treatment.

### 7.2 Materials and Methods

2-DSA ([8a](#)), methyl 2-decylsulfonylacetate ([8b](#)), 2-decylsulfonylacetic acid ([10](#)), 2-decylsulfonylthioamide ([12](#)), INH and ETA were screened for immunomodulating effects *in vitro* using the adherent A549 and non-adherent Jurkat cell lines. Effects of these drugs on PGE$_2$ and LTB$_4$ were assessed using the A549 cell line and NF-kB, TNF-α, IL-2, and IL-6 using the Jurkat cell line.
7.2.1 Materials

A549 and Jurkat cell lines were obtained from ATCC (Manassas VA, USA). Complete Dulbecco’s Modified Eagle Medium (DMEM)/F12, high glucose RPMI 1640 medium, L-glutamine, sodium pyruvate, HEPES, and Penicillin-Streptomycin solution were purchased from Invitrogen (Victoria, Australia). All other chemicals and reagents were of analytical grade and sourced from Sigma Aldrich (St Louis, USA). MilliQ water was sterilized by autoclaving at 121°C for 20 min. 96 well plates used in this study were purchased from Nunc (New York, USA).

7.2.2 Test compound stock solutions

The synthesised compounds 8a, 8b, 10, 12 and ETA were dissolved in DMSO at a concentration of 1 mM. Stock solutions were aliquoted and stored at 4°C and used within one month of preparation. INH was dissolved in sterile PBS at a concentration of 750 mM and prepared fresh before each experiment.

7.2.3 Cell culture

Adherent A549 cells were grown and maintained at 37°C with 5% CO2 in complete DMEM/F12 supplemented with 10% FBS and 500 U/mL Penicillin-Streptomycin.

Jurkat cells were cultured in complete RPMI 1640 consisting of high glucose, L-glutamine, sodium pyruvate, phenol red, 10% FBS, 500 U/mL of penicillin and 500 μg/mL of streptomycin.
7.2.4 PGE₂ assay

PGE₂, a product of arachidonic acid (AA) metabolism, was assessed using the PGE₂ ELISA kit (Cayman chemical company, Ann Arbor, USA). All steps were performed according to manufacturer’s instructions. A549 cells were seeded at 7.5×10⁵ cells/mL in 25 cm² flasks and allowed to recover for 24 h. The test compounds were then added and after 24 h, the culture media were removed for assessment of PGE₂.

Plates were precoated with mouse anti-rabbit IgG. PGE₂ antibody was reconstituted in the buffer provided. Ellman’s reagent was reconstituted in 20 mL milliQ H₂O. Following the addition of the PGE₂ antibody the plates were incubated for 18 h at 4°C. The plates were then developed with Ellman’s reagent in the dark, shaking on an orbital shaker for 90 min. Absorbance was measured at 414 nm using the Titertek Multiskan FC absorbance plate reader (Thermo Scientific, Victoria, Australia).

7.2.5 Determination of inflammation – LTB₄ assay

LTB₄, a product of AA metabolism, was assessed using the LTB₄ ELISA kit (Cayman chemical company, Ann Arbor, USA). All steps were performed according to the manufacturer’s instructions. A549 cells were seeded at 7.5×10⁵ cells/mL in 25 cm² flasks and allowed to recover for 24 h. The test compounds were then added and after 24 h, the culture media were removed for assessment of LTB₄.

Plates were precoated with mouse anti-rabbit IgG. LTB₄ antibody was reconstituted in the buffer provided. Ellman’s reagent was reconstituted in 20 mL milliQ H₂O. Following the addition of LTB₄ antibody the plates were incubated for 90
min at room temperature. The plates were developed with Ellman’s reagent in the dark, shaking on an orbital shaker for 90 min. Absorbance was measured at 414 nm using the Titertek Multiskan FC absorbance plate reader (Thermo Scientific, Victoria, Australia).

7.2.6 NF-κB

NF-κB was measured using the NF-κB ELISA kit (Cayman chemical company, Ann Arbor, USA). All steps were performed according to manufacturer’s instruction.

Jurkat cells were seeded at 1.5×10^5 in 25 cm² flasks and allowed to recover for 24 h. The test compounds were then added and cells were incubated for a further 24 h. Cells were removed from the culture flasks by scraping and resuspended in ice-cold PBS/phosphataise inhibitor solution. Following centrifugation (300 g for 5 min), the cells were resuspended in ice cold 1×hypotonic buffer (provided by the kit) and incubated on ice for 15 min, after which 10% Nonidet p-40 was added. The cells were then pulse spun (14,000 g for 30 sec) at 4°C and after which the supernatant was removed. Cell pellets were resuspended in ice-cold complete nuclear extraction buffer (provided by the kit), vortexed for 15 s and then incubated in ice on an orbital shaker for 15 min. After centrifugation (14,000 g for 10 min at 4°C), the supernatant was removed and used for NF-κB detection.

Plates were precoated with consensus double stranded DNA. Anti-human NF-κB primary antibody (p50 or p65) was reconstituted in the buffer provided. Goat anti-rabbit HRP conjugate secondary antibody was reconstituted in the buffer provided. The developing and stop solutions were provided by the kit and used in the recommended
dilution. Between antibody incubations, the wells were washed with wash buffer provided. Following the addition of the stop solution, absorbance was measured at 450 nm using the Titertek Multiskan FC absorbance plate reader (Thermo Scientific, Victoria, Australia).

7.2.7 Determination of immune response– IL-2 assay

IL-2 is an important cytokine for the normal immune response and is secreted by T-cells upon action and was used as a marker for T-cell activation using the human IL-2 Duoset ELISA kit (R&D systems, Minneapolis, USA). All steps were performed according to manufacturer’s instructions.

Jurkat cells were cultured and treated with the test compounds as mentioned in 7.4.6. After 24 h the culture medium was removed for the IL-2 assay. Plates were coated with the IL-2 capture antibody (mouse anti-human) in PBS. IL-2 biotinylated goat anti-human detection antibody was reconstituted in tris-buffered saline with 0.1% BSA and 0.05% Tween 20. Streptavidin-HRP was used in the recommended dilution and the substrate solution was prepared at 1:1 ratio of tetramethylbenzidine and H₂O₂. Between antibody incubations, the wells were washed with PBS containing 0.05% Tween 20. Following the addition of the stop solution (concentrated sulphuric acid), absorbance was measured at 450 nm using the Titertek Multiskan FC absorbance plate reader (Thermo Scientific, Victoria, Australia).
7.2.8 Determination of immune response—IL-6 assay

IL-6 is an important cytokine for the normal immune response and is secreted by activated T-cells stimulating B-cell activation, proliferation and antibody secretion. IL-6 was measured using the IL-6 ELISA kit (Cayman chemical company, Ann Arbor, USA). All steps were performed according to manufacturer’s instructions.

Jurkat cells were cultured and treated with the test compounds as mentioned in 7.4.7. After 24 h, the culture medium was removed for the IL-6 assay. Plates were precoated with IL-6 capture antibody. IL-6 AChE Fab conjugate was reconstituted in the buffer provided. Ellman’s reagent was reconstituted in 20 mL milliQ H₂O. Following the addition of IL-6 AChE Fab’ conjugate the plates were incubated over night at 4°C. The plates were developed with Ellman’s reagent in the dark, shaking on an orbital shaker for 120 min. Absorbance was measured at 414 nm using the Titertek Multiskan FC absorbance plate reader (Thermo Scientific, Victoria, Australia).

7.2.9 TNF-α

TNF-α was measured using the TNF-α ELISA kit (Invitrogen, Camarillo, CA). All steps were performed according to manufacturer’s instructions.

Jurkat cells were cultured and treated with the test compounds as mentioned in 7.4.7. After 24 h, the culture medium was removed for the TNF-α assay. Plates were precoated with human TNF-α antibody. TNF-α biotin conjugated anti-human detection antibody was reconstituted in the buffer provided. Streptavidin-HRP was used in the recommended dilution and the substrate solution was stabilized chromogen provided by
the assay. Between antibody incubations, the wells were washed with PBS containing 0.05% Tween 20. Following the addition of the stop solution (provided by the assay kit), absorbance was measured at 450 nm using the Titertek Multiskan FC absorbance plate reader (Thermo Scientific, Victoria, Australia).

7.2.10 Data analysis

Results were calculated and represented as mean ± S.D. Statistical analysis was performed using GraphPad Instat 3 (GraphPad Software, San Diego, CA, USA). One-way ANOVA followed by the Dunnett’s test were used to assess statistical differences between treatments. Statistical significance was accepted at a probability level of $P < 0.05$.

7.3 Results

The synthesised compounds (8a, 8b, 10 and 12), INH and ETA effect on PGE$_2$ and LTB$_4$ levels were assessed in vitro using the A549 cell lines after 24 h exposure to the test compounds. Effect on NF-kB, TNF-α, IL-2, and IL-6 levels were assessed in the Jurkat cell line.

7.3.1 PGE$_2$ and LTB$_4$

The effects of the synthesised compounds, INH and ETA on the levels of PGE$_2$ and LTB$_4$ in the cell media of A549 cells treated with 0.1 mM concentrations for 24 h are presented in Figures 76 and 77, respectively.
Treatment with 0.1 mM concentrations of the test compounds had varied effects on the levels of PGE\(_2\) produced by the A549 cells. Compound 12 produced a maximum effect, significantly increasing PGE\(_2\) levels compared to vehicle control (526.83 pg/mL, \(P < 0.001\)). In contrast, compound 8b decreased PGE\(_2\) levels produced by the A549 cells, however this was not statistically significant (-12.04 ± 68.69 pg/mL, \(P > 0.05\)). Compounds 8a (84.02 ± 19.67 pg/mL) and 10 (41.67 ± 18.27 pg/mL) had little effect on PGE\(_2\) levels. INH (113.7 ± 41.36 pg/mL) and ETA (89.51 ± 19.21 pg/mL) were shown to cause a subtle increase in PGE\(_2\) levels compared to vehicle control (63.04 ± 26.23 pg/mL).

![Diagram showing PGE\(_2\) levels normalized to cell number](image-url)

**Figure 74.** PGE\(_2\) (pg/ml) level of A549 cells normalized to cell number. Each bar represents mean ± S.D. (n = 3). Symbol (***)) indicates statistical significance as compared to vehicle control \(P < 0.001\).
The test compounds also had varied effects on the levels of LTB₄ produced by the A549 cells. With exception of 8a, all synthesised compounds (8b, 10 and 12) tended to increase LTB₄ levels produced by A549 cells treated with 0.1 mM concentrations for 24 h. Due to the variability associated with vehicle controls, only the result obtained for compound 8b was statistically significant (74.67, \( P < 0.05 \)). Although not statistically significant, compound 8a reduced LTB₄ levels (-9.33 ± 5.5%). INH was shown to have little effect on the LTB₄ levels. In contrast, ETA had the greatest effect, increasing LTB₄ levels by more than 150.67% compared to vehicle controls (\( P < 0.001 \)).

![Figure 75](image.png)

**Figure 75.** Percentage change LTB₄ relative to vehicle control normalized to cell number. Each bar represents mean ± S.D. (n=3). Symbols (*) and (****) indicate statistical significance as compared to vehicle control \( P<0.05 \) and \( P<0.001 \).
7.3.2 NF-κB

The expression of NF-κB in Jurkat cells treated with the synthesised compounds (8a, 8b, 10 and 12), INH and ETA for 24 h is presented in Table 10. All compounds were tested at a concentration of 0.1 mM. Compounds 8a, 8b, 10 and 12 stimulated the increased generation of the mature NF-κB subunits p50 and p65 when compared to the appropriate vehicle controls. INH and ETA produced similar up-regulation of the NF-κB p50 and p65 subunits.

Table 10. NF-κB expression of p50 and p65 subunits induced by the test compounds

<table>
<thead>
<tr>
<th>Drug compounds (mM)</th>
<th>p50</th>
<th>p65</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound 4a (0.1)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Compound 4b (0.1)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Compound 5 (0.1)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Compound 6 (0.1)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>INH 0.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>INH 40</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ETA 0.1</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ indicates up regulation of p50 or p65

7.3.3 IL-2, IL-6 and TNF-α

The relative production of IL-2, IL-6 and TNF-α by Jurkat cells treated with the synthesised compounds (8a, 8b, 10 and 12), INH and ETA for 24 h is presented in Table 11.
Table 11. IL-2, IL-6, and TNF-α release of Jurkat cells induced by the test compounds

<table>
<thead>
<tr>
<th>Drug compound (mM)</th>
<th>IL-2</th>
<th>IL-6</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 4a 0.1</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Compound 4b 0.1</td>
<td></td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>Compound 5 0.1</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Compound 6 0.1</td>
<td></td>
<td>+</td>
<td>++++</td>
</tr>
<tr>
<td>INH 0.1</td>
<td></td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>ETA 0.1</td>
<td></td>
<td></td>
<td>++</td>
</tr>
</tbody>
</table>

+, ++, +++ and ++++ indicate a 0.5, 1, 2 and 3 fold increase in cytokine release relative to vehicle control; - indicates a 1 fold decrease of cytokine release relative to vehicle control

All compounds were screened at a concentration of 0.1 mM. The synthesised compounds demonstrated a varied effect on IL-2, IL-6 and TNF-α production when compared to vehicle controls. Compounds 8a and 12 induced a 0.5-fold increase in IL-2 production. A similar fold increase in IL-2 production was observed for 0.1 mM ETA. In contrast, compounds 8b, 10 and INH did have any effect on IL-2 production. Compounds 8a, 10, 12 and INH-induced a 0.5-fold increase in IL-6 production. Whereas compound 8b and ETA produced no change in IL-6 production relative to vehicle controls. Compounds 8b, INH and ETA produced a 1-fold increased in TNF-α levels when compared to controls. The greatest increases in TNF-α levels were observed for compounds 4a (2-fold) and 6 (3-fold). In contrast, compound 12 induced a 1-fold decrease in TNF-α levels.
7.4 Discussion

The *in vitro* inflammatory and/or immunomodulating effects of the synthesised compounds were investigated to assess their therapeutic potential, disease influence and ADR potential. PGE$_2$ and LTB$_4$ production by human alveolar adenocarcinoma (A549) cells was used as surrogate markers for inflammatory response while NF-κB, IL-2, IL-6 and TNF-α production by T-cell lymphoblast-like (Jurkat) cells were used as surrogate markers of T-cell immune response. Both cell lines have widely been used to study either drug-induced inflammatory responses [157, 330, 331] or effects on T-cell function [332-337].

Compound 12 was shown to be a potent inducer of PGE$_2$ production, while INH and ETA were shown to only slightly increase PGE$_2$ levels in A549 cells. The observed increase in PGE$_2$ production may be due to induction of COX or PGE synthase by compound 12, INH and ETA in A549 cells [338-340]. However, further studies would be needed to determine the exact mechanism by which PGE$_2$ production was increased and the consequence it may have. In contrast, compounds 8a and 10 did not have any effect on PGE$_2$ production in A549 cells. Therefore, it is unlikely that either of these compounds would have any direct effects on PGE$_2$ mediated inflammatory responses in respiratory epithelial cells. Increased PGE$_2$ production may adversely affect the control of TB infection through the suppression of T-cell activity, reduction of TNF-α, IL-1α and IFN-γ and suppression of iNOS expression as previously shown in other models [296, 300, 304, 312]. In addition increased PGE$_2$ production may potentially, affect bronchial smooth muscle contraction and relaxation of. Conversely increased PGE$_2$ may also suggest a protective response. Chen *et al* (2008) suggest that PGE$_2$ may protect cells against virulent mycobacterium tuberculosis [341]. Increase in PGE$_2$ has been
associated with the induction of apoptosis in infected macrophage. Apoptosis of infected macrophages has been shown to restrict bacterial infection and also allow the uptake of apoptotic vesicles by dendritic cells to present bacterial antigens to T-cells, leading to activation [341, 342]. The activation of naïve T-cells is essential in the formation of granuloma to control the spread of TB infection and to activate macrophage killing of intracellular TB [343-345].

By comparison to effects on PGE$_2$, all of the synthesized compounds with the exception of compound 8a increased LTB$_4$ production in A549 cells. Of the 2-DSA derivatives that increased LTB$_4$ production, compound 8b showed the greatest increase in production. Although, compound 12 increased LTB$_4$ production in A549 cells it was shown to be a more potent inducer of PGE$_2$ compared to LTB$_4$. The observed increase in LTB$_4$ production was possibly through the up-regulation of lipoxygenase enzyme [314]. LTB$_4$ is a potent inflammatory mediator and is a strong chemoattractant of neutrophils, which is important in the innate immune response to TB infection [295, 346]. Increased LTB$_4$ levels have been reported in TB patients where it up-regulates macrophage, T-cell and B-cell activity, which potentially has an important role in the control of the disease [294, 298, 301]. Interestingly Chen et al (2008) also reported the up-regulation of LXA$_4$ by TB, decrease in PGE$_2$ levels leading to infected macrophages undergoing necrosis, which aid in spread of the bacteria and evasion of the adaptive immune system [341]. The up-regulation of LTB$_4$ may also adversely affect asthma patients and enhance allergen-induced responses in the airway through the recruitment of immune cells involved in the allergy response [297, 298]. The observed effect the derivatives of 2-DSA on PGE$_2$ and LTB$_4$ production may also reflect the observed toxicity in A549 cells (Chapter 5). In A549 cells, compound 12 was the most toxic of
the 2-DSA compounds, which may relate to its potent effect on PGE\(_2\) production. PGE\(_2\) and LTB\(_4\) induction by compound 12 may indicate a non-specific inflammatory response, which shows a lack of therapeutic potential. Of the antitubercular drugs examined, INH did not affect LTB\(_4\) production, while ETA was shown to be a potent inducer. This is the first report of ETA’s inflammatory effect, however the implication of this on the immune response during treatment is currently unclear. It is also possible that the observed increase in PGE\(_2\) and LTB\(_4\) may be responsible for ETA’s toxic effect in A549 cells. In A549 cells, ETA was shown to reduce cell viability by 10\% at 500 μM concentration.

T-cells are known to play a central role in the control of \textit{M. tuberculosis} infection [316, 343, 344, 347-349]. 2-DSA derivatives were examined for their potential immunomodulating effect on T-cells using Jurkat cells. All compounds screened were shown to increase the nuclear level of both the p50 and p65 subunit of NF-κB, suggesting potential immunomodulating properties. Although NF-κB has been shown to regulate a variety of immune responses, the maturation of the p50 subunit has been previously shown to promote IL-2, IL-6 and TNF-α in T-cells [350, 351] [352]. However, as the p50 subunit lacks a transactivation domain, it is unable to activate DNA transcription on its own [353]. The formation of p50/p65 dimer allows p50 activation of cytokine transcription [353, 354]. The p65 subunit regulates production of IL-6, development of B-cells and stimulation of T-cell proliferation [321, 322]. The induction of in p50 and p65 in Jurkat cells by 2-DSA and its derivatives, INH and ETA indicates a potential immunomodulating effect. This may lead to a variety of T-cell mediated responses, such as T-cell proliferation, IL-2 production and IL-6 [355, 356]. Depending on the type of cytokines stimulated these effects may either enhance or
inhibit TB disease progression through the role T-cell plays in controlling disease state and possible immune mediated ADR [295].

IL-2 is produced by activated T-cells and is a potent inducer of T-cell proliferation [329]. Of the 2-DSA derivatives, compounds 8a and 12 were shown to increase IL-2 secretion in Jurkat cells consistent with the increased cell viability observed (Chapter 5). The observed increase in viability suggests an increase in Jurkat cell proliferation. Compounds 8b and 10 did not promote IL-2 production in Jurkat cells. Of the antitubercular drugs examined, ETA alone showed an increase in Jurkat cells IL-2 production. Apart from its effect on inducing T-cell proliferation, IL-2 is also essential for T-cell survival [329]. When deprived of IL-2, T-cells have been shown to undergo apoptosis [329]. In TB infection, T-cells play an important role in the elimination and control of disease progression, hence increase in IL-2 may increase T-cell number and possibly enhance disease elimination [295]. Compound 8a, 12 and ETA enhancement of IL-2 production in T-cells may enhance immune response to TB infection. It is possible that the observed increase in IL-2 production by the compounds tested was through NF-κB activation. However, whole organism studies would be required to confirm this.

In contrast to effects seen with IL-2 all of the synthesised compounds with the exception of compound 8b, increased IL-6 production. IL-6 secretion by T-cells has been shown to enhance B-cell proliferation and differentiation into antibody producing cells [357]. It also enhances T-cell activation, growth and differentiation. IL-6 also up-regulates IL-2 receptor α-chain expression in T-cells [357]. In TB infection, IL-6 has been reported to play an important role in promoting early immune response to the
pathogen [295]. In contrast, it has also been reported that IL-6 inhibits IFN-γ activation of macrophages in TB infection [358]. The reported dual role of IL-6 in enhancing and inhibiting TB immune function may involve interplay of other cytokines not studied here. Therefore, further studies are required to determine the effect of compounds 8a, 10 and 12 on IL-6 production during the course of a TB infection. Similarly while this study also found that INH increases IL-6 production, further study will be required to determine its effect in TB infection.

Compounds 8a and 10 were shown to be potent inducers of TNF-α, while compound 8b was shown to weakly induce TNF-α. In contrast, compound 12 decreased TNF-α production in Jurkat cells. The induction of TNF-α by compounds 8a and 10 may enhance immune control of TB infection through TNF-α induced immune response. TNF-α induces the release of cytokines necessary for the recruitment of immune cells to the site of infection [324]. The decrease in TNF-α level by compound 12 may lead to dissemination of M. tuberculosis due to improper maintenance of granuloma structure [323]. Both animal and human studies have reported that decreased TNF-α production results in inability to contain TB infection and the reactivation of latent infection [326-328]. However, an increase in TNF-α is not always beneficial, as it is often associated with host-mediated destruction of lung tissue and other immune mediated disease such as rheumatoid arthritis [295, 323].

The results from this study indicate the potential inflammatory and immunomodulating effects of 2-DSA (8a) and its derivatives, which may be of therapeutic value. 8a was shown to have little effect on the production of the
inflammatory markers PGE₂ and LTB₄ in A549 cells. It also had little to moderate
effect on T-cell activity as observed in Jurkat cells. Once again, indicating the
specificity of 2-DSA in its biological activity against TB. Highlighting the importance
of 2-DSA as a lead compound for the development of novel antitubercular drug.
Meanwhile, the increase in PGE₂ by compound 5, INH and ETA is of benefit to the
patient during TB infection by promoting the activation of the adaptive immune
response. Both compound 5 and ETA also increased LTB₄ production which may also
aid the patient in combating TB infection as LTB₄ has been shown to up regulate
macrophage, T-cell and B-cell activities. Although all the compounds tested increased
the nuclear level of NF-κB in Jurkat cells, different cytokine up regulation profile was
observed for the compounds tested. Compounds 4a, 5 and ETA were shown to enhance
IL-2 production, which may potentially enhance T-cell response to TB infection. Apart
from compound 4b, all the compounds synthesised in this study enhanced IL-6
production. This may in turn enhance B-cell and T-cell activation, but may inhibit INF-
γ activation of macrophages in TB infection. This study is the first to report INH
promotion of IL-6 production in vitro Jurkat cell model. Compounds 8a, 10, INH and
ETA were shown to enhance TNF-α production while compound 5 decreased TNF-α
production in Jurkat cells. This too may be of benefit to the host as a decrease in TNF-α
have been shown to lead to the dissemination of M. tuberculosis. This study is the first
to identify that INH and ETA were able to moderately enhance TNF-α production in
Jurkat cells. However, further studies would be required to determine whether the
synthesised compounds’ effect on PGE₂, LTB₄, NF-κB, IL-2, IL-6 and TNF-α
production were of therapeutic value and its possible effect during a M. tuberculosis
infection. Furthermore, through chemical modification it may be possible to enhance or
to reduce these inflammatory and immunomodulating effects of 2-DSA (8a) creating
novel anti-inflammatory or immune enhancing drugs.
Chapter 8 Conclusion and Future Directions

A rational series of derivatives, based on the novel antitubercular compound 2-DSA, were synthesised in an attempt to study their biological activity. The compounds synthesised included 2-DSA (8a), methyl 2-decylsulfonylacetate (8b), 2-decylsulfonylacetic acid (10), 2-decylsulfonyl-N-prop-2-ynyl-acetamide (11) and 2-decylsulfonylthioamide (12). The synthesised compounds were screened for antitubercular, antibacterial, mammalian cell cytotoxicity, hepatotoxicity, inflammatory and immunomodulating properties.

Using methods adapted from Jones et al [27], the acetamide moiety of 2-DSA was modified and replaced with a methyl ester (8b) or carboxylic acid (10) functional groups. The optimized methods produced excellent yields. A thioamide derivative of 2-DSA (12) was synthesised to determine whether the addition of a thioamide functional group would enhance its antitubercular properties. There are various thioamide compounds, including ETA, with antitubercular and antibacterial properties. Compounds with a long fatty acyl tail, such as 2-DSA (8a) and its derivatives, are known to have low solubility, limiting their clinical application. Therefore its solubility must be enhanced for clinical applications. One method of enhancing the 2-DSA’s (8a) solubility would be the conjugation of 2-DSA (8a) to a water-soluble compounds, such as cyclodextrins. In this study, we theorized that the addition of alkyne moiety to 2-DSA (8a) would allow the conjugation of 2-DSA (8a) to water soluble compounds such as cyclodextrin through copper catalysed azide alkyne coupling chemistry (click chemistry) [107, 108, 131]. Therefore enhancing the solubility profile of 2-DSA (8a). Using common peptide coupling reagents HOBt and EDC the alkyne derivative (11) of 2-DSA was synthesised. In addition, click chemistry also allows the conjugation of
organ specific/cell specific molecules targeting 2-DSA to specific sites of action. For example, the conjugation of a receptor agonist such as the macrophage-inducible C-type lectin (mincle) to compound 11 it becomes possible to target 2-DSA toward TB infected macrophages [359, 360]. The chemistry explored in this study provides a good framework for the synthesis of other 2-DSA homologs. The alkyne derivative of 2-DSA provides a method for the synthesis of various 2-DSA prodrugs using click chemistry [110, 111]. Based on the findings of this study, this structural modification would significantly alter biological activity.

The antitubercular activity of 2-DSA (8a) in this current study was complementary to that reported by Jones et al [27]. A difference in activity was observed between the results of this study and that reported by Jones et al [27]. However, this was most likely due to the different M. tuberculosis strain and test methods used in this current study. Jones et al [27] used the virulent M. tuberculosis H37Rv strain, while the attenuated M. tuberculosis H37Ra strain was used in this study. Nevertheless, 2-DSA (8a) demonstrated high antitubercular activity against the attenuated M. tuberculosis H37ra strain. Interestingly, the thioamide derivative (12), showed significantly lower antitubercular activity. Furthermore, the lack of activity from the methyl ester (8b) and carboxylic acid (10) derivatives indicates that the acetamide functional group is essential for activity. Therefore, it is possible that the untested alkyne derivative (compound 11) may also decrease antitubercular activity. However, further investigation is required to confirm this.

Interestingly, while the acetamide moiety was essential for optimal antitubercular activity, the thioamide derivative showed the highest antibacterial activity.
against *S. aureus* (ATCC 25923). This observed antibacterial activity was comparable to that of gentamicin. 2-DSA (8a), compounds 8b and 11 also demonstrated some activity against *S. aureus* (ATCC 25923), although not as potent as compound 12. This was in agreement with reports of the antibacterial properties for thioamide-based compounds against *S. aureus* [100, 101].

The reported antibacterial activity of some amphiphilic compounds is often associated with membrane disruption. The selective antibacterial and antitubercular activities observed in this study suggests other possible mechanisms of action. One suggestion is that the antibacterial activity of 2-DSA (8a) and its synthesised derivatives is through inhibition of enzymes involved in the FASII pathway. 2-DSA was designed as an inhibitor of the KAS enzyme in mycobacterium FASII pathway involved in mycolic acid synthesis [27]. It is possible that the same mechanism of action was involved in its antibacterial activity as many of the FASII enzymes are conserved across bacterial species [147]. Further structural and biological studies would be required to determine if this was the case or if other mechanisms were involved.

2-DSA (8a) and its derivatives are amphiphiles. Some amphiphiles were shown to be toxic to mammalian cells through disruption of cell membranes. Therefore, the synthesized compounds were also screened for possible toxicity against mammalian cells using A549, HeLa, MCF-7 and Jurkat cells. 2-DSA and its derivatives displayed variable toxicity. Although compound 10 was the least toxic, it was also the least biologically active. The thioamide derivative (compound 12) was shown to be the most toxic. 2-DSA (8a) was shown to be only slightly toxic toward the cell lines examined, while being the most biologically active. Of the synthesised compounds, 2-DSA (8a)
appears to have the best balance between activity and toxicity. For this reason, it is proposed that it still the best candidate for further development of a novel antitubercular drug. If compound 12’s toxicity could be reduced, it has the potential to be developed into a novel antibacterial compound against *S. aureus*. Membrane disrupting effect of amphiphiles is unlikely to be the cause of the observed activities. The observed specificity and selectivity of the synthesised compounds indicate a more specific mechanism. Further investigation would be needed to determine its mechanism of toxicity.

The major limitation of this explorative study was that mechanisms underlying the observed effects were not investigated directly. Future studies would need to address this shortfall. This study does, however, provide the platform for producing a large variety of compounds of structure activity relationship investigations.

The hepatotoxicity of current antitubercular drugs is well documented. Therefore, 2-DSA and its derivatives were screened for potential hepatotoxicity in HepG2 cells and compared to two current antitubercular drugs, namely: INH and ETA. Of the synthesised 2-DSA derivatives, compound 12 was shown to have greatest hepatotoxic potential. Indicating, the substitution of the oxygen atom within the acetamide moiety with a sulfur atom dramatically increases the compounds toxicity. 2-DSA and compound 10 were shown to be the least toxic. These observations reinforce the importance of the acetamide group, not just for antitubercular activity, but also for reducing toxicity toward mammalian cells. Compared to INH, 2-DSA (8a) and its derivatives were significantly less toxic to HepG2 cells. However, since INH is a prodrug, it needs to be metabolised into its toxic metabolite HYD before maximum cell
toxicity is observed. Hence, HYD-induced toxicity in HepG2 was a better comparison of 2-DSA toxicity. Of concern, 2-DSA was shown to possess slightly more hepatotoxic potential than HYD and ETA, a second-line antitubercular drug. The difference in hepatotoxicity profile between the synthesised compounds suggests a specific mechanism of toxicity toward HepG2 cells. GSH was shown to be protective against the toxic effect of synthesised compound in HepG2 cells. Similarly, this was also observed in INH, ETA and HYD treated cells. Furthermore, intracellular lipid accumulation (a marker for steatosis) in HepG2 cells was also observed upon the reduction of intracellular GSH levels. GSH is an important antioxidant and has also been shown to play a key role in protecting the cell from xenobiotic toxicity by reducing ROS production and preventing MPT [248, 250]. This study identified that ROS production and MPT played an important role in the toxicity of the synthesised compounds towards HepG2 cells. It was found that certain of the synthesised compounds reduced ROS or MPT in HepG2 cells. This may be due to the incomplete depletion of intracellular GSH leading to sequestering of GSH within the mitochondria. This phenomenon was also observed with INH- and ETA-induced toxicity. The finding from this study is in agreement with the reported role ROS production and MPT in INH-induced toxicity in HepG2 cells [241, 261, 262]. An increase in ROS production, MPT and reduction of intracellular GSH have been reported to lead to apoptosis, autophagy or cellular senescence [207, 223]. It was observed that the mechanism of cell death for all the synthesized compounds, INH, ETA and HYD was apoptosis. However, some of the synthesised compounds induced autophagy and promoted premature cellular senescence. It is likely that some of the test compounds can induce more than one mechanism of cell death. The literature suggests that apoptosis can occur in parallel with autophagy and cellular senescence. Depending on a number of factors, cells can choose any path of cell death [223, 282]. Compounds 8b and 10 showed an increase in
AVO formation, an indicator of autophagy. However, further investigation is required to conclusively establish autophagy involvement. Interestingly, compound 10 increased β-galactosidase staining. Indicating some cells may have chosen to undergo autophagy and/or cellular senescence as an alternative to apoptotic cell death. Interestingly, upon the reduction of intracellular GSH, ETA, HYD, compound 8b, 11 and 12 treated HepG2 cells decreased β-galactosidase staining compared to untreated cells. The reduction of intracellular GSH may lead to an increase in cellular damage that the cell deems too severe for its continued survival. The observed toxicity and mechanism of toxicity from this study demonstrated that the substitution of the acetamide functional group of 2-DSA (8a) is able to dramatically alter the hepatotoxic profile of the synthesized compounds. For example the substitution of the acetamide functional group with a carboxyl acid functional group in compound 10 reduces HepG2 toxicity. In addition the difference in ROS production, MPT, autophagy and cellular senescence by the different synthesized compounds further reinforces the importance of the hydrophilic head groups in its biological properties. These findings indicate the possibility of reducing 2-DSA’s hepatotoxicity potential through the chemical modification of its hydrophilic head groups. This alteration would, however, need to maintain biological activity. This was not observed for any of the compounds synthesized in this study. Future study utilizing dihydrorhodamine 123 (DHR123), a mitochondria specific ROS probe will allow us to investigate the relationship between ROS production, MPT and the possible GSH sequestering by HepG2 cells s observed in this study [361].

Lastly, this study investigated the inflammatory and immunomodulating effects of 2-DSA and its derivatives. The preliminary finding from this study was used to assess the therapeutic potential, disease influence and potential for ADR of 2-DSA and
its derivatives. Surrogate markers for inflammation (PGE<sub>2</sub> and LTB<sub>4</sub>) produced by A549 cells and immune activation (NF-κB, IL-2, IL-6 and TNF-α) produced by Jurkat cells were used. 2-DSA and its derivatives demonstrated varied inflammatory and immunomodulating effects. The different hydrophilic head groups were shown to play an important role in determining its effect on A549 and Jurkat cells. In A549 cells compound 12 was shown to be a potent inducer of PGE<sub>2</sub> production. The observed increase in PGE<sub>2</sub> may deregulate immune reaction during TB infection by suppressing T cell activity [296]. In contrast, compound 8b increased LTB<sub>4</sub> production, which may cause chemotactic attraction of neutrophils; enhance B lymphocyte activation and antibody production; stimulate T-cell proliferation and IL-2 production, which may enhance the body’s immune response during TB infection [294]. Although, compounds 8a and 10 did not significantly affect PGE<sub>2</sub> production, 8a did reduce LTB<sub>4</sub> production. In comparison, INH and ETA were shown to only subtly increase PGE<sub>2</sub>. However, ETA was found to be a potent inducer of LTB<sub>4</sub> production. The test compounds’ induction of PGE<sub>2</sub> and LTB<sub>4</sub> release may not necessary indicate an enhanced or reduced inflammatory response in humans. It does however demonstrate that these compounds have some direct influence in lung epithelial cell’s production of PGE<sub>2</sub> and LTB<sub>4</sub>. The increase in PGE<sub>2</sub> may potentially be of benefit to the patients infected with TB. Various studies have shown that PGE<sub>2</sub> production may protect the host against virulent M.tuberculosis [341, 342]. During TB infection an increase in PGE<sub>2</sub> will induce apoptosis in infected macrophages. Unlike necrosis which helps the spread of the bacterium, apoptosis of infected macrophage restricts the spread of TB and also allows the uptake of apoptotic vesicles by dendritic cells [341]. This allows the dendritic cells to present bacterial antigens to naïve T-cells, leading to the activation of the adaptive immune system [341, 342]. Studies have shown that the activation of of naïve T-cells is essential in the formation of granuloma to control the spread of TB infection and to
activate macrophage killing of intracellular TB [343-345]. Further investigation using animal models will provide a better indication what the observed effect in \textit{in vitro} models means \textit{in vivo}.

T-cells play a central role in the control of \textit{M. tuberculosis} infection and the reduction of T-cell number in HIV patients have been shown to increase the risk of reactivation of latent TB [6]. All of the compounds screened increased NF-κB production in Jurkat cells demonstrating its potential effect on T-cell function. Compound 8a and 12 increased IL-2 production and this may be of benefit to TB infected patients through increased T-cell activation and proliferation. Only compound 8b did not increase IL-6 production. Of the antitubercular drugs tested, INH was also shown to increase IL-6 production. This may be of benefit to TB infected patients, as IL-6 has been shown to inhibit IFN-γ activation of macrophage killing of intracellular TB [358]. Complex animal based immune model would be required to determine what the observed \textit{in vitro} increase in IL-6 production means \textit{in vivo}. TNF-α production was also increased in Jurkat cells treated with compounds 8a, 8b and 10. TNF-α is an important cytokine in TB infection as it is secreted by activated T-cells to promote macrophage activation and killing of intracellular \textit{M. tuberculosis}. The limitation of this current study is that it only examined the direct \textit{in vitro} effect of these compounds on PGE$_2$, LTB$_4$, NF-κB, IL-2, IL-6 and TNF-α production and did not take into account the complex cellular interaction that occurs \textit{in vivo}. Hence, to better understand the possible effect of these compounds in humans, the combined effect of these compounds on immune response and inflammation would need to be investigated using animal models.
These studies demonstrated that modification of the hydrophilic head group of 2-DSA was able to alter its biological activity. The acetamide functional group was shown to be of importance to 2-DSA’s antitubercular properties. The chemistry explored in this study will allow the synthesis of other 2-DSA derivatives for future studies. Through the substitution of the hydrophilic groups it is possible to enhance or alter 2-DSA’s biological activity and reduce its toxicity toward mammalian cells. One recommendation is the creation of a 2-DSA prodrug using PEG. This can be achieved through conjugation of the amine functional group to PEG through a linker that can enzymatically be activated to release PEG from 2-DSA. The alteration of the lipid chain length may also alter its antibacterial activity. Further investigation of its inflammatory and immunomodulating properties, would require the use of *in vivo* animal models.
References


188. Henderson, M.C., L.K. Siddens, J.T. Morre, S.K. Krueger, and D.E. Williams, Metabolism of the anti-tuberculosis drug ethionamide by mouse and human


199. Brandon, E.F., T.M. Bosch, M.J. Deenen, R. Levink, E. van der Wal, J.B. van Meerveld, M. Bijl, J.H. Beijnen, J.H. Schellens, and I. Meijerman, Validation of in vitro cell models used in drug metabolism and transport studies; genotyping of cytochrome P450, phase II enzymes and drug transporter polymorphisms in the human hepatoma (HepG2), ovarian carcinoma (IGROV-1) and colon


247. Adam, I. and A.A. Hagelnur, Artesunate plus sulfamethoxypyrazine/pyrimethamine for the treatment of cutaneous


